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DRUG METABOLISM & DISPOSITION

GLUTATHIONE CONJUGATION OF TRICHLOROETHYLENE IN RATS AND MICE: SEX-, SPECIES-, AND TISSUE-DEPENDENT DIFFERENCES

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ABSTRACT:

Glutathione (GSH) conjugation of trichloroethylene (Tri) to form *S*-(1,2-dichlorovinyl)glutathione (DCVG) has been implicated in the nephrotoxicity and nephrocarcinogenicity of Tri. Marked sex- and species-dependent differences exist, however, in the susceptibility to Tri-induced renal toxicity, with the male rat being the most susceptible. The present study, therefore, focuses on potential differences in the initial step of the GSH pathway. Rates of DCVG formation were measured in suspensions of isolated renal cortical cells and isolated hepatocytes from male and female Fischer 344 rats and in kidney and liver microsomes and cytosol from male and female Fischer 344 rats and B6C3F1 mice to determine if sex- and species-dependent differences in GSH conjugation correlate with susceptibility to renal toxicity from Tri. Rates of γ-glutamyltransferase (GGT) with γ-glutamyl-*p*-nitroanilide and glycylglycine as substrates and GSH *S*-transferase (GST) with 1-chloro-2,4-dinitrobenzene as substrate were also measured in liver and kidney subcellular fractions to provide further information on the biochemical basis of susceptibility to Tri. Rates of DCVG formation in rat kidney cells and kidney subcellular fractions were 5- to 20-fold lower than those in rat hepatocytes and liver subcellular fractions. Rates of DCVG formation in kidney cells and subcellular fractions were comparable in male and female rats with the exception of male rat kidney microsomes, where DCVG formation was below the limit of detection, and those in liver cells and subcellular fractions were >3-fold higher in male rats than in female rats. Rates of DCVG formation in mouse kidney subcellular fractions were approximately 10-fold higher than in corresponding fractions from the rat, whereas those in mouse liver subcellular fractions were 4- to 8-fold higher than in corresponding rat tissues, with rates in male mouse liver cytosol and microsomes being modestly higher than in corresponding fractions from female mice. GGT activity was barely detectable in livers, was about 20-fold higher in rat kidneys than in mouse kidneys, and was slightly higher in female rat kidneys than in male rat kidneys. GST activity with 1-chloro-2,4-dinitrobenzene as substrate exhibited tissue-, sex-, and species-dependent patterns that were generally similar to those with Tri as the substrate. These results suggest that the higher susceptibility to Tri-induced renal toxicity of male rats as compared with female rats correlates with rates of DCVG formation. The high rates of DCVG formation in mice, however, indicate that other factors, possibly including differences in activities of cysteine conjugate β-lyase or *N*-acetyltransferase, may also be important determinants of the susceptibility to Tri.

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Tri[1] (also known as trichloroethene) is a major environmental contaminant and is an occupational concern because of its widespread industrial use (Davidson and Beliles, 1991). Tri produces toxicity and tumors in several tissues, with the target organ specificity varying significantly among species, different strains of the same species, and between males and females of the same strain. Most Tri toxicity is dependent on bioactivation, which occurs by two pathways, P-450-dependent oxidation and GSH conjugation. The nephrotoxicity and nephrocarcinogenicity of Tri have been attributed to formation of reactive sulfur-containing metabolites generated by GSH conjugation and subsequent metabolism by GGT, dipeptidase, and the β-lyase (Anders *et al.*, 1988; Goeptar *et al.*, 1995). The initial step in the overall pathway is catalyzed by GSTs (fig. 1). This is followed by hydrolysis reactions catalyzed by GGT and dipeptidases that cleave the glutamyl and glycyl residues to form DCVC. DCVC can then either undergo *N*-acetylation to form the mercapturate NAcDCVC or can undergo a β-elimination reaction catalyzed by the β-lyase to form a reactive thiol. This thiol rearranges to form potent acylating species. Subsequent acylation of proteins and DNA may lead to cytotoxicity and mutagenesis (Anders *et al.*, 1988; Goeptar *et al.*, 1995). Although NAcDCVC is a detoxication product, it can be deacetylated to regen-

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[1] Abbreviations used are: acivicin, L-(αS,5S)α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; β-lyase, cysteine conjugate β-lyase; P-450, cytochrome P-450; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tri, trichloroethylene; GSH, glutathione; GST, glutathione *S*-transferase; DCVG, *S*-(1,2-dichlorovinyl)glutathione; GGT, γ-glutamyltransferase; DCVC, *S*(1,2-dichlorovinyl)-L-cysteine; β-lyase, cysteine conjugate β-lyase; NAcDCVC, *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine.

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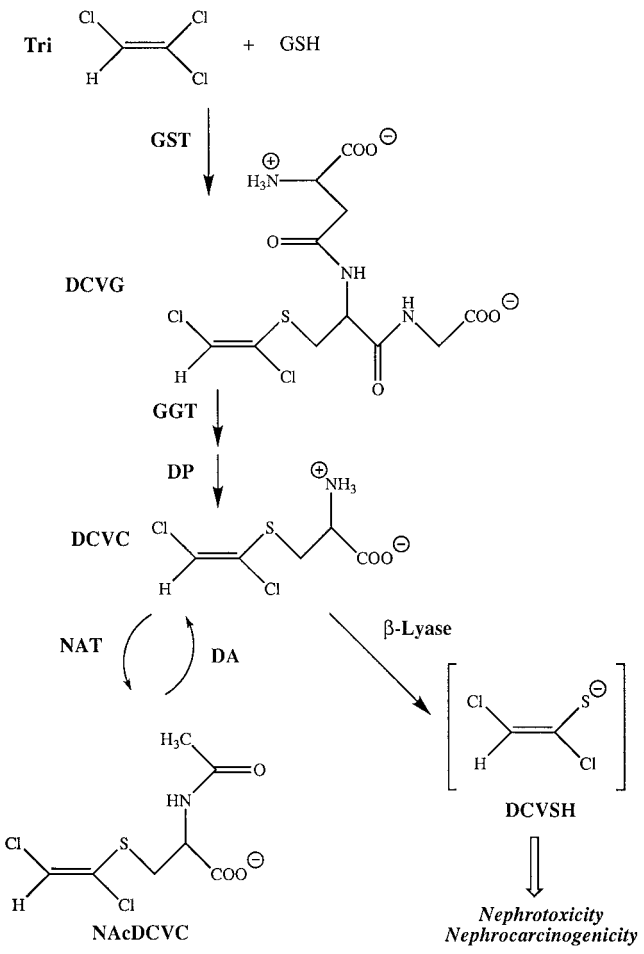

FIG. 1. *Scheme of Tri metabolism by the GSH conjugation pathway.*

erate DCVC. Although marked sex- and species-dependent differences also exist in P-450-dependent metabolism of Tri, there is no evidence that bioactivation by P-450 plays a role in the renal effects of Tri. Rather, the oxidative pathway of metabolism is responsible for effects of Tri in the liver, lung, and other extrarenal tissues.

