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Enzymes and Transport Systems Involved in the Formation and Disposition of Glutathione S-Conjugates

ROLE IN BIOACTIVATION AND DETOXICATION MECHANISMS OF XENOBIOTICS

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I. Introduction

Conjugation to the nucleophilic thiol-group of the tripeptide glutathione (GSH) is an important route of metabolism for a large number of lipophilic xenobiotics and endogenous compounds possessing an electrophilic center (fig. 1) (Boyland and Chasseaud, 1969; Chasseaud, 1979; Jakoby, 1978, 1980, 1990). The first observation pointing to GSH-conjugation was obtained before the discovery of GSH. More than 100 years ago, mercapturic acids were identified as sulfur-containing metabolites in the urine of dogs treated with bromobenzene (Baumann and Preusse, 1879; Jaffe, 1879). GSH, initially called "philothion," was isolated in 1888 (de Rey-Pailhade, 1888), but its structure was elucidated only 40 years later (Hopkins, 1929). The relationship between formation of mercapturic acids and conjugation to GSH was described in 1959 (Barnes et al., 1959; Bray and James, 1960). For this reason, GSH-conjugation is also frequently referred to as the mercapturic acid pathway. Because the excretion of mercapturic acids reflects exposure to electrophilic agents, quantification of urinary excretion of mercapturic acids is used frequently as a biological monitoring method to assess the exposure to electrophilic agents (Vermeulen, 1989; Van Welie et al., 1992). However, at present, it is known that in addition to mercapturic acids, several other metabolites derived from GSH-conjugation may also be excreted in urine, such as cysteine S-conjugates, 3-mercaptopyruvic acid S-conjugates, 3-mercaptolactic acid S-conjugates, 2-mercaptoacetic acid S-conjugates, methylthioether-compounds, as well as their corresponding sulfoxides. The relative amounts of these products will largely depend on the specific activities of the more than 15 enzyme systems that now are known to be active in the formation and disposition of GSH-conjugates. Whereas these enzymes were studied originally because of their involvement in mercapturic acid biosynthesis, they are currently of increasing toxicological and clinical interest because of their role in detoxification and bioactivation mechanisms and because of their involvement in several aspects of anticancer therapy.

The initial GSH-conjugation reaction may, depending on the nature of the electrophilic substrate, be nonenzymic or be catalyzed by GSTs (Coles, 1985). Because GSH-conjugation leads to interception of electrophilic species, which are thereby precluded from reacting with nucleophilic centers in essential cellular macromolecules, such as proteins, DNA and RNA, GSH-conjugation can be regarded as an extremely important detoxication mechanism. Over the last decade, however, it has been demonstrated that the cofactor GSH and the enzymes involved in disposition of GSH-conjugates may also play an important role in the activation of chemicals to biologically active or even toxic metabolites, as described in several reviews (Igwe, 1986; Van Bladeren, 1988; Anders et al., 1988, 1992; Lock, 1988; Commandeur and Vermeulen, 1990a; Monks et al., 1990; Koob and Dekant, 1991; Dekant and Vamvakas, 1993).

GSH can play several roles in the activation of xenobiotics (*a*) by forming reactive, direct-acting GSH-conjugates, (*b*) by functioning as a transporter molecule that releases reversibly bound electrophiles at distant target tissues, (*c*) by forming GSH-conjugates that are bioactivated by subsequent catabolism of the GSH-moiety or by activation of the xenobiotic-derived moiety, and (*d*) by reductive bioactivation mechanisms. In section II of this review, an overview of these different types of GSH-dependent activation pathways will be presented.

Because GSTs and the enzymes involved in the catabolism of GSH-conjugates seem to play important roles in

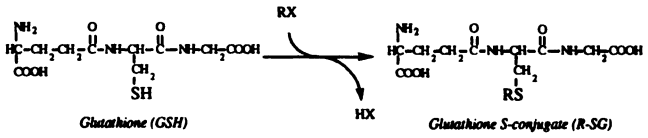

FIG. 1. Structure of GSH and general conjugation reaction to transform an electrophilic compound RX to a glutathione conjugate (R-SG).

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Abbreviations: **GSH**, γ-L-glutamyl-L-cysteinylglycine; **GST**, glutathione-S-transferase; **DNA**, deoxyribonucleic acid; **RNA**, ribonucleic acid; **GGT**, γ-glutamyltransferase; **2-GEMA**, N-acetyl-S-[2-(N^7-guanyl)ethyl]-L-cysteine; **GSSG**, oxidized glutathione; **BCNU**, N,N'-bis-(2-chloroethyl)-N-nitrosourea; **RS^-**, thiolate anion; **$Hb^{II}O_2$**, oxyhemoglobin; **RS•**, thiyl radical; **$RS^-S^•R$**, disulfide anion radical; **R-S-S-R**, disulfide; **RSM**, thiol; **R-SOM**, sulfenic acid; **$O_2^{•-}$**, superoxide anion radical; **H_2O_2**, hydrogen peroxide; **MMC**, mitomycin C; **cDNA**, complementary DNA; **HCBD**, hexachlorobutadiene; **HFP**, hexafluoropropene; **TFE**, tetrafluoroethylene; **1,2-DCV-G**, S-(1,2-dichlorovinyl)glutathione; **CTFE**, chlorotrifluoroethylene; **1,2-DCV-NAC**, N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine; **2,2-DCV-NAC**, N-acetyl-S-(2,2-dichlorovinyl)-L-cysteine; **1,2-DCV-Cys**, S-(1,2-dichlorovinyl)-L-cysteine; **1,2-DCV-HCys**, homocysteine analog of 1,2-DCV-Cys; **HFP**, hexafluoropropene; **K/L**, kidney/liver; **mRNA**, messenger ribonucleic acid; **DNP-G**, S-(2,4-dintrophenyl)glutathione; **PCBD-G**, 1-(glutathiou-S-yl)-1,2,3,4,4-pentachlorobuta-1,3-diene; **GHB**, γ-L-glutamyl-4-hydroxybenzene; **I-GHB**, iodinated GHB; **i.v.**, intravenous; **PLP**, pyridoxal phosphate; **CAT**, cysteine-conjugate α-ketoglutarate transaminase; **PCBD-Cys**, S-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine; **PCBD-NAC**, N-acetyl-S-(1,2,3,4,4-pentachlorobutadiene)-L-cysteine; **CTFE-Cys**, S-(2-chloro-1,1,2-trifluorethyl)-L-cysteine; **CTFE-G**, CTFE-glutathione; **TFE-Cys**, S-(1,1,2,2-tetrafluoroethyl)-L-cysteine; **BCDFE-Cys**, S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine; **BCDFE-G**, BCDFE-gluathione; **AT-125**, acivicin; **AOAA**, aminooxyacetic acid; **TCV-Cys**, S-(1,2,2-trichlorovinyl)-L-cysteine; **TFE-Cys**, S-(tetrafluoroethyl)-L-cysteine; **DCDFE**, 2,2-dichloro-1,1-difluoroethylene; **DCDFE-NAC**, N-acetyl-S-DCDFE-L-cysteine; **DCTFP-Cys**, S-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine; **P-Cys**, S-(6-purinyl)-L-cysteine; **TCV-NAC**, N-acetyl-S-TCV; **TFE-NAC**, N-acetyl-S-TFE; **CTFE-NAC**, N-acetyl-S-CTFE; **DBDFE-NAC**, N-acetyl-S-DBDFE; **FMO**, flavin-containing monooxygenase; **OP-NAC**, S-(3-oxopropyl)-N-acetyl-L-cysteine; **GS-X**, GSH S-conjugate export; **PAH**, *p*-aminohippuric acid; **BCH**, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid; **L-BSO**, L-buthionine sulfoximine.

both detoxication and bioactivation reactions, it is clear that the relative activities of these enzymes in tissues may determine the susceptibility of these tissues to toxic agents. In section III of this review, many features of the different enzyme systems, such as tissue distribution and substrate selectivity of (iso)enzymes (and their role in the bioactivation of xenobiotics), will be addressed. More or less selective inhibitors of these enzymes will be discussed, because these are very useful tools in the elucidation of the role of different enzyme systems in the bioactivation or detoxication of xenobiotics. Potent and selective inhibitors may also have important clinical applications. For example, selective inhibitors of GST-isoenzymes may help to sensitize tumors with acquired multidrug resistance to cytostatic drugs by blocking the inactivation of these drugs by their increased level of GSTs.

The relative amounts and activities of some of these enzymes in tissues are very species-dependent and thus may give rise to large interspecies differences in the susceptibility to toxic effects of xenobiotics. Therefore, in order to improve human risk assessment based on animal toxicity data, knowledge of the interspecies differences and similarities of the enzymes involved in activation and detoxication reactions is very important. In this section, the species-differences of the enzymes involved in the disposition of GSH-conjugates will also be discussed.

Next to interspecies differences in enzyme activities, some of the enzymes that will be discussed also show very large interindividual variations. These interindividual variations may be caused by exposure to enzyme inducers or inhibitors, the action of sex hormones, or to the individual genetic constitution. At present, at least two GST isoenzymes have been shown to display a genetic polymorphism in human population studies. Because of the important role of GSTs in detoxication and activation reactions, genetically impaired GST-activity may have important toxicological consequences, such as increased susceptibility to the development of tumors.

The knowledge of GSH-dependent bioactivation mechanisms is used to develop new strategies to improve the treatment of malignant tumors. GSH, GSTs, and GGT are frequently elevated in tumor cells. Several examples of prodrugs will be discussed; prodrugs are selectively activated to cytostatic compounds in the tumor cells because of their increased levels of GSH, GST, or GGT.

GSH-dependent bioactivation mechanisms can also be used to prevent undesired toxic side-effects of antitumor drugs. Coadministration of chemoprotectors that are activated by GSH, such as sodium selenite or ebselen, protect against the nephrotoxicity of cisplatin without affecting the antitumor activity in rats and mice.

Next to tissue distribution of enzymes, the tissue concentration of the substrates for these enzymes is a very important factor that determines the activity of a specific enzyme in a particular tissue. Because GSH-derived S-conjugates are charged molecules under physiological conditions, they rely on active transporters for their distribution throughout the body. Therefore, next to tissue distribution of enzymes, as mentioned earlier, the tissue distribution of transport systems will also play an important role in the organ selectivity of toxicants activated by GSH-dependent mechanisms, because this will determine whether a GSH-derived S-conjugate has actual access to activating enzymes in a particular cell type. In section IV, the characteristics and tissue distribution of different carriers active in the transport of toxic and nontoxic GSH-derived S-conjugates will be reviewed.

Finally, in section V, some concluding remarks and future perspectives will be given.

II. Glutathione-dependent Bioactivation of Xenobiotics

Conjugation of GSH, by definition, takes place on electrophilic sites in organic molecules, thereby precluding the reaction of these electrophiles with biological macromolecules. In a number of cases, the GSH S-conjugation has been identified as a bioactivation pathway leading to GSH S-conjugates that possess biological activities themselves or that have to undergo further metabolism to become biologically active. GSH S-conjugates that cause toxicity have been divided in three groups (Van Bladeren, 1988): *a*) reactive, direct-acting GSH-conjugates, *b*) GSH-conjugates in which GSH functions as a transporter molecule by releasing reversibly bound electrophilic compounds, and *c*) GSH-conjugates that require further bioactivation, either by catabolism of the GSH-moiety or by activation of the xenobiotic-derived moiety. In addition to conjugation reactions, GSH may also bioactivate xenobiotics by reductive mechanisms.

In the next paragraphs, examples of the different GSH-dependent bioactivation mechanisms will be discussed.

A. Direct Acting Glutathione S-Conjugates

Dihalomethanes, such as dibromo-, dichloro-, and diiodomethane are mutagenic in *Salmonella typhimurium TA 100* in the presence of GSH and GST-containing liver fractions (Van Bladeren et al., 1980). Substitution of one halogen by GSH yields a S-(halomethyl)-glutathione conjugate (fig. 2A). The reactivity and stability of these GSH-conjugates depend on the nature of the halogen: S-(chloromethyl)glutathione rapidly decomposes in an aqueous solvents, whereas S-(fluoromethyl)glutathione is more stable and could be identified as an intermediate during GSH-conjugation to chlorofluoromethane (Blocki et al., 1994). S-(Halomethyl)-glutathione conjugates may possess reactivity similar to halomethyl methyl ethers or bis-halomethyl ethers that are known carcinogens (Leong et al., 1971) and therefore are capable of alkylating various tissue nucleophiles (Anders et al.,

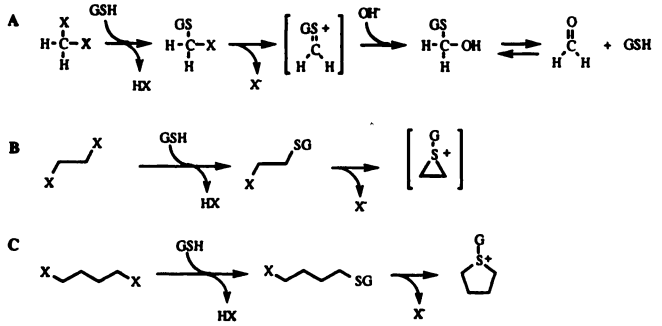


FIG. 2. Bioactivation mechanisms of dihalogenated alkanes by conjugation to GSH: (A) dihalomethanes; (B) 1,2-dihaloethanes; (C) 1,4-dihalobutanes.

1977). The hydrolysis product, S-(hydroxymethyl)glutathione, has been shown to dissociate to GSH and formaldehyde, which is also a potentially DNA-reactive metabolite (Ahmed and Anders, 1978; Casanova et al., 1992). It is suggested that S-(halomethyl)glutathione is responsible for DNA single-strand breaks, whereas formaldehyde causes DNA-protein cross-links (Graves et al., 1994). It has also been shown that formaldehyde inhibits DNA repair processes, a finding that explains why formaldehyde can potentiate the mutagenicity of physical and chemical carcinogens (Grafstrom et al., 1993). Next to GSH-conjugation, a dihalomethane may be activated by cytochrome P-450 to formyl halide, which decomposes to carbon monoxide, or may act as an acylating agent of tissue nucleophiles (Gargas et al., 1986).

Conjugation of GSH to vicinal dihalogenoalkanes leads to formation of sulfur half-mustard compounds (fig. 2B). These conjugates may form very reactive electrophilic episulfonium ions by internal displacement of the second halogen by anchimeric assistance of the thioether sulfur atom (fig. 2B) (Bland and Stammer; 1983; Vermeulen et al., 1989). Compounds that are bioactivated via this mechanism include 1,2-dichloroethane and 1,2-dibromoethane (Rannug et al., 1978; Van Bladeren et al., 1980; Shih and Hill, 1981), 1,2-dibromo-3-chloropropane (Pearson et al., 1990; Humphreys et al., 1991), 1,2,3-trichloropropane (Weber and Sipes, 1992), tris(2,3-dibromopropyl)phosphate (Inskeep and Guengerich, 1986), and ethylene dimesylate (Edwards et al., 1970).

Biotransformation of haloalkanes often involves both direct GSH-conjugation as well as oxidative metabolism by cytochrome P450; both biotransformation mechanisms may lead to formation of toxic metabolites. By studying the metabolism of tetradeutero-1,2-dibromoethane in rats, it was estimated that the ratio of direct GSH-conjugation and oxidative metabolism is approximately 1:4 (Van Bladeren et al., 1981). Oxidative metabolism results in formation of the highly reactive bromoacetaldehyde, which is able to alkylate DNA (Guengerich et al., 1981). Direct GSH-conjugation, however, seems to be more important for binding to DNA in vitro and has been used to screen for GST activity in human fetal tissues (Mitra et al., 1992). Binding of 1,2-dibromoethane to DNA in hepatocytes was decreased by prior GSH-depletion but was not influenced by inhibition of cytochrome P450 (Sundheimer et al., 1982). An increase in the occurrence of 1,2-dibromoethane-induced tumor formation in rats after inhibition of oxidative metabolism also points to direct GSH-conjugation as the major genotoxic pathway in vivo (Wong et al., 1982). The major DNA-adduct formed from 1,2-dibromoethane turned out to be S-[2-(N^7-guanyl)ethyl]glutathione and constituted up to 95% of all adducts formed, both in isolated hepatocytes and in vivo (Koga et al., 1986; Inskeep and Guengerich, 1986; Inskeep et al., 1986). Another reaction product, S-(2-(N1-adenyl)ethyl)glutathione, was suggested to be a minor adduct (< 5%) in RNA and DNA of rats exposed to 1,2-dibromoethane (Kim et al., 1990). This indicated that no further metabolism of the GSH-conjugate was necessary for covalent binding to DNA. Interestingly, an adduct excreted in the urine was the mercapturic acid of the glutathione DNA-adduct, 2-GEMA (Kim and Guengerich, 1989). The excretion in urine of 2-GEMA as well as the DNA-adduct formation was dose-dependent after administration of 1,2-dibromoethane.

α,ω-Dihalogenoalkanes (X = Cl, Br, I) with higher chain lengths also may be bioactivated by GSH-conjugation (Buys, 1985). Mutagenicity of the corresponding S-conjugates seemed to depend on chain length. 1,3-Dihalogenopropanes become mutagenic in the presence of rat liver S100. α,ω-Dihalopentanes and α,ω-dihalohexanes are also bioactivated by GSH-conjugation (Buys, 1985; Onkenhout et al., 1986). However, 1,4-dihalogenobutanes, which are direct-acting mutagens themselves, are inactivated by S100. GSH-conjugation of the latter compounds leads to formation of cyclic sulfonium ions (fig. 2C), which readily decompose to tetrahydrothiophene; thus, this pathway can be considered as a deactivation process.

After conjugation of electrophiles to GSH, the S-bound moiety of GSH-conjugates itself may still possess electrophilic properties. Thus, oxygenation of the hepatotoxin and nephrotoxin 1,1-dichloroethylene by microsomal cytochromes P-450 leads to formation of 2-chloroacyl chloride (fig. 3A) (Liebler and Guengerich, 1984). Reaction of this highly reactive intermediate with the thiol-group of GSH results in formation of S-(2-chloroacetyl) glutathione, which is more stable than the acyl chloride but still capable of alkylating protein thiol groups by nucleophilic substitution of the chlorine-atom of the chloroacetyl-moiety (Liebler et al., 1988). Because of the presence of the GSH-moiety, covalent binding of the chloroacetyl-moiety is directed to proteins containing specific binding sites for GSH: for example, hepatic GSTs (Moslen and Reynolds, 1985) and hepatic canicular membrane proteins (Kanz and Reynolds, 1986). S-(2-

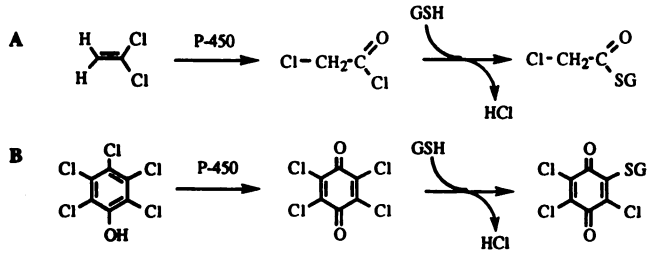


FIG. 3. Examples of electrophilic GSH-conjugates formed by GSH-conjugation to electrophilic oxidative metabolites of (A) 1,1-dichloroethene and (B) pentachlorophenol.

chloroacetyl)glutathione, however, is not cytotoxic to mouse hepatocytes (Kainz et al., 1993), indicating that alkylation of these specific GSH-binding sites is not lethal for this type of cells.

The GSH-conjugate formed from tetrachloro-1,4-benzoquinone, an oxidative metabolite of pentachlorophenol, also still possesses direct alkylating abilities (fig. 3B) (Van Ommen et al. 1988, 1991). Similar to S-(2-chloroacetyl)glutathione, the alkylation by this GSH-conjugate may be directed to proteins containing a GSH-binding site, as demonstrated by the irreversible inhibition of purified glutathione S-transferases.

Recently, it has also been shown that after reaction of GSH with aromatic nitroso-compounds, electrophilic sulfenamide cations are formed rapidly by hydrolysis (fig. 4). The phenacetin metabolite 4-nitrosophenetol was reduced by GSH to 4-phenetidine under the formation of GSSG. When the availability of GSH is restricted, 4-phenetidine competes with GSH as a nucleophile leading to the formation of bicyclic products (Galleman and Eyer, 1993a, b). In human red blood cells, the bicyclic products were produced to a relatively high extent. Because these compounds are capable of forming several equivalents of ferrihemoglobin, this mechanism may contribute to the methemoglobinemia occasionally observed after incorporation of nitrobenzenes and aromatic amines, both of which are metabolized to the corresponding nitrosoarenes.

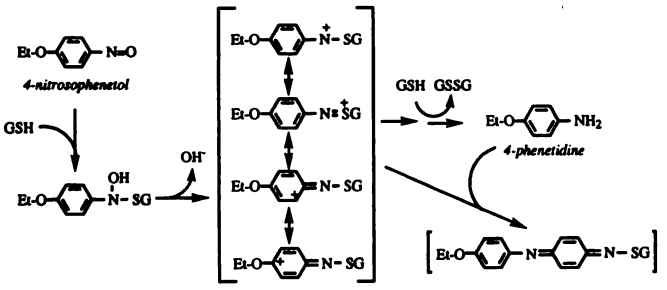


FIG. 4. Bioactivation of the phenacetin metabolite 4-nitrosophenetol by GSH-conjugation. 4-Phenetidine is the major product at high GSH-concentrations; at restricted GSH-availability, formation of bicyclic products becomes more important (modified from: Galleman and Eyer, 1993a, b).

B. Glutathione as Transporter of Reversibly Bound Electrophiles

Some GSH-conjugates can cause toxicity by transferring electrophiles to distant nucleophilic centers of essential biomacromolecules (Baillie and Slatter, 1991). Relatively labile GSH-conjugates may again dissociate to the parent electrophilic compound and GSH; the electrophile subsequently can react with endogenous nucleophiles to thermodynamically more favored adducts. Also, the S-bound electrophile may be transferred directly from the S-conjugate by nucleophilic substitution of the GSH-moiety; in this case, the parent electrophile is not liberated as such. Meanwhile, several classes of electrophiles have been shown to undergo reversible conjugation to GSH (fig. 5).

The GSH-conjugates of benzylisothiocyanate and allylisothiocyanate, S-(N-benzylthiocarbamoyl)glutathione and S-(N-allylthiocarbamoyl)glutathione, are cytotoxic, presumably because of their dissociation to GSH and the corresponding free isothiocyanates (fig. 5A) (Bruggeman et al., 1986; Temmink et al., 1986).

GSH-conjugation is a major pathway of metabolism of isocyanates (fig. 5B) (Slatter et al., 1991). It has been suggested that the cardiovascular, gastrointestinal, and muscoskeletal toxicity observed in survivors of the Bophal catastrophe was caused by the GSH-conjugate of methyl isocyanate, S-(N-methylcarbamoyl)glutathione (Bhattacharya et al., 1988). Both S-(N-methylcarbamoyl) glutathione and S-(N-methylcarbamoyl)cysteine are capable of carbamoylating proteins and peptides (Pearson et al., 1991). Release of methyl isocyanate was proposed to explain this observation. However, the fact that inhibition of GSH reductase occurred at a rate that was much faster than that of the decomposition to methyl isocyanate, indicates that the S-conjugates per se, rather than the free methyl isocyanate, are the carbamoylating species (Jochheim and Baillie, 1994).

The reversibility of GSH S-conjugates was demonstrated with S-(N-(1-methyl-3,3-diphenylpropyl)glutathione, which released the parent isocyanate and GSH

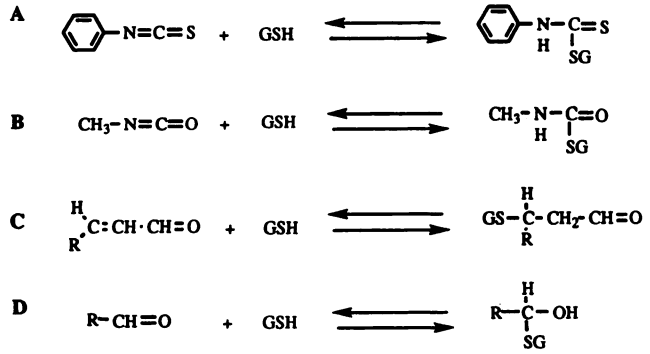


FIG. 5. Examples of GSH-conjugates of electrophiles that may act as transporter molecules of the electrophiles by dissociation at sites distant from the site of formation. (A) isothiocyanates; (B) isocyanates; (C) α,β-unsaturated aldehydes; (D) aldehydes.

under mild acidic conditions (Mutlib et al., 1990). The antitumor drug BCNU decomposes spontaneously under physiological conditions to afford a number of products, one of which, 2-chloroethyl isocyanate, is believed to be responsible for the inhibition of glutathione reductase by BCNU (Karplus et al., 1988). The labile S-linked GSH-conjugate of 2-chloroethyl isocyanate, which has been identified as a biliary metabolite of BCNU in the rat, is equipotent with BCNU as an inhibitor of GSH reductase in vitro in rat hepatocytes (Davis et al., 1993). Moreover, in the metabolism of fotemustine, a new antitumor drug, a reversible GSH-conjugate of an isocyanate metabolite has also been demonstrated (Brakenhoff et al., 1994). Whether this GSH-conjugate plays a role in the unexpected lung toxicity which is observed after the sequential administration of dacarbazine and fotemustine to patients with melanoma, however, remains to be established (Gerard et al., 1993).

α, β-Unsaturated aldehydes and ketones have long been known to form conjugates with GSH, both spontaneously and enzymatically catalyzed (Boyland and Chasseaud, 1968; Witz, 1989). Several thiol adducts of α,β-unsaturated aldehydes exhibit biological activity similar to that observed for the parent aldehyde. Thus, the 1:2 crotonaldehyde-cysteine adduct and the 1:1 trans-hydroxypentenal-cysteine adduct exhibited cytostatic activity when given to mice implanted with tumor cells (Conroy et al., 1977; Tillian et al., 1978). In order to explain the biological activity of these adducts, it has been suggested that the Michael addition of thiols to these electrophiles is reversible (fig. 5C) (Witz, 1989). Such a process might potentially generate reactive intermediates at target sites. This hypothesis offers a mechanism that might explain how trans,trans-muconaldehyde is transported to the bone marrow from the liver, if it is indeed formed from benzene during hepatic metabolism (Witz et al., 1985).

The veterinary drug furazolidone has been shown to be reduced in swine liver microsomes to a cyano-substituted α,β-unsaturated ketone, which was reactive toward mercaptoethanol (Vroomen et al., 1987, 1988). The chemical exchange of bound mercaptoethanol when GSH is added demonstrated that binding of this compound to the thiol-groups is reversible. The GSH-conjugates of E-2-hexenal and E-2,Z-6-nonadienal decomposed to GSH and the free aldehydes with halflives of 1–2 hours (Goelzer et al., 1994). The GSH-conjugate of the diuretic drug ethacrynic acid is able to transfer its ethacrynic acid-moiety to the thiol of N-acetylcysteine or to a thiol-group of glutathione S-transferase P1–1 (Ploemen et al., 1994). This reversible binding to glutathione S-transferase P1–1 was used to explain the efficient inhibition of this enzyme by ethacrynic acid, especially when the GSH-concentration is low.

The last category of compounds that form reversible GSH-conjugates are aldehydes (fig. 5D). The thiohemiacetal formed after conjugation of GSH to formaldehyde may fall apart again. However, it also may react with a second molecule of GSH and perhaps with nitrogen nucleophiles in DNA (Fennell et al., 1988).

C. Glutathione S-Conjugates Requiring Further Bioactivation

1. Bioactivation by catabolism of the glutathione-moiety. GSH-conjugates are usually not excreted as such in urine or feces. Instead, the tripeptide moiety of the conjugates undergoes efficient catabolism, yielding different types of S-containing metabolites (fig. 6). By studying the toxicity of intermediate metabolites and by using selective inhibitors of enzymes involved in the catabolism of the tripeptide-moiety, the role of several GSH-derived S-conjugates in toxicity has been well established.

In principle, all GSH S-conjugates may cause toxicity by mechanisms similar to those described in sections A and B. The GSH-conjugate of 1,2-dichloroethane, which is formed exclusively in the liver, is relatively stable, compared with the corresponding GSH-conjugate of 1,2-dibromoethane (fig. 2B) (Foureman and Reed, 1987). Hydrolysis by peptidases results in the formation of the corresponding cysteine S-conjugate, S-(2-chloroethyl)-L-cysteine, which has more alkylating power than the parent GSH-conjugate (Vadi et al., 1985; Elfarra et al., 1985). Concentration of S-(2-chloroethyl)-L-cysteine in the kidney, followed by formation of a reactive episulfonium ion, has been proposed as the mechanism responsible for the nephrotoxicity caused by 1,2-dichloroethane. The cysteine S-conjugates formed by hydrolysis of S-(N-benzylthiocarbamoyl)glutathione and S-(N-allylthiocarbamoyl)glutathione also displayed a higher cytotoxicity than did the corresponding GSH-conjugates (Bruggeman et al., 1986). However, the relative contribution of GSH and cysteine S-conjugates to the toxicity of isothiocyanates is not known yet.

β-Elimination (fig. 6, step d) of L-cysteine S-conjugates derived from GSH S-conjugates of haloalkenes results in the formation of thiol compounds, which may be very reactive themselves or which may rearrange to form other highly reactive intermediates such as thionoacyl halides, thioketenes, and/or thiiranes (fig. 7) (Dekant et al., 1987a, 1988a, 1991; Commandeur et al., 1989). Because of the accumulation of S-conjugates in the kidney and the relatively high activity of β-elimination in this organ, this mechanism is responsible for the severe nephrotoxicity caused by a number of halogenated olefins (Commandeur and Vermeulen, 1990a). Very recently, it has been suggested that the observed neurotoxicity of dichloroacetylene may also be linked to uptake and bioactivation in the brain of S-(1,2-dichlorovinyl)glutathione and/or S-(1,2-dichlorovinyl)-L-cysteine, the GSH-conjugate of dichloroacetylene and its corresponding L-cysteine S-conjugate metabolite (Patel et al., 1993, 1994).

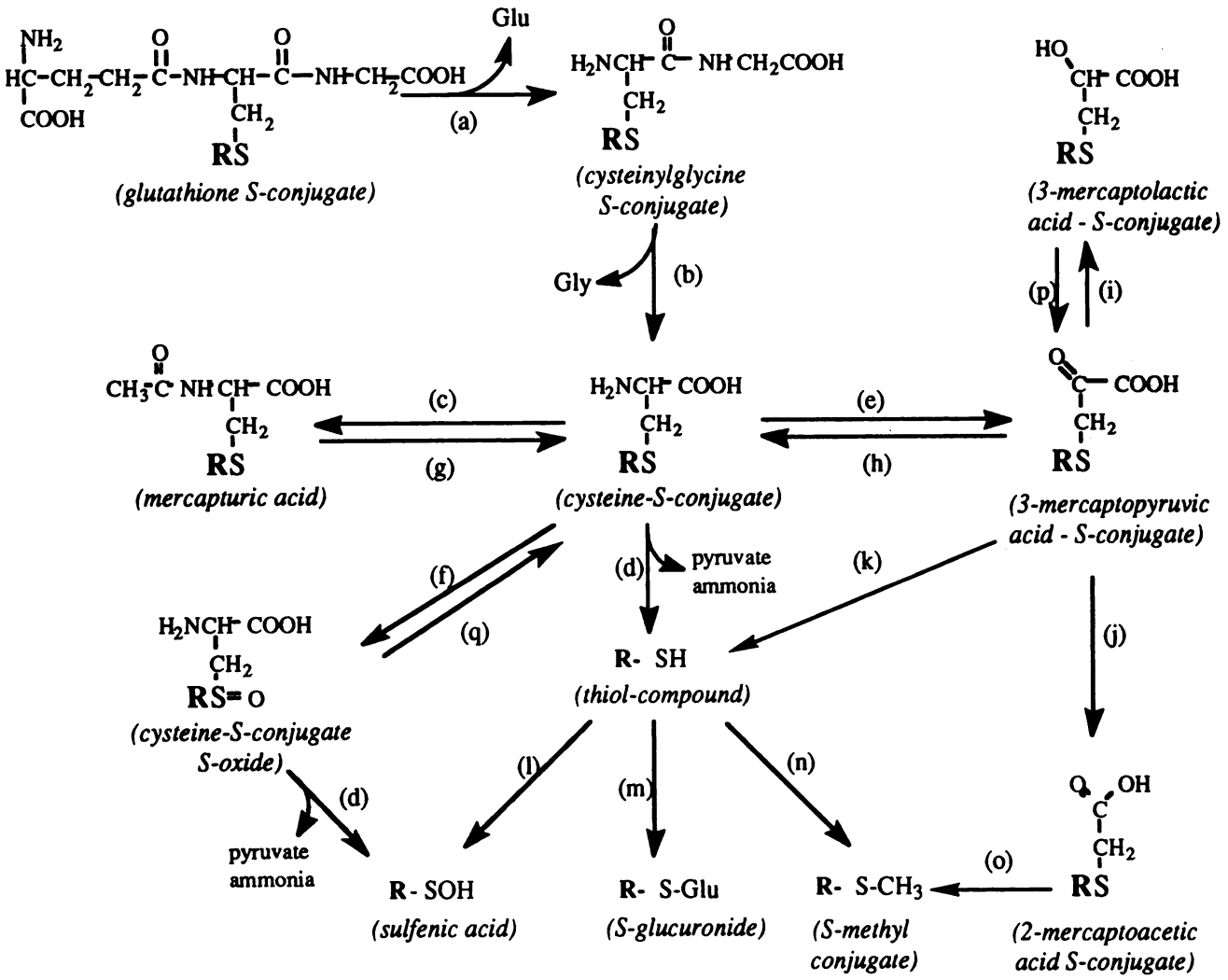


FIG. 6. Possible routes of catabolism of glutathione S-conjugates (I). Steps are catalyzed by; (a) γ-glutamyltranspeptidase; (b) dipeptidases: cysteineglycine dipeptidase and aminopeptidase M; (c) cysteine conjugate N-acetyltransferase; (d) cysteine conjugate β-lyase; (e) cysteine conjugate transaminase and L-amino acid oxidase; (f) cysteine conjugate S-oxidase; (g) N-deacetylase; (h) transaminases; (i) 3-mercaptopyruvic acid S-conjugate reductase; (j) decarboxylase; (k) enzyme not yet characterized (Tomisawa et al., 1986b); (l) S-oxygenase; (m) uridine diphosphate-glucuronyl transferase; (n) S-methyl transferase; (o) decarboxylase; (p) 3-mercaptolactic acid S-conjugate oxidase; (q) sulfoxide reductase.

