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GLUTATHIONE CONJUGATION OF TRICHLOROETHYLENE IN HUMAN LIVER AND KIDNEY: KINETICS AND INDIVIDUAL VARIATION

LAWRENCE H. LASH, JOHN C. LIPSCOMB[1], DAVID A. PUTT, AND JEAN C. PARKER

Department of Pharmacology, Wayne State University School of Medicine, Detroit, Michigan (L.H.L., D.A.P.); U.S. Air Force, Air Force Research Laboratory, Toxicology Branch, Wright-Patterson Air Force Base, Dayton, Ohio (J.C.L.); and National Center for Environmental Assessment, U.S. Environmental Protection Agency, Washington, D.C. (J.C.P.)

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ABSTRACT:

Isolated human hepatocytes exhibited time-, trichloroethylene (Tri) concentration-, and cell concentration-dependent formation of *S*-(1,2-dichlorovinyl)glutathione (DCVG) in incubations in sealed flasks with 25 to 10,000 ppm Tri in the headspace, corresponding to 0.011 to 4.4 mM in hepatocytes. Maximal formation of DCVG (22.5 ± 8.3 nmol/120 min per 10^6 cells) occurred with 500 ppm Tri. Time-, protein concentration-, and both Tri and GSH concentration-dependent formation of DCVG were observed in liver and kidney subcellular fractions. Two kinetically distinct systems were observed in both cytosol and microsomes from pooled liver samples, whereas only one system was observed in subcellular fractions from pooled kidney samples. Liver cytosol exhibited apparent K_m values (μM Tri) of 333 and 22.7 and V_{max} values (nmol DCVG formed/min per mg protein) of 8.77 and 4.27; liver microsomes exhibited apparent K_m values of 250 and 29.4 and V_{max} values of 3.10 and 1.42; kidney cytosol and microsomes exhibited apparent K_m values of 26.3 and 167, respectively, and V_{max} values of 0.81 and 6.29, respectively. DCVG formation in samples of liver cytosol and microsomes from 20 individual donors exhibited a 6.5-fold variation in microsomes but only a 2.4-fold variation in cytosol. In coincubations of pooled liver cytosol and microsomes, addition of an NADPH-regenerating system produced marked inhibition of DCVG formation, but addition of GSH had no effect on cytochrome P-450-catalyzed formation of chloral hydrate. These results indicate that both human kidney and liver have significant capacity to catalyze DCVG formation, indicating that the initial step of the GSH-dependent pathway is not limiting in the formation of nephrotoxic and nephrocarcinogenic metabolites.

Trichloroethene (Tri)[2] is a major environmental contaminant and is an occupational concern because of its widespread industrial use (Davidson and Beliles, 1991). Tri is an established animal carcinogen that produces tumors in a variety of tissues. Due to the possibility of human exposure, Tri has been extensively evaluated in rodent cancer bioassays, and the U.S. Environmental Protection Agency is currently performing a new human health risk assessment for Tri (Maull and Lash, 1998). A complicating factor in the evaluation of the risk of Tri exposure for humans is that susceptibility and target organ specificity for Tri exhibit marked species and sex dependence. For example, Tri produces lung and liver tumors in mice (National Cancer Institute, 1976; Fukuda et al., 1983; National Toxicology Program, 1983; Forkert et al., 1985) and nephrotoxicity and renal tumors in rats (National Toxicology Program, 1983, 1987; Maltoni et al., 1988), with males of each species exhibiting greater susceptibility than females. Epidemiological studies in occupationally or environmentally exposed humans, however, have given conflicting results with regard to susceptibility and target organ specificity. In spite of this, the International Agency for Research on Cancer (IARC) has recently revised their classification of Tri and have declared it a Class 2A carcinogen ("Probably Carcinogenic to Humans"; IARC, 1995).