The initial reaction of the GSH conjugation pathway, catalyzed by GSTs, occurs predominantly in the liver. The DCVG that is formed is rapidly secreted into bile and/or plasma (Lash *et al.*, 1995). The DCVG formed in the liver eventually gets to the kidneys as DCVG, DCVC, or NAcDCVC through interorgan translocation pathways (Lash *et al.*, 1988). GSTs are found in both cytosolic and microsomal fractions. Due to the tissue distribution of membrane transport systems and GGT, subsequent reactions of the pathway occur within the kidney, thereby generating the reactive and toxic species. Alternatively, this pathway can occur within the kidney, removing the need for interorgan translocation of DCVG and its derivatives (Lash *et al.*, 1995).

Controversy exists concerning the relevance for human health hazard assessment of certain animal data showing the kidneys as a target organ of Tri (Bloemen and Tomenson, 1995; Henschler *et al.*, 1995a, 1995b; Swaen, 1995). Two factors in this controversy are 1) that kidney tumors are most frequently seen in male rats but rarely in female rats or in other animal species of either sex and 2) that flux through the GSH conjugation pathway is thought to represent only a minor fraction of total Tri metabolism (Davidson and Beliles, 1991). Regarding the first factor, the mechanisms for this sex and species specificity are not clear but may involve differences in metabolism. As for the second factor, that of the relative flux of Tri through the two pathways, a more complete quantitation of Tri metabolism is needed, and a more accurate method of assessing flux through the GSH conjugation and β-lyase pathways is needed. Whereas metabolites generated from Tri by the P-450 pathway (*e.g.* tri- and dichloroacetate, chloral, and trichloroethanol) are chemically stable and generally are measured easily, those generated from Tri by the GSH conjugation and subsequent β-lyase pathways are chemically unstable and difficult to quantify (Anders *et al.*, 1988). Accordingly, relatively small amounts of these unstable metabolites may produce a disproportionately high toxic response. Many of the conclusions about the lack of significance of Tri metabolism by GSH conjugation and subsequent metabolism by the β-lyase, particularly in humans, have been based on relative recoveries of NAcDCVC and oxidative metabolites (*e.g.* trichloroacetate, trichloroethanol) in urine. Ratios of oxidative metabolites to mercapturate in urine of 100:1 to as high as 3000:1 have been reported (Bernauer *et al.*, 1996; Birner *et al.*, 1993; Davison and Beliles, 1991). Because NAcDCVC represents a detoxication product of DCVC and not the metabolite responsible for Tri-induced nephrotoxicity or nephrocarcinogenicity (see below), it would seem inappropriate to imply any toxicological conclusions to levels of its recovery without at least knowing how it correlates with reactive metabolite formation from DCVC.

We previously determined rates of DCVG formation from Tri in isolated renal cortical cells and hepatocytes and liver microsomes and cytosol from male F344 rats (Lash *et al.*, 1995). DCVG formation was demonstrated to occur in rat kidney cells but at rates that were only 5 to 20% of those in rat hepatocytes. DCVG formation in both liver and kidney was time-, substrate concentration-, and cell- or protein concentration-dependent.

The objectives of the present study were to extend these findings by determining rates of DCVG formation in kidney and liver cells from male and female F344 rats and kidney and liver microsomes and cytosol from male and female F344 rats and B6C3F1 mice. In addition, activities of GGT and GST with other substrates were determined in kidney and liver subcellular fractions to help assess further the importance of differences in GSH-dependent metabolism in susceptibility to Tri. By determining sex-, species-, and tissue-dependent differences in rates of DCVG formation and comparison with the known susceptibility of male and female rats and mice to Tri-induced nephrotoxicity and nephrocarcinogenicity, the role of differences in metabolism of Tri by GSTs in determining renal injury can be better assessed. Collection of these metabolism data is critical for improvement in our ability to assess the role of the GSH conjugation pathway in Tri-induced nephrotoxicity and the risk of nephrotoxicity, particularly in humans. The results from the present investigation indicate that rates of DCVG formation in rats correlate with the greater susceptibility of male rats to renal toxicity due to Tri. Although DCVG formation in mice was much faster than in rats, and although mice are not as susceptible to Tri-induced renal injury, the markedly higher rate of GGT in rats as compared with mice could partially explain this discrepancy. Hence, differences in both rates of GSH conjugation and those of subsequent steps in DCVG biotransformation may help explain the sex and species specificity of susceptibility to Tri.

Materials and Methods

Materials. Tri (reported to be 99.9% pure, as judged by electron ionization mass spectrometry), collagenase type I and type IV, bovine serum albumin (type V), acivicin, and L-γ-glutamyl-L-glutamate were purchased from Sigma. DCVG was synthesized as previously described (Elfarra *et al.*, 1986). Purity (>95%) was determined by HPLC analysis, and identity was confirmed by

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proton NMR spectroscopy. All other chemicals were of the highest purity available and were obtained from commercial sources.

Animals. Male and female F344 rats (150–300 g; Charles River Laboratories, Wilmington, MA) and male and female B6C3F1 mice (18–27 g; Charles River Laboratories, Wilmington, MA) were used in these studies and were housed in a controlled room on a 12-hr light/dark cycle and were given commercial rat chow and water *ad libitum*.