Thiols formed from nonhalogenated cysteine S-conjugates are chemically stable. Although a number of thiol-compounds, such as GSH, coenzyme A and lipoic acid, are essential for life, many body-foreign thiols, even the simplest member of the group, hydrogen sulfide, are extremely toxic to mammalian cells (Evans, 1972). These toxic phenomena include neurotoxicity, anemia, nephrotoxicity, and nephrocarcinogenicity. The neurotoxicity of methyl chloride and methyl iodide is suggested to be caused by the formation of methanethiol as a result of β-lyase-mediated degradation of the initially formed metabolite, S-methylglutathione (Kornbrust and Bus, 1983; Johnson, 1966; Honma et al., 1985; Repko and Lasley, 1979). Recently, it was suggested that the antitumor agent, 6-chloropurine, also is activated by initial GSH-conjugation and subsequent degradation to 6-mercaptopurine (Hwang and Elfarra, 1993).

Many thiol-compounds are toxic, because they may readily be autooxidized under physiological conditions, with subsequent formation of thiyl radicals and activation of molecular oxygen to reactive oxygen species (reviewed by Munday, 1989). The availability of a suitable metal catalyst may account for the tissue specificity of these toxic compounds. The ability of oxyhaemoglobin-bound iron to initiate one-electron thiol oxidation, scheme 1, accounts for susceptibility to anemia caused by aliphatic thiols, such as methanethiol, in species with oxyhaemoglobin relatively fragile erythrocytic defense systems. Secondary reactions may lead to formation of disulfides (scheme 1, reactions 3 and 6). The formation of mixed disulphides with proteins and other endogenous mercaptans, although relatively short-lived, may also be of relevance to toxicity caused by thiols (Damani, 1987; Lock and Schnellmann, 1990). Alternatively, superoxide

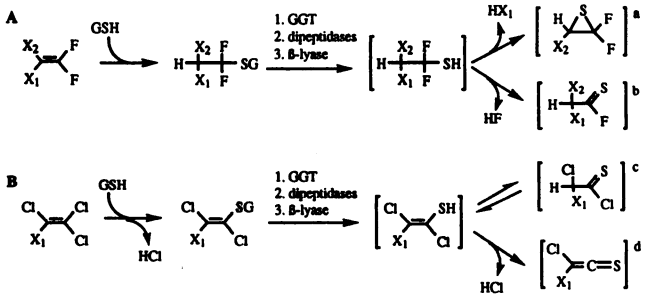


FIG. 7. Bioactivation mechanisms of halogenated alkenes leading to two different types of reactive thiol-compounds. (A) Saturated S-conjugates formed from 1,1-difluoroalkenes are cleaved to ethanethiol-compounds that may decompose to (a) thiiranes and/or (b) thionoacyl fluorides. (B) Unsaturated S-conjugates formed from 1,1-dichloroalkenes are cleaved to ethenethiol-compounds that may tautomerize to (c) thionoacyl chlorides or that may decompose to (d) thioketenes.

anion radicals and hydrogen peroxide formed in these reactions can react by the Haber-Weiss reaction to form highly reactive and extremely toxic hydroxyl radicals.

Scheme 1. Mechanism of thiol oxidation initiated by oxyhaemoglobin-bound iron.

$$RS^- + Hb^{II}O_2 + 2H^+ \rightarrow RS^\bullet + Hb^{III} + H_2O_2 \quad (1)$$

$$RS^- + RS^\bullet \rightarrow RS^-S^\bullet R \quad (2)$$

$$RS^-S^\bullet R + O_2 \rightarrow RSSR + O_2^{-\bullet} \quad (3)$$

$$O_2^{-\bullet} + RSH + H^+ \rightarrow H_2O_2 + RS^\bullet \quad (4)$$

$$H_2O_2 + RSH \rightarrow H_2O + RSOH \quad (5)$$

$$RSOH + RSH \rightarrow RSSR + H_2O \quad (6)$$

Chemically stable thiols can also be enzymatically bioactivated to toxic compounds (fig. 6, steps 1). Thus, enzymic S-oxygenation of thiols leads to highly reactive sulfenic acids, which may react rapidly with thiols to form disulphides or dimerize to thiolsulfinates (Davis et al., 1986). Formation of a sulfenic acid by hepatic cytochrome P450 and flavin-containing monooxygenase is proposed as the bioactivation mechanism responsible for the hepatotoxicity caused by the antitumor and immunosuppressant drug 6-mercaptopurine (fig. 8) (Van den Broek et al., 1990).

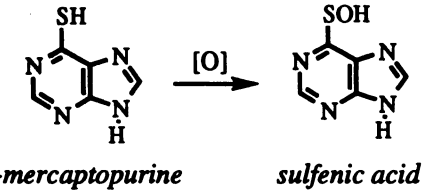


FIG. 8. Oxidative bioactivation mechanism of 6-mercaptopurine leading to formation of a reactive sulfenic acid.

2. Bioactivation by metabolism of the xenobiotic-derived moiety. The GSH-conjugates of 2-bromohydroquinone (Monks et al., 1985, 1988), 1,4-benzoquinone (Lau et al., 1988), 1,4-naphtoquinone (Lau et al., 1990a) and *p*-aminophenol (Fowler et al., 1991) all cause severe nephrotoxicity after administration to rodents. The toxication mechanism of these S-conjugates is proposed to involve intracellular oxidation of the hydroquinone moiety to an alkylating quinone moiety (fig. 9), which subsequently may react covalently with protein thiols and with nonsulfur nucleophiles (Weller and Hanzlik, 1991; Monks et al., 1992). Inhibition of γ-glutamyl transpeptidase by AT-125 results in protection against the nephrotoxicity of the GSH-conjugates of 2-bromohydroquinone and 1,4-benzohydroquinone, indicating that catabolism of the GSH-moiety is also involved in the bioactivation of these toxic GSH-conjugates. AT-125-pretreatment, however, did not protect against nephrotoxicity of *p*-aminophenol, suggesting that catabolism of the GSH-moiety of its GSH-conjugate is not required for the induction of this toxicity (Anthony et al., 1993).

An alternative mechanism explaining the toxicity of glutathione S-conjugates of quinones may be redox-cycling or autoxidation of the (hydro)quinone-moiety, leading to the production of reactive oxygen species (Wefers and Sies, 1983; Monks et al., 1992). Thus, it is suggested that the nephrotoxicity in the perfused kidney caused by the GSH-conjugate of menadione, thiodione (Redegeld et al., 1991), results from redox-cycling of the (hydro)quinone moiety (fig. 10A). The GSH-conjugate of menadione, however, did not produce renal proximal tubular necrosis when administered to rats in vivo (Lau et al., 1990a).

Recent evidence has also suggested that aberrant oxidations of 5-hydroxytryptamine (5-HT, serotonin) occur in the central nervous system of individuals with Alzheimer's disease. Compound I is formed as a result of 5-HT within serotonergic nerve terminals or axons and is expected to be conjugated rapidly by intraneuronal GSH to give 7-S-glutathionyl-tryptamine-4,5-dione (compound 2, fig. 10B) (Wong et al., 1993). Compound 2 was shown to be lethal to mice at very low doses when injected in the brains. It is proposed that the redox-cycling proper-

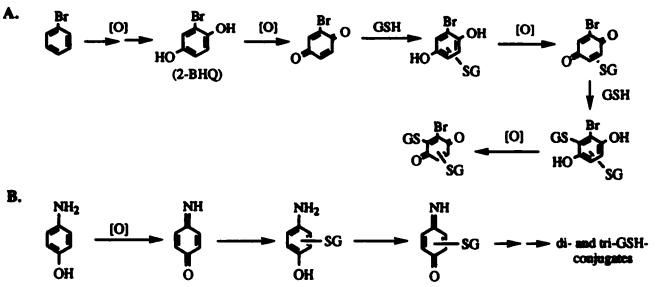


FIG. 9. Proposed GSH-dependent bioactivation mechanisms responsible for the nephrotoxicity of bromobenzene (A) and *p*-aminophenol (B).

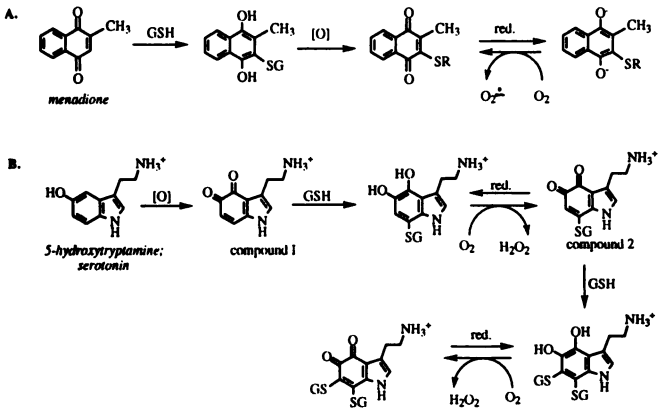


FIG. 10. Formation of reactive oxygen species by redox-cycling or autoxidation of GSH-conjugutes of quinone compounds formed from menadione (A) and serotonine (B).

ties of compound 2 and its facile reactions with cellular nucleophiles such as GSH may represent mechanisms that contribute to the toxicity of this drug.

The synthetic food anti-oxidant, butylated hydroxyanisole, causes forestomach cancer in rats. The mechanism responsible for this mode of action is suggested to be oxidation by cytochrome P-450 to a quinone metabolite and subsequent conjugation to GSH, which leads to a hydroquinone conjugate that can redox-cycle efficiently, forming reactive oxygen species. (Van Ommen et al., 1992).

The GSH-conjugate of acrolein causes nephrotoxicity when administered to rats (Horvath et al., 1992). Different mechanisms have been proposed to explain the toxicity of this conjugate. Dissociation of the S-conjugate may release the toxic parent aldehyde, followed by subsequent reaction of acrolein with critical cellular targets. An alternative mechanism involving sulfoxidation and subsequent release of acrolein has also been postulated (Hashmi et al., 1992). A third proposed mechanism involves reaction of the aldehyde group of the intact conjugate with a cysteine moiety or amino group of a protein, thereby altering the protein function. Finally, a mechanism is proposed that involves formation of oxygen radicals. The enzymes xanthine oxidase and aldehyde dehydrogenase were found to interact with the GSH conjugate to produce superoxide anion radicals and hydroxyl radicals (Adams and Klaidman, 1993).

D. Reductive Bioactivation by Glutathione

1. Reductive bioactivation of antitumor agents. Several selenium compounds, such as selenite, possess inhibitory effects on mammalian cell growth, particularly tumor cells (Shamberger, 1985). It has been suggested that selenite is activated by interaction with reduced GSH, because the cytotoxicity of selenite is enhanced in tumor cell lines with high levels of GSH (Batist et al., 1986a; Leyva et al., 1994). Selenite is reduced nonenzymatically by four equivalents of GSH to form GSSG and selenodiglutathione (GS-Se-SG), which has been proposed to be the major metabolite of inorganic selenium compounds in mammalian tissues (fig. 11A) (Shamberger, 1985). Selenodiglutathione is one of the most effective selenium compounds inhibiting the growth of neoplastic cells (Poirier and Milner, 1984). Selenodiglutathione is metabolized by glutathione reductase and/or thioredoxin reductase, which leads to formation of hydrogen selenide, which subsequently is methylated to methylselenol (fig. 11A) (Ganther, 1971; Björnstedt et al., 1992). Methyl selenol is methylated further to dimethyl selenide and trimethyl selenonium, respectively (Palmer et al., 1969; Foster et al., 1986). The rate of degradation via the methylated intermediates may be an important determinant in selenium chemoprevention (Ip and Ganther, 1990; Ip et al., 1991). It has been suggested that induction of apoptosis in tumor cells by methyl selenol or a closely related metabolite explains the selenium-mediated inhibition of tumorigenesis (Lu et al., 1993, 1994; Thompson et al., 1994). Selenite also inhibits the process of initiation by blocking the formation of carcinogen-DNA adducts (Liu et al., 1991). Whether the strongly nucleophilic selenol intermediates that are formed upon reaction of selenite with GSH are the protective agents remains to be elucidated.

KW-2149 is an analogue of mitomycin C (fig. 12), which shows superior inhibitory activity against a number of tumor cell lines and is effective against mitomycin C-resistant and mitomycin C-sensitive tumors in vitro and in vivo (Tsuruo et al., 1990; Morimoto et al., 1991). 7-N-{{2-{[2-(γ-L-glutamylamino)ethyl]dithio}ethyl}} mitomycin C (KW-2149) is now undergoing clinical trials in Europe. It was shown recently that physiological concentrations of GSH and L-cysteine significantly enhance the cytotoxicity of KW-2149 in human colon carcinoma HT-29 cells and increase DNA-adduct formation of KW-2149 in isolated nuclei (Lee et al., 1994). It was concluded that KW-2149 is activated nonenzymatically by cellular thiol molecules. Although the molecular mechanism of KW-2149 activation by GSH is not yet clear, it was suggested that GSH reduces the disulfide moiety, followed by activation of the quinone ring to the corresponding semiquinone through an intramolecular reaction. Once the semiquinone is formed, it may react with

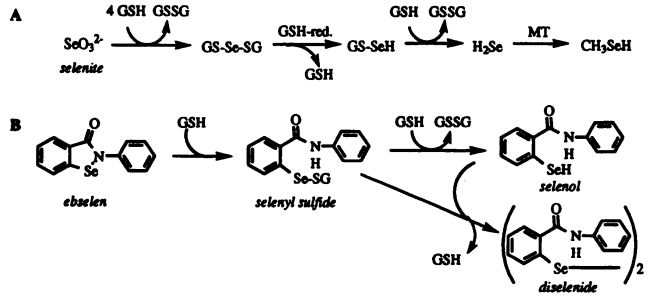
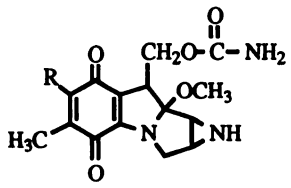


FIG. 11. GSH-dependent reductive activation of selenite (A) and ebselen (B) to selenols. GSH-red., GSH-reductase; MT, methylthiol transferase.



MMC: R = NH_2
KW-2149: R = γ-L-Glu-NH-$(CH_2)_2SS(CH_2)_2$NH

FIG. 12. Chemical structure of mitomycin C (MMC) and KW-2149.

DNA (Lee et al., 1994). Multidrug resistance has often been encountered with increased cellular GSH levels (Kramer et al., 1988). Because KW-2149 is activated by GSH, this compound may be an interesting compound to overcome the drug resistance caused by elevated GSH levels.

2. Reductive bioactivation of chemoprotectors. In addition to its antiproliferative action, selenite can act as a chemoprotector against cisplatin nephrotoxicity. Selenite protected rodents against the nephrotoxicity of cisplatin without reducing the antitumor activity of this drug (Naganuma et al., 1984; Baldew et al., 1989). It has been proposed that selenol-metabolites, which are formed upon GSH-dependent bioactivation of selenite (fig. 11A), may react with cisplatin or its reactive aquated metabolites, resulting in detoxication of these compounds. Also, liberation of platinum from platinum-protein bonds by the strongly nucleophilic selenols may play a role in the mechanism of chemoprotection of selenite. The fact that selenite concentrates in the kidney and that GSH-levels in the kidney are higher than those in tumors may favor the selective inactivation of cisplatin in the kidney after administration of selenite (Vermeulen et al., 1993).

A relatively nontoxic selenium compound that is also activated by reduction by GSH is the anti-inflammatory drug ebselen (fig. 11B). Ebselen has been shown to be an extremely potent GSH-peroxidase-mimetic compound that is able to catalyse the reduction of hydrogen peroxide and smaller organic hydroperoxides in the presence of GSH (Sies, 1993). Ebselen reacts with GSH to form a selenylsulfide, which is subsequently converted by GSH to a selenol and GSSG (fig. 11B) (Haenen et al., 1990; Cotgreave et al., 1992). The selenol formed may react with the selenylsulfide, thus forming ebselen diselenide and GSH. It has been suggested that the selenol intermediate may be more important for the peroxidase-activity of ebselen than is the diselenide (Morgenstern et al., 1992). Ebselen also protects against the nephrotoxicity of cisplatin without reducing the antitumor activity of the drug (Baldew et al., 1990). The strongly nucleophilic selenol compound, which is formed in the kidney upon reduction of ebselen by GSH, was suggested to protect against the reactive hydrolysis products of cisplatin. Ebselen was also shown to protect hepatocytes of mice and rats against paracetamol-induced cytotoxicity (Harman et al., 1992; Li et al., 1994). The selenol of ebselen reduces the reactive intermediate N-acetyl-parabenzoquinoneimine back to paracetamol instead of forming an adduct between the selenol and N-acetyl-parabenzoquinoneimine (Li et al., 1994).

In conclusion, GSH may be involved in different types of bioactivation mechanisms, which may be divided into conjugative and reductive mechanisms. A GSH-S-conjugate formed may have direct-acting properties and cause toxicity in situ. A GSH-conjugate may also function as a transporter molecule transferring an electrophile or a toxicophoric group to organs or tissues distant from the site of initial conjugation. In the latter case, covalent binding is generally directed to places where GSH-derived S-conjugates are concentrated and/or to places where subsequent bioactivation takes place. Bioactivation of GSH S-conjugates is the third possibility by which GSH is associated with toxicity. For example, halogenated alkenes are bioactivated by four subsequent activation steps to form reactive thiol compounds. However, the activity of competing biotransformation routes (N-acetylation, S-oxygenation, and deamination) and the localization of transport mechanisms also may be important. Activities and tissue distribution of these enzymes may determine relative toxicity and target organ specificity. Next to activation of xenobiotics to toxic metabolites, GSH-dependent bioactivation mechanisms may be beneficial by activating antitumor agents or chemoprotectants used during anticancer therapy.

The characteristics and tissue distribution of the various enzymes and transport mechanisms involved in the GSH-dependent toxication and detoxication of xenobiotics will therefore be reviewed in the next sections.

III. Enzymes Involved in the Formation and Degradation of Glutathione S-Conjugates

A. Glutathione S-Transferase

The first step in the mercapturic acid pathway in the metabolism of xenobiotics is conjugation to GSH. It was shown for the first time by Booth et al. (1960), that conjugation to GSH was catalyzed enzymatically by rat liver cytosol. The first GST (EC 2.5.1.18) to be purified was isolated as a binding protein and called ligandin (Ketterer et al., 1967). Using 1-chloro-2,4-dinitrobenzene as substrate, four different GSTs were isolated from rat liver cytosol by gradient elution from a carboxymethylcellulose ion-exchange matrix (Jakoby et al., 1976, 1978). Nowadays, a much larger number of GSTs have been isolated. Because of their pivotal role in the protection against toxic and carcinogenic xenobiotics, these enzymes have been studied extensively. GSH and GST activity is present in different subcellular fractions of most tissues and blood cells of the mammalian organism (table 1).

GST consists of a superfamily of multiple isoenzymes that have been catalogued under one of five species-independent gene classes. These include four classes of cytosolic enzymes referred to as alpha, mu, pi and theta, and a class of microsomal enzymes (table 2). Multiple isoenzymes now also have been identified in human liver (Mannervik et al., 1992; Hayes et al., 1987).

1. Cytosolic glutathione S-transferases.

a. MULTIPLICITY. All the cytosolic GST-isoenzymes are dimeric proteins composed of two identical or nonidentical subunits with apparent molecular weights between 23 and 28 kDa (Mannervik and Danielsson, 1988). Whereas class pi and class theta seem to be represented by homodimeric isoenzyme forms, the alpha and mu classes are much more complex and display multiplicity of homodimeric and heterodimeric isoenzyme forms (table 2). To date, at least 13 different subunits have been identified in GSTs of the rat (Mannervik et al., 1985; Mannervik and Danielsson, 1988). Heterodimers may form between different subunits from the same gene class, but intergene class heterodimers have not been observed. Different nomenclatures have been used to cope with the heterogeneity of GSTs (table 2), which may give rise to much confusion. A new nomenclature of human GSTs that is indicative for the class and subunit composition has recently been presented (Mannervik et al., 1992). A prefix may be used to indicate the species (r, rat; h, human; b, bovine; p, pig; m, mouse; rb, rabbit; gp, guinea pig) whereas A, P, M, and T indicate gene class alpha, pi, mu, and theta. For example, the acronym rGST A1–1 refers to a rat GST isoenzyme belonging to the alpha class and is a dimer of two type-1 subunits.

Sequence analysis of the cDNA of cytosolic GSTs has revealed that subunits of class theta are highly conserved throughout evolution; proteins with high identity have been demonstrated in procaryotes, plants, yeasts, fungi, insects, and animals (Pemble and Taylor, 1992). The very early occurrence of GST theta probably relates to the evolution of GSH in purple bacteria and cyanobacteria to protect against oxygen toxicity. A class theta enzyme is also identified in the matrix of mitochondria (Harris et al., 1991), which is presumed to have arisen from bacterial endosymbioses in eukaryotes. It is suggested that classes alpha, mu, and pi arose from a duplication of a class theta gene. Class mu, however, diverged from the common alpha/mu/pi predecessor before alpha and pi diverged.

b. TISSUE DISTRIBUTION. GST-isoenzymes may be expressed to a very different extent, both qualitatively and quantitatively, in different tissues (Mannervik et al., 1985; Ketterer, 1986; Sundberg et al., 1993). In human liver, alpha class isoenzymes represent more than 80% of total GSTs (Van Ommen et al., 1990). Three subunits (A1, A2, and Ax), forming two homodimers and three

TABLE 1

Tissue-distribution of enzymes involved in formation and disposition of GSH-derived S-conjugates in the rat

	[GSH]*	GSH S-transferase†		γ-Glutamyl-transferase‡	Dipep-tidase§	Amino-peptidase M‖	β-Lyase¶	N-Acetyl-transferase#	Acylase I**	Acylase III††	3-Mercapto-pyruvic acid S-conjugate reductase‡‡
		(cytosolic)	(microsomal)								
liver	7.7	1400	126	2	1.3	2.1	0.84	0.46	926	4.4	100
kidney	4.1	336	8.5	560	4.5	10.2	3.05	2.92	8900	40	33
small intestine	2.9	429	60	2–5	0.5	3.1	—	0.04	—	—	17
lung	1.5	79	15	2	12.1	1.2	0.11	—	—	—	6
brain	2.1	190	7.9	1	1.3	1.3	0.21	—	210	0.7	38
spleen	3.4	56	9	2	2	1.1	0.23	—	123	0.6	—
testis	—	3850	129	< 1	—	—	0.4	—	69	0.3	81
adrenal	—	253	52	2	—	—		—	—	—	—
heart	1.1	93	7.2	4	—	—	0.47	—	—	—	—
thymus	—	46	4.4	2	—	—		—	219	0.5	—
pancreas	1.8	—	—	115	0.8	1.1	0.14	—	730	1.5	—
muscle	0.8	—	—	< 1	—	—	0.44	—	—	—	—
bone marrow	—	—	—	—	—	—	0.03	—	—	—	—

* μmol per gram tissue (Meister, 1983).

† nmol conjugate per minute per mg protein using 1-chloro-2,4-dinitrobenzene as substrate (DePierre and Seidegard, 1983).

‡ nmol product per hour per mg protein using γ-glutamylanilide as substrate; the activities in different parts of the small intestine are 2 (duodenum), 5 (jejunum) and 4 (ileum) (Goldbarg et al., 1960).

§ nmol product per minute per mg protein using glycyldehydrophenylalanine as substrate (Hirota et al., 1986).

‖ nmol product per minute per mg protein using S-benzyl-L-cysteine *p*-nitroanilide as substrate (Hirota et al., 1986).

¶ nmol product per minute per mg protein using S-(1,2-dichlorovinyl)-L-cysteine as substrate (Jones et al., 1988).

nmol product per minute per mg protein using S-benzyl-L-cysteine as substrate (Inoue et al., 1987).

** nmol product per hour per 10 mg tissue using N-acetylmethionine as substrate (Endo, 1978).

†† nmol product per hour per 10 mg tissue using N-acetyl-L-tryptophan as substrate (Endo, 1978).

‡‡ Relative activity in 700 g supernatant compared with that in the liver (10 nmol per minute per μL); substrate S-(4-bromophenyl)-3-mercaptopyruvic acid (Tomisawa et al., 1990).

TABLE 2
*Classification of cytosolic GSTs in rat and human**

Class (isoelectric point)	Relative selective substrate for rat isoenzyme†	
Alpha (basic)		
rat		human
1–1 (YaYa; B_1; L_2)	Δ^5-androstene-3,17-dione	α
1a-1a (Ya_1Ya_1)		β
1b-1b (Ya_2Ya_2)		GSTA-1 (ϵ, B1B1)
1–2 (YaYc; $YaYc_1$; B2; BL)		GSTA1–2 (δ, B1B2)
2–2 (YcYc; Yc_1Yc_1; AA; B2)	cumene hydroperoxide	GSTA2–2 (γ, B2B2)
8–8 (K, YkYk)	4-hydroxynonenal	GSTA3–3
10–10 (Yc_2Yc_2)	aflatoxin B_1-8,9-epoxide‡	skin "9–9"
		"GST 5.8"§
Mu (neutral)		
rat		human
3–3 (Yb_1Yb_1; A; A2)	4-chloro-7-sulfobenzofurazan‖	GST M1a-1a (μ)
3–4 (Yb_1Yb_2; C; AC)		GST M1a-1b (μ/ψ)
4–4 (Yb_2Yb_2; D; C2)	trans-stilbene oxide	GST M1b-1b (ψ)
3–6 (Yb_1Yn; P)		GST M2–2
4–6 (Yb_2Yn; S)		GST M3–3
6–6 (Yb_3Yb_3; Yb_1Yn_1; M_T)		GST M4–4
9–9		GST M5–5
11–11		
'HTP-II'¶		
Pi (acidic)		
rat		human
7–7 (YfYf; YpYp; P)	B(a)P-DE	GST P1–1 (π)
Theta		
rat		human
5–5 (E)	EPNP	GST T1–1 (θ)
12–12	methylchloride#	GST T2–2**
13–13		
Microsomal form		

* Mannervik and Danielsson, 1988; Mannervik 1985; Meyer 1991b.
† Compiled from Mannervik and Mannervik, 1988; except for the following:
‡ Hayes et al., 1991.
§ Singhal et al., 1994.
‖ Bolton et al., 1994.
¶ Funk et al., 1994.
# Schröder et al., 1992.
** Hussey and Hayes, 1992.
EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; B(a)P-DE, benzo(a)pyrene-7,8-diol-9,10-epoxide.

heterodimers, were identified; the homodimer A1–1 was the predominant GST-isoenzyme (Wheatley et al., 1994). In the liver of male rats, the major subunits are mu-class subunits 3 (29% of total GST-protein) and 4 (27%), and alpha class subunits 2 (21%), 1a (10%) and 1b (6%); pi class subunit 7 is only present in significant amounts (4%) in rat liver after induction with ethoxyquin (Parola et al., 1993). In contrast, the pi class GST π is the major isoenzyme in mouse liver (Sharma et al., 1993). A pi class GST is the major component of human nasal tissue (Aceto et al., 1989), bovine erythrocytes (Hu and Hultquist, 1992), human colon (Singhal et al., 1992), and bovine placenta (Schäffer et al., 1988). Alpha class GSTs are expressed predominantly in bovine and human adrenal glands (Meikle et al., 1992). The alpha class GST 10–10 is mainly found in the epididymus of the rat; however, antioxidant compounds, such as ethoxyquin and butylated hydroxyanisole, strongly induce this isoenzyme in rat liver (McLellan et al., 1994). Also, within an organ the GSTs may be selectively localized in different regions. For example, in the human and rat kidney the class alpha GSTs are selectively expressed in the proximal tubules, whereas the class mu and pi GSTs are expressed in the thin loop of Henle, distal tubules, and collecting ducts (Hiley et al., 1989; Rozell et al., 1993). The qualitative and quantitative differences in the occurence of GST isoenzymes in different organs are of particular interest, as they might cause differential susceptibility of tissues to the toxic effects of xenobiotics.

Some tumors display increased levels of certain GST isoenzymes (Tsuchida and Sato, 1992). There is strong evidence that GSH and its associated enzymes play a role in cellular resistance to anticancer drugs (Black and Wolf, 1991). The overexpression of specific forms of GST has been linked to an increased cytostatic drug resistance in certain tumor cells (Cowan et al., 1986; Batist et al., 1986b). The pi class GSTs are the most prevalent in human tumors. Recent results revealed that in six out of nine human tumor cell lines and immortalized human hepatocytes (Chang liver), the pi class GST P1–1 was the predominant isoenzyme (Hao et al., 1994). Transfection experiments in yeast have demonstrated that increased pi class GST confers resistance of cells to doxorubicin and chlorambucil (Black et al., 1990). The isoenzymes GST A1–1 and GST M1–1 were the major GST components in Hep G2 and HeLa cells, respectively. There is also evidence that alpha class GST has a particular role in the cellular resistance to the alkylating agent melphalan and chlorambucil (Lewis et al., 1988; Tew et al., 1990), whereas nitrosourea detoxication may be mediated by the mu class of GST-enzymes (Smith et al., 1989).

Human blood cells also contain GST isoenzymes. A major acidic GST-isoenzyme ρ (Marcus et al., 1978) and a minor basic GST-isoenzyme σ (Awasthi and Singh, 1984) have been isolated. GST ρ is either identical or closely related to the human pi-isoenzyme (Thumser and Ivanetich, 1993). However, although the kinetic mechanisms of these two isoenzymes seem to be identical, it is hypothesized that they exhibit subtle differences in their solution structure, resulting in different acessibility of haem-peptides to the nonsubstrate binding sites (Thumser and Adams, 1994). Recently, a class theta GST-isoenzyme, GST T1–1, has been isolated from human erythrocytes (Schröder et al., 1992). The cDNA of this isoenzyme has 82% identity to rat GST subunit 5 (Pemble et al., 1994a, b). GST-isoenzymes are also present in plasma of cancer patients (Hao et al., 1994). The majority of patients with bladder cancer were found to have increased levels of both GST A1–1 and GST P1–1, whereas patients with renal cancer showed increases only in GST P1–1. Measurement of alpha class enzymes in plasma is also used for detection of hepatocellular damage, because it is a more accurate and sensitive index than alanine and aspartate aminotransferase (Beckett and Hayes, 1987; Howie et al., 1992).

c. SEX DIFFERENCE. Sex differences in hepatic GST activities have been shown in the rat and mouse, but as yet, not in humans, hamster, guinea pig, duck, frog, goldfish, lizard, and marmoset (Igarashi and Satoh, 1989). In the adult rat, GST activity has been demonstrated to be significantly higher in males than in females with dichloronitrobenzene as substrate, which is a good substrate for mu class isoenzymes (Igarashi et al., 1985). Indeed, GST 3–3 was shown to be three-fold more predominant in male than in female rat liver, whereas concentration of GSTs 3–4 and 4–4 were approximately 30 and 40%, respectively: higher in male rat liver. On the contrary, alpha class GSTs were more predominant in female than in male rat liver; the concentration of GST 1–2 was almost five-fold higher in female than in male rat liver (Igarashi et al., 1985). This may explain the higher GSH peroxidase activities observed in female rat liver.