The toxicity of Tri is dependent on its metabolism, which occurs by either cytochrome P-450 (P-450)-dependent oxidation or GSH conjugation. Metabolites derived from P-450 metabolism, including chloral hydrate, trichloroacetate, and dichloroacetate, have been associated with the pulmonary and hepatic toxicity of Tri (Davidson and Beliles, 1991). In contrast, reactive metabolites derived from GSH conjugation of Tri, with subsequent metabolism by γ-glutamyltransferase (GGT), dipeptidases, and cysteine conjugate β-lyase (β-lyase), are associated with the nephrotoxicity of Tri (Anders et al., 1988; Goeptar et al., 1995). The cysteine conjugate DCVC may also be *N*-acetylated to form the mercapturate, which represents a detoxification mechanism. The mercapturate is then either deacetylated to reform DCVC or is excreted in the urine.

One of the major controversies about human risk of Tri exposure

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[1] Present address: National Center for Environmental Assessment, U. S. Environmental Protection Agency, 26 W. Martin Luther King Drive, MC-190, Cincinnati, OH 45268.

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

Send reprint requests to: Lawrence H. Lash, Ph.D., Department of Pharmacology, Wayne State University School of Medicine, 540 East Canfield Ave., Detroit, MI 48201. E-mail: l.h.lash@wayne.edu

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concerns the kidney as a target organ (Bloemen and Tomenson, 1995; Henschler et al., 1995a,b; Swaen, 1995). Three observations that have been interpreted to indicate that the nephrotoxicity and nephrocarcinogenicity of Tri may not be relevant to humans are that: 1) kidney tumors are most frequently observed in male rats but are rarely seen in female rats or in males or females of other species, 2) kidney toxicity or renal tumors are rarely observed in humans exposed to Tri, and 3) flux through the GSH conjugation pathway is thought to represent only a minor fraction of total Tri metabolism (Green et al., 1997).

The initial step in the GSH conjugation pathway, which is catalyzed by glutathione *S*-transferases (GSTs) found in the cytosol and microsomes of most tissues, occurs predominantly in the liver (Lash et al., 1988). GSH *S*-conjugates formed in the liver are then readily translocated into bile and small intestine or plasma. Biliary or intestinal GSH *S*-conjugates then undergo subsequent metabolism to the corresponding cysteine *S*-conjugates or mercapturates. Through interorgan pathways, these metabolites are extracted by the kidneys and are metabolized further or excreted. Plasma GSH *S*-conjugates are also delivered to the kidneys, where they are metabolized further or excreted as mercapturates. The selective tissue distribution of the different enzymes of the GSH conjugation pathway and of plasma membrane transporters determines the renal selectivity of GSH-derived metabolites of chemicals such as Tri (Lash et al., 1988).

In addition to interorgan metabolism, Tri may undergo GSH conjugation and subsequent reactions within the kidneys, representing an intraorgan metabolic pathway (Lash et al., 1995). Although male rats exhibit markedly higher rates of both hepatic and renal GSH conjugation of Tri than female rats (Lash et al., 1995, 1998), which agrees with the higher susceptibility of male rats to Tri- or DCVC-induced nephrotoxicity and nephrocarcinogenicity, male and female mice exhibit markedly higher rates of GSH conjugation of Tri than either sex of rats (Lash et al., 1998). Although human kidney cytosol has β-lyase, its activity is present at only about 10% of levels found in rats (Lash et al., 1990), suggesting that this step may be limiting in humans. Furthermore, Green et al. (1997) measured GSH conjugation of Tri and reported rates that were severalfold lower than those in rats, and they concluded that flux through the entire pathway in humans is minor compared with that through the P-450 pathway.

In the present study, we quantitated activity and determined kinetics of GSH conjugation of Tri in isolated hepatocytes and liver and kidney cytosol and microsomes from human tissue donors. The method of analysis involved measurement of *S*-(1,2-dichlorovinyl) glutathione (DCVG) by HPLC, allowing direct confirmation of conjugate formation. The results showed that rates of GSH conjugation of Tri in human liver and kidney are comparable to those found in male rats, demonstrating that the initial step in the pathway is not limiting in the generation of potentially nephrotoxic metabolites.