Preparation of Isolated Renal Cortical Cells from Rats. Suspensions of renal cortical cells from rats were prepared by the method of Jones *et al.* (1979) as modified by Lash (1989). Before surgery, animals were anesthetized with ip injections of sodium pentobarbital (0.11 mg/100 g body weight). The aorta was cannulated below the renal arteries with a 19-gauge steel cannula, and kidneys were perfused *in situ* at 8 ml/min with calcium-free, EGTA-containing Hank's buffer. After 10 min, the kidneys were then perfused in a recirculating manner with Hank's buffer supplemented with 4 mM $CaCl_2$ and collagenase type I (0.15%, w/v) at 5 ml/min for 15–18 min. All buffers were continuously bubbled with 95% O_2/5% CO_2 and were maintained at 37°C. Cells were released into Krebs-Henseleit buffer supplemented with 25 mM Hepes (pH 7.4), 0.2% (w/v) bovine serum albumin, 2.5 mM $CaCl_2$, 5 mM glucose, and 5 mM glutamine. Cell concentration was estimated by counting on a hemacytometer, and cell viability was estimated by determining the fraction of cells that excluded trypan blue (0.2%, w/v) on a hemacytometer or by determining the fraction of cells that released lactate dehydrogenase. By either method, cell viability was >85%.

Preparation of Isolated Hepatocytes from Rats. Suspensions of hepatocytes from rats were prepared by the method of Moldéus *et al.* (1978). Before surgery, animals were anesthetized with ip injections of sodium pentobarbital (0.11 mg/100 g body weight). Briefly, two sutures were placed around the portal vein as far apart as possible. A third ligature was placed around the vena cava close to the kidneys. A 16-gauge steel cannula was inserted into the portal vein between the two sutures. The liver was perfused *in situ* with Hank's buffer supplemented with 25 mM $NaHCO_3$, 25 mM Hepes (pH 7.4), 0.5 mM EGTA, and 0.2% (w/v) bovine serum albumin (Hank's I) at a flow rate of 10 ml/min for 5–7 min. All buffers were continuously bubbled with 95% O_2/5% CO_2 and were maintained at 37°C. The liver was then perfused in a recirculating manner with Hank's buffer supplemented with 4 mM $CaCl_2$ and collagenase type IV (0.1%, w/v) at 5 ml/min for 10–12 min. Cells were released by gentle rubbing of the liver surface with a glass rod and were resuspended in Krebs-Henseleit buffer, supplemented with 25 mM Hepes (pH 7.4), 0.2% (w/v) bovine serum albumin, and 2.5 mM $CaCl_2$. Cell concentration and viability (>90%) were determined as above.

Isolation of Renal and Hepatic Microsomes and Cytosol from Rats and Mice. Renal and hepatic microsomes were isolated from homogenates as described by Sharer *et al.* (1992). Before surgery, rats were anesthetized with ip injections of sodium pentobarbital (0.11 mg/100 g body weight), and mice were killed by carbon dioxide asphyxiation. Livers or kidneys were removed, rinsed with buffer (250 mM sucrose, 10 mM triethanolamine, 1 mM EDTA·Na_2, pH 7.6), and homogenized in 3 ml of buffer/g tissue. Homogenates were initially centrifuged at 15,000*g* for 5 min. The postmitochondrial supernatant was then centrifuged for 60 min at 105,000*g*. The resulting supernatant (cytosolic fraction) was stored at −80°C until used. The resulting pellets were resuspended in buffer and centrifuged an additional 60 min at 105,000*g* to produce the "washed" microsomal fraction. Microsomal pellets were resuspended in buffer containing 10% (v/v) glycerol and were stored at −80°C until used. Enzymatic activities were normalized to protein concentrations, which were determined by a Coomassie blue G dye-binding assay (Read and Northcote, 1981) with bovine serum albumin as standard. Purity of microsomal and cytosolic fractions were estimated by measurement of marker enzymes for several subcellular compartments as described previously (Lash *et al.*, 1995). Results indicated that cross-contamination of microsomal and cytosolic fractions was <5%, and contamination of these two fractions with mitochondria, lysosomes, and plasma membranes was <2%.

Assay of Tri Metabolism by GSH Conjugation Pathway in Isolated Cells and Subcellular Fractions. All incubations were performed in 25-ml polypropylene Erlenmeyer flasks on a Dubnoff metabolic shaking incubator (60 cycles/min) at either 37°C (cells) or 30°C (subcellular fractions). The lower temperature was chosen for assays with subcellular fractions because more consistent results were obtained than at the higher temperature used with the intact cells. Isolated renal cortical cells and renal microsomes were preincubated for 15 min with 0.25 mM acivicin to inhibit GGT activity before performing incubations to measure DCVG formation. Previous studies in isolated renal cortical cells and renal plasma membrane vesicles showed that this procedure irreversibly inhibited GSH or GSH *S*-conjugate degradation by >95% (Lash, 1989; Lash and Jones, 1985). A similar preincubation of renal cytosol, liver cells, or liver subcellular fractions with acivicin was not necessary because these biological samples do not contain significant GGT activity; there was no effect of acivicin pretreatment on DCVG formation in these biological samples. Cells or subcellular fractions were then incubated with 5 mM GSH and 1 or 2 mM Tri (stock solution made in acetone; final concentration <1%, v/v). The concentration of GSH was chosen because it is the normal, physiological concentration of GSH in renal and hepatic cells; the two concentrations of Tri were chosen to obtain measurable rates of product formation and a concentration-dependent effect. In some cases, 0.5 mM Tri was also used. With isolated cells, Triton X-100 (0.1%, v/v, final concentration) was added to solubilize the plasma membranes. After incubations for various lengths of time, reactions were terminated by addition of perchloric acid (10%, w/v, final concentration), and samples were processed for analysis of DCVG as described previously (Lash *et al.*, 1995) using the HPLC method described by Fariss and Reed (1987).

Assay of GSH-Dependent Enzymes in Cells and Subcellular Fractions. GGT activity was measured with L-γ-glutamyl-*p*-nitroanilide as substrate and glycylglycine as γ-glutamyl acceptor (Orlowski and Meister, 1963). Formation of *p*-nitroanilide was quantified by the increase in absorbance at 410 nm using $\epsilon^{410} = 8800\ M^{-1}\ cm^{-1}$. GST activity with 1-chloro-2,4-dinitrobenzene as substrate was measured by quantifying formation of 2,4-dinitrophenol as increases in absorbance at 365 nm (Habig *et al.*, 1974).

Data Analysis. All values are means ± SE of measurements made on the indicated number of separate cell preparations or subcellular fractionations. Significant differences between means for data were first assessed by a one-way analysis of variance. When significant *F* values were obtained, the Fisher's protected least significance *t* test was performed to determine which means were significantly different from one another, with two-tail probabilities <0.05 considered significant.

Results

Sex and Species Dependence of GSH-Dependent Enzymes in Rat and Mouse Kidney and Liver. Before making direct measurements of DCVG formation in isolated cells and subcellular fractions from kidney and liver of rats and mice of both sexes, measurements of two key enzymes involved in GSH conjugate metabolism were determined using standard spectrophotometric procedures (tables 1 and 2).