Male CD-1 mice had about 1.6-fold higher GSH-concentration, two-fold higher GST-activity (with 1-chloro-2,4-dinitrophenyl as substrate), and almost three-fold higher GST-protein concentration in their livers as compared with the female mice (Sharma et al., 1993). Quantitation of the isoenzymes revealed that GST-π and GST-μ both were about 2.5-fold more abundant in male liver. A pronounced sex difference was also observed in the expression of alpha class subunits in kidney of BALB/c mice, with the female mice expressing approximately four-fold greater levels of subunits Ya and Yk than male mice (McLellan et al., 1992). Lower levels of GSTs in female mouse liver, and also to some extent in lung, suggest the possibility of females being more susceptible in these organs to toxic response to electrophilic compounds that require the GSH/GST system for their detoxification.

Recent studies have indicated that human colon and skin contain relatively higher amounts of class pi isoenzyme(s) in females as compared with males (Singhal et al., 1992; Sharma et al., 1993). Activity of the alpha-class GST in male colon was approximately two-fold higher than the corresponding isoenzyme in female colon (Singhal et al., 1992). It is not known yet whether these differences among males and females are constitutive or inductive. Nonetheless, these differences may contribute to the observed sex-related differences in the incidence of certain types of cancers (Million et al., 1989).

Qualitative differences of GST-isoenzymes also have been observed between different sexes. The specific activity of alpha class GST from female mouse kidney was found to be about two-fold higher than the corresponding isoenzyme from male mouse kidney (Srivastava et al., 1993). In contrast, the specific activity of the pi class isoenzyme from male mouse kidney was about 1.4-fold higher as compared with that from female kidney. Also, the pi class isoenzyme from male mouse kidney is more heat stable than the enzyme from female mouse kidney. In contrast, the pi class GST isolated from female human colon was more thermostable as compared with the corresponding male isoenzyme (Singhal et al., 1992).

d. SUBSTRATE SELECTIVITY. Next to physicochemical and immunochemical differences, cytosolic GST-enzymes have been shown to differ greatly in substrate selectivity (Mannervik and Danielsson, 1988; Vos and Van Bladeren, 1990; Armstrong, 1991). The substrate selectivity most probably is determined by three-dimensional features of the binding site of the electrophilic

substrates, the so-called H-site. Because of the catalytic diversity of the cytosolic isoenzymes, a very broad range of structurally diverse electrophilic substrates, as well as potentially harmful organic (hydro)peroxides, can be detoxified by GSH.

The class alpha isoenzymes have high activity in the reduction of organic hydroperoxides, such as linoleic acid hydroperoxide, and are thus mainly responsible for the nonselenium-dependent glutathione peroxidase activity. Rat GST 8–8 (Stenberg et al., 1992) and its human ortholog GST 5.8 (Singhal et al., 1994) were particularly active in the detoxication of the highly cytotoxic α,β-unsaturated 4-hydroxyalkenals that are generated during the peroxidation of polyunsaturated fatty acids.

Class mu and pi isoenzymes have high activity toward many epoxides. The genotoxic metabolite of benzo(a)pyrene, (+)-anti-benzo(a)pyrene-7,8-diol-9,10-oxide, is a good substrate for GSTs M1–1, M2–2 and M3–3, a better substrate for GST P1–1, but a poor substrate for GSTs A1–1 and A2–2. Class mu isoenzymes catalyze the reduction of DNA hydroperoxides and 5′-hydroxymethyluracil (Ketterer et al., 1990).

The theta class rat liver GST 5–5 shows high sequence homology with the dichloromethane dehalogenases, denoted as groups A and B, which have been isolated from bacteria that metabolize dichloromethane as their sole source of carbon and energy (Scholtz et al., 1988). Rat GST 5–5 and bacterial group B dehalogenase show comparable kinetic parameters with dichloromethane (Blocki et al., 1994). However, GST 5–5 is very active with other substrates, including 1,2-epoxy-3-(*p*-nitrophenoxy)propane, cumene hydroperoxide, methyl chloride, 1,2-dichloroethane and 1,1-dichloroethane, whereas the bacterial GSTs are inactive. To date, only dihalomethanes have been observed to be substrates for the bacterial transferases.

In addition to their role as detoxicating enzymes, GSTs may also have important physiological catalytic functions, e.g., by catalyzing the GSH-dependent isomerization of 3-ketosteroids (Benson et al., 1977) and the biosynthesis of leukotriene A_4 (Tsuchida et al., 1987) and hepoxilin A_3 (Pace-Asciak et al., 1990). Cytosolic GSTs not only display catalytic activity, they also function as very versatile ligand-binding proteins, also known as ligandins. Their abundance in cells (up to 10% of total cytosolic protein), as well as their binding properties, suggest that they mediate the intracellular storage and transport of hormones, metabolites, drugs, and a great variety of other hydrophobic nonsubstrate compounds (Listowsky, 1993). GSTs of the alpha, mu, and pi classes are sensitive to inhibition to GSH S-conjugates (i.e., product), whereas the theta class GST 5–5 is not (Meyer, 1993). Therefore, GSTs of the former classes apparently have evolved toward increased product binding at the expense of catalytic efficiency. It may be concluded that GSTs cooperate with the export system for GSH S-conjugates in order to reduce the free intracellular concentration of GSH S-conjugates.

e. REGIOSELECTIVITY AND STEREOSELECTIVITY. In addition to substrate selectivity, the cytosolic GSTs also differ in stereoselectivity or regioselectivity (Te Koppele and Mulder, 1991). For example, the rat alpha class isoenzymes GST 1–1 and GST 2–2 preferentially catalyze the conjugation of the (S)-enantiomer of α-bromoisovalerylurea, whereas mu class isoenzymes GST 3–3 and GST 4–4 prefer the (R)-enantiomer (Te Koppele et al., 1988). In contrast to rat class mu GSTs, the human GST M1a-1a preferentially conjugates the (S)-isomer of α-bromoisovalerylurea, indicating that related isoenzymes in different species may have a different stereospecificity (Mulders et al., 1993).

In a study with purified rat mu class GSTs, it was shown that subunit 4 was more stereoselective in the conjugation with arene and aza-arene epoxides (with more than a 99% attack at the oxirane carbon with the R configuration) than subunit 3 (Cobb et al., 1983). Stereoselectivity of GSH-conjugation most probably is the result of orientation of substrates in the binding sites for the substrate (H-site) and the binding site for GSH (G-site) (Mannervik et al., 1985; Danielson and Mannervik, 1985). Stereoselective biotransformation, in general, and stereoselective GSH-conjugation, in particular, may have important toxicological consequences (Van Bladeren et al., 1979; Vermeulen, 1987). Human GSH S-transferase GST M1–1 is equally active with the (+) and (-) enantiomers of 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-benzo(a)pyrene, whereas GST P1–1 is stereospecific for the more potent carcinogenic (+) enantiomer (Robertson et al., 1986a, b).

Aflatoxin B_1 is enzymically oxygenated to both an *exo*-epoxide and an *endo*-epoxide. The product ratio depends upon the species from which the microsomes are derived. In all species, the *exo* product predominates, however, human microsomes give larger proportions of the *endo* product. Purified GSTs from rat and human have strongly different selectivities toward the two isomers (Raney et al., 1992). The *exo*-epoxide was conjugated to GSH by most of the GSTs investigated, albeit with different efficiency. However, the *endo*-epoxide was only conjugated by the polymorphic human GST M1–1 and the rat GSTs 4–4 and 4–6. Very recently, it was shown that the *exo*-epoxide is a potent mutagen, whereas the *endo*-epoxide was at least 500-fold less active (Iyer et al., 1994). The reason for this difference is that the *exo*-epoxide reacts efficiently with DNA, whereas the *endo*-epoxide is incapable of this reaction.

f. GENETIC POLYMORPHISMS. Unlike the class alpha GSTs, which are found in all human livers, the major liver class mu isoenzyme is found only in about half of the adult white caucasian population (Board, 1981). This enzyme is encoded at the GSTM1 locus, which displays three alleles: *GSTM1a* (encoding GST-μ), *GSTM1b* (encoding GST-ψ), and *GSTM1-null*. Individuals who are homozygous *GSTM1–0* lack GST-activity toward trans-stilbene oxide in their leukocytes (Seide-

gard and Pero, 1985) and do not express either GST M1a or GST M1b in their livers (Seidegard et al. 1987; Van Ommen et al. 1990). The GST M1–0 gene seems to be a deletion of the gene that encodes GST M1a/b. Glutathione S-transferase cDNAs from four additional class mu loci have now been described: *GST M2*, *GST M3*, *GST M4*, and *GST M5* (Pearson et al., 1993). Of these four loci, only *GST M5* also displays polymorphism by RFLP-analysis, a finding that does not correlate with GST M1-polymorphism.

The human isoenzymes GST M1a/b have a pronounced activity toward genotoxic epoxides, such as styrene-7,8-oxide and benzo(a)pyrene-4,5-dihydro-4,5-oxide (Warholm et al., 1983), and products of reactive oxygen species attack DNA to form 5-hydroxymethyluracyl and DNA hydroperoxide (Ketterer et al., 1990). These isoenzymes also have the highest catalytic efficiency in the detoxication of 4-hydroxyalkenals, which are produced as a result of free radical-initiated lipid peroxidation (Berlane et al., 1994). It has been proposed that individuals who are homozygous GST M1–0 may be more sensitive to certain genotoxic and carcinogenic substances (Board, 1981; Warholm et al., 1983). Epidemiological studies indicate that individuals deficient in this may be more prone to develop lung cancer (Seidegard et al., 1986, 1990). However, in view of the relatively high content of GST P1–1 and GST M3–3 in the lung and the low occurrence of GST M1–1, any effect of GST M1-expression on susceptibility to lung cancer most probably originates from an extrapulmonary compartment such as the liver (Anttila et al., 1993). Deficiency of GST M1-activity has also been related to susceptibility to bladder cancer (Bell et al., 1993), stomach cancer (Harada et al., 1992), colorectal cancer (Zhong et al., 1993), and prolactinoma (Fryer et al., 1993).

Recently, a second polymorphism of GST-activity has been identified (Schröder et al., 1992; Hallier et al., 1993). The theta class GST T1–1 in human erythrocytes was shown to be absent in 25 to 40% of the human population. Like the GST M1 gene, the GST T1 has a null allele (Pemble et al., 1994a, b). The genotype of GST M1 and GST T1 are not linked, because individuals who are GST T1-null are not necessarily GST M1-null and vice versa (Pemble et al., 1994b). The polymorphic isoenzyme GST T1–1 seems to have a high activity toward a number of low molecular weight genotoxic substrates, such as methyl chloride, methyl bromide, methyl iodide, dichloromethane, and ethylene oxide. The lack of this GST-isoenzyme in the 'non-conjugators' was associated with a marked increase in genotoxicity (sister chromatid exchanges) of these compounds in lymphocytes of this part of the population (Hallier et al., 1993). A considerable interindividual variation in GSH-conjugation of methylene chloride in human liver fraction, with individuals having no activity whatsoever, suggests that this polymorphism is also expressed in human liver (Bogaards et al., 1993). As yet, the significance of this polymorphism remains to be elucidated.

g. THREE-DIMENSIONAL STRUCTURES. Recently, considerable effort has been devoted to the elucidation of the three-dimensional structure of GSTs, as reviewed recently (Sinning et al., 1993; Gilliland, 1993; Dirr et al. 1994). This has lead to the x-ray quality crystals of three of the main GST family groups, namely human GST A1–1 (Cowan et al., 1989), pig and human GST P1–1 (Parker et al., 1990; Reinemer et al., 1991, 1992), and rat GST 3–3 (Sesay et al., 1987; Ji et al., 1992). The detailed three-dimensional protein structures derived from these crystals have greatly enhanced the knowledge of the protein structures and the active sites of the enzymes. Recently, the three-dimensional structures of crystallized GSTs of different classes have been compared (Sinning et al., 1993; Dirr et al., 1994). Each subunit has an active site that can be separated into two distinct functional regions: a hydrophilic site for the binding of the physiological substrate GSH (G-site) and an adjacent hydrophobic site (H-site) for the binding of structurally diverse hydrophobic electrophilic substrates. Although the active sites of GSTs are catalytically independent, the fully functional active site is formed by the structural elements from both subunits of the dimer. Considerable progress has been made in understanding the mechanism whereby the thiol group of GSH is activated (by lowering its pKa) in the G-site, a rate-enhancement strategy shared by all soluble GSTs. The GSTs are proposed to form a hydrogen bonding pair between the hydroxyl group of a tyrosine residue and enzyme-bound GSH stabilizing the thiolate anion at neutral pH (Liu et al., 1992). Also, significant differences could be demonstrated in the structure of the H-site, which may explain differences in the substrate-selectivity of the different GST-isoenzymes. By cocrystallization of rat GST 3–3 with substrate, a transition-state analogue and product, respectively, a reaction path profile of the catalytic mechanism of conjugation of GSH to 1-chloro-2,4-dichlorobenzene was deduced (Ji et al., 1993). The fact that crystallized GSTs retain their catalytic ability demonstrates that the three-dimensional structure of the crystals may well reflect the conformation of the enzyme in aqueous solution.

Before crystal-structures and site-specific mutagenesis experiments provided insight into the nature of the G-site, the G-site of various isoenzymes was mapped by using a series of closely related GSH analogues (Adang et al., 1990). From these studies, it was concluded that the G-site of each isoenzyme possesses unique characteristics. The γ-glutamyl moiety of GSH serves as the cosubstrate's major binding determinant, whereas the glycine moiety seemed to be the least restrictive group for GSH recognition. Using these insights, a number of selective inhibitors of GSTs differing in the amino acid composition of the γ-glutamyl moiety were designed (Mulder et al., 1991; Adang et al., 1991).

2. Microsomal glutathione S-transferase. GST-activity has also been shown in microsomal fractions (Morgenstern et al., 1982, 1983, 1985). A microsomal enzyme, distinct from the cytosolic isoenzymes, has been purified from rat liver (Morgenstern et al., 1983). Rat liver microsomal GST has a molecular mass of 17.3 kDa. Cross-linking studies suggest that the microsomal GST is a trimer in situ in the endoplasmic reticulum (Lundqvist et al., 1992). The liver microsomal GSTs from rat and human exhibit 95% similarity to each other, and it appears that there is a single microsomal GST gene in the rat genome (DeJong et al., 1988). Neither rat nor human microsomal GST sequences show significant similarity to the sequences of any of the soluble GST families.

The microsomal GST can account for as much as 3% of the microsomal protein (Morgenstern et al., 1983), and may be important in the protection against lipid peroxidation by reducing lipid hydroperoxides or by functioning as a GSH-dependent vitamin E radical reductase (Haenen and Bast, 1983; Morgenstern 1990). The activity of the rat liver microsomal GST is strongly enhanced by alkylation of its thiol group, e.g., by N-ethylmaleimide (Morgenstern and DePierre, 1983; Morgenstern et al. 1988). This potentiation of microsomal GST-activity might be an adaptive mechanism increasing GST-activity when it is needed, i.e., when alkylating agents are produced. When activated, the microsomal GST was also shown to protect against lipid peroxidation (Mosialou et al., 1993a). The human microsomal GST is active toward a phospholipid hydroperoxide, a feature unique to this enzyme compared with the cytosolic GSTs (Mosialou et al., 1993b). One of the toxic second-messenger products of the process of lipid peroxidation, 4-hydroxynon-2-enal, was also shown to activate microsomal lipid peroxidation (Haenen et al., 1988). Recently, it has been shown that microsomal GST is the predominant binding site of leukotriene C_4 in cellular membranes (Metters et al., 1994).

3. Mitochondrial and nuclear glutathione S-transferases. Different GSTs have been demonstrated in the mitochondria. A soluble GST has been isolated from the matrix from rat liver mitochondria (Harris et al., 1991). Partial protein sequencing suggested that this GST, designated as GST 13–13, belongs to the theta class of GSTs. More recently, a GST was purified from the matrix fraction of mouse liver mitochondria which exhibited an immunological relationship to the cytosolic alpha class of GSTs (Addya et al., 1994). A transferase similar to the microsomal GST has also been identified in the outer membrane of the mitochondria of rat liver (Nishino and Ito, 1990).

GSTs from the mu class are also present in the nucleus and nucleolus (Bennett et al., 1986). Recent cellular fractionation has shown that cytosolic GSTs are able to penetrate nuclear pores, in contrast to the selenium-dependent GSH peroxidase. Therefore, although the GSTs reduce polyunsaturated fatty acid and thymine hydroperoxides less efficiently than the selenium-dependent GSH peroxidase, they will probably be responsible for the protection of DNA and other nuclear components against oxidative damage by hydroperoxide formation (Ketterer, 1994).

4. Inhibition of glutathione S-transferases. Because of their extensive involvement in the metabolism of xenobiotics, the inhibition of GSTs has received considerable attention over the past years (Van Bladeren and Van Ommen, 1991). Apart from providing insights into the mechanism of the enzyme actions, selective in vivo GST inhibitors could conceivably be applied in clinical practice. Selective inhibition of tumor GSTs, for instance, may be a tool to increase the sensitivity of tumors to cytostatics. Inhibition of elevated GST levels in cells by the inhibitor ethacrynic acid has been shown to potentiate the cytotoxicity of several cancer drugs in cell culture lines (Hansson et al., 1991; Smith et al., 1989; Tew et al., 1988). Phase-I clinical trials of this relatively nonselective GST inhibitor have not revealed any severe side effects attributable to transient depression of GST levels by more than 50% as measured in peripheral white blood cells (O'Dwyer et al., 1991).

Inhibitors of GSTs can be divided into two groups: reversible and irreversible. Of the reversible inhibitors, GSH-analogs have been used to probe the G-site of the enzyme. By changing or deleting functional groups in the γ-glutamyl moiety, a difference in the G-site between alpha- and mu class GSTs was determined (Adang et al., 1990). Recently, a diverse set of peptide-analogs of GSH S-conjugates with variations in the C-terminal amino acid and with different S-bound substituents have been tested as isoenzyme-specific inhibitors of human GSTs (Flatgaard et al., 1993). In this study, a number of highly potent inhibitors for the GST M1a-1a and GST P1–1 have been identified, showing 10- to 20-fold selectivity over the tested GST-isoenzymes of other classes. The most selective inhibitors for the M1a-1a isoenzyme have beta-alanine as the C-terminal amino acid in the GSH-moiety, whereas the most selective inhibitors for the P1–1 isoenzyme have phenylglycine as the C-terminal amino acid. Also, potent inhibitors toward GST A1–1, albeit with lower selectivity, were identified. An analysis of *n*-alkyl GSH-analogs as probes of the hydrophobicity binding parameter shows that the binding strength increases with the chain length of the alkyl group: the IC_{50}-value decreasing gradually from 1 to 2 mM concentration to approximately 1 μM when the length of the alkyl-chain increases from S-methyl- to an S-decyl-group (Flatgaard et al., 1993).

A second class of reversible GST inhibitors are non-peptide compounds which inhibit by blocking the second substrate (H-site) site of GST (for a review, see Mannervik and Danielsson, 1988). Very potent inhibitors of GSTs with relatively low selectivity are Cibacron blue, ethacrynic acid, and Eosin b. Other organic compounds show higher selectivity, such as Eosin y

(GST A1–1), gossypol acetic acid and hematin (GST A1–1 and M1a-1a), and doxorubicin (GST A1–1) (Flatgaard et al., 1993).

Irreversible inhibition of GSTs has been observed after exposure to tetrachloro-1,4-benzoquinone. By adding a GSH moiety the inhibition halftime was reduced, showing a targeting of the inhibitor to the GST enzymes (Van Ommen et al., 1991). These inhibitors could be used to modify the biotransformation of certain xenobiotics in vivo, or to improve the efficacy of certain antitumor drugs by GST inhibition.

5. Bioactivation of halogenated compounds by glutathione S-transferase.

a. HALOALKENES. GSH-conjugation of haloalkenes in vivo has been established by identification of the respective GSH-conjugates in bile of rats treated with HCBD (Nash et al., 1984; Jones et al., 1985), HFP (Koob and Dekant, 1990) and tetrachloroethylene (Odum and Green, 1987). Administration of collected bile of HCBD-treated rats to other rats caused a nephrotoxicity that was similar to the toxicity caused by chemically synthesized GSH-conjugates of HCBD (Nash et al., 1984). In bile of HCBD-treated rats a mono- and a bis-GSH-conjugate, as well as the corresponding cysteinylglycine conjugates, have been identified, both representing about 65% of the biliary HCBD-metabolites (Jones et al., 1985). Cannulation of the bileduct completely protected rats from the nephrotoxic action of HCBD (Nash et al., 1984), suggesting that GSH-conjugation of HCBD predominantly takes place in the liver. Bile cannulated rats exposed to HFP excreted S-(1,2,3,3,3-pentafluoropropenyl)glutathione into bile while simultaneously N-acetyl-S-(1,1,2,3,3,3-hexafluoropropyl)-L-cysteine was excreted in urine. These findings indicate not only a different reaction mechanism for the GSH-conjugation for the liver and the kidney but also a liver-independent GSH-conjugation of HFP in the kidney (Koob and Dekant, 1990). The intact GSH-conjugate could not be detected in bile of rats exposed to the potent nephrotoxin TFE, probably because of the high activity of biliary peptidases (Odum and Green, 1984)..

GSH-conjugation of a number of nephrotoxic haloalkenes has also been studied in vitro using different subcellular fractions of rat liver and rat kidney; the results of these studies are summarized in table 3. Specific activities of GSH-conjugation in hepatic fractions were much higher than in renal fractions. Because the total protein content of the liver is also higher than that of the kidney, this indicates that hepatic GSH-conjugation is more important for bioactivation of haloalkenes than is renal GSH-conjugation. Conjugation of GSH to dichloroacetylene has been shown to yield 1,2-DCV-G, a conjugate identical to that presumed to be formed after conjugation of GSH to trichloroethylene (Patel et al., 1993). Conjugation of GSH to dichloroacetylene in vitro was approximately equal for renal and hepatic fractions, so for this compound, extrahepatic GSH-conjugation also might significantly contribute to the observed nephrotoxicity and nephrocarcinogenicity (Kanhai et al., 1989). Because biliary cannulation did not influence the excretion of the corresponding mercapturic acid (N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine), it was concluded that in vivo GSH-conjugation of dichloroacetylene predominantly occurs in the kidney (Kanhai et al. 1991). However, more recently, it was shown that GSH-conjugation to dichloroacetylene may also occur in other extrahepatic tissues, because GSH-conjugation proceeds nonenzymatically at a relatively high rate (Patel et al., 1994). Enzymic GSH-conjugation could only be demonstrated in the presence of liver microsomes and mitochondria, increasing the GSH-conjugation-rate approximately two-fold. It was suggested that the local nonenzymic GSH-conjugation in the brain, as well as uptake of the corresponding S-conjugates in the brain, may contribute to the neurotoxicity of dichloroacetylene, because high β-lyase-activities have been demonstrated in the rat brain (Patel et al., 1993; Cooper et al., 1993).

For most haloalkenes tested, the specific activity of GSH-conjugation was significantly higher in rat liver microsomes than in cytosol (table 3). Very high ratios of microsomal versus cytosolic activities were observed in hepatic fractions obtained from human, monkey and hamster, especially because cytosolic activities are very low in these species (table 4). When comparing specific activities of GSH-conjugation with HCBD in hepatic microsomes and cytosol obtained from different species, microsomal activity was comparable in livers of human, monkey and rat (table 4). Much lower activities were observed in liver microsomes from rabbit, hamster, mouse and, in particular, guinea pig. A large species difference was also observed in the GSH-conjugation of tetrachloroethylene (Green et al. 1990). The rate of conjugation in rat liver cytosol was five- to six-fold greater than that in mouse liver cytosol. However, in human liver fractions, no GSH-conjugation could be detected in either microsomes or cytosol. Based on the limit of detection of the assay used, the human rate was estimated to be at least an order of magnitude less than that in the rat. These results suggest that different GST-isoenzymes are active in the conjugation of HCBD and tetrachloroethylene.

The fact that microsomal HCBD conjugation was not activated by N-ethylmaleimide was suggested to point to the involvement of as yet unidentified microsomal GSTs in GSH-conjugation of HCBD (Oesch and Wolf, 1989). The higher activity of microsomal GST to halogenated alkenes may be explained by accumulation of these lipophilic substrates in membranes, resulting in a high local substrate concentration for microsomal enzymes.

The relatively hydrophilic monoconjugate of HCBD cannot accumulate in membranes and therefore is conjugated more efficiently by the cytosolic enzyme (Dekant et al., 1988c). When conjugation of HCBD was studied using purified cytosolic rat liver GSTs, the specific ac-

TABLE 3

In vitro conjugation of GSH to nephrotoxic haloalkenes by subcellular fractions of rat liver and rat kidney

Substrate (concentration)	[GSH] (mM)	Gender	Subcellular fraction	Activity#		Reference
				Liver	Kidney	
A. Conjugation by addition/elimination mechanism						
hexachloro-1,3-butadiene (2.8 mM)	10	male	microsomes	0.175	0.089	*
			cytosol	0.290	0.049	
		female	microsomes	0.575	0.122	
			cytosol	0.168	0.143	
PCB-G (2 mM)	10	male	microsomes	0.69	0.003	†
			cytosol	0.83	0.17	
tetrachloroethylene (4 mM)	10	male	microsomes	0.23		‡
			cytosol	0.13		
		female	microsomes	0.21		
			cytosol	0.14		
1,1,2-trichloro-3,3,3-trifluoro-1-propene (4 mM)	10	male	microsomes	530	<12	§
			cytosol	120	<12	
		female	microsomes	513	<12	
			cytosol	161	<12	
hexafluoropropene (1 mM)	10	male	microsomes	240	<3	‖
			cytosol	<3	<3	
B. Conjugation by addition mechanism						
tetrafluoroethylene (1 atm, 79%)	0.25	male	microsomes	3.0		¶
			cytosol	0.7		
chlorotrifluoroethylene (3 atm, 100%)	5	male	microsomes	175	<5	#
			cytosol	91	<5	
hexafluoropropene (1 mM)	10	male	microsomes	36	<10	‖
			cytosol	136	46	
dichloroacetylene (5 mM)	5	male	microsomes	2923	2838	**
			cytosol	705	129	

* Wolf et al., 1984.
† Dekant et al., 1988b.
‡ Dekant et al., 1987.
§ Vamvakas et al., 1989.
‖ Koob et al., 1990.
¶ Odum and Green, 1984.
# Dohn et al., 1985.
** Kanhai et al., 1989.
Abbreviations: PCB-G, S-(1,2,3,4,4-pentachlorobutadienyl)glutathione; PFP-G, 1-(1,2,3,3,3-pentafluoropropenyl)glutathione.
#, expressed in nmol/min/mg protein.

tivity was highest with the isoenzymes GST 1–2 (YaYc) and GST 3–3 (Yb_1Yb_1), but two-fold lower with the isoenzymes GST 3–4 (Yb_1Yb_2) and GST 4–4 (Yb_2Yb_2) (table 5). All isoenzymes formed both monoconjugates and diconjugates in a 2:1 ratio, except for GST 4–4, which formed both conjugates in a ratio of 1:1. The human cytosolic alpha- and mu classes, however, did not demonstrate HCBD conjugating activity at all, which may explain the very low cytosolic activity (table 5) (Oesch and Wolf, 1989).

GSH-conjugation of CTFE leads to the formation of two diastereomeric conjugates because of the introduction of a chiral centrum (fig. 13A). Upon incubation of CTFE with cytosol, these conjugates were formed in a 1:1 ratio. Interestingly, upon incubation with the microsomal fraction, the ratio of the S- to R-isomer, was 4.5 to 5:1 (Dohn et al., 1985b; Hargus et al., 1990). With HCBD as well as 1,1,2-trichloro-3,3,3-trifluoro-1-propene, GSH-conjugation by an addition/elimination mechanism results in formation of both *cis* and *trans* isomers (Wolf et al., 1984; Vamvakas et al., 1989). The relative contribution of the different stereoisomers to nephrotoxicity, however, remains to be established.

GSH-conjugation of trichloroethylene leads to the formation of two regioisomeric GSH-conjugates, as demonstrated by the urinary excretion of the two regioisomeric mercapturic acids 1,2-DCV-NAC and 2,2-DCV-NAC (fig. 13B) (Commandeur and Vermeulen, 1990b; Birner et al., 1993a). Because of the very high cytotoxicity and mutagenicity of the corresponding cysteine-conjugate 1,2-DCV-Cys, this regioisomer will be mainly responsible for the toxic effects caused via the mercapturic acid pathway (Commandeur et al., 1991a). Glutathione conjugation to trichloroethylene as well as its alkaline decomposition product dichloroacetylene, leads to formation of identical 1,2-dichlorovinyl-S-conjugates (Kanhai et al.,

TABLE 4

Species-differences in the activities of enzymes involved in formation and disposition of GSH-derived S-conjugates

	Liver				Gallbladder		Kidney				
	GSH S-transferase*		γ-Glutamyl-transferase†	Dipep-tidase‡	γ-Glutamyl-transferase†	Dipep-tidase‡	γ-Glutamyl-transferase†	Dipep-tidase‡	β-Lyase§		
	(microsomal)	(cytosolic)							V_{max}‖	K_m (mM)	V_{max}/K_m
rat	1.39	0.350	3.8	3.3	n.d.¶	nd¶	3325	65	4.00 (3.64)	0.68 (1.26)	5.88 (2.88)
mouse	0.16	0.109	1.5	5.4	2.4	9.4	619	31	1.15 (1.66)	5.69 (4.43)	0.20 (0.37)
rabbit	0.14	0.042	4.8	0.7	8.4	2.1	482	48	—	—	—
guinea pig	0.03	0.038	19.8	5.5	5.8	14.3	294	155	—	—	—
hamster	0.25	0.011	—	—	—	—	—	—	—	—	—
pig	—	—	6.5	8.3	3.8	3.6	123	78	—	—	—
monkey	1.39	0.074	9.7	3.7	10.9	4.1	454	60	—	—	—
human	1.17	0.031	—	—	—	—	—	—	0.49 (0.64)	2.53 (2.67)	0.21 (0.24)

* nmol conjugate per minute per mg protein using hexachlorobutadiene (HCBD) as the substrate (Oesch and Wolf, 1989).
† nmol product per minute per mg protein using γ-glutamyl-p-nitroanilide as substrate (Hinchman and Ballatori, 1990).
‡ nmol product per minute per mg protein using alanine-p-nitroanilide as substrate (Hinchman and Ballatori, 1990).
§ enzyme kinetics of cytosolic β-lyase using S-(1,2,2-trichlorovinyl)-L-cysteine as substrate; values in parentheses show the corresponding data from female gender (Green et al., 1990).
‖ nmol product per min per mg protein.
¶ n.d., not determined (rats do not have a gallbladder).

TABLE 5

*Activity of conjugation of HCBD by purified cytosolic GSTs from rat liver**

GST-isoenzyme	Enzyme activity†		ratio
	monoconjugate	diconjugate	
GST 3–3 (Yb_1Yb_1)	3.92	1.61	2.4
GST 3–4 (Yb_1Yb_2)	2.22	1.11	2.0
GST 4–4 (Yb_2Yb_2)	1.68	1.40	1.2
GST 1–2 (YaYc)	4.38	2.11	2.1

* Oesch and Wolf, 1989.
† nmol/min/mg protein.

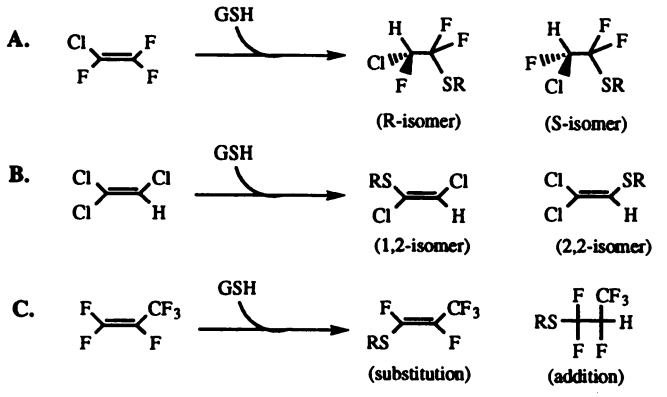


FIG. 13. Examples of GSH-conjugation of polyhaloalkenes leading to different S-conjugates: chlorotrifluoroethylene is conjugated to two diastereomeric conjugates (A); trichloroethylene is conjugated to two regioisomeric conjugates (B); and, hexafluoropropene is conjugated to a saturated and an unsaturated conjugate (C).