Experimental Procedures

Materials. Tri (reported to be 99.9% pure, as judged by electron ionization mass spectrometry), collagenase type IV, *L*-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin), and L-γ-glutamyl-L-glutamate were purchased from Sigma. DCVG was synthesized as previously described (Elfarra et al., 1986) and was a gift from Dr. Adnan A. Elfarra (University of Wisconsin, Madison, WI). Purity (>95%) was determined by HPLC analysis and identity was confirmed by proton nuclear magnetic resonance spectroscopy. All other chemicals were of the highest purity available and were obtained from commercial sources.

Preparation of Isolated Hepatocytes. Human liver and kidney samples were purchased from the Human Cell Culture Center, Inc. (Anatomic Gift Foundation, Folkston, GA). Hepatocytes were isolated from six liver donors by collagenase perfusion (Dorko et al., 1994; Strom et al., 1996). After surgical isolation, the left hepatic lobe was prepared by cannulating the hepatic vein and warming to 37–39°C. The liver was initially perfused with a calcium-free buffer to help separate intercellular junctions. The second perfusion buffer contained calcium, collagenase, and 0.5% (w/v) bovine serum albumin (Dorko et al., 1994). Perfusion was typically initiated within 18 to 24 h of clinical death and isolated hepatocytes were immediately suspended and shipped on ice-cold University of Wisconsin medium. Cells were used fresh without cryopreservation and time from organ removal to in vitro experiment was $\leq$ 36 h. Cell viability was assessed by trypan blue exclusion and cells were discarded if viability was <65%. Cells were then centrifuged, resuspended, and incubated at a density of 0.25 to 2×10^6/ml in Chee's modified medium in 30-ml screw-capped Erlenmeyer flasks. Flasks were purged with 95% O_2/5% CO_2. The volume of each flask (38 to 42 ml) was gravimetrically determined with water and the volume of the cell suspension (kept constant at 3.0 ml) used was just enough to ensure that the bottom of the flask remained covered when the flask was oscillated at 50 rpm.

TABLE 1

Donor information for hepatic cytosol and microsomes

Human Liver Cytosol			Human Liver Microsomes		
Sample #	Demographics[b]	COD[c]	Sample #	Demographics[b]	COD[c]
10	33 CM	GSW	53	50 CF	ICH
11	24 CM	GSW	59	50 CF	ICH
17	63 BF	SAH	60	26 CF	CHI
19	60 CF	ANO	65	46 CF	ICH
29	24 CM	GSW	67	50 CF	CHI
31	44 CM	CHI	78	38 CM	MI
32	42 CM	CHI	79	40 CM	CHI
33	35 CF	GSW	84	22 HF	ICH
34	26 CF	CHI	98	43 BF	ICH
37	35 CM	CHI	100	47 BM	ICH
39	52 CF	SAH	102	51 CF	ICH
40	26 CM	GSW	126	31 CM	ICH
41	45 BF	SAH	127	36 CF	ICH
42	36 CM	SAH	138	41 CF	GSW
43	44 CM	CHI	142	39 CM	ANO
44[a]	63 PF	SAH	155	42 CF	ICH
45[a]	63 PF	SAH	177	47 CF	ICH
46	22 CF	ANO	207	60 CF	ICH
47	39 BF	MI	211	65 CF	ICH
48	22 CF	ANO/DRO	212	45 CF	ICH

[a] These represent duplicated lots from the same liver donor.
[b] Demographics: Samples are identified by age in years; sex, M = male, F = female; ethnic background: C = Caucasian, B = Black, H = Hispanic, P = Filipino.
[c] COD, cause of death; GSW, gunshot wound; SAH, subarachnoid hemorrhage; CHI, closed head injury; MI, myocardial infarction; ICH, intracranial hemorrhage; ANO, anoxia; DRO, drowning.

Preparation of Liver and Kidney Subcellular Fractions. Liver cytosol and microsomes (see Table 1 for donor table) and kidney cytosol and microsomes from pooled donors were prepared from tissue homogenates by differential centrifugation (Guengerich, 1989). Both pooled samples and individual samples were used for liver cytosol and microsomes. Information on human liver cytosol and microsome donors are given in Table 1. The cause of death in all individuals was unrelated to liver function; the donor population exhibited a wide range of ages (24 to 63 years for cytosol, 26 to 65 years for microsomes) and ethnic groups and comprised both males and females. Pooled samples were used for kidney cytosol and microsomes. For measurement of GGT activity in human kidney and liver subcellular fractions, kidney cytosol and microsomes and liver cytosol were pooled from donor tissues and were obtained from the source described above. Pooled human liver microsomes used for GGT assays were a gift from Dr. Paul F. Hollenberg (University of Michigan, Ann Arbor, MI).