GGT is the major enzyme that catalyzes the hydrolysis of the γ-glutamyl peptide bond of GSH and GSH *S*-conjugates. It is localized predominantly to the renal brush-border plasma membrane, which is recovered with the microsomes in the subcellular fractionation procedure used here (table 1). GGT is present at undetectable to very low levels in the livers of most mammalian species (Hinchman and Ballatori, 1990), which was confirmed here. The rat has been reported to have the highest GGT activity of mammals, and this finding was supported by measurements in this study. Specific activity of GGT in both male and female rat kidney microsomal fractions was approximately 20-fold higher than that in the corresponding fractions from mice. Whereas renal GGT activity was about 40% higher in male mouse kidney microsomes than in female mouse kidney microsomes, it was about 20% higher in female rat kidney microsomes than in male rat kidney microsomes.

GST activity with 1-chloro-2,4-dinitrobenzene as an alternative substrate to Tri was measured (table 2). Activity was found predominantly in the cytosol of both liver and kidney, with liver cytosols generally having significantly higher specific activities than kidney cytosols. GST activity in male rat liver cytosol was 2-fold higher than in male kidney cytosol, whereas that in female rats was similar in the

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TABLE 1

GGT activity in cells and subcellular fractions from male and female F344 rat and B6C3F1 mouse liver and kidney

GGT activity was measured spectrophotometrically with γ-glutamyl-*p*-nitroanilide as substrate and glycylglycine as γ-glutamyl acceptor. Results are means ± SE of measurements from three tissue fractionations.

Sex	Species	Tissue	Fraction	Activity (milliunits/mg protein)
Male	Mouse	Liver	Homogenate	ND[a,b]
			Cytosol	0.07 ± 0.04
			Microsomes	0.05 ± 0.02[a]
Male	Mouse	Kidney	Homogenate	102 ± 16[c,d]
			Cytosol	1.63 ± 0.85[e]
			Microsomes	92.6 ± 15.6[d]
Female	Mouse	Liver	Homogenate	ND
			Cytosol	0.10 ± 0.06
			Microsomes	0.03 ± 0.02[a]
Female	Mouse	Kidney	Homogenate	24.3 ± 2.3[d]
			Cytosol	0.79 ± 0.46[e]
			Microsomes	69.3 ± 14.0[d]
Male	Rat	Liver	Homogenate	ND[a]
			Cytosol	ND
			Microsomes	ND[a]
Male	Rat	Kidney	Homogenate	323 ± 4[c]
			Cytosol	ND[d]
			Microsomes	1570 ± 100[c]
Female	Rat	Liver	Homogenate	ND[a]
			Cytosol	ND
			Microsomes	ND[a]
Female	Rat	Kidney	Homogenate	386 ± 34
			Cytosol	ND[e]
			Microsomes	1840 ± 40

[a] Statistically significant ($p < 0.05$) difference from kidney from same sex and species (kidney *vs.* liver).
[b] ND, not detectable; limit of detection was 0.02 milliunits/mg protein.
[c] Statistically significant ($p < 0.05$) difference from corresponding tissue and fraction from female of same species (male *vs.* female).
[d] Statistically significant ($p < 0.05$) difference from corresponding tissue, fraction, and sex from rats (mouse *vs.* rat).
[e] Statistically significant ($p < 0.05$) difference from microsomes from same sex, species, and tissue (cytosol *vs.* microsomes).

TABLE 2

GST activity with 1-chloro-2,4-dinitrobenzene as substrate in cells and subcellular fractions from male and female F344 rat and B6C3F1 mouse liver and kidney

GST activity was measured spectrophotometrically with 1-chloro-2,4-dinitrobenzene as substrate. Results are means ± SE of measurements from three tissue fractionations.

Sex	Species	Tissue	Fraction	Activity (milliunits/mg protein)
Male	Mouse	Liver	Homogenate	0.21 ± 0.02
			Cytosol	0.78 ± 0.28[a]
			Microsomes	0.13 ± 0.01
Male	Mouse	Kidney	Homogenate	0.13 ± 0.01[b]
			Cytosol	0.70 ± 0.09[a,c]
			Microsomes	0.08 ± 0.04
Female	Mouse	Liver	Homogenate	0.17 ± 0.01
			Cytosol	0.49 ± 0.03[a]
			Microsomes	0.02 ± 0.01
Female	Mouse	Kidney	Homogenate	0.05 ± 0.01
			Cytosol	0.34 ± 0.04[a]
			Microsomes	0.02 ± 0.01
Male	Rat	Liver	Homogenate	0.21 ± 0.10
			Cytosol	1.10 ± 0.44[a,c,d]
			Microsomes	0.18 ± 0.04
Male	Rat	Kidney	Homogenate	0.44 ± 0.20
			Cytosol	0.55 ± 0.10[a,c]
			Microsomes	0.10 ± 0.04
Female	Rat	Liver	Homogenate	0.17 ± 0.03
			Cytosol	0.28 ± 0.06[a]
			Microsomes	0.04 ± 0.02
Female	Rat	Kidney	Homogenate	0.24 ± 0.09
			Cytosol	0.34 ± 0.08[a]
			Microsomes	0.02 ± 0.01

[a] Statistically significant ($p < 0.05$) difference from microsomes from same sex, species, and tissue (cytosol *vs.* microsomes).
[b] Statistically significant ($p < 0.05$) difference from corresponding tissue, fraction, and sex from rats (mouse *vs.* rat).
[c] Statistically significant ($p < 0.05$) difference from corresponding tissue and fraction from female of same species (male *vs.* female).
[d] Statistically significant ($p < 0.05$) difference from kidney from same sex and species (kidney *vs.* liver).

two tissues. GST activity in liver and kidney cytosol of male mice and male rats was 60% to more than 100% higher than that in the corresponding fractions of female mice and rats. The only significant difference between GST activity in mice and rats was that activities in male mouse liver cytosol and male mouse kidney homogenates were significantly lower than corresponding fractions in male rats.