1991). The analysis of 2,2-DCV-NAC in urine therefore offers a biological monitoring method that may discriminate between exposure to trichloroethylene and dichloroacetylene.

GSH-conjugation of HFP was shown to result in two GSH S-conjugates: one resulting from addition/elimination and one resulting from addition (fig. 13C) (Koob and Dekant, 1990). Cytosolic GSTs seem to exclusively form the addition-product S-(1,1,2,3,3,3-hexafluoropropyl)glutathione, whereas the microsomal GST preferentially forms the addition/elimination product, S-(1,2,3,3,3-pentafluoropropenyl)glutathione (table 3). It was suggested that abstraction of a proton of water by the carbanion formed initially after the reaction of the thiolate (GS^-) to HFP may predominate in the cytosolic fractions because of the higher local water concentration. Because only the corresponding mercapturic acid from S-(1,1,2,3,3,3-hexafluoropropyl)glutathione was detected in urine of rats exposed to HFP, it was concluded that intrarenal GSH-conjugation may be responsible for HFP-induced nephrotoxicity (Koob and Dekant, 1990). However, it cannot be excluded that S-(1,2,3,3,3-pentafluoropropenyl)glutathione is completely metabolized via the β-lyase-pathway that is the ultimate bioactivation step. Therefore, the relative role of the two GSH-conjugates in the nephrotoxicity of HFP still remains to be established.

b. HALOALKANES. Dihalomethanes and dihaloethanes also are bioactivated by glutathione conjugation (fig. 2A). Rates of GSH-conjugation of dichloromethane are considerably lower in rats and humans than in mice. This information, along with physiologically based pharmacokinetic models, has been used to predict that humans, like rats, will be considerably less prone than mice to develop tumors from dichloromethane exposure (Andersen et al., 1987; Reitz et al., 1989). Enzymes of the GST class theta, especially rat GST 5–5, are proficient in the conjugation of dichloromethane (Meyer et al., 1991; Blocki et al., 1994). The specific activity of purified rat liver GST 5–5 was about 500-fold higher than that for the class mu isoenzymes GSTs 3–3, 3–4,

and 4–4. The rat liver alpha class GSTs were inactive (Blocki et al., 1994). Given the much higher prevalence of mu versus theta isoenzymes in rat liver, it is possible that mu isoenzymes contribute significantly to the in vivo metabolism of dichloromethane. By introducing a plasmid expression vector containing a cDNA clone for rat GST 5–5 into a standard tester bacterium *Salmonella typhimurium* TA1535, it was shown that this enzyme is active in the bioactivation of dihalomethanes, like dibromomethane and dichloromethane, as well as dihaloethanes, such as 1,2-dibromoethane (Thier et al., 1993). Intracellular activation of dihalomethanes was essential for mutagenicity, because addition of GST 5–5 externally did not result in mutagenesis. Because GST T1–1 seems to be the human orthologue of rat GST 5–5, it is possible that individuals positive for this gene may be at a greater risk of cancer if exposed to chemicals such as methylene chloride (Schröder et al., 1992).

By using purified human GST isoenzymes, it was shown that alpha class isoenzymes have highest activity toward 1,2-dibromoethane (Cmarik et al., 1990). Little or no activity was observed with human pi class isoenzyme. Consistent with this, it was demonstrated recently that mutagenicity of both 1,2-dibromoethane and 1,2-dichloroethane was increased in *Salmonella typhimurium* strains expressing human GST A1–1 but not in cells expressing GST P1–1 (Simula et al., 1993a). The mutagenicity and nephrotoxicity of 1,2-dibromo-3-chloropropane and tris(2,3-dibromopropyl)phosphate has also been linked to GSH-dependent bioactivation mechanism similar to 1,2-dibromoethane (Humphreys et al., 1991; Inskeep and Guengerich, 1984). However, GST-expression in *Salmonella* strains did not potentiate the mutagenicity of these compounds in the absence of rat liver microsomes, indicating that cytochrome P450-mediated metabolism was a prerequisite for GST-mediated potentiation (Simula et al., 1993b). In the presence of microsomes, GST P1–1 was more active in potentiating the mutagenicity of tris(2,3-dibromopropyl)phosphate than GST A1–1. Both enzymes had comparable effects on the mutagenic frequency of dibromo-3-chloropropane.

6. Bioactivation of antitumor agents by glutathione S-transferase. GST and GSH are frequently elevated in many tumors relative to surrounding healthy tissue (Tew and Clapper, 1987). Isoenzyme GST P1–1, for example, was elevated in lung, colon and stomach cancers, compared with the normal tissues (Howie et al., 1990). Multidrug resistance may partially be explained by the increase of GST-levels (Kramer et al., 1988). Antitumor agents that are bioactivated by GST therefore may be effective chemotherapeutic agents for the treatment of tumors with elevated levels of GST-isoenzymes. These compounds may also overcome drug resistance caused by increased GSH and GST levels.

Recently, different alkylating agents that are activated by GSTs have been designed and tested in cell cultures of cancer cells (Lyttle et al., 1994). S-Functionalized GSH analogs have been designed which, upon binding to GSTs, are bioactivated by proton-abstraction by a tyrosine-residue in the active site of GST (fig. 14). In this enzyme-catalyzed cleavage reaction, a highly cytotoxic phosphate-moiety is liberated. By modifying the GSH-moiety, substrates were developed that were bioactivated more or less selectively by human GST-isoenzymes. The compound with a C-terminal glycine, R = H (fig. 14) is activated efficiently by GST M1a-1a, whereas the analog with the C-terminal phenylglycine R = phenyl (fig. 14) was activated by GST P1–1 and GST A1–1 but not by GST M1a-1a (Lyttle et al., 1994). MCF-7 cancer cell lines that were transfected with GST P1–1 seemed to be much more sensitive to the cytotoxicity of the latter compound than MCF-7 cells not expressing GST P1–1.

B. γ-Glutamyltransferase.

The first step in the catabolism of GSH-conjugates involves either hydrolysis or transfer of the γ-glutamyl group to an appropriate acceptor by γ-glutamyltransferase (EC 2.3.2.2) (also known as γ-glutamyl transpeptidase, (5-glutamyl)-peptide: amino acid, 5-glutamyltransferase, GGT) (fig. 6, step a) (Meister and Tate, 1976; Curthoys and Hughey, 1979; Curthoys, 1986). GGT is an ubiquitous enzyme, which was first identified in kidney tissue and later shown to be present in serum and in all cells except muscle cells (Hanigan and Pitot, 1985). Some enzyme is present in the cytosol, but the larger fraction is membrane-bound, with its active site oriented on the outer surface of the cell membrane (Horiuchi et al., 1978).

GGT contains two nonidentical subunits (Curthoys, 1986). The amino-terminal portion of the heavy subunit (nonglycosylated, Mr = 41, 650) comprises a hydrophobic domain that serves to anchor the enzyme to membranes. The remainder of the large subunit is highly glycosylated and strongly associated to the small subunit. The light subunit (nonglycosylated, Mr = 19, 750), which is also heavily glycosylated, contains a γ-glutamyl binding site and performs the catalytic activity of the enzyme. The enzyme is the only protease that can cleave intact GSH and GSH-conjugates (Curthoys and Hughey, 1979). The extracellular degradation of GSH and GSH S-conjugates finally results in formation of L-cysteine

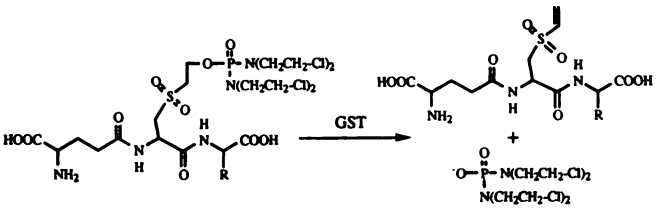


FIG. 14. Glutathione S-transferase-mediated bioactivation of S-functionalized GSH-analogs results in formation of a nontoxic vinyl sulfone compound and a cytotoxic tetrakis(chloroethyl)phosphorodiaminidate (Lyttle et al., 1994).

and corresponding cysteine S-conjugates that subsequently can be actively taken up by these cells. Therefore, GGT plays an important role in maintaining high intracellular GSH concentrations by enabling resorption of extracellular GSH catabolites. However, GGT also plays an important role in the intracellular exposure of cells to potentially toxic S-conjugates.

1. Tissue distribution. In the rat, the kidney possesses the highest levels of GGT activity (table 1), followed by the pancreas, which in the rat has 20% of the kidney level, and then the seminal vesicles, which has approximately 2% of the kidney level (Goldbarg et al., 1960). It is estimated that more than 80% of GSH in plasma is extracted by the kidney, because of the high GGT-activity in this organ (Bartoli et al., 1978; McIntyre and Curthoys, 1980). In the rat kidney, GGT is localized on both the luminal (brush border) and, to a lesser extent, basolateral membranes (Spater et al., 1982). Along the nephron, GGT is localized in the proximal tubules. The highest GGT activity is present in the S_3-segment (Heinle and Wendel, 1977; Shimada et al., 1982; Abbott et al., 1984). All other tissues have less than 1% of the renal activity in the rat. Cells expressing a high activity of GGT are pancreatic acinar and ductile epithelial cells, glandular epithelium of the breast, the primary follicle in the ovary and epithelial cells of jejunum, bile duct, epididymis, seminal vesicles, choroid plexus, ciliary body, and retinal epithelium (Meister et al., 1976; Tate and Meister, 1981).

Interestingly, a number of tumors have been shown to contain elevated levels of GGT (Black and Wolf, 1991). Studies on the GGT activity of normal and malignant hepatocytes demonstrated that isolated hepatocytes exhibit low GGT activity, whereas hepatomas have substantially increased enzyme activity (Fiala et al., 1972). Increased GGT, therefore, may be used as a marker for putative liver preneoplastic and neoplastic hepatic cells (Cameron et al., 1978). Human breast cancers, squamous-cell carcinomas, adenocarcinomas of the lungs, and melanomas also have substantially increased GGT levels when compared with the normal tissue (Bard et al., 1986; Hu and Buxman, 1979; Tateishi et al., 1976). It has been proposed that the increased level of GGT observed in preneoplastic and neoplastic lesions leads to a local increase in the hydrolysis of GSH from serum, with a corresponding increase in the intracellular levels of the amino acids required to synthesize GSH (Hanigan and Pitot, 1985). The increased level of GSH decreases the stress imposed on a transformed cell by electrophilic carcinogens, and may play an important role in the process of cell division. Up to 30-fold elevation of serum GGT is seen as a result of liver damage (hepatobiliary disease), enzyme induction, and neoplasms.

2. Species differences. When comparing tissue distribution of GGT activities in different species, it seemed that the rat and mouse have a very high K/L ratio of GGT activity (table 4). A large fraction of GSH synthesized in the rat liver is released into the bile, and only a small fraction of it is metabolized by GGT as it travels within the biliary tree (Ballatori et al., 1988). Much lower K/L ratios are observed in other species because of the higher GGT activity in the liver. A consequence of this high hepatic GGT activity is that nearly all the GSH secreted into guinea pig or rabbit bile is degraded within the biliary tree (Ballatori et al., 1988). Estimates of K/L ratios from human liver and kidney GGT activities indicated that the relative distribution of activities resemble that of the guinea pig or macaque rather than the rat or mouse (Shaw et al., 1978). Therefore, the rat and mouse may not be the best species to use as models of the catabolism of GSH and GSH-conjugates in humans (Hinchman and Ballatori, 1990). The species variations of GGT not only result from differences in concentrations of GGT but also from species differences in the GGT-protein. The apparent molecular weights of GGT subunits differ between different species (Tate et al., 1988), probably as a consequence of differences in protein glycosylation (Yamashita et al., 1989).

Recent work indicates that in a single species, differences in GGT also exist between tissues (Antoine et al., 1989; Courtay et al., 1994). Although only one GGT gene has been demonstrated to exist in rat, at least four types of mRNA are transcribed from this single gene (Darbouy et al., 1991). In humans, the genomic organization of the GGT gene seems to be considerably different and more complex than in rat. At least five GGT genes seem to be expressed in a tissue-specific manner (Courtay et al., 1994). The different forms of GGT may have different specificity for γ-glutamyl acceptor substrates (Tate and Meister, 1974; Tate and Ross, 1977; Tate et al., 1988). The multiplicity of GGT therefore should be taken into account when investigating the role of GGT in different species or tissues.

Histochemical techniques have demonstrated large species differences in distribution of GGT within the liver. In the rat liver, GGT activity is selectively localized to canalicular membranes and the lumen of biliary epithelia (Albert et al., 1961; Abbott and Meister, 1986). Because rats have no detectable sinusoidal GGT activity, most GSH or GSH S-conjugate present in the plasma will be degraded in the kidney. In contrast, the guinea pig shows faint staining along the bile canaliculi and intense sinusoidal staining (Hinchman and Ballatori, 1990; Lanca and Israel, 1991). Sinusoidal membranes of the human liver also contains abundant GGT-activity (Purucker and Wernze, 1990). The abundant sinusoidal GGT-activity indicates that a significant amount of GSH and GSH S-conjugates in the circulation may be degraded in the guinea pig liver. Recently, it has been demonstrated that the glutathione-conjugate DNP-G is rapidly eliminated from the perfusate in the isolated perfused liver from guinea pig (half time 3.7 min) (Hinchman et al., 1993). Addition of AT-125 (acivicin; NCS-163501; L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-

isoxazoleacetic acid) (fig. 15), an inhibitor of GGT (Reed et al., 1980; Schasteen et al., 1983), resulted in a marked decrease in the elimination of DNP-G (half time 36 min), suggesting that in the guinea pig liver, clearance of this GSH S-conjugate from the plasma is strongly GGT-dependent. In the perfused rat liver, elimination of DNP-G from the perfusate is much slower (half time 35 min) than in perfused guinea pig liver and seems not to be GGT-dependent (Hinchman et al., 1993).

In an effort to produce stable in vivo inhibitors of GSH S-transferase, GSH analogues were synthesized that had to withstand GGT degradation. The ability of GGT to hydrolyze the the γ-glutamylcysteine peptide bond is largely because of the specificity of the enzyme to recognize γ-glutamyl, yet the activity also depends on the amino acids to which γ-glutamine is bound. D-Amino acids are not accepted and therefore form the basis of several stable in vivo GST inhibitors (Adang et al., 1990).

3. Involvement in the toxicity of glutathione S-conjugates. The involvement of GGT in the bioactivation of nephrotoxic GSH-conjugates has primarily been established using specific inhibitors of this enzyme both in in vivo and in in vitro studies. Pretreatment of rats with 10 mg/kg AT-125 reduced GGT-activity in rat kidney to less then 10%. AT-125 inactivates GGT by binding covalently to the active site of the enzyme (Reed et al., 1980). AT-125 is more effective in inhibiting the GGT bound to the plasma membrane side (basolateral) than that bound to the brush border membrane (apical, luminal side) (Welbourne and Dass, 1982; Dass and Welbourne, 1982). As a result of inhibited GGT activity, both renal and urinary concentrations of GSH were elevated (Monks et al., 1985; Kramer et al., 1987).

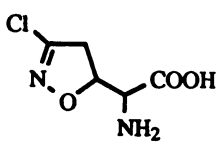
Pretreatment of rodents with AT-125 has been shown to influence the toxicity of a number of nephrotoxicants. When AT-125 was given 1 hour before the treatment of rats with 1,2-DCV-G, the nephrotoxicity of this compound was shown to be markedly reduced (Elfarra et al., 1986a). Rats were also effectively protected by AT-125 from the nephrotoxicity of 2-bromohydroquinone (Monks et al., 1985), 2-bromo-(diglutathion-S-yl)hydroquinone (Monks et al., 1988), and 2,3,5-triglutathion-S-yl)hydroquinone (Lau et al., 1988). GGT is the first step leading to the formation of 2-benzoquinol-cysteine conjugates which are more readily (aut)oxidized to the corresponding quinone-compounds when compared with the parent GSH-conjugates. Recently, it was demonstrated that AT-125 also protects against the nephrotoxicity of the GSH-conjugate of acrolein (Horvath et al., 1992).

AT-125-pretreatment completely protected male Fischer 344 rats against the nephrotoxicity of the GSH-conjugate of *p*-aminophenol (Fowler et al., 1994). However, AT-125 did not protect against the nephrotoxicity of *p*-aminophenol itself (Anthony et al., 1993); in contrast, even a slight potentiation of nephrotoxicity by AT-125 has been observed (Fowler et al., 1993). Potentiation of toxicity by AT-125-pretreatment has also been observed with several nephrotoxicants such as hexachlorobutadiene (Davis, 1988) and the GSH S-conjugates of chlorohydroquinones (Mertens et al., 1991). These findings suggest that for a number of compounds, degradation by GGT may be a detoxication mechanism. Alternatively, accumulation of intact GSH S-conjugates in the kidney cells can also lead to an increase in toxicity when the GSH S-conjugates themselves are more toxic than their breakdown products.

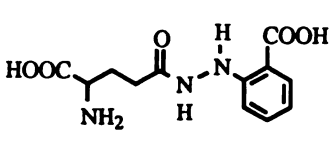
Involvement of GGT in the bioactivation of GSH-conjugates was also demonstrated in vitro. A concentration of 0.25 mM of AT-125 nearly completely protected isolated proximal tubular cells against the cytotoxicity of 1,2-DCV-G (Lash and Anders, 1986), S-(chlorotrifluoroethyl)glutathione (Dohn et al., 1985a), and the GSH-conjugate of *p*-aminophenol (Klos et al., 1992). Recently, it has been shown that the GSH-conjugate of *p*-aminophenol is not toxic to isolated rat renal proximal tubules (Lock et al., 1993). This may be explained by the fact that in this in vitro model, the rat renal cells are only exposed to the GSH-conjugate via the basolateral side, which has a very low GGT-activity (Hinchman et al., 1993).

Another GGT-inhibitor, anthglutin (1-γ-l-glutamyl-2-(2-carboxyphenyl)-hydrazine), also protected isolated proximal tubular cells against PCBD-G (Jones et al., 1986). When cells were incubated in the presence of glycinylglycine, a suitable acceptor substrate for GGT-catalyzed deglutamination, the cytotoxicity of 1,2-DCV-G was increased because of stimulation of GGT-activity (Lash and Anders, 1986). AT-125 also protected a monolayer of the pig renal epithelial cell line LLC-PK_1 against cytotoxicity induced by 1,2-DCV-G (Stevens et al., 1986a) and S-(2-bromo-2-chloro-1,1-difluoroethyl) glutathione (Finkelstein et al., 1992), which is the GSH-conjugate of the anesthetic halothane (Cohen et al., 1975; Wark et al., 1990).

4. Oxidative damage caused by γ-glutamyltransferase. Despite its protective function against electrophiles and oxidants, extracellular GSH has been shown to be mutagenic to bacteria (Stark et al., 1987, 1988) and genotoxic to mammalian cells (Thust and Bach, 1985; Thust, 1988). The mutagenicity of GSH depends on the activity of GGT, is oxidative in its nature, and involves free radicals leading to oxidative damage (Stark et al. 1987, 1988). The metabolism of GSH by GGT in preneoplastic



AT-125



anthglutin

FIG. 15. Chemical structure of the GGT-inhibitors AT-125 and anthglutin.

liver foci is suggested to create a radical-rich environment and oxidative damage. Such damage may contribute to the process by which cells within such foci progress to malignancy (Stark et al., 1993, 1994). Lipid peroxidative-positive staining of liver sections coincided with the focal areas containing high GGT-activity. Inhibition of GGT by AT-125 protected against lipid peroxidation. GGT-dependent peroxidation is dependent on iron from iron-sources such as transferrin. Hemoglobin and ferritin did not support GSH-GGT-driven lipid peroxidation in vitro (Stark et al., 1994). It is proposed that oxidative damage by the GSH-GGT system may occur in vivo at or near the plasma membranes of other GGT-rich cells. Treatment of rats and mice with chelated iron resulted in renal proximal tubular carcinomas, acute damage, and lipid peroxidation in the proximal tubular region of the kidney cortex; the initial damage occurred on the lumenal side of microvilli (Ebina et al., 1986; Li et al., 1987). Under normal circumstances, the iron concentration in the glomerular filtrate is very low, and oxidative damage does not occur. It is suggested that metabolism of lumenal GSH by renal GGT in the presence of high concentrations of iron thus seems to stimulate oxidative damage and renal carcinomas (Stark et al., 1994).

5. Activation of antitumor agents by γ-glutamyltransferase. As indicated earlier, GGT activity is increased in a number of tumors. In human ovarian adenocarcinoma, cell lines derived from a patient before and after the onset of drug resistance to cisplatin, chlorambucil and 5-fluorouracil, GGT-activity exhibited a four- to six-fold elevation (Lewis et al., 1988). Furthermore, Godwin et al. (1992) showed that cisplatin resistance in human ovarian carcinoma cell lines correlated well with their GGT levels. Leukemia cells resistant to killing by L-phenylalanine mustard also exhibited increased GGT activity (Ahmad et al., 1987). Transfection studies have indicated that an increased expression of GGT in tumor cells is associated with enhanced tumorigenic capacity of papilloma-producing epidermal cell lines (Yoshimi et al., 1992).

Recently, a strategy was developed using the elevated GGT-activity in tumor cells as a target for antitumor compounds (Prezioso et al., 1994). GHB and its iodinated analog, I-GBH, demonstrated high antitumor activity in human and in murine melanoma cell lines. The growth-inhibitory activity of these compounds in melanoma cells was blocked by the GGT-inhibitor, AT-125. In the Chinese hamster, ovary cells, transfected with cDNA of GGT, GHB and I-GHB, were significantly more cytotoxic than in nontransfected cells. These data suggest that GGT-catalyzed hydrolysis of these antitumor prodrugs to the corresponding 4-aminophenols mediates the expression of antitumor activity. Because of their selective cytotoxic activity with tumor populations exhibiting elevated GGT-levels and tumorigenetic capacity, GHB and I-GHB may have broad clinical applications as antitumor agents.

C. Cysteinylglycine Dipeptidase and Aminopeptidase-M

Two enzymes have been shown to catalyze the degradation of cysteinylglycine S-conjugates to the corresponding cysteine S-conjugates (fig. 6, step b), namely cysteinylglycine dipeptidase (dipeptidase; microsomal dipeptidase; dehydropeptidase-I; EC 3.4.13.11) (Hughey et al., 1978; Hirota et al., 1985, 1986) and aminopeptidase-M (EC 3.4.11.2) (Kozak and Tate, 1982). Both enzymes are homodimers and are associated with the plasma membrane with the active site exposed to the extracellular compartment (Curthoys, 1986; Hirota et al., 1987a).

Cysteinylglycine dipeptidase is a membrane-associated zinc metalloproteinase and has been identified in the kidney and lung of rat (Hirota et al., 1986), mouse (Satoh et al., 1993), and sheep (Campbell et al., 1990) and in the kidney of pig (Campbell et al., 1963) and human (Campbell et al., 1984; Adachi et al., 1989). Analysis of apparent molecular weight under reducing and nonreducing conditions suggested that dipeptidase exists in a two-subunit structure linked by disulfide bonds (Adachi et al., 1989; Satoh et al., 1993). The number of amino-acid residues among different species is almost the same, and the deduced amino-acid sequence in different species is more than 70% identical (Satoh et al., 1993). However, the apparent molecular weight of the subunits in different species ranges from 42,000 to 62,000 Da. Dipeptidases were shown to be glycoproteins, and it was revealed that the differences in molecular mass among various species depend on different degrees of glycosylation (Hooper et al., 1990). Renal dipeptidase hydrolyzes a wide range of dipeptides to its constituent amino acids (Kozak and Tate, 1982; Hirota et al., 1986). In addition to its action on cysteinylglycine S-conjugates, dipeptidase is responsible for the hydrolytic scission of the lactam bond in carbapenems, potent broad-spectrum antibiotics that are resistant to the action of microbial β-lactamases (Kropp et al., 1982). Cilastin (fig. 16) is a relative selective inhibitor of dipeptidase (Kahan

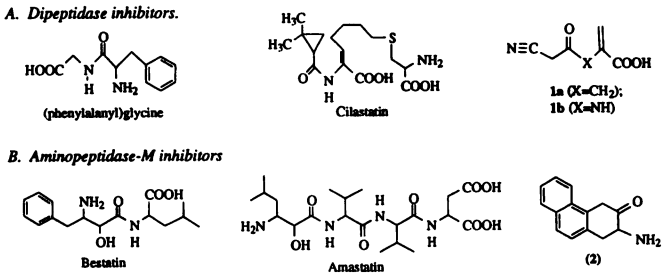


FIG. 16. Chemical structures of inhibitors of dipeptidase (A) and aminopeptidase-M (B). Compounds **1a** and **1b** are mechanism-based inhibitors of dipeptidase (Wu and Mobashery, 1991). Compound **2** is the 3-amino-2-tetralone-derivate that is the most potent inhibitor of aminopeptidase-M (K_i = **20 nm**) (Schalk et al., 1994).

et al., 1983). 1,10-Phenanthrolin, a metal chelating agent, and thiol-compounds, such as dithiotreitol and L-penicillamine, are less selective inhibitors of dipeptidase (Kozak and Tate, 1982). Enzymic turnover of carbapenems by dipeptidase in vivo poses a serious obstacle to clinical efficacy of these bactericidal agents (Kropp et al., 1982); therefore, specific inhibitors for this enzyme are widely sought. Recently, two mechanism-based inactivators, compounds 1a, b (fig. 16) were synthesized, which were specifically designed for porcine renal dipeptidase (Wu and Mobashery, 1991).

Aminopeptidase-M is also a zinc-containing metallopeptidase and is composed of two large subunits with an apparent molecular weight of 110 to 120 kDa (Hirota et al., 1985; Malfroy et al., 1989). Aminopeptidase-M is widely distributed in mammalian tissues including the central nervous system and is particularly abundant in the kidney and intestinal microvilli. Aminopeptidase-M exhibits greater activity with oligopeptides than with dipeptides. Aminopeptidase-M is strongly inhibited by 1,10-phenanthroline and the natural compounds bestatin and amastasin (fig. 16); however, these inhibitors are relatively poorly selective. Recently, it was shown that 3-amino-2-tetralone-compounds are very potent and more selective inhibitors of aminopeptidase-M, which make them interesting tools in delineating the role of aminopeptidase-M in the metabolism of endogenous compounds and GSH-derived S-conjugates (Schalk et al., 1994). Aminopeptidase-M is more abundant than the dipeptidase, however, the latter most probably is more important in the metabolism of GSH-derived S-conjugates. S-Methyl-L-cysteinylglycine is hydrolyzed with a more than 250-fold specific activity by renal dipeptidase than by aminopeptidase-M (Kozak and Tate, 1982). In isolated rat renal cells, N-ethylmaleimide-S-cysteinylglycine was rapidly hydrolyzed to N-ethylmaleimide-S-cysteine, and subsequently acetylated to the corresponding mercapturic acid (Hirota et al., 1987a). Cilastatin, a selective inhibitor of dipeptidase, strongly inhibited the hydrolysis of N-ethylmaleimide-S-cysteinylglycine, whereas bestatin, an inhibitor of aminopeptidase-M, only had a small inhibitory effect. Similar results were obtained when the hydrolysis of leukotriene-D_4, an S-substituted cysteinylglycine derivative of arachidonic acid, was studied. Purified rat renal dipeptidase was at least 16,000-fold more active in the hydrolysis of leukotriene-D_4 than was purified aminopeptidase-M (Kozak and Tate, 1982). These results indicate that dipeptidase may play a more important role in the metabolism of GSH and its conjugates than aminopeptidase-M does. Comparison of a series of dipeptides demonstrated that dipeptidase is particularly active against peptides in which the amino-terminal amino acid contains a hydrophobic side chain. Cysteinylglycine S-conjugates with a hydrophobic S-bound moiety, therefore, can be anticipated to be good substrates for this enzyme (Hughey et al., 1978).

1. Tissue distribution. The intertissue and intratissue distribution of the two peptidases has been investigated, among others by enzyme activity measurements, by immunostaining procedures, and by mRNA analysis. In the rat, dipeptidase activity was highest in the lung, followed by kidney, spleen and liver, brain, pancreas, and small intestine (table 1). The specific activity in lung was more than twice as high as that in the kidney. The enzymes of both organs were shown to be immunologically identical and to have similar catalytic properties, molecular masses, and pH optima (Hirota et al., 1986). The high activity of dipeptidase in the lung may indicate that the lung is a physiologically important organ for the metabolism of GSH and its S-conjugates.

Using immunostaining, it was found that dipeptidase in the kidney is present on both the brush border and the basolateral membranes of proximal tubular cells (Hirota et al., 1987b). The fact that some parts of the brush border of the proximal tubules were strongly stained, whereas other parts were not stained at all, suggests that dipeptidase is not evenly distributed along the proximal tubules. The immunohistochemical observations are in good agreement with the observed distribution profiles of enzyme activity in the kidney (Curthoys and Shapiro, 1975; Dass et al., 1981; Sochor et al., 1980; Spater et al., 1982). The highest activity is localized in the outer stripe region of the renal medulla (Hughey et al., 1978), which corresponds with the S_3-segment of the proximal tubule. In the liver, the peptidases are present in the canalicular membrane of hepatocytes, the luminal membrane of the biliary epithelium (Inoue et al., 1983a; Meier et al., 1984; Ballatori et al., 1986), and in pancreatic secretions in the bile (Abbott et al., 1984; Hirata and Takahashi, 1981).

In the rat, aminopeptidase-M activity was highest in the kidney, followed by brain, small intestine, liver, and other tissues including the lung (table 1).

When N-ethylmaleimide-S-glutathione was injected i.v. into nephrectomized rats, a significant amount of N-ethylmaleimide-S-cysteine was detected in the blood, demonstrating that the GSH S-conjugate could be metabolized to its cysteine S-conjugate by extrarenal GGT and dipeptidase (Hirota et al., 1987c). In the isolated liver perfusion, both N-ethylmaleimide-S-cysteinylglycine and N-ethylmaleimide-S-cysteine were formed from N-ethylmaleimide-S-glutathione. It was shown that N-ethylmaleimide-S-cysteinylglycine was converted to N-ethylmaleimide-S-cysteine in plasma, although N-ethylmaleimide-S-glutathione was not metabolized by the plasma. Therefore, the liver and plasma also contribute to the metabolism of GSH S-conjugates in vivo.

2. Species differences. When comparing activities of dipeptidase in liver, kidney, and gall bladder of different species, the highest activity was found in the kidney in all species (table 4) (Hinchman and Ballatori, 1990). Much smaller differences in K/L ratios were observed than with GGT activities in the corresponding species.

As already mentioned, the highest activity of dipeptidase in the rat was present in the lung. Dipeptidase forms leukotriene-E_4 from leukotriene-D_4, and these leukotrienes are produced mainly in the lung as the slow-reacting substance of anaphylaxis. Therefore, if the expression of dipeptidase is regulated in lung by some substrate derived from the disease as anaphylaxis, the expression of dipeptidase in lung may be variable. In the mouse, however, it was shown by Northern blotting hybridization analysis that mRNA of dipeptidase was expressed strongly in the kidney, but at much lower levels in the lung (Satoh et al., 1993). Northern blotting hybridization of human tissues showed that mRNA of dipeptidase is only transcribed in the kidney (Satoh et al., 1994).

3. Involvement in the toxicity of cysteinylglycine-S-conjugates. Few data pointing to the possible involvement of dipeptidases in bioactivation mechanisms are available. Inhibitors of the dipeptidases, however, have demonstrated some involvement of dipeptidases in the nephrotoxicity of haloalkenes. It was shown that 1,10-phenanthroline (Okajima et al., 1981), as well as (phenylalanyl)glycine, a competitive inhibitor of cysteinylglycine dipeptidase (Jones et al., 1979), protected isolated rat kidney cells from the cytotoxicity of both 1, 2-DCV-G and S-(1, 2-dichlorovinyl)cysteinylglycine (Lash and Anders, 1986).

D. Cysteine-S-conjugate β-Lyase

Cysteine-S-conjugate β-lyases (EC 4.4.1.13; β-lyase; C-S lyase) are PLP-dependent enzymes that catalyze the cleavage of the C-S bond of S-conjugates of L-cysteine; S-conjugates of D-cysteine are not cleaved (Tateishi et al., 1978; Tateishi, 1983). The products of the C-S cleavage are thiol compounds and dehydroalanine, which very rapidly hydrolyzes to ammonia and pyruvic acid (fig. 17). In analogy with other PLP-dependent enzymes, a mechanism of action has been proposed for β-lyase by Stevens et al. (1986b). In this proposal, a Schiff base is formed between the L-cysteine S-conjugate and the coenzyme PLP. The resulting aldimine subsequently eliminates a thiol-compound, and the eneamine formed rapidly hydrolyses into pyruvic acid, ammonia, and free PLP (fig. 17, route A). The catalytic action of β-lyase most probably involves deprotonation of the cysteine S-conjugate at the α-carbon position, which is facilitated by delocalization of the resulting free electron pair over the PLP-moiety, which may be regarded as an electron sink (Snell, 1986). Because the ketimine, which is one of the mesomeric forms of the deprotonated aldimine, may be hydrolyzed to pyridoxamine phosphate and a 3-mercaptopyruvic acid S-conjugate, cysteine conjugate β-lyase may also function as a cysteine conjugate transaminase (fig. 17, route B) (Stevens et al., 1989; Miles, 1985).

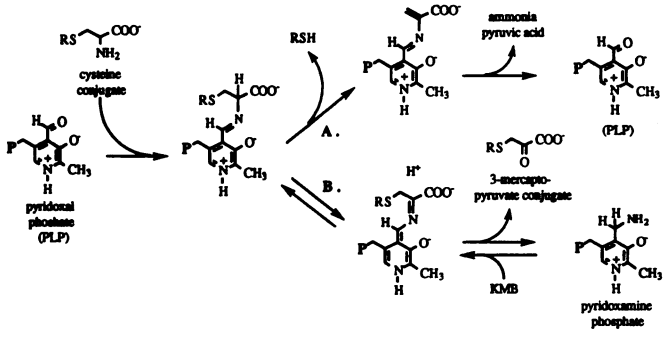


FIG. 17. Schematic representation of the mechanism of β-elimination (A) and transamination (B) reactions catalyzed by pyridoxal phosphate and pyridoxamine phosphate-dependent enzymes. KMB, α-keto-γ-methiolbutyric acid; P, phosphate-group.

1. Tissue distribution. β-Lyase-activity was initially demonstrated to be present in the cytosol of rat liver (Tateishi et al., 1978). Rat hepatic β-lyase was shown to be identical with kynureninase (EC 3.7.1.3) (Stevens, 1985a). The physiological role of this enzyme therefore may be participation in the conversion of L-tryptophan into biosynthetic precursors of nicotinamide ribonucleotides. A cytosolic β-lyase was also purified from human liver (Tomisawa et al., 1986a; Buckberry et al., 1992a). This hepatic β-lyase was shown to be a homodimer consisting of subunits with an apparent molecular weight (by sodium dodecyl sufate-polyacrylamide gel electrophoresis) of 37,000 Da (Buckberry et al., 1992a). In contrast to the rat liver enzyme, β-lyase from human liver cytosol was not able to cleave kynurenine to anthranilic acid and alanine (Tomisawa et al., 1986a; Buckberry et al., 1992b). Instead, the human hepatic cytosolic β-lyase was shown to function as a kynurenine aminotransferase. In the human liver, significant β-lyase activities were also demonstrated in mitochondrial and microsomal fractions (Buckberry et al., 1992a; Blagbrough et al., 1992).

Studies demonstrated that β-lyase activity was also present in other mammalian tissues (Stevens and Jakoby, 1983; Jones et al., 1988) (table 1) as well as in intestinal microflora (Suzuki et al., 1982; Larsen and Stevens, 1985; Larsen, 1985). In the rat, the highest specific activity of β-lyase toward 1,2-DCV-Cys was found in the kidney. In the rat kidney, β-lyase is present mainly in cytosol and to a lesser extent in the mitochondrial outer membrane (Stevens et al., 1986b, 1988). Renal cytosolic and mitochondrial β-lyase both seem to be identical to glutamine transaminase K (EC 2.6.1.64) (Stevens et al., 1986b; Cooper, 1978). The cytosolic and mitochondrial enzymes, however, possess different substrate selectivity; the cytosolic enzyme has a higher activity with 1,2-DCV-Cys as substrate, whereas the mitochondrial form has a higher activity with S-(benzothiazolyl)-L-cysteine (Stevens, 1985b). By comparing the activities of cytosolic and mitochondrial β-lyase toward cysteine S-conjugates of five halogenated alkenes, it seems that PCBD-Cys is the worst substrate for the cytosolic enzyme but the best

substrate for the mitochondrial enzyme (Hayden and Stevens, 1990) (table 6).

Rat renal β-lyase is a homodimer of two subunits with apparent molecular weights of approximately 45,000 to 50,000 Da (Stevens et al., 1986b; Abraham and Cooper, 1991). In immunological experiments, it was shown that antibodies against the renal enzyme was not cross-reactive to the hepatic enzyme (Stevens and Jakoby, 1983).

Using a nondenaturating polyacrylamide gel electrophoresis method combined with activity staining, it was shown that liver, skeletal muscle, and heart possess a glutamine transaminase K/β-lyase activity with identical mobility during electrophoresis as the rat renal enzyme (Abraham and Cooper, 1991). Using this method, a second protein having an apparent molecular weight of 330,000 Da, which showed β-lyase activity toward 1,2-DCV-Cys, was identified in rat renal cytosol. Whether this enzyme represents a higher multimer of the purified glutamine transaminase/β-lyase or a protein containing higher molecular weight subunits remains to be established.

Rat renal glutamine transaminase/β-lyase transaminates glutamine with a suitable α-keto acid acceptor to yield α-ketoglutaramate and L-amino acid. The best α-keto acid acceptors are the α-keto acids analogs of methionine (α-keto-γ-methiolbutyric acid) and the aromatic amino acids; pyruvate is a poor substrate, and α-ketoglutarate is even less readily transaminated (Cooper and Meister, 1981). α-Keto-γ-methiolbutyric acid also seems to stimulate the β-elimination reaction of cysteine S-conjugates several-fold. This can be been explained by the conversion of pyridoxamine phosphate, which results from the intrinsic transaminase activity of β-lyase, to PLP, which is the active form in β-lyase activity (fig. 17) (Stevens et al., 1986b). Transamination can be seen as a competing route for the bioactivation of cysteine S-conjugates by β-lyase, and thus may have an important impact on the relative nephrotoxicity of these conjugates. To date, however, no data are available on the structure-dependency of the competing transamination reaction.

Cytosolic and mitochondrial β-lyase activity was also demonstrated in human kidney using S-(benzothiazolyl)-L-cysteine and 1,2-DCV-Cys as substrates (Lash et al., 1990a; Buckberry et al., 1990). The highest β-lyase activity was present in the cytosolic fraction. From human cytosol, two isoenzymes of β-lyase, which display physicochemical and biochemical properties with glutamine transaminase K, were purified (Buckberry et al., 1990). The specific activity of human kidney cytosol, however, was only 10% of that present in rat kidney cytosol (Lash et al., 1990a). Using the cysteine S-conjugate of tetrachloroethylene, TCV-Cys, as a substrate gave comparable results: cytosolic β-lyase activity was again much lower in human renal fractions than in rat renal fractions (Green et al., 1990) (table 4). Combined with the extremely low activity of GSH-conjugation of tetrachloroethylene in human liver fractions, it was concluded that the presence of renal β-lyase in human kidney is of no toxicological significance for tetrachloroethylene (Green et al., 1990).

In contrast to the rat, considerable β-lyase activity was also found in human kidney microsomes (Buckberry et al., 1990). The nature of the renal microsomal β-lyase has not yet been investigated in detail.

By immunohistochemical examination, rat kidney β-lyase was shown to be evenly distributed along the different segments of the proximal tubule (S_1, S_2 and S_3), but it was absent in the glomeruli and the distal tubule (Jones et al., 1988). Using a highly purified antibody against renal cytosolic β-lyase, however, only the S_3-segment was shown to contain β-lyase (MacFarlane et al., 1989). The reasons for these contradictory results are not known yet, however.

To examine the factors controlling the expression of rat renal cytosolic β-lyase, a cDNA for β-lyase has been isolated from a rat kidney cDNA library using immunological and hybridization screening. This led to the sequencing of a full length cDNA from which the amino acid composition of the protein, its molecular weight, and the PLP binding site was predicted (Perry et al., 1993). The predicted amino acid composition was confirmed after the purification of the enzyme from rat kidney cytosol (Yamauchi et al., 1993). A dose-dependent induction of β-lyase was found after dosing rats with PCBD-NAC (MacFarlane et al., 1993). β-Lyase ac-

TABLE 6

Substrate selectivities of rat renal cytosolic and mitochondrial β-lyase and cytosolic L-amino acid oxidase toward nephrotoxic cysteine conjugates

cysteine conjugate	cytosolic β-lyase*	mitochondrial β-lyase†	cytosolic L-amino acid oxidase‡
S-(1,1,2,2-tetrafluoroethyl)-	590	9.5	1.8
S-(2-chloro-1,1,2-trifluoroethyl)-	560	8.0	2.6
S-(1,2-dichlorovinyl)-	360	7.1	6.5
S-(1,1,2,3,3,3-hexafluoropropyl)-	230	4.2	2.8
S-(1,2,3,4,4-pentachlorobutadienyl)-	110	14.4	28.4

* nmol per minute per mg protein using purified rat renal cytosolic β-lyase; conjugate concentration, 1 mM (Hayden and Stevens, 1990).
† nmol per 60 min per mg protein using isolated rat renal mitochondria; conjugate concentration, 0.1 mM (Hayden and Stevens, 1990).
‡ nmol per min per mg protein using purified rat renal cytosolic L-amino acid oxidase; conjugate concentration, 1 mM (Stevens et al., 1989).

tivity and protein levels were raised two- to three-fold and were accompanied by an increase in the levels of mRNA, as demonstrated by a cDNA probe for rat renal cytosolic β-lyase. The induction of β-lyase activity and protein seemed to be specific for the cytosolic form: no change in the levels of mitochondrial β-lyase was observed, either in the activity or in the protein levels. Higher doses of PCBD-NAC decreased rather then increased the β-lyase levels, probably because of toxicity of the inducer.

2. Gastrointestinal cysteine conjugate β-lyase. Metabolism of xenobiotics by the microflora of the gut may be extensive and highly diverse, and may contribute in the bioactivation of dietary and environmental mutagens and carcinogens (Chadwick et al., 1992). Cysteine conjugate β-lyase was detected in 24 of 43 gastrointestinal bacteria (Larsen, 1985). The bacterial β-lyase localized in the gut of mammals has a broader substrate specificity than the mammalian enzymes (Tateishi, 1983). Both S-aryl- and S-alkyl-linked cysteine S-conjugates, which are very poor substrates for liver or kidney β-lyase, are efficiently cleaved by the bacterial enzymes. This may explain the role of the gut flora in the production of thiomethyl metabolites from a variety of cysteine-S-conjugates, for example, the cysteine S-conjugate of propachlor, which are not substrates for the mammalian enzymes (Rafter et al., 1983; Bakke and Gustafsson, 1984). For methylation, the thiols produced by the intestinal microflora require translocation into the intestinal mucosa, the liver or the kidney, because S-methyltransferase is not found in the intestinal lumen (Larsen, 1985). The role of the intestinal microflora in the production of methylthiol-metabolites has been studied by comparing the urinary metabolites of conventional rats and germfree rats (Bakke and Gustafsson, 1984; Bakke et al., 1990a). In conventional rats, pentachlorothioanisole was excreted in feces mainly as 1,4-bis(methylthio) tetrachlorobenzene, as a result of degradation of the GSH S-conjugate formed initially (Bakke et al., 1990a). In germ-free rats, however, bis(methylthio)tetrachlorobenzene could not be demonstrated in feces. Instead, the GSH S-conjugates formed were excreted in feces as mercapturic acid.

3. Bioactivation by mammalian β-lyase.

a. IN VIVO STUDIES. The first example of bioactivation by β-lyase was discovered nearly 30 years ago. 1,2-DCV-Cys (Terracini and Parker, 1965) was identified as the toxic factor in trichloroethylene-extracted soybean meal responsible for fatal aplastic anemia in cattle after a single dose of 2.5 to 3 mg/kg (McKinney et al., 1959). Other species, such as the rat, mouse, rabbit, and turkey were much more resistant against 1,2-DCV-Cys-induced toxicity. However, at doses higher than 25 mg/kg 1,2-DCV-Cys induced severe nephrotoxicity in these species (Schulze et al., 1962). It was demonstrated that enzymes present in kidney, liver, and many species of bacteria cleaved 1,2-DCV-Cys to reactive sulfur-containing fragments that bind covalently to proteins and DNA that is believed to initiate the toxicity (Anderson and Schulze, 1965; Bhattacharya and Schulze, 1967, 1972). Other cysteine-S-conjugates also found to cause nephrotoxicity in vivo are CTFE-Cys (Dohn et al., 1985a), TFE-Cys (Odum and Green, 1984), and BCDFE-Cys (Finkelstein et al., 1992).

The involvement of β-lyase in vivo has been demonstrated by studies using the potent β-lyase-inhibitor AOAA, which may rapidly depress β-lyase activity in rat kidney by more than 90% (Elfarra et al., 1986b). The nephrotoxicity of 1,2-DCV-Cys (Elfarra et al., 1986b), CTFE-Cys (Dohn et al., 1985a), and BCDFE-Cys (Finkelstein et al., 1992) was inhibited efficiently by AOAA. It was also shown that AOAA protected against the nephrotoxicity of the corresponding GSH-conjugates, 1,2-DCV-G, CTFE-G, and BCDFE-G. Additional evidence for the involvement of β-lyase in the nephrotoxicity of the haloalkene derived S-conjugates was obtained by dosing rats with α-methyl-L-cysteine S-conjugates of 1,2-DCV-Cys, CTFE-Cys and BCDFE-Cys that cannot be metabolized by β-lyase and consequently were not nephrotoxic (Elfarra et al., 1986b; Dohn et al., 1985a; Finkelstein et al., 1992).

The nephrotoxicity of S-(2-chloroethyl)-L-cysteine was unaffected by AOAA-pretreatment, supporting the hypothesis that this cysteine-S-conjugate is a direct-acting nephrotoxin (Dohn et al., 1985a). AOAA also did not protect against the nephrotoxicity of 2,3,5-(triglutathion-S-yl)hydroquinone (Lau et al., 1988) or 2-bromo-(diglutathion-S-yl)hydroquinone (Monks et al., 1988).

b. IN VITRO STUDIES. 1,2-DCV-Cys, PCBD-Cys, S-(1,1,2,3,3,3-hexafluoropropyl)-L-cysteine, TCV-Cys and TFE-Cys also caused toxicity to rat kidney slices, as determined by inhibition of organic anion and cation transport, and all caused release of equimolar amounts of pyruvic acid and ammonia, indicating β-lyase dependent metabolism (Green and Odum, 1985). Using similar rat kidney slices, the β-lyase mediated nephrotoxicity of TFE-Cys, CTFE-Cys, DCDFE-Cys, DBDFE-Cys, and of the L-cysteine S-conjugates of three regioisomers of chlorophenyl-β, β-difluoroethylene has been examined and compared to the in vivo toxicity of the corresponding mercapturic acids (Stijntjes et al., 1993).

1,2-DCV-Cys (Lash and Anders, 1986), CTFE-Cys (Dohn et al., 1985a), PCBD-Cys (Jones et al., 1986), TFE-Cys, CTFE-Cys, DCDFE-Cys and DBDFE-Cys (Boogaard et al., 1989) appeared to be cytotoxic to isolated rat kidney proximal tubular cells, as assessed by lactate dehydrogenase leakage, trypan blue exclusion, and α-methylglucose uptake. AOAA almost completely protected against the cytotoxicity of these cysteine-S-conjugates. AOAA also blocked cytotoxicity of the GSH-conjugates CTFE-G (Dohn et al., 1985a), PCBD-G (Jones et al., 1986), DCV-G (Lash and Anders, 1986), S-(1, 2-dichloro-3, 3, 3-trifluoro-1-propenyl)glutathione (Vamvakas et al., 1989) and S-(2-bromo-2-chloro-1, 1-difluoro-

ethyl)-glutathione (BCDFE-G) (Finkelstein et al., 1992), as well as the S-(1, 2-dichlorovinyl)cysteinylglycine conjugate (Lash and Anders, 1986). The activity of peptidases was not affected by AOAA, further indicating that inhibition of β-lyase provided protection against cytotoxicity. Addition of the β-lyase stimulator α-ketomethiolbutyric acid to the medium increased cytotoxicity of 1,2-DCV-Cys in isolated rat proximal tubular cells (Elfarra et al., 1987). Similar results were obtained with the LLC-PK_1 renal cell line. 1,2-DCV-Cys (Stevens et al., 1986a; Wallin et al., 1992), 1,2-DCV-G (Stevens et al., 1986a), PCBD-Cys (Mertens et al., 1990), PCBD-G (Mertens et al., 1988), BCDFE-Cys and BCDFE-G (Finkelstein et al., 1992) all were shown to be cytotoxic to this cell line, and this toxicity could be blocked by AOAA.

Using the Ames test for mutagenicity, some nephrotoxic cysteine S-conjugates of haloalkenes were found positive while others were not. The chlorinated conjugates PCBD-Cys, 1,2-DCV-Cys, and TCV-Cys produced a marked increase in revertants, while the fluorinated cysteine S-conjugates TFE-Cys, CTFE-Cys, and S-(1,1,2,3,3,3-hexafluoropropyl)-L-cysteine did not (Green and Odum, 1985). Studying the regioisomeric cysteine S-conjugates 1,2-DCV-Cys and 2,2-DCV-Cys for their mutagenicity, it was found that 1,2-DCV-Cys was much more mutagenic than 2,2-DCV-Cys (Commandeur et al., 1991a). The involvement of β-lyase in mutagenicity was demonstrated by inhibition with AOAA (Dekant et al., 1986a). The difference in mutagenicity of the regioisomeric 1,2-DCV-Cys and 2,2-DCV-Cys was suggested to originate from the 3 to 4 times higher β-lyase activity of 1,2-DCV-Cys, as well as from the higher reactivity of the reactive intermediate formed (Commandeur et al., 1991a). The mutagenicity of S-(2-chloroethyl)-L-cysteine was not affected by AOAA, again supportive of the hypothesis of direct alkylating ability of this cysteine S-conjugate (Elfarra et al., 1985).

Due to the lipophilic nature of both PCBD-Cys and 1,2-DCV-Cys, the mitochondrial matrix was found to be the primary target for these reactive electrophiles (Wallin et al., 1987; Hayden et al., 1990). The formation of mitochondrial adducts of TFE-Cys and CTFE-Cys was shown using antibodies against halothane-derived trifluoroacetyllysine adducts (Hayden et al., 1991). Using ^{35}S-labeled substrates, Hayden et al. (1992) have recently shown that mitochondrial phospholipid adducts are major products of the β-elimination reaction of TFE-Cys, CTFE-Cys, 1,2-DCV-Cys, and PCBD-Cys. This covalent binding leads to a cascade of events including depletion of cellular nonprotein sulfhydryls, increased cellular free calcium, and lipid peroxidation that may ultimately be responsible for cell death (Groves et al., 1991; Chen et al., 1992).

Renal β-lyase activity toward nephrotoxic cysteine S-conjugates has meanwhile been found in cytosol and in mitochondria (table 6). The relative contribution of these two fractions to nephrotoxicity remains to be established. Because mitochondria are proposed to be the initial targets of β-lyase-mediated toxicity, the mitochondrial enzyme may be critically important for toxicity (Hayden et al., 1990).

Glutamine transaminase K/β-lyase has also been demonstrated to be widespread throughout the rat brain; the highest specific activity of this enzyme was found in the choroid plexus and in astrocytes (Cooper et al., 1993; Makar et al., 1994). In the brain, the enzyme is mostly mitochondrial. It was suggested that the localization of this enzyme in the brain plays a role in the reported neurotoxicity of halogenated xenobiotics such as dichloroacetylene. Both 1,2-DCV-Cys and 1,2-DCV-G are readily taken up intact across the rat blood-brain barrier (Patel et al., 1993).

4. Bioactivation by β-lyase from intestinal microflora. In principle, relatively stable toxic thiol-compounds formed by β-lyase in the gastrointestinal microflora can be taken up by the organism and produce toxicity at distant sites. Both the glutathione and the cysteine S-conjugate of 1-nitropyrene 4,5-oxide have been shown to be mutagenic in the Ames-test (Kinouchi et al., 1993). The mutagenicity of the cysteine S-conjugate was 10-fold higher than that of the GSH-conjugate. Also, the in vitro DNA binding of the cysteine conjugate was enhanced by addition of purified bacterial β-lyase and was inhibited by aminooxyacetic acid, a β-lyase inhibitor. It was shown that after administration of the GSH-conjugate of 1-nitropyrene oxides to conventional mice, three DNA-adducts could be detected in the lower intestinal mucosa by the ^{32}P-postlabeling method (Kinouchi et al., 1993). In antibiotic-treated mice, which are germ-free, these DNA-adducts could not be detected, suggesting that the intestinal microflora play an important role in the bioactivation of these GSH-conjugates. It was postulated that the β-lyase-induced metabolites of the cysteine conjugates of 1-nitropyrene oxides, nitrothiol-derivates, can be further activated by nitroreductase of the intestinal microflora, producing activated hydroxylamino-derivates or aminothiol-derivates. After absorption of the aminothiol-derivates, the metabolites may be activated by tissue enzymes such as cytochrome P-450.

The nephrotoxicity of HCBD has also been investigated in germ-free rats and conventional rats (Wallin et al., 1993). According to morphological changes, it seemed that germ-free rats were slightly more susceptible to HCBD and its glutathione and cysteine conjugates. Therefore, the presence of an intestinal microflora tend to protect against the nephrotoxicity of HCBD. This may be explained by the high chemical reactivity of the thiol-compound formed that will react locally and therefore will not reach the kidney.

5. The nature of reactive intermediates formed by β-lyase. Elucidation of the chemical structure of the reactive intermediates formed upon β-elimination of nephrotoxic cysteine S-conjugates has been the goal of a

number of studies involving the nephrotoxic cysteine conjugates. Because of the very high reactivity of the reactive intermediates, these compounds cannot be analyzed directly. Therefore, the nature of the reactive intermediates must be deduced from the nature of the covalent adducts formed by these electrophiles. TCV-Cys and 1,2-DCV-Cys were shown to be bioactivated to thioacylating agent(s) by β-lyase, presumably thionoacyl chlorides or thioketenes (Dekant et al., 1988a). Using α-haloalkenyl 2-nitrophenyl disulfides as precursors for the biological reactive intermediates of S-(1,2-dichloro-3,3,3-trifluoro-1-propenyl)-L-cysteine, TCV-Cys, and PCBD-Cys, it was recently shown that thioketene formation is the most likely route after β-elimination (fig. 7B) (Dekant et al., 1991). The reactive intermediates formed from the fluorinated conjugate TFE-Cys have been shown to be difluorothionoacylating agents, presumable difluorothionoacyl fluoride (fig. 7A) (Commandeur et al., 1989; Hayden et al., 1991; Harris et al., 1992). A corresponding reactive intermediate, chlorofluorothionoacyl fluoride, is formed upon β-elimination of CTFE-Cys (Dekant et al., 1987a; Commandeur, 1991; Fisher et al., 1993). However, analysis of reaction products of CTFE-Cys-incubations by ^{19}F-nuclear magnetic resonance demonstrated a high abundance of fluoride anion relative to the chlorofluorothionoacyl-derived products (Commandeur, 1991; Fisher et al., 1993). This may partly be explained by defluorination of the thioamide adduct, because in 4 days, 50% of a synthetically Na-acetyl-L-lysine adduct was hydrolyzed (Fisher et al., 1993). Another explanation, however, may be that next to the thionoacyl fluoride, a second reactive intermediate is formed that rapidly defluorinates upon hydrolysis or binding. Thiiranes have been postulated as possible alternative reactive intermediates that may be formed from fluorinated cysteine conjugates (fig. 7A) (Dohn et al., 1985b; Commandeur, 1991). Ab initio calculations of the free enthalpies of formation of the two possible decomposition products of fluorinated ethanethiol products show that for TFE-Cys formation of a difluorothionoacyl fluoride is energetically favorable, whereas for CTFE-Cys, formation of the thiirane was energetically more favorable (Commandeur, 1991). However, this pathway still has not been conclusively demonstrated or disproven.

Because of their high instability and reactivity, the secondary metabolism of the thiols formed is not expected to play an important role in the bioactivation of this type of nephrotoxic cysteine S-conjugate (fig. 6, steps l, n and m). However, the corresponding sulphenic acid (Nash et al., 1984) and S-methyl conjugate (Reichert et al., 1985), which may result from enzymic S-oxygenation and S-methylation of 1-mercapto-1,2,3,4,4-pentachloro-1,3-butadiene, respectively, have been detected as urinary metabolites of HCBD (fig. 11). Because sulphenic acids usually are highly reactive compounds, the occurrence of a sulphenic acid in urine of rats treated with HCBD is rather surprising (Van den Broek et al., 1990). The sulphenic acid, however, was not detected directly but as a trimethylsilyl-ester after derivatization of urine (Nash et al., 1984). Recently, it was demonstrated that an identical trimethylsilyl-ester is formed upon trimethyl-silylation of the sulfoxide of HCBD-S-conjugates (Commandeur, unpublished results). As an alternative route leading to formation of the S-methyl conjugate of HCBD not involving formation of reactive thiols, decarboxylation of the 2-mercaptoacetic acid S-conjugate of HCBD has been proposed (fig. 6, step o) (Reichert et al., 1985).

6. Bioactivation of antitumor agents by β-lyase. Selective nephrotoxicity of a number of cysteine conjugates, can be explained by the selective renal uptake and metabolism of cysteine S-conjugates by renal β-lyase. Recently, it has been proposed that this mechanism might be used to selectively deliver sulfur-containing antitumor prodrugs to the kidneys (Hwang and Elfarra, 1989; Elfarra and Hwang, 1993). Currently, treatment of renal cell carcinoma is surgery because of the absence of an effective chemotherapeutic agent. P-Cys was designed as a potential kidney-selective prodrug of the antitumor and immunosuppressant drug 6-mercaptopurine (fig. 18). 6-Mercaptopurine is known to have anticancer activity against solid tumors in laboratory animals. However, the use of 6-mercaptopurine in patients with solid tumors is limited because of the severe bone marrow and hepatic toxicity associated with 6-mercaptopurine toxicity (Van Scoik et al., 1985). The development of tissue-selective prodrugs of 6-mercaptopurine may enhance the therapeutic index of the compound. When rats were given P-Cys, both 6-mercaptopurine and further metabolites were detected in the kidneys, liver, or plasma. However, concentrations in the kidneys were 100-fold greater than those in plasma and were nearly 2.5-fold greater than those in the liver (Hwang and Elfarra, 1993). The reduction of the renal metabolite concentrations after pretreatment with aminooxyacetic acid provides evidence for the role of renal β-lyase in the renal selectivity of P-Cys. In vitro metabolism of P-Cys by renal mitochondria occurred at a four-fold higher rate than that of renal cytosol (Hwang and Elfarra, 1989). The specific activity in renal fractions were nearly three-fold higher than that in liver fractions, indicating that differences between the rates of metabolism may contribute to the higher accumulation of 6-mercaptopurine

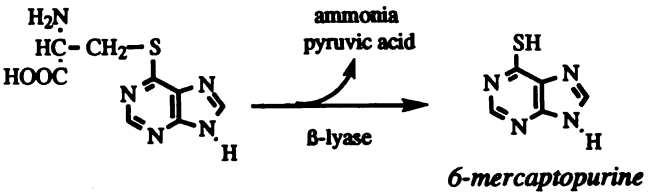


FIG. 18. β-Lyase-dependent bioactivation of S-(6-purinyl)-l-cysteine to the antitumor-agent 6-mercaptopurine.

in the kidney when compared with the liver. The observation that P-Cys was not acutely nephrotoxic suggests that this compound may be useful in the treatment of renal cell carcinoma and/or as an immunosuppressant in kidney transplants.

E. Cystathionase

The enzyme cystathionase (EC 4.4.1.1 or cystationine γ-lyase), also referred to as homoserine dehydratase, cleaves the C-S linkage in several derivates of L-methionine and L-cysteine by α,γ- and in a few instances, α,β-elimination reactions, thus stochiometrically forming the corresponding thiol products, α-keto acids, and ammonia (Tomisawa et al., 1988b). The enyzme, which is found in most tissues, consists of four identical subunits with molecular weight of 40,000 Da. The substrate selectivity is different from that of purified β-lyase. Cystathionase is active toward L-cysteine and S-alkyl (n-propyl, i-propyl-, n-butyl-, t-butyl-, cyclohexyl-) conjugates of L-cysteine, whereas β-lyase is not (Tateishi, 1983; Tomisawa et al., 1988b). S-(*p*-Bromophenyl)-L-cysteine and 1,2-DCV-Cys, two good substrates for mammalian β-lyase, were not cleaved by cystathionase. It was shown that the active center of rat liver cystathionase contained two binding sites referred to as the C_3- and C_4-sites (Billy et al., 1975). L-Cysteine and S-alkyl-L-cysteine conjugates bind to the C_3-site, whereas L-homoserine and L-homocysteine bind to the C_4-site. This explains why dithiotreitol, which has a high affinity to the C_3-site, inhibits activity with L-cysteine and S-alkyl-L-cysteine as substrates but is without effect with L-homoserine as a substrate (Tomisawa et al., 1988b).

Whether cystathionase is involved in the bioactivation of toxic S-conjugates is at present not known. Although the hepatic enzyme was not active toward 1,2-DCV-Cys, it has been suggested that a renal cystathionase would be involved in the bioactivation of the very nephrotoxic 1,2-DCV-Cys-analog, 1, 2-DCV-HCys (Elfarra et al., 1986b). 1,2-DCV-HCys is first transaminated to give a 2-oxo acid that subsequently decomposes nonenzymatically via a retro-Michael mechanism to give a reactive thiol and 2-oxo-3-butenoic acid (Lash et al., 1990b). Other enzymes that are also active in this bioactivation mechanism are purified bovine kidney glutamine transaminase K/β-lyase and L-amino acid oxidase from snake venom.

F. Cysteine S-conjugate N-acetyltransferase

The final step in the mercapturic acid pathway is N-acetylation of L-cysteine S-conjugates by cysteine S-conjugate N-acetyltransferase (fig. 6, step c) (Barnsley et al., 1969; Green and Elce, 1975). A cysteine S-conjugate N-acetyltransferase (EC 2.3.1.80) has been isolated from rat kidney microsomes (Duffel and Jakoby, 1982). The enzyme requires acetyl coenzyme A as a cosubstrate and has an estimated molecular weight of 34,000 Da. The efficiency of catalysis by the purified rat renal N-acetyltransferase increases with the lipophilicity of the sulfur-bound substituent; a very high correlation (r = 0.99) of the Hansch π constants, as a measure of lipophilicity, with V_m/K_m as a measure of catalytic efficiency was observed (Duffel and Jakoby, 1982). The hydrophilic substrate S-carboxymethyl-L-cysteine is not acetylated by this enzyme. S-Conjugates of D-cysteine and the L- and D-isomers of cysteine were not substrates. N-Acetyltransferase activity is inhibited by organic anions both in vivo and in vitro. Probenecid, a potent inhibitor of the organic anion transporter, significantly inhibited the N-acetylation of S-benzyl-L-cysteine (Okajima et al., 1984; Inoue et al., 1984b). Cysteine S-conjugate N-acetyltransferase is located at the endoplasmatic membrane in different tissues (table 1), with the active site faced to the cytoplasmic surface (Okajima et al., 1984). Cysteine S-conjugate N-acetyltransferase is specific for L-cysteine S-conjugates and not for substrates of the cytosolic amine N-acetyltransferases. The polymorphism associated with cytosolic N-acetyltransferases in the human population has so far not been shown to be related to microsomal cysteine conjugate N-acetyltransferase.

A considerable species variation has been observed in the urinary excretion of mercapturic acids. In the rat and hamster, N-acetylation was by far the most important route of metabolism of S-pentyl-L-cysteine (50 to 80%); the guinea pig, however, was found to excrete only 2% of the dose as the mercapturic acid, indicating a low activity of N-acetylation compared with the alternative routes of cysteine conjugate biotransformation (James and Needham, 1973).

In the kidney, N-acetyltransferase activity is located in cells of the proximal tubule (Hughey et al., 1978); it was shown that the N-acetyltransferase capacity was localized in the proximal tubular region. The capacity for acetylation and/or secretion in the straight part of the proximal tubule was almost twice that of the convoluted part (Heuner et al., 1990, 1991). When comparing specific activities of the N-acetyltransferase, with S-benzyl-L-cysteine as a substrate, in different organs, the highest specific activity was observed in the kidney being six-fold higher than that in the liver and more than 70-fold higher than in the small intestine (table 1) (Inoue et al., 1984b). However, i.v. administration of S-benzyl-L-cysteine resulted in a rapid acetylation of the cysteine S-conjugate in liver and kidney to a similar extent: partial hepatectomy (75%) resulted in a 50% decrease in urinary excretion of the corresponding mercapturic acid (Okajima et al., 1984). The relatively high contribution of the liver may be explained by the higher amount of protein in liver when compared with the kidney, resulting in an higher total activity in this organ (Inoue et al., 1984b).