Metabolism of Tri in Rat Kidneys. Tri undergoes GSH conjugation in isolated kidney cells from male rats to form DCVG. After an

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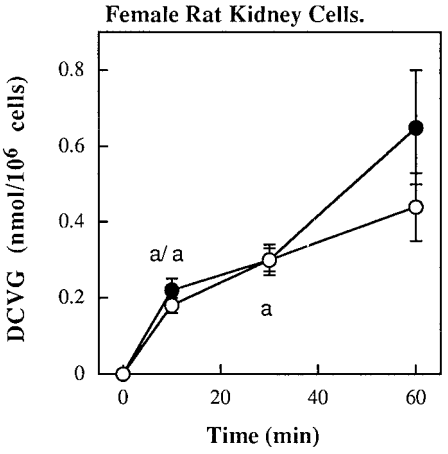

FIG. 2. *Time and concentration dependence of DCVG formation in suspensions of isolated kidney cells from female F344 rats.*

Isolated renal cortical cells (2×10^6 cells/ml) were incubated with 1 (○) or 2 (●) mM Tri and 5 mM GSH and 0.1% (v/v) Triton X-100 at 30°C for the indicated times. Metabolism was measured by quantitation of DCVG formation by HPLC after derivatization. Results are the means ± SE of three experiments. *a*, statistically significant difference ($p < 0.05$) from DCVG content in corresponding samples (*i.e.* same time, concentration of Tri, and tissue sample) in males (as compared with data from ref. 4).

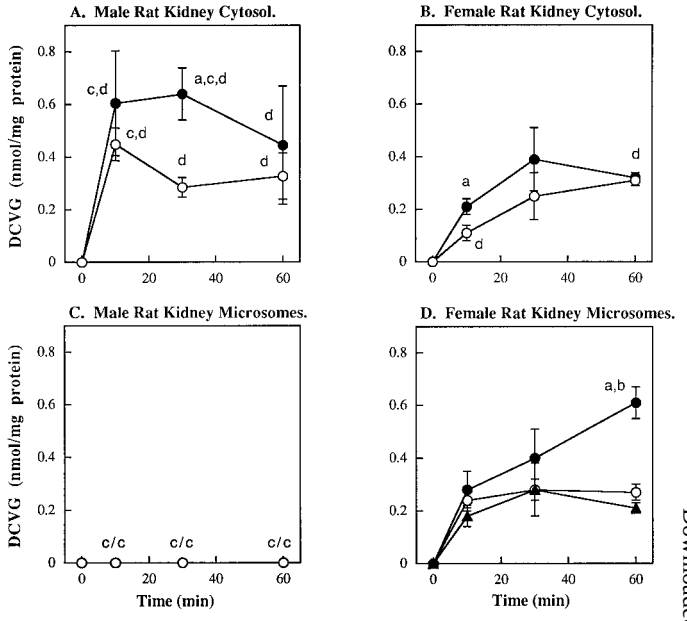

FIG. 3. *Time and concentration dependence of DCVG formation in kidney cytosol (*A, B*) and microsomes (*C, D*) from male (*A, C*) and female (*B, D*) F344 rats.*

Isolated renal cortical cytosol and microsomes (0.5–2 mg of protein/ml) were incubated with 1 (○) or 2 (●) mM Tri in the presence of 5 mM GSH at 30°C for the indicated times. Metabolism was measured by quantitation of DCVG formation by HPLC after derivatization. Results are the means ± SE of three experiments. *a*, statistically significant difference ($p < 0.05$) from DCVG content with 1 mM Tri at the same time point in the same sex, species, and tissue sample. *b*, statistically significant difference ($p < 0.05$) from DCVG content with 0.5 mM Tri at the same time point in the same sex, species, and tissue sample. *c*, statistically significant difference ($p < 0.05$) from DCVG content in corresponding samples (*i.e.* same time, concentration of Tri, and tissue sample) in females. *d*, statistically significant difference ($p < 0.05$) from DCVG content in corresponding incubations with microsomes at the same time point and concentration of Tri.

apparent lag period, less than 0.5 nmol of DCVG was formed during a 60-min incubation (Lash *et al.*, 1995). Similar rates of DCVG formation were observed in female rat kidney cells (fig. 2), although no apparent lag period in metabolism occurred. Hence, significantly higher amounts of DCVG were formed in female rat kidney cells at the 10- and 30-min time points due to the absence of the lag; no difference between males and females was observed by the 60-min time point.

DCVG formation was next examined in renal subcellular fractions from male and female rats (fig. 3). DCVG formation in kidney cytosol was approximately twice as fast in male rats (fig. 3*A*) as in female rats (fig. 3*B*). However, whereas rates of DCVG formation that were comparable with those in the cytosol were observed in female rat kidney microsomes (fig. 3*D*), no detectable DCVG (<0.05 nmol/mg protein) was formed in male rat kidney microsomes (fig. 3*C*). Several attempts were made to ensure that DCVG formation in male rat kidney microsomes was not inadvertently missed due to an assay artifact or subsequent metabolism of the product. These attempts included addition of DCVG to samples to assess recovery and inclusion of acivicin to inhibit degradation of GSH conjugates. Nonetheless, no DCVG formation was ever detected in male rat kidney microsomes. Because the total amount of microsomal protein is much smaller than that of the cytosol, capacity for renal DCVG formation is comparable in male and female rats.

Metabolism of Tri in Rat Liver. DCVG formation in isolated hepatocytes from male rats was reported previously to be 5- to 10-fold more rapid than in isolated kidney cells from male rats (Lash *et al.*, 1995). Similarly, DCVG formation in isolated hepatocytes from female rats (fig. 4) was approximately 5-fold higher than that in isolated kidney cells from female rats (*cf.* fig. 2) but was approximately one-third of that in male rat hepatocytes (*cf.* ref. 4). Amounts of DCVG formed in male rat hepatocytes were significantly greater than those in female rat hepatocytes at all time points and with both concentrations of Tri. With female rat hepatocytes, only the DCVG content at 60 min was significantly higher with 2 mM Tri than with 1 mM Tri. Overall, the total capacity of GSH conjugation (liver cells + kidney cells) was higher in male than in female rats.

Distribution of GSH conjugation activity of Tri in liver was examined further by measurement of DCVG formation in hepatic cytosol and microsomes (fig. 5). In contrast to the findings in intact hepatocytes, measured DCVG formation at 15 or 30 min was significantly, although only slightly, higher in cytosol from female rat liver than in cytosol from male rat liver (fig. 5*A* and Lash *et al.*, 1995), and DCVG formation in microsomes from male and female rat liver was similar (fig. 5*B* and Lash *et al.*, 1995).