Kinetic analysis revealed that the major part of orally administered S-benzyl-L-cysteine was transferred to the liver and acetylated predominantly in this organ (Inoue et al., 1987). Therefore, hepatic N-acetylation seems to

play a significant role in the formation of mercapturic acids in vivo. From the fact that the GSH S-conjugate of paracetamol was metabolized to the mercapturic acid by isolated kidney cells, but not by hepatocytes, it was concluded that the kidney was the site of N-acetylation (Jones et al., 1979). However, this observation also might be explained by a low GGT-activity in hepatocytes, as a result of which insufficient formation of cysteine S-conjugate occurred.

Mercapturates formed in the liver are transported to the plasma by a probenecid-sensitive mechanism, and only to a small extent to the bile (Inoue et al., 1987). In the plasma, the mercapturates are bound to albumin and transported to the kidney and actively excreted by the organic anion system.

1. N-Acetylation of nephrotoxic cysteine S-conjugates. N-acetyl-L-cysteine S-conjugates are not substrates for β-lyase. Therefore, N-acetylation of nephrotoxic cysteine S-conjugates can be regarded as a detoxification pathway. As seen with S-benzyl-L-cysteine (table 1), the specific activity of N-acetylation of cysteine S-conjugates of fluoroethylenes in kidney is higher than in the liver (table 7). Because the kidney is the target-organ for toxicity of these S-conjugates, other factors, such as a higher β-lyase-activity and concentration of the cysteine S-conjugate, apparently are more important factors in determining the organ selectivity of toxicity. The concentration of the cysteine S-conjugate will partially depend on the local balance between N-deacetylation and N-acetylation and seem to be higher in the kidney (table 7). In addition, the activity of uptake systems active in the uptake of S-conjugates may be higher in the kidney when compared with the liver.

N-acetylation is the main route of metabolism of 1,2-DCV-Cys in rats; 41.5% of a given dose of 1,2-DCV-Cys was excreted within 24 hours in urine as its mercapturic acid, 1,2-DCV-NAC (Derr and Schulze, 1963). Other compounds in urine were unchanged 1,2-DCV-Cys (18.5%) and inorganic sulfate (34%), which is believed to be derived from a reactive thiol formed by the β-lyase pathway. The corresponding mercapturic acid could not be identified in urine of calves (Derr et al., 1963), because the activity of N-acetyltransferase in calf tissue was only 10% of that in the corresponding rat tissues (Bakke et al., 1990b). These low levels of N-acetyltransferase activities in calf tissues may explain the high susceptibility of calves to 1,2-DCV-Cys-induced toxicity (Terracini and Parker, 1965). In calves, 1,2-DCV-Cys produces aplastic anemia; whether this type of toxicity is also mediated by β-lyase is not known. A higher rate of N-acetylation also has been proposed to contribute to the lower nephrotoxicity of 1,2-DCV-Cys in rats when compared with corresponding homocysteine S-conjugate (Elfarra et al., 1986b). However, the homocysteine S-conjugate may also be more toxic because of the bioactivation to both a sulfur-containing reactive intermediate as well as a highly reactive vinylglyoxylate (Cooper et al., 1989).

1,2-DCV-NAC has been identified in urine of rats exposed to dichloroacetylene (10% of exposed dose) (Kanhai et al., 1989, 1991) and in trace amounts in urine of rats treated with trichloroethylene (Dekant et al., 1986b, 1990; Commandeur and Vermeulen, 1990b). Up to 60% of the dose of DCDFE was excreted in the urine of rats in the form of DCDFE-NAC (Commandeur et al., 1987).

Both the cysteine S-conjugate and the mercapturic acid, PCBD-NAC have been identified in comparable amounts in urine of mice treated with HCBD (Dekant et al., 1988c.). PCBD-NAC has also been identified in urine of rats treated with HCBD (Reichert and Schutz, 1986). The N-acetylcysteine S-conjugate of HCBD was detected as the major metabolite in urine and in perfusate of isolated rat kidneys perfused with the cysteine S-conjugate or glutathione conjugate of HCBD (Schrenk et al., 1988). The identification of TCV-NAC in urine of rats treated with tetrachloroethylene also indicated N-acetylation of the potent nephrotoxin TCV-Cys in vivo (Dekant et al., 1986c). TCV-NAC represents 1% of urinary metabolites of tetrachloroethylene, whereas dichloroacetic acid, which is presumed to result from β-lyase catalyzed activation of TCV-Cys, constituted 5% of urinary metabolites. This might point to a preference of β-lyase acti-

TABLE 7

*Specific activities of enzymes active in the bioactivation and inactivation of S-conjugates of fluorinated alkenes, in relation to their nephrotoxicity**

S-conjugated moiety	Liver				Kidney				Lowest nephrotoxic dose‖
	N-Deacetylase† (A)	N-acetyl-transferase‡ (B)	Ratio (A)/(B)	β-lyase§	N-Deacetylase† (A)	N-acetyl-transferase‡ (B)	Ratio (A)/(B)	β-lyase§	
S-(1,1,2,2-tetrafluoroethyl)-	15	10	1.50	5.7	116	60	1.93	24	50
S-(2-chloro-1,2,2-trifluoroethyl)-	8	26	0.31	4.6	66	88	0.75	23	25
S-(2,2-dichloro-1,1-difluoroethyl)-	1.7	26	0.07	4.4	11	80	0.14	15	75
S-(2,2-dibromo-1,1-difluoroethyl)-	1.5	40	0.04	3.7	13	58	0.22	10	100

* Commandeur et al., 1989; Commandeur, 1991.

† nM per minute per mg cytosolic protein using the N-acetylcysteine S-conjugate (4 mM) as substrate.

‡ nM per minute per mg microsomal protein using the cysteine S-conjugate (4 mM) as substrate.

§ nM per minute per mg cytosolic protein using the cysteine S-conjugate (4 mM) as substrate.

‖ lowest nephrotoxic dose of corresponding N-acetyl S-conjugate when administered intraperitoneally to male Wistar rats.

vation of TCV-Cys when compared with deactivation by N-acetylation.

G. N-Deacetylases

In several tissues, enzymes are present that antagonize the action of the N-acetyltransferases by catalyzing the deacetylation of mercapturic acids. Because the regenerated cysteine S-conjugates again can be bioactivated by β-lyase, N-deacetylation may be regarded as a bioactivation step. N-Deacetylase activity toward S-alkyl-, S-aralkyl-, and S-aryl-mercapturic acids have been demonstrated in the liver and kidney of the rat, rabbit and guinea pig; the specific activity of kidney fractions was greater than that of liver fractions (Bray and James, 1960). This activity is also present in the intestinal mucosa (Tateishi, 1983). Both in rat kidney and liver, multiple enzymes are known to catalyze N-deacetylation of mercapturic acids. In the kidney, two enzymes, acylase I (EC 3.5.1.14 or aminoacylase) and acylase III, seemed to be active toward mercapturic acids. Renal acylase I exhibited high activity toward S-alkyl mercapturic acids but much lower or no activity toward S-aralkyl and S-aryl conjugates. Acylase I-like activity was also found in other tissues, with the highest activity found in the kidney (table 1) (Endo, 1978). Acylase III has higher activity toward S-aryl and S-aralkyl mercapturic acids and much lower activity toward S-alkyl mercapturic acids. Acylase III seems to be present in many tissues and has the highest specific activity in the kidney (table 1) (Endo, 1978). A deacetylase has also been purified from rat liver (Suzuki and Tateishi, 1981). The molecular weight of rat hepatic deacetylase, 145.000 Da, seems to be much higher than rat renal acylase III, which is estimated to be 55,000 Da. The hepatic enzyme seems to be a tetramer of subunits with molecular weights of 35,000 Da.

1. N-deacetylation of nephrotoxic mercapturic acids. Several in vivo studies demonstrate the involvement of N-deacetylases in the nephrotoxicity of S-conjugates. The mercapturic acid PCBD-NAC appeared to cause nephrotoxicity similar to the parent compound HCBD (Nash et al., 1984). This was attributed to the metabolism of PCBD-NAC to the corresponding cysteine S-conjugate, and subsequent bioactivation by renal β-lyase. N-Deacetylation of PCBD-NAC was demonstrated both with renal and hepatic cytosol of the rat (Vamvakas et al., 1987; Pratt and Lock, 1988). The activity in renal fractions was two times higher than in hepatic fractions. Similarly, the mercapturic acids of TFE (TFE-NAC), CTFE (CTFE-NAC), CDCFE (DCDFE-NAC), and DBDFE (DBDFE-NAC) were all very potent nephrotoxins when administered to male Wistar rats (table 7) (Commandeur et al., 1988).

The role of deacetylation in the nephrotoxicity of the mercapturic acids of halogenated alkenes was studied in vivo by treating rats with N-trideuteroacetyl mercapturates (Commandeur and Vermeulen, 1990b; Commandeur et al., 1991b). The highly toxic mercapturic acids TFE-NAC and CTFE-NAC were shown to be efficiently deacetylated; less than 1% of the dose was excreted in urine as mercapturic acid. The less toxic mercapturic acids, DCDFE-NAC and DBDFE-NAC, however, were excreted unchanged, i.e., still containing the N-trideuteroacetyl-group, up to 17 and 31% of the dose, respectively. Significant amounts of unlabeled mercapturic acids were detected in case of DCDFE-NAC and DBDFE-NAC, that is, 48 and 28% of the initial dose, respectively. This demonstrates that after deacetylation of the parent mercapturic acid, a significant fraction of the cysteine S-conjugates formed are reacetylated again by cysteine conjugate N-acetyltransferase.

The specific activity of N-deacetylases toward some mercapturic acids of fluorinated ethylenes is shown in table 7. As with N-acetyltransferase, the highest specific activity of N-deacetylase again was present in the renal fractions, being approximately seven- to eight-fold higher than in hepatic fractions. As can be seen from table 7, the nature of the halogens present in the S-bound moiety has a much higher effect on the rate of N-deacetylation of the corresponding mercapturic acids than on the rate of N-acetylation and β-elimination reactions of the corresponding cysteine S-conjugates. A very high N-deacetylation/N-acetylation ratio, combined with a higher β-lyase activity therefore may explain the higher nephrotoxicity of TFE-NAC and CTFE-NAC when compared with that of DCDFE-NAC and DBDFE-NAC (table 7). The differences in N-deacetylation rates are apparently mainly responsible for the differences in urinary excretion of these mercapturic acids.

By studying the metabolism of TFE-Cys in renal 9000 g-supernatant, containing both N-acetyltransferase activity and N-deacetylase activity, it was shown that a time-dependent formation of the corresponding mercapturic acid was measurable despite the relatively high N-deacetylase activity (Commandeur et al., 1989). The availability of acetyl coenzyme A as a cosubstrate, however, seems to be limiting after several minutes of incubation, leading to deacetylation of the mercapturic acid formed. Whether availability of acetyl coenzyme A also is limiting in the in vivo situation is not yet known, however. A similar role for the balance between N-deacetylating and N-acetylating enzymes in determining the relative toxicities of S-conjugates has been suggested in the observed species differences of the toxicity of 2-bromo-(diglutathion-S-yl)-hydroquinone (Lau et al., 1990b).

The two regioisomeric mercapturic acids derived from trichloroethylene, N-trideuteroacetyl-1,2-DCV-NAC) and N-trideuteroacetyl-2,2-DCV-NAC), were shown to be excreted in rat urine in approximately equal amounts unchanged and as nonlabeled mercapturic acids that result from deacetylation/acetylation cycling (Commandeur et al., 1991b). Although the availability of cysteine S-conjugates to β-lyase is approximately comparable, the 1,2-

DCV-isomer is probably more relevant for the observed nephrotoxicity and suspected nephrocarcinogenicity of trichloroethylene than the 2,2-DCV-isomer. Firstly, the β-lyase-activity is higher with 1,2-DCV-Cys than with 2,2-DCV-Cys as substrate. Secondly, the reactive intermediates formed from 1,2-DCV-Cys (thionoacyl chloride and/or thioketene) most probably will be much more reactive than that formed from 2,2-DCV-Cys (thioaldehyde). Indeed, both the nephrotoxicity and mutagenicity of the 1,2-DCV-conjugates were shown to be much higher than that of the corresponding 2,2-DCV-conjugates (Commandeur et al., 1991a). The fact that 1,2-DCV-NAC has recently been identified in urine of workers exposed to high levels of trichloroethylene indicates that humans are also exposed to the mutagenic cysteine S-conjugate, which may have important implications for the risk assessment (Birner et al., 1993a). However, because the balance of N-acetylation and N-deacetylation in humans is not yet known, the availability of 1,2-DCV-Cys to human β-lyase is also not known. Because renal β-lyase activity in human kidney is only 10% of that in rat kidney (Lash et al., 1990a), however, humans may equally well be at much lower risk.

H. Cysteine Conjugate Deamination Enzymes

An alternative route of metabolism of cysteine S-conjugates, next to C-S cleavage and N-acetylation, is deamination to yield the corresponding 3-mercaptopyruvic acid S-conjugates (fig. 6, step e). Because the guinea pig and rabbit are poor excretors of mercapturic acids, excretion of deamination products was shown to be more important after administration of benzyl isothiocyanate to these species (Görler et al., 1982). Deamination reactions can be divided into two categories, namely, transamination and oxidative deamination. These reactions are catalyzed by different enzyme systems, respectively transaminases and oxidases.

1. Cysteine conjugate transaminase. Transamination reactions of amino acids are catalyzed by a variety of PLP-dependent enzymes. The cofactor PLP functions as an acceptor of the amine-group, thereby converting into the pyridoxamine phosphate form (fig. 12). Pyridoxamine phosphate subsequently can donate its amine group to an acceptor, α-keto acid, thereby returning to the active PLP-form. The PLP-dependent enzyme, cysteine conjugate β-lyase from rat kidney cytosol, was shown to catalyze deamination of 1,2-DCV-Cys to the corresponding α-keto acid S-(1,2-dichlorovinyl)-3-mercaptopyruvic acid (Stevens et al., 1986b). Addition of α-keto acids to the purified enzyme resulted in higher yields of 3-mercaptopyruvic acid S-conjugate by competitive inhibition of the reversed reaction.

From rat liver cytosol, three enzymes (CAT-I, CAT-IIA and CAT-IIB) that are active in the transamination of cysteine S-conjugates, have been purified and characterized (Tomisawa et al., 1988a). The main cysteine S-conjugate, α-ketoglutarate transaminase (CAT-I), has a molecular weight of 64,000 Da as determined by gel filtration and was active toward S-(*p*-bromophenyl)-L-cysteine, S-benzyl-L-cysteine, S-phenyl-L-cysteine, S-(*n*-propyl)-L-cysteine, and S-(*n*-butyl)-L-cysteine. S-Methyl-, S-ethyl-, S-(*i*-propyl)-, S-(*t*-butyl)-, S-cyclohexyl)-, 1,2-DCV-, and S-(2-chloroethyl)-L-cysteine were inactive as substrates. All three transaminases showed no or very low activity toward endogenous amino acids, except for CAT-IIB, which was active toward L-aspartic acid and L-cysteinesulphinic acid. α-Ketoglutaric acid had the highest activity as an amino-acceptor. The two minor aminotransferases, CAT-IIA and CAT-IIB, closely resembled the main aminotransferase with respect to molecular weight and substrate specificity. However, the ability to catalyze the reverse reaction, e.g., amination of 3-thiopyruvic acid S-conjugates, was higher with CAT-IIA and CAT-IIB when compared with CAT-I. The three enzymes did not catalyze β-elimination reactions of cysteine S-conjugates.

Recently, an additional enzyme with transaminase activity, distinct from β-lyase, was identified in the rat kidney cytosol (Abraham and Cooper, 1991). This enzyme has an apparent molecular weight of 330,000 Da and shows a lower transamination activity toward 1,2-DCV-Cys when compared with β-lyase. Additional PLP-dependent transaminases active toward cysteine S-conjugates of xenobiotics, but distinct from β-lyase, have been purified from rat liver (Tomisawa et al., 1988a). In contrast to the renal β-lyase, these enzymes did not catalyze β-elimination reactions.

The involvement of transaminases in the bioactivation of cysteine S-conjugates was indirectly demonstrated by the ability of 1,2-DCV-D-cysteine to induce nephrotoxicity (Wolfgang et al., 1989a). This cysteine S-conjugate, which is not a substrate for β-lyase, can be deaminated to the corresponding 3-mercaptopyruvic acid conjugate, which, in turn, can be transaminated again to the corresponding nephrotoxic L-cysteine-isomer.

2. L-Amino acid oxidase. The second mechanism of deamination of cysteine S-conjugates to 3-mercaptopyruvic acid S-conjugates is oxidative deamination by L-amino-acid oxidases (EC 1.4.3.2), which are flavin-dependent enzymes present in peroxisomes, mitochondria, and cytosol (Cromartie and Walsh, 1975; Stevens et al., 1989). In the rat kidney, at least three oxidases are present in cytosol (Cromartie and Walsh, 1975). The major isoenzyme was purified and seemed to have a tetrameric structure with subunits of M_r 47,500. This isoenzyme seems to be identical with the single isoenzyme found in the mitochondrial fractions. L-Amino acid oxidases require molecular oxygen for activity and oxidize amino acids and L-α-hydroxy acids to the corresponding α-keto acids, ammonia, and hydrogen peroxide. From rat kidney cytosol, an L-amino acid oxidase was purified, which metabolized 1,2-DCV-Cys selectively by oxidative deamination (Stevens et al., 1989). Other nephrotoxic cysteine S-conjugates also are deami-

nated by the purified enzyme. Interestingly, the chlorinated alkenyl S-conjugates are better substrates than the fluorinated alkyl S-conjugates (table 6). The participation of these enzymes in deamination of 1,2-DCV-Cys by rat kidney homogenate was estimated by using selective inhibitors of PLP-dependent transamination (AOAA) and L-amino-acid oxidase (2-hydroxy-3-butynoate) (Stevens et al., 1989). Deamination seemed to be the major route of metabolism of 1,2-DCV-Cys: 65% of total metabolism. Both L-amino-acid oxidase and β-lyase were reported to contribute to the deamination, 45 and 55%, respectively. However, because AOAA also inhibits other PLP-dependent enzymes, β-lyase is not necessarily the only enzyme that accounts for transamination of 1,2-DCV-Cys in renal homogenates. From the fact that inhibition of L-amino-acid oxidase did not reduce covalent binding of 1,2-DCV-Cys in rat kidney homogenate and the fact that, upon incubation of 1,2-DCV-Cys with purified oxidase, no covalent binding occurred, it was concluded that the α-keto acid of 1,2-DCV-Cys is not reactive toward cellular macromolecules.

The nephrotoxicity caused by 1,2-DCV-D-cysteine is believed to result from an initial oxidative deamination by renal D-amino acid oxidases, followed by transamination of the 3-mercaptopyruvic acid conjugate to form 1,2-DCV-Cys (fig. 6, step h). The L-cysteine S-conjugate, in contrast to the corresponding D-isomer, can be bioactivated by β-lyase (Wolfgang et al., 1989a).

A cyclic mercaptopyruvic acid was identified in urine upon oral administration of the cysteine S-conjugate of benzyl isothiocyanate to guinea pigs and rabbits (Görler et al., 1982). The intramolecular cyclization prevents further metabolism of the mercaptopyruvic acid moiety. However, 3-mercaptopyruvic acid S-conjugates are usually excreted only in extremely low amounts, caused by secondary metabolism. Reactions that may occur are transamination, again forming the corresponding cysteine S-conjugates, reduction to 3-mercaptolactic acid S-conjugates, and decarboxylation to a mercaptoacetic acid S-conjugates.

I. 3-Mercaptopyruvic Acid S-Conjugate Reductase

An important reaction of 3-mercaptopyruvic acid conjugates is reduction to the 3-mercaptolactic acid conjugates. To date, 3-mercaptopyruvic acid S-conjugate reductase activities have been demonstrated in several rat tissues (table 1) (Tomisawa et al., 1990). The highest of this reductase activity was observed in the liver followed by testis, brain, and kidney. From rat liver cytosol, three different 3-mercaptopyruvic acid S-conjugate reductases with different substrate specificity were isolated. A considerable species variation was seen in this type of cysteine S-conjugate metabolism. When rodents are treated with S-butyl-L-cysteine and S-pentyl-L-cysteine, the corresponding 3-mercaptolactic acid S-conjugates can be detected in urine of mouse, rabbit, and guinea pig, but only in trace amounts in urine of rat and hamster (James and Needham, 1973; Harris et al., 1968). When the rat was dosed with S-pentyl-3-mercaptopyruvic acid, only very small amounts of unchanged compound and the corresponding 3-mercaptolactic acid S-conjugate were present in urine. The major metabolite was N-acetyl-S-(pentyl)-L-cysteine, indicating that transamination to the cysteine S-conjugate followed by N-acetylation is the main route of biotransformation in the rat (James and Needham, 1973). In line with this, only trace amounts of 3-mercaptolactic acid S-conjugates have been detected in urine of rats treated with E- or Z-1,3-dichloropropene, compounds known to be predominantly metabolized by GSH-conjugation (Onkenhout et al., 1986). Recently, an endogenous 3-mercaptolactic acid S-conjugate derived from a GSH-conjugate of urocanic acid, a L-histidine catabolite, was identified in human urine (Kinuta et al., 1994).

J. 3-Mercaptolactic Acid S-Conjugate Oxidase

Recently, it was shown that incubation of rat liver homogenate with a 3-mercaptolactic acid S-conjugate gave the corresponding cysteine S-conjugate via sequential enzymic oxidation and transaminaton (fig. 6, step p and h) (Tomisawa et al., 1992). From rat liver cytosol, two distinct 3-mercaptolactic acid S-conjugate oxidases (3-mercaptolactic acid S-conjugate oxidase-I and 3-mercaptolactic acid S-conjugate oxidase-II) were purified. The two enzymes showed a molecular mass of 160 and 250 kDa and were composed of four and six subunits of 41 and 39 kDa, respectively. Both enzymes contain flavin mononucleotide as their prosthetic group and oxidized several aromatic and aliphatic S-substituted L-3-mercaptolactic acid S-conjugates. These enzymes provide another route by which nephrotoxic cysteine S-conjugates can be generated. To date, however, no clear evidence exists of the involvement of these enzymes in the toxicity of cysteine S-conjugates.

K. Decarboxylation

Small amounts of 2-mercaptoacetic acid S-conjugates, possibly resulting from decarboxylation of the corresponding 3-mercaptopyruvic acid conjugates (fig. 6, step j), have been identified in urine of rats treated with either S-methyl-L-cysteine (Barnsley, 1964; Sklan and Barnsley, 1968; Mitchell et al., 1984), HCBD (Reichert et al., 1985, 1986), vinylidene chloride (Reichert et al., 1979), or vinyl chloride (Green and Hathway, 1977). Decarboxylation of α-keto acids might be enzymic, catalyzed by lipoic acid-dependent enzymes, or nonenzymic, by hydrogen peroxide. L-Amino acid oxidase produces, in addition to the 3-mercaptopyruvic acid S-conjugate, ammonia and hydrogen peroxide. The very high activity of L-amino acid oxidase toward PCBD-Cys, followed by decarboxylation, might explain the relatively high abundance of the mercaptoacetic acid S-conjugate of HCBD in rat urine (Reichert et al., 1985, 1986). A second decarboxylation reaction of mercaptoacetic acid S-conjugates

(fig. 6, step o) was suggested to be an alternative, β-lyase-independent route to the formation of methylthiolated metabolites (Reichert et al., 1985).

Incubation of S-(*p*-bromophenyl)-3-mercaptopyruvic acid in the presence of rat renal cytosol has been shown to result in the formation of the corresponding thiol compound, 1-bromo-4-mercaptobenzene (fig. 6, step k) (Tomisawa et al., 1986b). This observation suggests the presence of an alternative route of formation of potentially toxic thiol compounds. However, whether cleavage of the 3-mercaptopyruvic acid S-conjugate to the thiol is direct or proceeds via sequential transamination and β-elimination steps (fig. 6, steps h and d) is not yet clear.

L. S-Oxygenating Enzymes

Sulfoxides of cysteine S-conjugates, mercapturic acids, 3-mercaptolactic acid S-conjugates, and mercaptoacetic acid S-conjugates have been identified as metabolites in vivo. Treatment of rats with S-methyl-L-cysteine (Barnsley, 1964) and S-carboxymethyl-L-cysteine (Waring and Mitchell, 1982) led to excretion of the corresponding cysteine S-conjugate sulfoxides in urine. The main urinary metabolites of S-carboxymethyl-L-cysteine in humans were thiodiacetic acid and its sulfoxide (Staffeldt et al., 1991). Thiodiacetic acid is formed by subsequent deamination and decarboxylation of S-carboxymethyl-L-cysteine. Small amounts of mercapturic acid sulfoxides have been detected in urine of rodents treated with allyl halides (Kaye et al., 1972), S-alkyl-L-cysteine S-conjugates (James and Needham, 1973; Barnsley, 1964), and the mercapturic acid of propachlor (Bakke et al., 1981; Rafter et al., 1983). The sulfoxide of a 3-mercaptolactic acid conjugate has been identified in urine of rats treated with S-methyl-L-cysteine (Sklan and Barnsley, 1968).

Sulfoxidation of cysteine S-conjugates (fig. 6 step f) is catalyzed by cytosolic as well as microsomal fractions. S-Carboxymethyl-L-cysteine is sulfoxidized by a cytosolic enzyme in the liver; no activity was shown in microsomal fractions. Interestingly, approximately 30% of the population is deficient in this sulfoxidation activity (Mitchell and Waring, 1989). From twin and family studies, it was concluded that impaired sulfoxidation is genetically determined; however, the final proof must await the isolation and characterization of specific gene products. No relation has been observed between polymorphisms of S-carboxymethyl-L-cysteine and debrisoquine 4-hydroxylase (Haley et al., 1985).

S-Benzyl-L-cysteine is sulfoxidized by microsomal (but not by cytosolic) fractions of rat kidney and liver (Sausen and Elfarra, 1990a). The intracellular localization of sulfoxidation may depend on the lipophilicity of the cysteine S-conjugates. The microsomal S-oxidation of cysteine S-conjugates was shown to be catalyzed by FMOs (Sausen et al., 1990a). Sulfoxidation of S-benzyl-L-cysteine was observed with both hepatic and renal microsomes, the renal fraction having a two-fold higher specific activity (Sausen et al., 1993). A marked sex difference in S-oxidase activities was shown; in male rat liver and kidney microsomes, S-oxidase activities were nearly 2.5- and 3.3-fold higher, respectively, than those detected in the corresponding female tissues (Sausen et al., 1993).

Based on the primary structures of isolated FMO-isoenzymes, the FMOs have been divided into five classes: 1A1, 1B1, 1C1, 1D1, and 1E1 (Ziegler, 1993). Analysis of genomic DNA suggests that all five genes are present in humans, rats, mice, guinea pigs, rabbits, hamsters, and pigs. Purified cysteine conjugate S-oxidases from rat liver and kidney had apparent molecular weights of approximately 56,000 and were shown to be immunoreactive, with antibodies raised against the pig liver FMO 1A1 isoenzyme. Also, the amino-terminal amino acid sequence was comparable to that of pig and rabbit liver FMO 1A1 isoenzymes. Recently, a second FMO, FMO 1D1, was shown to be capable of sulfoxidizing S-benzyl-L-cysteine (Elfarra et al., 1993).

Purified pig liver FMO 1A1 was active in the sulfoxidation of both cysteine S-conjugates and N-acetylcysteine S-conjugates (Park et al., 1992). The cysteine S-conjugates were preferred substrates and N-acetylation decreased activity. Chemical sulfoxidation of S-conjugates results in the formation of two diastereomeric sulfoxides. However, pig liver FMO 1A1 was shown to be diastereoselective, and diastereoselectivity was much greater for cysteine S-conjugates than for mercapturic acids.

Very recently, FMO 1A1 was shown to have a high affinity and activity in the sulfoxidation of farnesyl-L-cysteine methyl ester (Park et al., 1994). The *ras* protein possesses a farnesylated L-cysteine methyl ester at the C-terminus that is important for membrane association. If this group is also a substrate for FMO, the S-oxygenated farnesylated *ras* protein may not be stable and may cleave the farnesyl group to provide an inactivated protein that would not be associated with the plasma membrane. This activity may be an important physiological role of FMO that requires further studies.

1. Role of sulfoxidation of S-conjugates in toxicity. Evidence is accumulating that sulfoxidation may play an important role in the toxicity of S-conjugates. L-Cysteine S-conjugate sulfoxides have also been shown to be substrates for β-lyase. Specific activity of human hepatic β-lyase was higher with S-phenyl-L-cysteine sulfoxide than with S-phenyl-L-cysteine as the substrate (Tomisawa et al., 1986a). Cleavage of a L-cysteine S-conjugate sulfoxide by β-lyase would lead to formation of a sulphenic acid (fig. 6 step d), which, as mentioned previously, might contribute to toxic effects. In presence of glutathione, β-lyase-catalyzed degradation of the sulfoxide of S-(*p*-bromophenyl)-L-cysteine resulted in the formation of *p*-bromothiophenol (fig. 19) (Tomisawa et al., 1993). It was suggested that the sulphenic acid formed initially is rapidly converted to a thiosulphinate by in-

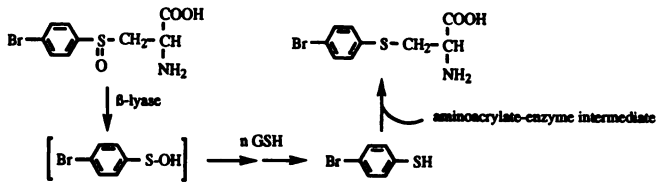


FIG. 19. Proposed mechanism of the β-lyase-mediated reduction of the sulfoxide of S-(*p*-bromophenyl)-l-cysteine in presence of glutathione.

termolecular dehydration, which, in the presence of glutathione, liberates the thiol by thiol-disulphide exchange. The thiol-compound released may associate with the aminoacrylate-enzyme intermediate resulting in formation of a cysteine-conjugate. This route therefore may be regarded as an alternative route producing potentially toxic thiol-compounds and/or as a sulfoxide reductase pathway.

The S-oxide of 1,2-DCV-Cys causes nephrotoxicity in rats (Sausen and Elfarra, 1990b). The sulfoxide of 1,2-DCV-Cys is also a better substrate of renal β-lyase of bovine origin than 1,2-DCV-Cys itself (Anderson and Schultze, 1965). However, β-lyase seems not to be involved in this bioactivation mechanism, because the S-oxide was shown to be a direct-acting electrophile (fig. 20A). Glutathione adducts were shown to be present in the bile of rats treated with 1,2-DCV-Cys sulfoxide (Sausen and Elfarra, 1991). As yet, no evidence was found for sulfoxidation of 1,2-DCV-Cys in the rat (Derr and Schultze, 1963). However, the fact that 1,2-DCV-Cys was able to inhibit sulfoxidation of S-benzyl-L-cysteine, suggests that 1,2-DCV-Cys may act as a substrate for FMO (Sausen and Elfarra, 1990a). Also, the fact that methimazole, a potent inhibitor of FMO, blocked the nephrotoxicity of 1,2-DCV-Cys in rats may support this suggestion (Sausen et al., 1992). However, the mechanism of protection may also involve the antioxidant properties of methimazole.

The GSH-conjugate of acrolein also is nephrotoxic when administered to rats (Horvath et al., 1992). The

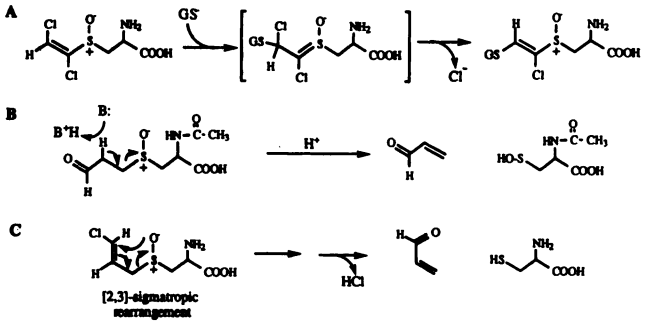


FIG. 20. Examples of sulfoxides of S-conjugates that have direct alkylating abilities (A) (S-oxide of 1,2-DCV-Cys), or that decompose to the toxic metabolite acrolein by general base catalysis (B) (S-oxide of OP-NAC) or by [2,3]-sigmatropic rearrangement (C) (S-oxide of S-(3-chloropropenyl)-l-cysteine))

corresponding mercapturic acid, OP-Nac, was cytotoxic in LLC-PK$_1$ cells and in isolated rat renal proximal tubular cells (Hashmi et al., 1992). The fact that a potent inhibitor of FMO, methimazole, inhibited the toxicity of OP-NAC in isolated kidney cells suggested involvement of sulfoxidation in the bioactivation of S-conjugates of acrolein. Indeed, the sulfoxide of OP-NAC was cytotoxic to isolated kidney cells, and this toxicity was not reduced in the presence of methimazole. It was demonstrated that acrolein was released from the sulfoxide of OP-NAC, most likely by a general-base-catalyzed elimination mechanism (fig. 20B) (Hashmi et al., 1992).

The cysteine S-conjugates and mercapturic acids of *cis*-1,3-dichloropropene and *trans*-1,3-dichloropropene all were cytotoxic in LLC-PK$_1$ cells and in isolated rat renal proximal tubular cells (Park et al., 1992). The cytotoxicity of these conjugates could be reduced with methimazole, again suggesting bioactivation by FMO enzymes. It was postulated that a [2,3]-sigmatropic rearrangement of the allylic sulfoxide may result in release of acrolein (fig. 20C). However, in the presence of microsomes from rat kidney, sulfoxidation of these S-conjugates could not be demonstrated.

PCBD-NAC sulfoxide has been identified as a metabolite of HCBD in urine of male rats (Birner et al., 1993b). Interestingly, female rats did not excrete this metabolite. In vitro, only liver microsomes of male rats catalyzed the formation of the sulfoxide, indicating that a sex difference in sulfoxidation is responsible for the difference in urinary excretion. Female rats are four times more sensitive to the nephrotoxicity of HCBD than male rats (Hook et al., 1983). Apparently, sulfoxidation represents a detoxication route in the case of the S-conjugates of HCBD. However, both PCBD-NAC and its sulfoxide were cytotoxic to isolated rat renal tubular cells. Therefore, the role of sulfoxidation in the disposition and toxicity of S-conjugates of HCBD is still not clear.

IV. Transport of GSH-derived S-Conjugates

From Section III, it is clear that the enzymes involved in the biotransformation of GSH-derived S-conjugates are distributed throughout the whole body (table 1). The interorgan and intraorgan selective toxicity of several GSH-derived S-conjugates can in part be explained by the relatively high activity of activating enzymes in the target cells of toxicity. However, another very important factor that may determine the organ selectivity of the biological activity of S-conjugates is the distribution of the S-conjugates within the body. The distribution of S-conjugates throughout the body depends primarily on the activity of a variety of active transport systems that have been identified in several tissues (table 8).

Efficient disposal of GSH S-conjugates from cells is of extreme importance, because accumulation of GSH-conjugates in a cell can lead to inhibition of GSH S-transferases and GSH reductase. This becomes especially critical during conditions of oxidative stress accompa-

TABLE 8

Characteristics of systems that are active in the transport of GSH-derived S-conjugates

Transporter	Substrate characteristics	Specific substrates	Transport characteristics	S-conjugates transported (localization)
GS-X pump	anionic amphiphilic compounds	MK 571	ATP-dependent	DNP-G (cLPM; HL60/ADR cells; heart sarcolemma; erythrocytes§§
	glucuronate, sulfate conjugates	LTC_4, GSSG	Na^+-independent	
GSH carrier	organic anions	GSH	electrogenic	$CH_3Hg.SG$ and S-methyl-glutathione (cLPM)‖‖
			Na^+-independnet trans-stimulation	DNP-G and BSP-G (BLM of liver)¶¶
				NB-G and S-ethyl-glutathione (BBM of intestine)##
GSH carrier	γ-glutamyl-compounds	GSH, GSSG probenecid	Na^+-dependent	1,2-DCV-G (BLM of kidney cells)***
system A	small aliphatic amino acids	MeAIB	Na^+-dependent	1,2-DCV-Cys and 1,2-DCV-HCys (BBM and BLM of kidney cell)†,‡
	N-methylated amino acids		trans-inhibition	S-methyl-L-cysteine (hepatocyte)§
system L	amino acids with preferentially branched and aromatic side chains	BCH cycloleucine	Na^+-independent strong trans-stimulation	1,2-DCV-Cys (BLM of kidney cell and LLC-PK_1-cell; brain)†,§,‖,¶
				1,2-DCV-HCys (BLM of kidney cell)‡
system ASC	amino acids with preferentially 3 to 5 carbon chain no N-methylated amino acids	AIB	Na^+-dependent trans-stimulation	1,2-DCV-HCys (BLM of kidney cells)‡
				S-methyl-L-cysteine (hepatocyte)§
system T	aromatic and bicyclic amino acids	D-tryptophan	Na^+-independent weak trans-stimulation	PCBD-Cys (BLM of LLC-PK_1-cells)#
organic anion transporter	organic anions	PAH, probenecid	indirectly Na^+-dependent*	1,2-DCV-NAC, 1,2-DCV-HCys and 1,2-DCV-Cys‡ (BLM of kidney cell)**,‡; PCBD-NAC (in vivo)††

* Dependent on dicarboxylate gradient that is driven by the sodium gradient.
† Schaeffer and Stevens, 1987b.
‡ Lash and Anders, 1989.
§ Kilberg et al., 1981.
‖ Schaeffer and Stevens, 1987a.
¶ Patel et al., 1993.
# Mertens et al., 1990.
** Zhang and Stevens, 1989.
†† Lock and Ishmael, 1985.
‡‡ Lash and Anders, 1986.
§§ Ishikawa, 1992.
‖‖ Dutczak and Ballatori, 1994.
¶¶ Hinchman et al., 1993.
Vincenzini et al., 1991.
*** Lash and Jones, 1984.

BSP-G, bromosulphtalein-glutathione; NB-G, S-(*p*-nitrobenzyl)-GSH; CH_3HgSG, methylmercury-glutathione complex; BCH, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid; MK 571, 3-(([3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl]-(3-dimethylamino-3-oxopropyl)-thio)-methyl]thio)propanoic acid; BLM, basolateral membrane; BBM, brush border membrane); cLPM, canalicular liver plasma membrane; LTC_4, leukotriene C_4; AIB, α-aminoisobutyric acid; McAIB, (N-methylamino)α-isobutyric acid.

nied by increased canalicular GSSG-transport. GSSG can depress transport of the S-conjugates, and the accumulation of the S-conjugate could then be amplified by its inhibitory effect on GSSG reductase (Akerboom and Sies, 1989).

GSH S-Conjugates can be transported by at least three different transport sytems, each with different substrate characteristics and transport characteristics (table 8). An ATP-dependent GS-X pump is present in different organs and cell types in the body (Ishikawa, 1992; Oude Elferink et al., 1993). This transporter exhibits a broad substrate specificity toward different types of GSH S-conjugates, and GSSG and seems to be determined by the cysteinylglycine residue and the hydrophobicity of the adduct (Kobayashi et al., 1990; Oude Elferink et al., 1990, 1991). Other substrates of this transporter are anionic amphiphilic compounds, such as glucuronide and sulfate conjugates. Because of its broad substrate specificity, this protein is also known as the multispecific organic anion transporter (Akerboom et al., 1991).

The GS-X pump may also function in the elimination of cellular metabolites of anticancer drugs from tumor cells (Ishikawa and Ali-Osman, 1993). It has been identified as a component of a nonP-glycoprotein-mediated resistance to anticancer drugs such as anthracyclines, Vinca alkaloids,

and epipodophyllotoxins in several cell lines (Jedlitschky et al., 1994). Increased excretion of a cytotoxic GSH-platinum complex formed in tumor cells exposed to cisplatin may contribute to the resistance of tumor cells to cisplatin. Recently, it was shown that transport of DNP-G by the GS-X pump can be increased by heat shock, showing the physiological significance of this system when cells are exposed to stress (Kondo et al., 1993).

Two other transport systems that are active in the transport of GSH S-conjugates are also active in the transport of GSH. One of these systems is Na^+-dependent and is active to γ-glutamyl-compounds as well as to probenecid (table 8) and is demonstrated in basolateral membranes in the kidney (Lash and Jones, 1984) and intestine (Lash et al., 1986). The other GSH-transport system is Na^+-independent and is possibly driven by a membrane potential. Because of its capacity for transstimulation, this transporter may exchange GSH S-conjugates or organic anions with GSH. GSH-transporters with these characteristics have been demonstrated in brush border membranes of intestinal cells (Vincenzini et al., 1991) and in both canalicular and sinoidal membranes of liver cells (Dutczak and Ballatori, 1994; Hinchman et al., 1993).

The catabolites of GSH-conjugates are believed to be transported by carriers that are also active in the transport of amino acids and dipeptides. A large number of different systems that are active in the transport of amino acids and peptides across the plasma membrane has been characterized (recently reviewed in McGivan and Pastor-Anglada, 1994). Cysteine S-conjugates are transported both by Na^+-dependent (systems A and ASC) as by Na^+-independent (systems L and T) that are localized in several cell-types.

Mercapturic acids are transported by the well-characterized organic anion transporter in the kidney (table 8). This transporter is indirectly Na^+-dependent, because it is driven by a dicarboxylate gradient, which in its turn is generated carriers driven by the Na^+-gradient. Other transport systems that are less well-characterized, however, are shown to exist in the liver. Possibly dependent on its molecular weight, mercapturic acids formed in the liver may be excreted in the bile or in the blood.

In the remainder of this section, the role of these transport mechanisms in the disposition of GSH-derived S-conjugates in different organs will be discussed in more detail.

A. Hepatic Transport Mechanisms

1. Transport of GSH S-conjugates. As discussed previously, the liver plays a central role in the biosynthesis and disposition of GSH and GSH S-conjugates. Because of its relatively high GSH S-transferase activity, the liver is the predominant site for the conjugation of GSH with a wide variety of both endogenous compounds and xenobiotics. In order to undergo further degradation by γ-glutamyltranspeptidases, which are localized in the bile canalicular membrane of the hepatocyte, the luminal membrane of the biliary epithelium, and the small intestine epithelium, the GSH S-conjugates have to be extruded from the hepatic cells. On the other hand, GSH S-conjugates that are formed extrahepatically may reach the liver via the blood and can be taken up by liver cells. Different transport systems have been shown to be active in the transport of GSH, GSSG, and GSH-derived S-conjugates (fig. 21).

a. EXCRETION. Excretion of GSH S-conjugates occurs across either the canalicular or sinusoidal membrane. The characteristics of the transport across the canalicular and sinusoidal poles are quite different and will be discussed separately.

i. Canalicular efflux. Hepatic transport mechanisms for GSH conjugates have been studied extensively in recent years, with DNP-G as a model GSH conjugate. GSH-conjugates synthesized in the liver seem to be excreted preferentially into bile because of their high molecular weight (Hinchman et al., 1991; Wahllander and Sies, 1979). The most important canalicular transporter that excretes GSH-conjugates into bile is ATP-dependent (fig. 21). The canalicular transport of DNP-G is inhibited competitively by GSSG and other GSH-conjugates but not by GSH (Akerboom et al., 1991) or the low-molecular-weight GSH S-conjugate, S-methyl glutathione (Kobayashi et al., 1990). As the carbon skeleton of an S-substituted GSH-adduct increases, the conjugate becomes a better substrate for the GS-X pump. The GS-X pump is impaired in hepatocytes of TR^--mutant

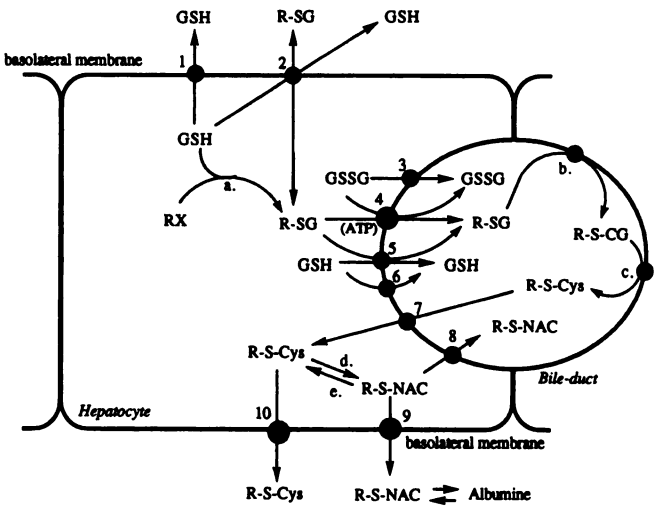


FIG. 21. Cellular localization of enzymes and transport mechanisms of GSH, GSSG, and GSH-derived S-conjugates in the hepatocyte. *Transporters*: (1) high affinity sinusoidal GSH-transporter; (2) low affinity sinusoidal GSH-transporter; (3) ATP-independent GSSG-transporter; (4) multispecific organic anion transporter (GS-X pump); (5) high affinity canalicular GSH-transporter; (6) low affinity canalicular GSH-transporter; (7) canalicular cysteine-conjugate transporter(s); (8) canalicular organic anion transporter; (9) sinusoidal organic anion transporter; (10) sinusoidal cysteine-conjugate transporter. *Enzymes*: a. GST; b. GGT; c. dipeptidases; d. N-acetyltransferase; e. acylases.

rats (Kitamura et al., 1990; Oude Elferink et al., 1989). Hepatobiliary excretion of the leukotriene GSH-conjugate, leukotriene C_4, was as low as 1.8% of the control value in TR^--rats, suggesting that this may be a physiological substrate for the GS-X pump (Huber et al., 1987).

Canalicular transport of GSH is reported to be electrogenic and independent on either a Na^+-gradient (Inoue et al., 1983a, b) or ATP (Fernandez-Checa et al., 1992). Both a high (K_m = 0.24 mM) and a low (K_m = 17 mM) affinity component for GSH transport has been detected (fig. 21) (Dutczak and Ballatori, 1994). The high affinity component of GSH transport is inhibited by GSH S-conjugates and other γ-glutamyl compounds, indicating that it might function to transport these compounds into bile by an ATP-independent mechanism. Methylmercury transport across liver canalicular membranes into bile is dependent upon intracellular GSH. It has been shown that a GSH-methylmercury complex is transported by the two canalicular carriers that also transport GSH; this complex is not a substrate for the ATP-dependent GS-X pump (Dutczak and Ballatori, 1994). Uptake of the GSH-methylmercury complex by rat liver plasma membrane vesicles could be trans-stimulated when vesicles were preloaded by GSH or the GSH-methylmercury, indicating that this carrier functions bidirectionally. Because GSH-transporters are present in all mammalian cells, they may provide a common mechanism for the removal of univalent metals that form 1:1 GSH complexes. In contrast, divalent metals, such as inorganic mercury, zinc, copper, lead and cadmium, are believed to be secreted into bile as multivalent GSH-complexes; thus, it is more likely that these complexes are transported by the GS-X-pump.

ii. Sinusoidal efflux. In addition to canalicular transport, transport of GSH S-conjugates also occurs in rat liver basolateral plasmamembrane vesicles (Kobayashi et al., 1990). This transport is not ATP-dependent but seems to be driven by the membrane potential. In contrast to the canalicular transporter, the basolateral transport of GSH S-conjugates is inhibited by GSH, suggesting that transport is mediated by a sinusoidal GSH-transporter. In sinusoidal membrane vesicles of rat liver, kinetic analysis of GSH-transport revealed two systems, a high affinity system (K_m, 0.3 mM, V_{max} 4.2 nM/min/mg) and a low affinity system (K_m, 3.3 mM, V_{max} 11.2 nM/min/mg) (Inoue et al., 1984c). Thus, both systems are probably saturated at physiological hepatic GSH concentrations. The kinetics of the low affinity system seem to correspond to those observed in the intact organ; the apparent K_m for GSH efflux was about 3 μM/g. Sinusoidal transport of GSH can be inhibited by GSH S-conjugates, such as DNP-G, S-(benzyl)glutathione and the GSH S-conjugate of sulfobromophtalein and by organic anions, such as bilirubin and sulfobromophtalein (Ookhtens et al., 1988). The GSH-transporter operates bidirectonally, exhibiting trans-stimulation of cell GSH efflux as well as uptake of intact GSH (Aw et al., 1987; Garcia-Ruiz et al., 1992; Sze et al., 1993). It was concluded that the basolateral transporter was not of physiological importance in excretion of GSH S-conjugates, because it has a much higher Michaelis-Menten constant for DNP-G than the canalicular transporter (1 mM versus 4 μM) and because it will be inhibited by the concentration of GSH found within the hepatocyte (Kobayashi et al., 1990). However, at high intracellular GSH S-conjugate concentrations, when biliary transport is saturated, efflux from the hepatocyte via the sinusoidal membrane to the blood may also occur (Inoue et al., 1984c).

iii. Hepatic excretion of toxic glutathione S-conjugates. Biliary excretion of GSH S-conjugates participates in the process leading to selective toxicity caused by a number of nephrotoxic xenobiotics. GSH-conjugates were identified in bile of rats given *p*-aminophenol (Gartland et al., 1990), HCBD (Nash et al., 1984; Gietl and Anders, 1991), trichloroethylene (Dekant et al., 1986b, 1990), tetrachloroethylene (Dekant et al., 1986c, 1987b), 1,1,2-trichloro-3,3,3-trifluoropropene (Vamvakas et al., 1989), HFP (Koob and Dekant, 1990), and dichloroacetylene (Kanhai et al., 1991). The role of the biliary GSH S-conjugates in the toxicity was demonstrated by protection against nephrotoxicity by cannulation of the bile-duct. This procedure was shown to protect rats from nephrotoxicity following an oral dose of HCBD (Nash et al., 1984). Recently, however, a similar study showed only partial protection of bile-duct cannulation against HCBD-induced toxicity (Payan et al., 1993). Similarly, cannulation only partially protected against *p*-aminophenol-induced nephrotoxicity (Gartland et al., 1990). The fact that cannulation does not protect completely against nephrotoxicity may be explained by a sinusoidal efflux of GSH S-conjugates from the liver. Gietl and Anders (1991) showed that at a low dose of HCBD, the GSH-S-conjugate was excreted exclusively into the bile, whereas at high doses, significant amounts were excreted in the perfusate. Alternatively, incomplete protection by biliary cannulation may also indicate the contribution of extrahepatic GSH conjugation (Kanhai et al., 1991; Vamvakas et al., 1989). Biliary excretion of 1,2-DCV-NAC in urine of rats exposed to dichloroacetylene was not affected by bile-cannulation. N-Acetyl-S-(1,1,2,3,3,3-hexafluoropropyl)-L-cysteine was the exclusive urinary metabolite in HFP-treated rats, while the main biliary metabolite found was S-(1, 2, 3, 3, 3-pentafluoropropenyl)GSH. From these observations, it was concluded that N-acetyl-S-(1,1,2,3,3,3-hexafluoropropyl)-L-cysteine possibly resulted from local GSH-conjugation in the kidney.

b. UPTAKE. Recently, in isolated perfused livers from rat and guinea pig, a basolateral transporter was characterized that was able to transport DNP-G from the perfusate into the hepatocyte (Hinchman et al., 1993). DNP-G was found in the bile at a concentration 100-fold

higher than that in the perfusate, indicating that the liver is capable of transporting the intact conjugate across both sinusoidal and canalicular membrane. Uptake of intact DNP-G seems to be mediated by sinusoidal multispecific organic anion transport system(s). The sinusoidal transporter that is active in excretion of cellular GSH functions bidirectionally and exhibits transstimulation. Extracellular GSSG, ophthalmic acid, and sulfobromophtalein-G all stimulated GSH-efflux. It was suggested that the downhill transport of GSH out of the cell may be a driving force for the uptake of organic anions (Garcia-Ruiz et al., 1992; Sze et al., 1993). However, the fact that GSSG and ophthalmic acid did not *cis*-inhibit uptake of DNP-G in the perfused rat liver may argue against this mechanism (Hinchman et al., 1993). The GSH conjugate of HCBD is also taken up by rat liver (Koob and eDkant, 1992), but uptake of this relatively lipophilic adduct is probably caused by simple passive diffusion. The contribution of the liver to the disposition of GSH-conjugates synthesized extrahepatically is still unclear, however.

2. Transport of cysteine S-conjugates and mercapturic acids. After excretion into the bile, GSH S-conjugates will be exposed to biliary γ-glutamyltranspeptidase and subsequently to dipeptidase activities (fig. 21). For GSH, a substantial fraction of GSH excreted in the biliary tree undergoes hydrolysis with subsequent partial reabsorption of its constituents (Ballatori et al., 1986). This so-called hepatobiliary cycling may also occur in the case of GSH S-conjugates. The resultant cysteine S-conjugates formed after hydrolysis in the biliary tree may be reabsorbed and acetylated within hepatocytes to form mercapturic acids (Hinchman et al. 1991; Simmons et al., 1992). Isolated hepatocytes rapidly accumulate S-benzyl-L-cysteine by a carrier-mediated mechanism (Inoue et al., 1984b).

The amount of GSH-derived S-conjugates excreted in bile will strongly depend on the activity of hydrolases (γ-glutamyltranspeptidase and dipeptidases) as well as on the activity of reuptake transporters. Because of the large species-differences in the activities of these hydrolases (table 4), the disposition of hepatic GSH S-conjugates may differ strongly between species. For example, in the rat, DNP-G is excreted in bile mainly as the intact GSH-conjugate, and to a smaller extent as S-(2,4-dinitrophenyl)cysteinylglycine. However, biliary hydrolysis of GSH S-conjugates in rat may also strongly depend on the nature of the S-substituent; in bile of tetrafluoroethylene-treated rats, only the cysteine S-conjugate, TFE-Cys, could be identified (Odum and Green, 1984). In contrast to the rat, in the guinea pig DNP-G could not be measured in bile at all because of the much higher activities of hydrolases (Hinchman et al., 1993). The major biliary metabolites in the guinea pig were the mercapturic acid and, to a lesser extent, the cysteine conjugate. Because the localization of the N-acetyltransferase catalyzing the N-acetylation of the cysteine-conjugate is intracellular, the cysteine S-conjugate formed extracellularly has to be reabsorbed by the hepatocytes. It has been shown that multiple transport processes are active in the transport of cysteine S-conjugates. Sodium-dependent uptake of S-methyl-L-cysteine has both (N-methylamino)α-isobutyric acid-sensitive and (N-methylamino)α-isobutyric acid-insensitive components, suggesting involvement of both system A (trivial name for transporter that among others transports *a*lanine) and system ASC (trivial name for transporter of *a*lanine, *s*erine, and *c*ysteine) (Kilberg et al., 1981). The fact that S-methyl-L-cysteine and S-benzyl-L-cysteine did not affect uptake of 1,2-DCV-Cys in rat liver canalicular plasma membrane vesicles demonstrates that cysteine S-conjugate transport is a complex process that involves several carriers and that is also determined by the nature of the S-substituent group (Simmons et al., 1992).

Mercapturic acids formed in the liver may be secreted into the bile or blood depending on the physiochemical properties or the molecular weight of the substituent. After N-acetylation, the mercapturic acid derived from DNP-G apparently is excreted again in bile (Hinchman et al., 1993). However, it was also shown that mercapturic acid formed in the liver may leave the liver via the blood (Inoue et al., 1984b; Lock and Ishmael, 1985). It has been suggested that translocation of the mercapturic acid across the sinoidal membranes occurred by a facilitated mechanism in which binding to plasma albumin may function as a transport sink (Inoue et al., 1984b, 1987). Therefore, the lack of protection against nephrotoxicity by bile cannulation may in some cases be explained by efficient hepatobiliary cycling of the GSH-conjugate and excretion of mercapturic acid or cysteine S-conjugate to the blood before excretion in the bile.

S-Conjugates formed in the liver and excreted to the blood will be delivered to the kidney by the systemic circulation. However, S-conjugates excreted in the bile first have to be reabsorbed from the small intestine in order to be able to reach the kidney.

B. Intestinal Transport Mechanisms

1. Transport of glutathione S-conjugates. GSH and GSH S-conjugates excreted via the bile will be exposed to the luminal side of intestinal mucosal cells. Several mechanisms may be involved in the luminal utilization and uptake of these compounds by brush-border membranes of the intestine (fig. 22) (Vincenzini et al., 1992). GSH from the bile or from the diet can be transported intact across brush border membrane vesicles by a transport mechanism that is Na^+-independent, pH-dependent, and specifically activated by monovalent and bivalent cations, in particular Ca^{2+}. The efficient uptake of exogenous GSH protects GSH-depleted rat's small intestinal epithelial cells against injury induced by *t*-butylhydroperoxide or menadione (Lash et al., 1986). The GSH can subsequently be excreted intact into the mesenteric circulation (Hagen and Jones, 1987). There-

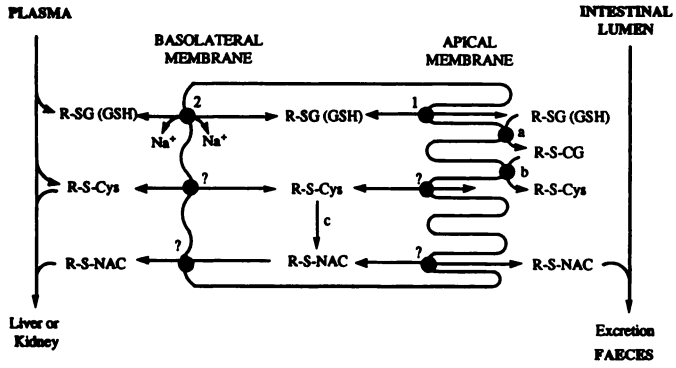


FIG. 22. Cellular localization of enzymes and transport mechanisms of GSH and GSH-derived S-conjugates in the intestinal cell. *Transporters:* (1) apical Na^+-independent transporter for GSH and GSH S-conjugates; (2) basolateral Na^+-dependent transporter for GSH and GSH S-conjugates. Transporters for cysteine S-conjugates, N-acetylcysteine S-conjugates remain to be characterized. *Enzymes:* (a) γ-glutamyltransferase; (b) cysteinylglycine dipeptidase; (c) N-acetyltransferase.

fore, orally administered GSH may be useful in the treatment of many disease states.

The uptake of GSH by brush border membrane vesicles is markedly and competitively inhibited by the GSH-S-conjugates, S-(*p*-nitrobenzyl)glutathione and S-ethyl glutathione (Vincenzini et al., 1991). The uptake of these GSH S-conjugates seems to be mediated by the Na^+-independent transporter of GSH (table 8). It is also supported by trans-stimulation of GSH-uptake; preloading brush border vesicles with GSH S-conjugates, increases the uptake of GSH by 60–80%. This mechanism may be important for the elimination of GSH S-conjugates from the cell. The luminal transporter is not inhibited by probenecid, γ-glutamyl compounds, or GSSG.

A GSH-transporting system with different characteristics is localized on the intestinal basolateral membrane. This system is a sodium-dependent active transporter. This transporter may provide GSH to the enterohepatic circulation, increase plasma GSH values, and supply GSH to epithelial cells or other cells where it may aid in cellular detoxication. Alternatively, because of the reversibility of these transport systems, the basolateral transporter may also function to transport GSH from the plasma to the intestinal cell. By administration of PCBD-G into the intestine of rats via a biliary cannula, it was demonstrated that the intact GSH-conjugate of HCBD was present in portal blood (Gietl et al., 1991). This demonstrates that transport systems for intact GSH S-conjugates are present in both brush border membrane and basolateral membrane.

The intestinal transport of GSH S-conjugates has been studied using the Caco-2 cell, a cultured human colonic adenocarcinoma cell line with many of the characteristics of enterocytes. It was shown that DNP-G, which was formed intracellularly by incubation with 1-chloro-2,4-dinitrobenzene, was secreted almost two-fold faster over the basolateral membrane than over the apical membrane (Oude Elferink et al., 1993). The secretion over both membranes was sensitive to ATP-depletion. The fact that secretion of DNP-G is not trans-stimulated by DNP-G in the medium suggests that the transporter has a highly asymmetric character and therefore probably is primarily, if not exclusively, involved in secretion of GSH S-conjugates rather than in uptake. The intestinal transport of PCBD-G was also investigated using polarized monolayers of Caco-2 cells. Upon apical exposure of the Caco-2 cells, the concentration of PCBD-G accumulated in the basolateral chamber, demonstrating that absorption of this conjugate also is not by passive diffusion but by active transport (Gietl et al., 1991).

2. Transport of cysteinylglycine S-conjugates and cysteine S-conjugates. GSH and GSH-conjugates excreted in the bile will be degraded by γ-glutamyltranspeptidases and dipeptidases present in biliary tree and in luminal membrane of the intestine (Kozak and Tate, 1982). The cysteinylglycine S-conjugates and cysteine S-conjugates present in the intestinal lumen may be excreted via various routes. They may be reabsorbed from the lumen and then transported to the liver and kidneys, where they may be metabolized into their corresponding mercapturic acids. The transport of the dipeptide probably is slower than the corresponding cysteine S-conjugate. Alternatively, the S-conjugates may be metabolized by the intestinal microflora and/or excreted in feces.

By administration of the GSH-conjugate into the intestine via a biliary cannula, it was demonstrated that next to the intact GSH-conjugate of HCBD, the corresponding cysteine S-conjugate was also present in portal blood in an approximately equal concentration (Gietl et al., 1991). This may be explained by enzymic hydrolysis of the GSH-conjugate by apical γ-glutamyltransferase and dipeptidases, followed by uptake of the cysteine S-conjugate from the intestinal lumen. PCBD-Cys formed enzymatically or added directly to the apical chamber of polarized monolayers of Caco-2 cells equilibrated on both sides of the Caco-2 cells. This may indicate passive diffusion of PCBD-Cys without active apical or basolateral transport (Gietl et al., 1991).

C. Renal Transport Mechanisms

Because β-lyase activity is only present intracellularly, nephrotoxic S-conjugates that require β-lyase-mediated bioactivation must be transported into the renal cell. The S-conjugates can enter the renal cells from plasma via the basolateral (also, contraluminal, peritubular) membrane or from the tubular lumen (after glomerular filtration) via the brush border (also, apical, luminal) membrane. The localization of the different transport mechanisms that have been characterized and shown to be active in the transport of the S-conjugates is shown in figure 23.

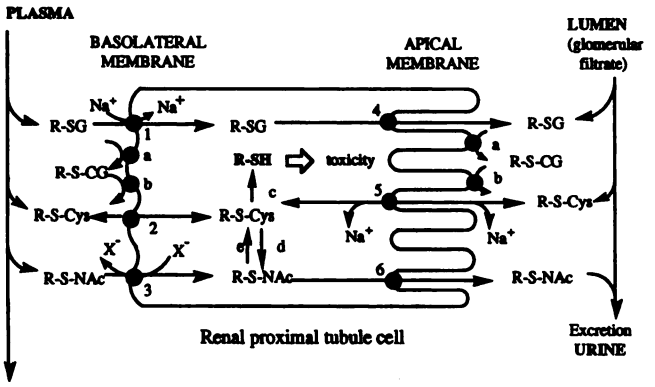


FIG. 23. Cellular localization of enzymes and transport mechanisms of GSH-derived S-conjugates in proximal tubular cells of the kidney. *Transporters:* (1) Na^+-coupled transport of glutathione S-conjugate across the basolateral membrane; (2) Na^+-dependent and Na^+-independent transport of cysteine S-conjugate; (3) Na^+-coupled and probenicid-sensitive transport of N-acetyl-cysteine S-conjugate across the basolateral membrane; (4) translocation of R-SG and excretion across the brush border membrane into the tubular lumen; (5) Na^+-coupled transport of cysteine S-conjugate into the renal proximal tubular cell; (6) excretion of the mercapturic acid N-acetyl-cysteine S-conjugate into the tubular lumen. *Enzymes:* (a) GGT-mediated catabolism of R-SG and formation of cysteinylglycyl S-conjugate; (b) Cysteinylglycine dipeptidase catalyzed formation of cysteine S-conjugate; (c) β-lyase mediated formation of thiol-compound; (d) N-acetyltransferase catalyzed formation of mercapturic acid; (e) formation of cysteine S-conjugate through decetylation of mercapturic acid. Abbreviations: R-SG, glutathione S-conjugate; R-S-CG, cysteinylglycyl S-conjugate; R-S-Cys, cysteine S-conjugate; R-S-NAc, N-acetyl-cysteine S-conjugate; R-SH, xenobiotic derived thiol; X^-, dicarboxylate.

1. Transport of glutathione S-conjugates. As much as 80% of the plasma clearance of GSH is renal clearance (Häberle et al., 1979). Glomerular filtration is responsible for 25% of renal clearance of GSH, the rest of the circulating GSH is removed by nonfiltration mechanisms that involve transport across the basolateral membrane. A sodium-coupled, probenecid-sensitive transport system has been characterized in renal basallateral vesicles. It exhibits a broad substrate specificity and is able to transport both intact GSH and GSH-conjugates across the basolateral membrane (Lash and Jones, 1984; Ullrich et al., 1989). Uptake of 1,2-DCV-G by basolateral membrane vesicles was inhibited by GSH, GSSG, and γ-glutamylglutamate but not by the corresponding cysteine-conjugate 1,2-DCV-Cys, indicating that the transport system is specific for the γ-glutamyl-moiety.