Metabolism of Tri in Mouse Kidneys. In contrast to DCVG formation in rat kidney subcellular fractions, where maximal amounts of less than 1 nmol/mg protein were found (fig. 3), DCVG formation in male (fig. 6*A*) and female (fig. 6*B*) mouse kidney cytosol after 60-min incubations was approximately 6 and 4 nmol/mg protein, respectively. Similarly, amounts of DCVG formation in both male (fig. 6*C*) and female (fig. 6*D*) mouse kidney microsomes were markedly higher than those in the rat kidney microsomes. DCVG formation in male mouse kidney microsomes was comparable with that in the cytosol. The absence of DCVG formation in male rat kidney microsomes and the detection of relatively high levels of DCVG in incubations with male mouse kidney microsomes suggest that there is a difference in substrate specificity of the renal microsomal GST in the two species. DCVG formation in female mouse kidney microsomes was approximately 2.5-fold higher than that in the male mouse kidney microsomes, with differences at both concentrations of Tri and at all time points being statistically significant.

Metabolism of Tri in Mouse Liver. Similar to the findings of higher rates of DCVG formation in mouse kidney subcellular fractions as compared with those from the rat, DCVG formation in liver subcellular

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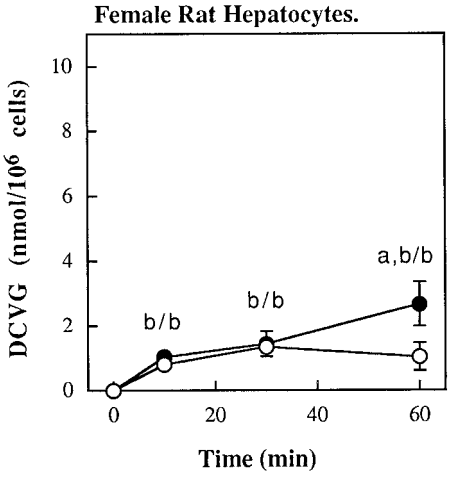

FIG. 4. *Time and concentration dependence of DCVG formation in suspensions of isolated hepatocytes from female F344 rats.*

Isolated hepatocytes (2×10^6 cells/ml) were incubated with 1 (○) or 2 (●) mM Tri and 5 mM GSH and 0.1% (v/v) Triton X-100 at 30°C for the indicated times. Metabolism was measured by quantitation of DCVG formation by HPLC after derivatization. Results are the means ± SE of three experiments. *a*, statistically significant difference ($p < 0.05$) from DCVG content with 1 mM Tri at the same time point in the same sex, species, and tissue sample. *b*, statistically significant difference ($p < 0.05$) from DCVG content in corresponding samples (*i.e.* same time, concentration of Tri, and tissue sample) in males (as compared with data from ref. 4).

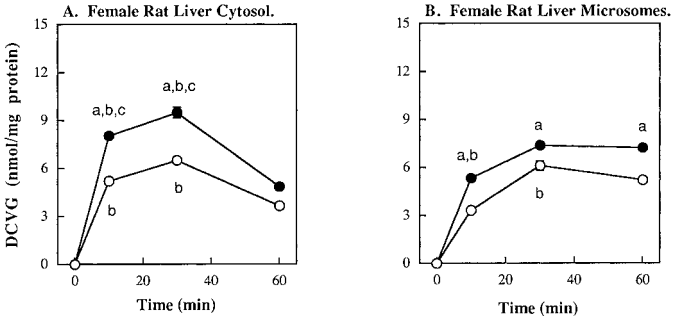

FIG. 5. *Time and concentration dependence of DCVG formation in liver cytosol (*A*) and microsomes (*B*) from female F344 rats.*

Isolated liver cytosol and microsomes (0.5–2 mg of protein/ml) were incubated with 1 (○) or 2 (●) mM Tri in the presence of 5 mM GSH at 30°C for the indicated times. Metabolism was measured by quantitation of DCVG formation by HPLC after derivatization. Results are the means ± SE of three experiments. *a*, statistically significant difference ($p < 0.05$) from DCVG content with 1 mM Tri at the same time point in the same sex, species, and tissue sample. *b*, statistically significant difference ($p < 0.05$) from DCVG content in corresponding samples (*i.e.* same time, concentration of Tri, and tissue sample) in males (as compared with data from ref. 4). *c*, statistically significant difference ($p < 0.05$) from DCVG content in corresponding incubations with microsomes at the same time point and concentration of Tri.

fractions from both male and female mice were 3- to 4-fold higher than that in corresponding liver subcellular fractions from male and female rats (fig. 7). DCVG formation in male mouse liver cytosol was similar to that in female mouse liver cytosol, except at 60 min and 1 mM Tri, where DCVG formation was significantly higher in males. In contrast, DCVG formation in male mouse liver microsomes at both concentrations of Tri and at all time points measured was significantly higher than that in corresponding samples from female mice.

Discussion

The selective tissue distribution of the various enzymes of the GSH conjugation pathway and of the plasma membrane transporters for the

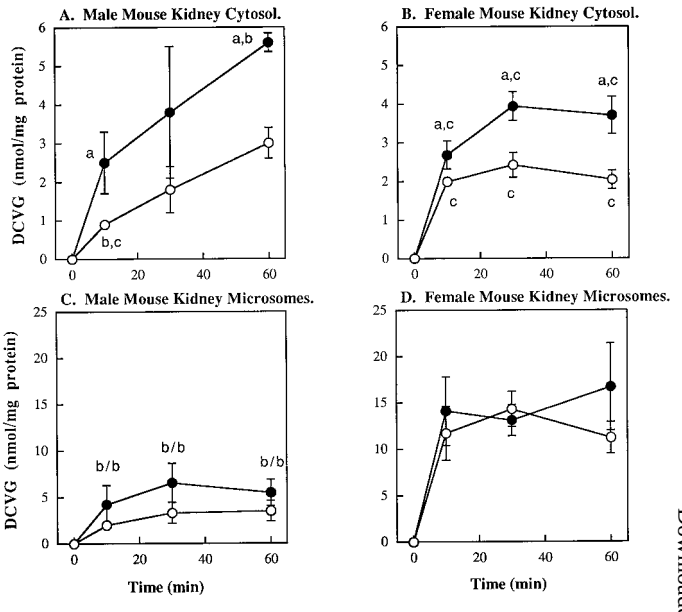

FIG. 6. *Time and concentration dependence of DCVG formation in kidney cytosol (*A, B*) and microsomes (*C, D*) from male (*A, C*) and female (*B, D*) B6C3F1 mice.*

Isolated renal cortical cytosol and microsomes (0.5–2 mg of protein/ml) were incubated with 1 (○) or 2 (●) mM Tri in the presence of 5 mM GSH at 30°C for the indicated times. Metabolism was measured by quantitation of DCVG formation by HPLC after derivatization. Results are the means ± SE of three experiments. *a*, statistically significant difference ($p < 0.05$) from DCVG content with 1 mM Tri at the same time point in the same sex, species, and tissue sample. *b*, statistically significant difference ($p < 0.05$) from DCVG content in corresponding samples (*i.e.* same time, concentration of Tri, and tissue sample) in females. *c*, statistically significant difference ($p < 0.05$) from DCVG content in corresponding incubations with microsomes at the same time point and concentration of Tri.

metabolites of this pathway play important roles in the target organ specificity of DCVG and the other GSH-derived metabolites of Tri. Hence, although most of the DCVG formation occurs in the liver, the rapid efflux of DCVG from the hepatocyte and interorgan translocation pathways deliver DCVG or one of its metabolites (*i.e.* DCVC or NAcDCVC) to the kidneys (Anders *et al.*, 1988; Lash *et al.*, 1988). The metabolites of DCVG are generated by biliary or intestinal metabolism. DCVG, DCVC, or NAcDCVC are then transported into proximal tubular cells and can undergo intracellular bioactivation. Additionally, Tri may be conjugated with GSH within kidney cells, leading to an intraorgan cycle of membrane transport and metabolism (ref. 4 and figs. 2, 3, and 6).

Recovery and identification of NAcDCVC as a urinary metabolite of Tri in rats, mice, and humans (Birner *et al.*, 1993; Commandeur and Vermeulen, 1990; Dekant *et al.*, 1986, 1990) suggest that the kidney is the primary site for the accumulation of DCVG and the subsequent metabolites before their excretion from the body. This is consistent with the interorgan pattern of GSH and GSH *S*-conjugate metabolism and transport. A complicating factor in understanding the significance of nephrotoxicity and nephrocarcinogenicity of Tri for humans is that sex- and species-dependent differences exist in target organ specificity and susceptibility.

The objective of the present study was to quantify hepatic and renal metabolism of Tri by GSH conjugation to assess whether rates of this pathway correlate with the known sex and species specificity of susceptibility to Tri-induced renal injury. Hence, because male rats are the most susceptible animal to renal injury from Tri (Davidson and Beliles, 1991), we expected that male rats would accordingly exhibit the highest rates of DCVG formation. Because a previous study of

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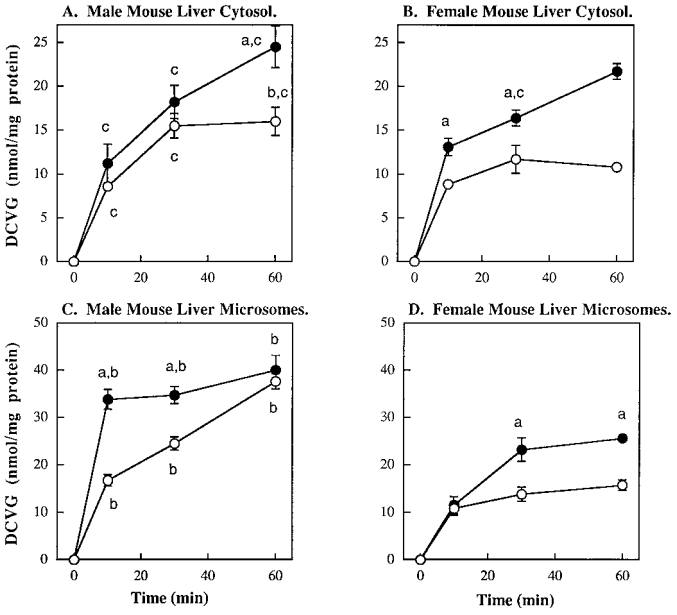

FIG. 7. *Time and concentration dependence of DCVG formation in liver cytosol (*A, B*) and microsomes (*C, D*) from male (*A, C*) and female (*B, D*) B6C3F1 mice.*

Isolated liver cytosol and microsomes (0.5–2 mg of protein/ml) were incubated with 1 (○) or 2 (●) mM Tri in the presence of 5 mM GSH at 30°C for the indicated times. Metabolism was measured by quantitation of DCVG formation by HPLC after derivatization. Results are the means ± SE of three experiments. *a*, statistically significant difference ($p < 0.05$) from DCVG content with 1 mM Tri at the same time point in the same sex, species, and tissue sample. *b*, statistically significant difference ($p < 0.05$) from DCVG content in corresponding samples (*i.e.* same time, concentration of Tri, and tissue sample) in females. *c*, statistically significant difference ($p < 0.05$) from DCVG content in corresponding incubations with microsomes at the same time point and concentration of Tri.

ours demonstrated that DCVG formation could be measured and occurred in a time- and protein concentration-dependent manner in preparations of liver and kidney of male F344 rats, this study extended our previous work to determine relative pathway flux in female rats and male and female mice.

A summary of metabolism data from figs. 2–7 and our previous metabolism study with tissue from male rats (Lash *et al.*, 1995) shows that marked sex- and species-dependent differences in DCVG formation occur (table 3). Amounts of DCVG formed during 60-min incubations in liver cells of male rats were significantly higher than those in livers of female rats. Although amounts of DCVG formed in kidney cells and subcellular fractions were comparable in male and female rats, the predominance of this pathway in the liver indicates that overall flux of Tri through GSH conjugation is significantly greater in male rats than in female rats. This finding is consistent with the greater susceptibility of male rats to Tri-induced nephrocarcinogenicity.

Based on the known species specificity of toxicity, the expectation was that rates of DCVG formation in mouse liver and kidney would be much lower than those in the rat. However, rates of DCVG formation were markedly higher in both male and female mouse tissues than in corresponding fractions from the rat (table 3). These results are consistent with the more rapid clearance and overall rate of Tri metabolism in mice as compared with rats (Davidson and Beliles, 1991; Goeptar *et al.*, 1995; Prout *et al.*, 1985). In terms of the potential toxicological consequences of these differences in rates of metabolism, studies by Bull and colleagues (Eyre *et al.*, 1995a, 1995b) also found an unexpected contrast between rats and mice regarding the actions of Tri or DCVC. Using radiolabeled Tri or DCVC, formation

TABLE 3

Summary of Tri metabolism to DCVG in kidney and liver cells or subcellular fractions from F344 rats and B6C3F1 mice

Results are means ± SE of measurements from three separate cell or tissue fractionation preparations and are for 2 mM Tri incubated with 5 mM GSH for 60 min. DCVG formation was measured after derivatization of acid extracts with iodoacetate and 1-fluoro-2,4-dinitrobenzene, separation by ion exchange, gradient HPLC on an amine column using a methanol-acetate mobile phase, and detection of *N*-dinitrophenyl DCVG at 365 nm.