GSH-conjugates are not degraded intracellularly, but they are excreted subsequently into the lumen, where degradation by brush-border peptidases to the corresponding cysteine-conjugates takes place (fig. 23). A second basolateral uptake mechanism has been suggested to be degradation by basolateral GGT and dipeptidase (Abbott et al., 1984), followed by uptake of the corresponding cysteine-conjugate. It has been shown that DCV-G and the GSH-conjugate of HCBD (PCBD-G) inhibited the basolateral transport of PAH, a compound selectively transported by the probenecid-inhibitable organic anion carrier (table 9) (Ullrich and Rumrich, 1988). However, whether this inhibition implies transport of these GSH-conjugates or only binding to the organic anion carrier is not known yet. The fact that L-glutamyl glutamate inhibits GSH uptake but not PAH-transport indicates that both probenecid-inhibitable processes, basolateral GSH-transport and the organic anion transport, are not carried out by the same transporter.

The precise contribution of the basolateral processes to removal of GSH-conjugates from plasma and in the onset of nephrotoxicity is not known. It might differ strongly between different types of GSH-conjugates. Treatment of rats with AT-125, an inhibitor of both basolateral and luminal GGTs, protected against the nephrotoxicity of the mono- and di-GSH-conjugates of 2-bromohydroquinone (Monks et al., 1985) and 1,2-DCV-G (Elfarra et al., 1986a), but it resulted in increased toxicity of S-(2-chloroethyl)glutathione (Kramer et al., 1987). The latter was explained by increased basolateral

TABLE 9

Interaction of nephrotoxic GSH-, cysteine- and N-acetyl-L-cysteine-S-conjugates with basolateral PAH transport in the proximal tubule of rat kidney (app. K_i, PAH mM)†*

Conjugate	L-glutathione	L-cysteine	N-acetyl-L-cysteine
S-(2-chloroethyl)-	> 5 (NS)		
S-(2-chloro-1,2,2-trifluoroethyl)-	0.66		
S-(2-chlorovinyl)-	1.10		
S-(1,2-dichlorovinyl)-	> 5 (NS)	0.82	0.46
S-(1,2,2-trichlorovinyl)-	1.30	0.41	0.22
S-(1,2,3,3,3-pentachloropropenyl)-	0.11		
S-(1,2,3,4,4-pentachlorobutadienyl)-	0.47	0.16	0.07
S-(1,2,3,4-tetrachlorobutadienyl)-bis-1,-	> 5 (NS)		
S-(2-bromo-hydroquinone)-5-	0.34		
S-(2-bromo-hydroquinone)-bis-3,5-	0.93		

* app. Ki, PAH (mM), concentration of S-conjugate at which inhibition of PAH-transport is half-maximal.
† Ulrich et al., 1988.
NS, no significant inhibition.

uptake of intact GSH-conjugate, which was suggested to be more important for nephrotoxicity of 1,2-dichloroethane than the corresponding cysteine-S-conjugate, S-(2-chloroethyl)-L-cysteine.

2. Transport of cysteine S-conjugates. Cysteine S-conjugates, delivered by glomerular filtration and by in situ degradation of GSH-conjugates by brush border GGT and dipeptidases, are reabsorbed in the proximal tubule from the lumen by transepithelial transport. Cysteine S-conjugates may also be delivered to the kidney cells by basolateral transport systems. The nature of the systems active in the transport of cysteine S-conjugates have been investigated by studying the effect of selective inhibitors of transport on the uptake of cysteine S-conjugates in freshly isolated kidney cells, rat kidney tubules, plasma membrane vesicles, kidney slices and the kidney cell line LLC-PK_1. From these studies, it is clear that multiple systems are able to transport cysteine S-conjugates across basolateral and brush border membranes. Both sodium-dependent as well as sodium-independent transporters have been identified. In isolated rat kidney cells, about 50% of the uptake of 1,2-DCV-Cys was sodium-dependent, whereas the other component was sodium-independent (Lash and Anders, 1989).

a. SODIUM-DEPENDENT TRANSPORT. Using selective competitive inhibitors of the transporters (table 8), it was shown that several sodium-dependent transporters may be active in the uptake of cysteine S-conjugates.

Sodium-dependent uptake of 1,2-DCV-Cys in isolated rat renal proximal tubular cells was inhibited by 80% by probenecid and PAH (Lash and Anders, 1989), indicating involvement of the basolateral organic anion transporter. Inhibition of PAH-transport in rat renal proximal tubules by 1,2-DCV-Cys and other cysteine S-conjugates (table 9) further supports the involvement of this organic anion transporter in cysteine S-conjugate-transport (Ullrich et al., 1989). Experiments with the isolated perfused rat kidney revealed that PCBD-Cys, the cysteine S-conjugate of HCBD, is excreted principally via probenecid-sensitive tubular secretion: clearance of PCBD-Cys was six-fold higher than inulin clearance. In the presence of probenecid, the fractional clearance approximates unity (Schrenk et al., 1988). Therefore, in vivo, the organic anion transporter seems to be the predominant system involved in the basolateral transport of PCBD-Cys.

The results of a number of studies argue against involvement of the organic anion transporter in basolateral uptake of 1,2-DCV-Cys. No sodium-dependent uptake was observed in basolateral membrane vesicles (Schaeffer and Stevens, 1987b). However, it has been shown that the apparent sodium-dependence of the organic anion transporter is caused by the coupling of a sodium-dependent transporter of dicarboxylates to the exchange of dicarboxylates with organic anions (Ullrich and Rumrich, 1988). Thus, the lack of activity of the organic anion transport in basolateral membrane vesicles may be caused by the lack of exchangable dicarboxylates in this system. Probenecid was not inhibitory in the renal uptake of 1,2-DCV-Cys in isolated rat renal proximal tubules (Zhang and Stevens, 1989) and rabbit kidney slices (Wolfgang et al., 1989b). The reason for these contradictory observations are not known yet.

Sodium-dependent uptake of 1,2-DCV-Cys in isolated rat renal proximal tubular cells could also be inhibited significantly by MeAIB (Lash and Anders, 1989), indicating involvement of system A, which is present both on the basolateral and brush border membrane (Murer and Gmaj, 1986). MeAIB strongly inhibits transport of 1,2-DCV-Cys in brush border membrane vesicles, indicating that this transporter may be involved in sodium-dependent apical uptake of cysteine S-conjugates (Schaeffer and Stevens, 1987b).

The lack of inhibition of uptake of 1,2-DCV-Cys in isolated rat renal proximal tubular cells by α-isobutyric acid, shows that system ASC is not involved in sodium-dependent transport of 1,2-DCV-Cys (Lash and Anders, 1989). In contrast, the uptake of the 1,2-DCV-Cys was strongly inhibited by α-isobutyric acid, demonstrating transport of this conjugate by system ASC.

b. SODIUM-INDEPENDENT TRANSPORT. The sodium-independent uptake of 1,2-DCV-Cys in isolated rat renal proximal tubular cells was inhibited by BCH, indicating participation of system L (*l*eucine *t*ransporter) (Lash and Anders, 1989) (table 8). System L also seemed to be involved in basolateral transport of 1,2-DCV-Cys in rabbit renal slices (Wolfgang et al., 1989b) and in the renal cell line LLC-PK_1 (Schaeffer and Stevens, 1987a). Uptake and toxicity of 1,2-DCV-Cys in monolayers of LLC-PK_1-cells was trans-stimulated (i.e., increased by preloading the cells with nontoxic S-cysteine conjugates), was not sodium-dependent, and was inhibited by BCH (Schaeffer and Stevens, 1987a). However, no trans-stimulation of 1,2-DCV-Cys-uptake was observed in isolated basolateral membrane vesicles (Schaeffer and Stevens, 1987b). As an explanation for this contradictional observation, these authors speculated that system L requires ultrastructural aspects of the cell for activity, or that it is rendered inactive during the preparation of vesicles.

An amino acid transporter with characteristics of system T (*t*ryptophan *t*ransporter) was involved in the basolateral transport of the PCBD-Cys in LLC-PK_1-cells (Mertens et al., 1990). System T resembles system L, but has a higher preference for aromatic and bicyclic amino acids. However, it is doubtful that whether the LLC-PK_1 cell line is a good model for the in vivo situation. For instance, no active transport of PAH could be demonstrated in LLC-PK_1-monolayers (Mertens et al., 1988).

3. Transport of mercapturic acids. Transport of N-acetylcysteine S-conjugates into kidney cells is better characterized than cysteine-conjugate transport. Kinetic analysis of transport of N-acetyl-S-benzyl-L-cysteine (Inoue et al., 1984b) and of the nephrotoxicant 1,2-DCV-NAC (Zhang and Stevens, 1989) fitted a single-compart-

ment model, indicating that only one transport system is involved. N-Acetylcysteine S-conjugates are actively secreted by the basolateral organic anion system, which is competitively inhibited by probenecid. In line with this, probenecid produced 80% inhibition of 1,2-DCV-NAC uptake as well as toxicity in rabbit renal slices. 1,2-DCV-NAC also inhibited accumulation of *p*-aminohippurate in rabbit renal slices (Wolfgang et al., 1989b; Zhang and Stevens, 1989) and rat renal proximal tubules (Ullrich et al., 1989). The basolateral organic anion transporter can function as an exchanger for dicarboxylates (Ullrich, 1994). The fact that transport of dicarboxylates is driven by the sodium-gradient may explain the previously observed sodium-dependency of organic anion transport (Lash and Anders, 1989). Microperfusion studies with isolated rabbit renal tubules revealed that within the tubule, the activity of organic anion transporter is highest in the S_2-segment; secretion by the S_1-segment is comparable to that of the S_3-segments (Woodhall et al., 1978). The K_m for PAH is not significantly different between the S_1-, S_2- and S_3-segments; however, maximal transport rates are approximately six- to seven-fold greater in the S_2-segment, suggesting an increased density of the number of PAH transporters in the S_2-segment (Shimomura et al., 1981). In the rat, microinfusion of isolated tubules showed that the N-acetyltransferase capacity and/or mercapturate excretion of the straight part is almost twice that of the convoluted part (Heuner et al., 1991). However, because the available dicarboxylate pool in this model might differ from that in the in vivo situation, it is not known whether it reflects the distribution of organic anion transport in vivo.

Uptake of mercapturic acids from the tubular lumen probably does not occur. Mercapturic acids are transported in blood bound to plasma albumin and therefore are not eliminated by glomerular filtration (Inoue et al., 1987).

D. Transport of S-Conjugates in the Brain

Dichloroacetylene induces neurotoxicity that is selective for the lower cranial nerve region of the brain (Reichert et al., 1976). Inhalation of dichloroacetylene may lead to pulmonary formation of 1,2-DCV-G, and 1,2-DCV-G present in the pulmonary circulation may be delivered to the cerebral circulation. 1,2-DCV-G may be subsequently hydrolyzed to 1,2-DCV-Cys by γ-glutamyltranspeptidase and dipeptidases in cerebral microvessel endothelial cells (Orlowski et al., 1974). Therefore, the brain may be exposed to 1,2-DCV-G and/or 1,2-DCV-Cys after inhalation of dichloroacetylene. Both S-conjugates are taken up intact by saturable carrier systems (Patel et al., 1993).

Inhibition of uptake by BCH, as well as the sodium-independence of uptake, indicated that 1,2-DCV-Cys is taken up by system-L, which represents the majority of the transport sites at the blood-brain barrier (Smith et al., 1981). 1,2-DCV-Cys uptake was not inhibited by probenecid, glycylglycine or α-(methylamino)-isobutyric acid, which argues against involvement of sodium-dependent anion transport, γ-glutamyltransferase-dependent transport, or sodium-dependent system-A transport in 1,2-DCV-Cys uptake.

The uptake of 1,2-DCV-G in the brain seemed to be less extensive; the brain uptake index was almost eightfold lower than that of 1,2-DCV-Cys (Patel et al., 1993). The uptake of 1,2-DCV-G was not affected by inhibitors of system-L, organic anion transporter, γ-glutamyltransferase-dependent transport, and system-A transport. Cysteine stimulated brain uptake of 1,2-DCV-G slightly, but the mechanism of this stimulatory effect is not yet understood. Because brain tissue contains significant amounts of glutamine transaminase K/cysteine conjugate β-lyase (Cooper et al., 1993), uptake of S-conjugates and subsequent bioactivation by β-lyase may be the mechanism responsible for the trigeminal neuropathy caused by dichloroacetylene.

E. Transport of S-Conjugates in Blood Cells

Erythrocytes contain both GSH and GSH S-transferases. Therefore, significant amounts of GSH-conjugates may be formed in erythrocytes. Two ATP-dependent components of DNP-G transport have been identified in the human erythrocyte, one of low K_m and low capacity, and another of high K_m and high capacity (Eckert and Eyer, 1986; Akerboom et al., 1992). The transport of DNP-G and GSH-conjugates from 4-dimethylaminophenol was strictly unidirectional and inhibited by sodium fluoride (Eckert and Eyer, 1986). The low-K_m transport of DNP-G is inhibited competitively by GSSG; the high-K_m transporter of DNP-G is not inhibited by GSSG and is sensitive to the action of sulfhydryl-reagents. The two components may represent separate transport systems with overlapping substrate specificities. The low-K_m system seems to be involved in the transport of GSH-conjugates at physiological concentrations and possibly also in the transport of divalent organic anions (Bartosz et al., 1993). Transport of monovalent organic anions, including bile acids and sulphated and glucuronidated drugs, might proceed via the high-K_m transport component, which represents an organic anion translocator with broader specificity.

Erythrocytes do not have a capacity for mercapturic acid formation and eliminate only GSH-conjugates across the plasma membrane without further metabolism (Awasthi et al., 1989). The role of erythrocytes in the distribution of other GSH-derived S-conjugates in plasma, however, remains to be established. Different transport-systems are present in human red blood cells that are related to transporters present in the kidney (Vadgama and Christensen, 1985; Rosenberg et al., 1980). Both system-L and system-T transporters known to be active in the transport of cysteine S-conjugates in the brain (Patel et al., 1993) and kidney (Mertens et al., 1990) are also present in human erythrocytes. Very recently, both the cysteine S-conjugate and the N-acetyl-

cysteine S-conjugates of propachlor were identified in erythrocytes of rats dosed orally with the herbicide propachlor (Davison and Larsen, 1993). The cysteine S-conjugate was the predominant metabolite in erythrocytes. In plasma, only the cysteine S-conjugate was present. Erythrocytes were the major transporter of propachlor metabolites in rat blood, whereas plasma was the major transporter of these metabolites in pig and calf (Davison and Larssen, 1993).

F. Interorgan Transport: What S-Conjugates Are Circulating?

Because formation and further processing of GSH-conjugates apparently is extremely complex and involves multiple enzymes and transport systems in different organs (fig. 24), an important question is this: to which S-conjugates are the target organs actually exposed? This question should be considered carefully before model compounds are chosen to elucidate the molecular mechanisms of S-conjugate-induced toxicity both in vivo and in vitro.

The most direct way to determine which S-conjugate will be delivered to a target organ is measuring these conjugates in blood. However, as yet, only limited data are available about circulating S-conjugates in blood. The cysteine S-conjugate was the major metabolite found in the systemic blood from rat, pig, and calf given propachlor via the stomach (Davison and Larsen, 1993). Erythrocytes were the major transporter of propachlor-S-cysteine in rat blood, whereas plasma was the major transporter of this metabolite in pig and calf. Blood did not further metabolize the cysteine S-conjugate.

Most of the information concerning the transport of S-conjugates throughout the body has been obtained indirectly by using specific inhibitors of transport systems or by using cannulation techniques. Both GSH-conjugates of trichloroethylene (1,2-DCV-G) and chlorotrifluoroethylene (CTFE-G) produce nephrotoxicity in vivo (Elfarra et al., 1986a; Dohn et al., 1985a) and are cytotoxic to isolated proximal tubular cells (Lash and Anders, 1986; Dohn et al., 1985a). However, whether these GSH-conjugates will reach the kidney after in vivo exposure to the parent ethylenes is not known. A very important factor might be the localization of GSH-conjugation, which in turn might depend upon the route of administration of the parent ethylenes. When GSH-conjugation predominantly takes place in the liver, the GSH-conjugates and catabolites will be present in the bile in relatively high concentrations (Rafter et al., 1983). GSH-conjugates are secreted into the bile via membrane potential-dependent transport systems (Inoue et al., 1984a). Because of the high activity of biliary and intestinal hydrolases, and because a GSH-conjugate may be too polar to be reabsorbed from the intestinal lumen, exposure of the kidney to intact GSH-conjugates via this route is not likely. However, at high intracellular GSH-conjugate concentrations, when biliary transport is saturated, efflux from the hepatocyte via the sinusoidal membrane to the blood may occur (Inoue et al., 1984c), and the kidney may be exposed to intact GSH-conjugates. Alternatively, intact GSH-conjugates may reach the kidney when initial GSH-conjugation takes place in extrahepatic tissues, such as the lung, the kidney, or in blood cells. GSH-conjugation of nephrotoxic haloalkenes is higher in hepatic than in renal subcellular fractions (table 3). Because cannulation of bile protected rats from nephrotoxicity caused by an oral dose of HCBD, hepatic GSH-conjugation seems to be the main route in the bioactivation of HCBD (Wolf et al., 1984). However, the fact that cannulation of bile did not block nephrotoxicity completely may point to sinoidal efflux of HCBD-conjugates (Payan et al., 1993). The excretion of the GSH-conjugate of HCBD in feces (Dekant et al., 1988c) supports the idea that intact GSH-conjugates are too polar to be reabsorbed from the intestinal lumen. The inability of AT-125 to protect against HCBD-induced nephrotoxicity, despite the fact that renal GGT-activity was reduced by 95% (Davis, 1988), tends to indicate that transport of intact GSH-conjugates to the kidney does not take place. Using an isolated perfused liver model, however, Koob and Dekant (1992) have shown that for PCBD-G, hepatic uptake was efficient, whereas biosynthesis of PCBD-NAC was only a very minor pathway. From this, it was suggested that at least some intact PCBD-G was reaching the kidney after in vivo exposure to HCBD.

For HFP, it was demonstrated in bile-canulated rats that two distinct routes of GSH-conjugation exist (Koob and Dekant, 1990). The main biliary metabolite was found to be S-(1,2,3,3,3-pentafluoropropenyl)-glutathione, whereas N-acetyl-S-(1,1,2,3,3,3-hexafluoropropyl)-

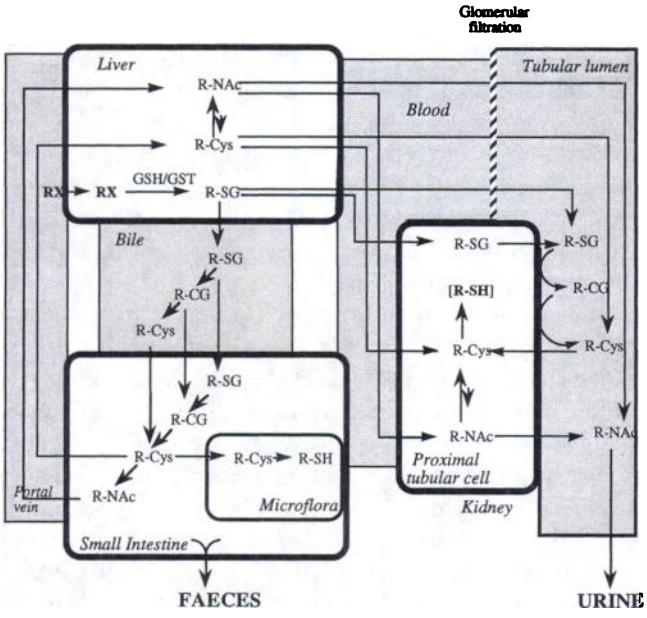


FIG. 24. Schematic representation of the interorgan transport of S-conjugates.

L-cysteine was the exclusive urinary metabolite. It was suggested that intact HFP must have reached the kidney, where local GSTs catalyze conjugation of HFP by an addition mechanism. Also, after exposure of rats to TFE by inhalation, excretion of S-conjugates in the bile was observed. Because of rapid biliary hydrolysis, the intact GSH-conjugate, S-(tetrafluoroethyl)glutathione, could not be detected (Odum and Green, 1984). However, except for renal conjugation in vitro, no data on extrahepatic GSH-conjugation of TFE are available, so renal exposure to the GSH-conjugate cannot be ruled out.

In addition to intact GSH-conjugates, the kidney might be exposed to hydrolysis products escaping from enterohepatic circulation. In addition to hydrolases, the small intestine also contains cysteine conjugate N-acetylase, which may participate in mercapturate biosynthesis in vivo (Grafstrom et al., 1979). However, because of the low activity when compared with the liver and kidney (table 1), this contribution is probably limited. After reabsorbtion via the portal vein, cysteine S-conjugates first pass the liver, which contains a high activity of cysteine conjugate N-acetyltransferases (Inoue et al., 1984b). Depending on the efficiency of hepatic acetylation, the kidney will be exposed to cysteine-S-conjugates and/or mercapturic acids. From the fact that probenecid protected against nephrotoxicity caused by HCBD, its cysteine- and N-acetylcysteine-conjugates (Lock and Ishmael, 1985), and by 1,2-DCV-Cys (Elfarra et al., 1986a), it was suggested that exposure of the kidney to mercapturic acid might be important for nephrotoxicity. However, it has been demonstrated that the cysteine conjugate of HCBD may also be transported by the probenecid-sensitive organic anion carrier (Schrenk et al., 1988). Mercapturic acids taken up by the kidney can be deacetylated by renal acylases to form the corresponding cysteine S-conjugates (Vamvakas et al. 1987; Pratt and Lock, 1988), which subsequently can be bioactivated by renal β-lyases.

V. Concluding Remarks and Future Perspectives

GSH S-transferases, which catalyze the first step in mercapturic acid biosynthesis, and several other enzymes that catalyze subsequent steps in the mercapturic acid formation have long been associated with the detoxication of mutagens, carcinogens, and other toxic substances. However, current evidence presented in this review has implicated a crucial role of GSH in the bioactivation of an increasing number of xenobiotics. Moreover, an increasing number of enzymes and transport mechanisms have now been demonstrated to be involved in the disposition of GSH S-conjugates and in bioactivation mechanisms of xenobiotics. The development of more selective inhibitors for the individual enzymes and transport systems will help to further elucidate the contribution of these systems in the mechanism of bioactivation of xenobiotics. In addition, these inhibitors may be used to prevent the toxicity of xenobiotics that are bioactivated by GSH S-conjugation.

The enzymes and transport systems that are present in the liver and kidney have been studied most extensively, because these organs are considered to be the major tissues involved in the formation and degradation of toxic GSH S-conjugates and, as a consequence, are the target tissues for toxicity. It is suggested however that GSH-dependent bioactivation mechanisms may also be responsible for toxicity in organs other than the liver and kidney. The neurotoxicity of halogenated alkenes (Cooper et al., 1993; Patel et al., 1993, 1994), as well as the pulmonary toxicity of isocyanate-compounds (Baillie and Slatter, 1991; Brakenhoff et al., 1993, 1994), may be explained by local GSH-dependent bioactivation mechanisms. Further studies are required to characterize enzyme and transport systems that are responsible for the organ-selectivity of these toxicities.

Because mercapturic acids are metabolic end-products of the conjugation of potentially toxic electrophilic chemicals and because the analysis of mercapturic acids in biological fluids has become routine, the measurement of urinary mercapturic acids has developed as a popular tool in the biological monitoring of exposure to electrophilic chemicals (Vermeulen, 1989; Van Welie et al., 1992). For at least 50 chemicals, mercapturic acids have been identified in rat and human urine. The half-life of urinary elimination of mercapturic acids in humans typically range from 1.5 to 9 hours. Urinary mercapturic acids are therefore frequently used as biomarkers of current exposure (Van Welie et al., 1992). In urine of rats exposed to 1,2-dibromoethane a DNA-adduct was excreted in urine as a mercapturic acid, 2-GEMA. Because this mercapturic acid is the result of covalent binding of the GSH-conjugate of 1,2-dibromoethane to DNA (Kim and Guengerich, 1989), 2-GEMA may be considered to be the first mercapturic acid possibly acting as a biomarker of effective dose.

From the present review, it is clear that mercapturic acids are not the only metabolic end-products of GSH-conjugation, because several other metabolic pathways may also be involved in the disposition of GSH S-conjugates. Therefore, a low amount of mercapturic acids in urine does not necessarily imply a low degree of exposure to electrophiles, because other routes of metabolism of cysteine S-conjugates, for example β-elimination, deamination, or sulfoxidation, may be more prevalent. Therefore, in addition to mercapturic acids, other potential metabolites may have to be analyzed in order to get a better estimate of the degree of GSH-conjugation.

Based on a higher ratio of mercapturic acids versus oxidative metabolites in urine, it was recently speculated that man is more at risk for developing renal tumors upon exposure to trichloroethylene than are rodents (Birner et al., 1993a). However, epidemiological studies on more than 15,000 individuals with a follow-up of more than 25 years have shown no evidence of an

association between human exposure to trichloroethylene and increased incidence of cancer or cancer mortality (Goeptar et al., 1995). Therefore, the relatively high level of mercapturic acids in human urine may also indicate a lower activity of routes competing with N-acetylation of cysteine conjugates, amongst others, β-elimination. In line with this, it has been shown that purified human kidney β-lyase was ten-fold less active in the bioactivation of 1,2-DCV-Cys than rat kidney β-lyase (Lash et al., 1990a). Quantification of urinary products derived from the β-elimination step is required to prove whether or not β-elimination is more or less prevalent in man when compared with rodents.

The value of urinary GSH-derived S-conjugates as biological monitoring parameters may decrease if enzymes that are involved in GSH-dependent biotransformation display genetic polymorphism. A number of enzymes described in this review demonstrate genetic polymorphism, e.g., GST M1a-1a, GST T1–1, and cytosolic cysteine S-conjugate sulfoxidase. When these enzymes are involved in the formation and disposition of electrophilic xenobiotics, very large interindividual differences in the excretion of S-conjugates can be anticipated at similar exposure levels. Therefore, the role of polymorphic enzymes in the disposition of electrophilic compounds requires further investigation. In a biological monitoring study of Z- and E-1,3-dichloropropene, no relationship was observed between phenotype of GST M1a-1a and the level of excretion of the corresponding mercapturic acids (Vos et al., 1991). However, a relationship with the recently discovered polymorphism of GST T1–1, which has a high catalytic activity toward several low molecular weight halogenated hydrocarbons, cannot be ruled out.

Genetically determined enzyme activity may also be an important factor determining the susceptibility of an individual to develop toxicity or cancer and therefore should be considered in risk assessment. From epidemiological studies, it seems that individuals who are deficient in GST M1a-1a may be more prone to develop lung cancer, bladder cancer, stomach cancer, colorectal cancer, and prolactinoma. This may be explained be a reduced capacity in the inactivation of genotoxic carcinogens. However, it is not known yet what the significance is of the polymorphisms of GST-isoenzymes for the susceptibility to the toxic effects of compounds that are bioactivated by GSH-conjugation.

The GSH-dependent bioactivation of dichloromethane and ethylene dibromide is catalyzed efficiently by class theta GSTs of rat and human. Significant activity of GST T1–1 can be measured in erythrocytes. Whether GSH-conjugation in erythrocytes can be regarded as a detoxicating or a toxicating pathway probably depends on the reactivity of the toxic GSH-conjugate formed. If the GSH-conjugate is highly reactive and binds instantaneously to hemoglobin, efficient conjugation in erythrocytes may result in a decreased toxicity, because it will lower the exposure of potential target tissues to the parent compound. In this case, individuals that are *GST T1-null* will be at higher risk. On the other hand, if the reactive GSH-conjugate is stable enough to be excreted by the erythrocyte and transported to the target tissues, GSH-conjugation in erythrocytes may be regarded as a bioactivation pathway. In that case, *GST T1-null* individuals will be at lower risk.

This review shows that GSH and the enzymes and transporters involved in the disposition of GSH S-conjugates also play an important role in several aspects of anticancer therapy. The concentration of GSH and the activity of GST and GGT is often increased in tumor cells and may contribute to the multidrug resistance observed in human tumors and cell lines. Different strategies are developed to improve the therapeutic efficiency of cytostatic drugs. One strategy used to sensitize tumor cells to alkylating agents is inhibition of GST-isoenzymes. Recently, the diuretic drug ethacrynic acid, a potent inhibitor of GST-isoenzymes, has been used in a phase-I clinical study with the cytostatic compound thiotepa (O'Dwyer et al., 1991). Other strategies use the elevated activities of GST and GGT in tumor cells as a target for activation of antitumor compounds. GSH S-conjugate analogs have been designed that are activated enzymatically by GST to antitumor compounds (Lyttle et al., 1994). γ-L-Glutamyl-4-hydroxybenzene and its iodinated analog γ-L-glutamyl-4-hydroxy-3-iodobenzene are bioactivated by GGT and possess high antitumor activity in human and murine melanoma cell lines (Prezioso et al., 1994).

Depletion of GSH by L-BSO has been used to define the role of intracellular thiols in mediating resistance to chemotherapeutic agents. The cytotoxicity of a number of drugs, including melphalan, doxorubicin, and bleomycin, is enhanced when GSH is depleted by L-BSO (Russo et al., 1984, 1986). This has been attributed, in part, to the ability of GSH to inactivate compounds and to quench the DNA cross-link precursors that these compounds produce. Clinical trials of L-BSO as a chemotherapy sensitizer are in progress (Bailey et al., 1992). The use of L-BSO together with chemotherapy has been of particular interest in the treatment of ovarian cancer (Ozols and Young, 1991). However, GSH-depletion has been shown to antagonize the cytotoxicity of taxol (Liebmann et al., 1993) and neocarzinostatin (DeGraff et al., 1985) in tumor cell lines. Therefore, attempts to modulate the activity of these compounds with L-BSO may results in a decrease in tumor response. Other antitumor compounds that depend on GSH for bioactivation to cytostatic compounds are selenite and KW-2149, an analogue of mitomycin C.

Although modulation of GSH and GST may sensitize tumor cells to the cytostatic activity of chemotherapeutic agents, it should be avoided, that healthy tissues are also sensitized to the toxic side-effects of these antitumor agents. This is an important consideration, because

the use of many antitumor agents is limited by the intrinsic toxicity of these compounds. It is therefore a challenge to develop compounds that may selectively modulate the sensitivity of tumor-tissues without affecting the healthy tissues. Similarly, it should be remembered that antitumor agents, which are activated by GST and GGT, are activated in tumor tissues and not in tissues that also have high activities of these enzymes. An approach to protect against side-effects of antitumor agents is through the use of chemoprotectors. For example, sodium selenite can protect rats against cisplatin-induced nephrotoxicity without reducing the antitumor activity of cisplatin (Baldew et al., 1989). It has been proposed that the selective tissue concentration of selenol compounds that are formed after the reductive activation by renal GSH may explain the protection by selenite. Organoselenium compounds that are less toxic than inorganic selenium compounds may even be more promising chemoprotectors. Ebselen has been shown to protect against cisplatin-induced nephrotoxicity, possibly after GSH-dependent activation (Baldew et al., 1990, 1992).

GSH-conjugation and subsequent β-lyase-dependent activation have been proposed to explain the antitumor activity of 6-chloropurine (Hwang and Elfarra, 1993). Based on the mechanisms underlying the kidney-selectivity of β-lyase-mediated toxicities, S-(6-purinyl)-L-cysteine has been designed to target the antitumor agent 6-mercaptopurine to the kidney (Hwang and Elfarra, 1989, 1993). The analogs of P-Cys, N-acetyl-S-(6-purinyl)-L-cysteine, and S-(6-purinyl)-L-homocysteine were even better prodrugs of 6-mercaptopurine (Elfarra and Hwang, 1993). The observation that these conjugates were not acutely nephrotoxic in rats suggests that these compounds may be useful in the treatment of renal cell carcinoma. However, it remains to be established whether this approach will be applicable in humans, inasmuch as renal β-lyase activity in human kidney is lower than in rat kidney.

In conclusion, whereas the GSTs and related enzymes were studied originally because of their involvement in mercapturic acid biosynthesis, these enzymes are also currently of interest because of their important role in the conversion of xenobiotics to biologically active compounds and because of their role in several aspects of anticancer treatments. From this review it is clear that a large number of enzymes and transport systems, differentially distributed over different tissues, may be involved in the formation and disposition of a GSH-derived S-conjugate. Because only limited data are available on the substrate-selectivity of most of these proteins, it is not yet predictable that enzymes or transporters will be active in the disposition of a specific GSH-conjugate. Because the enzyme-activities may be sex-dependent, inducible and/or genetically determined, large interindividual variations in GSH-related bioactivation and bioinactivation processes can occur, which by inference leads to large interindividual differences in susceptibility to toxicity. This is supported by epidemiological studies in which an overrepresentation of GST μ-negative individuals is observed in cancer patients. Also, large species-differences in enzyme activities should be taken into account when extrapolating animal toxicity data to humans.

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