	DCVG Formation (nmol/mg protein or 10^6 cells per 60 min)	
	Male	Female
Rat kidney cells	0.48 ± 0.02^{a}	0.65 ± 0.15
Rat hepatocytes	$9.70 \pm 0.29^{a,b}$	2.67 ± 0.69
Rat kidney cytosol	$0.45 \pm 0.22^{c,d}$	0.32 ± 0.02^{d}
Rat kidney microsomes	$ND^{b,c}$	0.61 ± 0.06
Rat liver cytosol	$7.30 \pm 2.80^{a,c}$	4.86 ± 0.14^{d}
Rat liver microsomes	$10.3 \pm 2.8^{a,c}$	7.24 ± 0.24
Mouse kidney cytosol	5.60 ± 0.24^{b}	3.70 ± 0.48^{d}
Mouse kidney microsomes	5.47 ± 1.41^{b}	16.7 ± 4.7
Mouse liver cytosol	24.5 ± 2.4^{d}	21.7 ± 0.9
Mouse liver microsomes	40.0 ± 3.1^{b}	25.6 ± 0.8

[a] Data derived from ref. 4.
[b] Significantly different ($p < 0.05$) from DCVG formation in same species and tissue sample in females.
[c] Significantly different ($p < 0.05$) from DCVG formation in corresponding sample in mice of same sex.
[d] Significantly different ($p < 0.05$) from DCVG formation in microsomes from same sex, species, and tissue.

of covalent adducts with protein were quantified in rat and mouse kidneys. Unexpectedly, acid-labile adduct formation from Tri or DCVC was 2- to 12-fold higher, respectively, in mouse kidneys. This seemed to correlate with higher rates of Tri- or DCVC-induced cell proliferation in mouse kidneys as compared with rat kidneys. Hence, they concluded that other factors may contribute to the greater sensitivity of the rat to the induction of renal carcinogenesis by Tri.

Rates of DCVG formation reported previously (Lash *et al.*, 1995) and in this study are severalfold lower than those reported for Tri metabolism by oxidative pathways. For example, Miller and Guengerich (1983) measured oxidative metabolism of 1 mM Tri in suspensions of isolated hepatocytes from male Osborne-Mendel rats and found that formation of total oxidative metabolites (trichloroethanol + trichloroacetic acid + CO_2) occurred at a rate of approximately 0.5 nmol/min/mg protein. In contrast, rates of formation of DCVG from incubations of isolated hepatocytes from F344 rats with 1 mM Tri and 5 mM GSH were approximately 0.07 and 0.02 nmol/min/mg protein in males and females, respectively (*cf.* ref. 4 and fig. 4). Similarly, Nakajima *et al.* (1993) reported that rates of formation of chloral hydrate, the initial oxidative metabolite generated from Tri, in liver microsomes from male Wistar rats were 0.62 and 1.51 nmol/min/mg protein with 0.2 and 5.9 mM Tri, respectively. They also quantified rates of chloral hydrate formation from Tri in liver microsomes from male B6C3F1 mice and found them to be 2- to 3-fold higher than those in rat liver microsomes (1.84 and 2.98 nmol/min/mg protein with 0.2 and 5.9 mM Tri, respectively). Hence, oxidative metabolism of Tri is severalfold faster than GSH-dependent metabolism of Tri, and rates of metabolism in mice for both pathways are 2- to 4-fold faster than in rats.

The measurements of GGT and GST activities with alternate substrates from DCVG and Tri, respectively, also showed some species-, sex-, and tissue-dependent differences that may provide additional explanation for the species susceptibility differences. The sex- and species-dependent differences in renal GGT activity would likely play a major role in determining the amount of substrate for the β-lyase, which in turn produces the ultimate, nephrotoxic metabolite. Hence, although mice of both sexes produced severalfold more DCVG than

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rats, the greater susceptibility of rats to the nephrocarcinogenic effect of Tri and DCVC may be partially explained by the nearly 20-fold greater activity of GGT in rat kidneys of both sexes. GGT activity may, therefore, be limiting in the mouse, thereby allowing much lower flux of Tri through the β-lyase. This explanation for the comparison of rats and mice would not apply to a comparison between male and female rats. In the latter case, in contrast, female rats are less susceptible than male rats to Tri- or DCVG-induced nephrotoxicity and nephrocarcinogenicity. However, GGT activity in female rat kidney microsomes was approximately 20% higher than that in male rat kidney microsomes. This discrepancy is not really inconsistent with the higher susceptibility of male rats, as GSH conjugate formation was markedly higher in males. The results on GST activity with 1-chloro-2,4-dinitrobenzene as substrate showed that, as with Tri as substrate, liver and kidney cytosols of both male rats and mice had higher activities than the corresponding samples in females.

In summary, rates of DCVG formation were quantified in isolated cells or subcellular fractions from liver or kidney from male and female F344 rats and B6C3F1 mice to determine if sex- and species-dependent patterns of Tri metabolism correlate with the known pattern of sensitivity to renal injury. Overall rates of DCVG formation in liver were severalfold faster in male rats than in female rats, although renal metabolism of Tri by this pathway was comparable in the two sexes. In contrast to expectations, rates of DCVG formation to form DCVG were nearly 10-fold higher in male or female mouse kidney than in corresponding subcellular fractions from rat kidney and were 3- to 4-fold higher in male or female mouse liver than in corresponding subcellular fractions from rat liver. These results suggest that sex dependence of susceptibility to Tri-induced nephrocarcinogenicity in the rat correlates with rates of DCVG formation (male $\gg$ female). However, the species-dependent patterns of Tri-induced nephrocarcinogenicity (rat $\gg$ mouse) do not correspond to rates of DCVG formation (mouse $\gg$ rat). Hence, differences in the activities of other enzymes, such as GGT, β-lyase, *N*-acetyltransferase, or deacetylase, are likely to contribute to the species dependence of Tri-induced nephrocarcinogenicity.

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