Incubations for Measurement of Metabolism of Tri by GSH Conjugation. For incubations with isolated hepatocytes, Tri was volatilized into tedlar bags containing known volumes of O_2/CO_2 and was diluted into sealed flasks to yield headspace concentrations of 25 to 10,000 ppm. Previous partitioning experiments (Lipscomb et al., 1998a) demonstrated that these concentrations of Tri in the headspace result in Tri concentrations of 0.011 to 4.4 mM in isolated hepatocytes. Preliminary studies indicated that this range of headspace concentrations produced detectable quantities of metabolites at the low end

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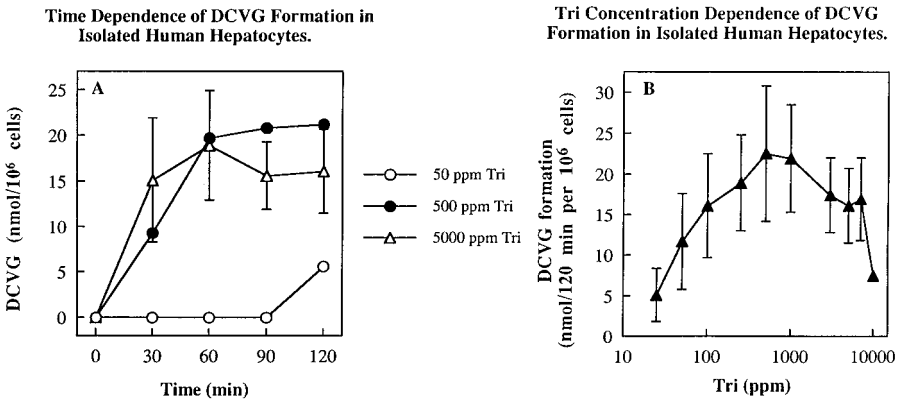

FIG. 1. *Time (A) and concentration (B) dependence of GSH conjugation of Tri in isolated human hepatocytes.*

Formation of DCVG in suspensions of isolated human hepatocytes was measured by incubating cells (2×10^6/ml) at 37°C with the indicated concentrations of Tri in the headspace. DCVG was measured after derivatization of acid extracts of samples by HPLC. Results are means ± S.E. of three separate measurements except for the time courses for 50 and 500 ppm Tri, which were measurements from single experiments.

while saturating the initial GST- or P-450-catalyzed reaction at the high end, as demonstrated by formation of DCVG or chloral hydrate, trichloroethanol, and trichloroacetate, respectively. Tri concentrations in the headspace were verified by manually injecting 0.1-ml samples of flask headspace on a Hewlett-Packard model 5890 Series II gas chromatograph equipped with a Supelco (Bellefonte, PA) 2–5320 Vocol capillary column (0.53 mm × 30 m) and interfaced with a flame ionization detector. Tri concentrations in flasks were verified by comparing area counts with those from an external standard curve of authentic Tri volatilized in crimp-sealed serum vials and analyzed simultaneously by the same system. Incubations were carried out for up to 2 h and were quenched by addition of 0.1 ml of 70% (v/v) perchloric acid to 0.5-ml aliquots of incubation samples. Quenched samples were rapidly frozen in liquid nitrogen and stored at −80°C until analysis. No significant loss of cell viability, as assessed by release of cytosolic enzymes into extracellular medium, occurred from hepatocytes during the incubations with Tri (data not shown).

Incubations of cytosol or microsomes were carried out for various times at 37°C under gentle rotation in a total volume of 1.0 ml in vials (screw caps with Teflon-lined rubber septa, total volume 1.9 ml) containing an atmosphere of room air. All cytosolic and microsomal incubations were carried out in 0.1 M potassium phosphate (pH 7.4), and contained 0.5 to 2.0 mg protein, 0.5 to 5 mM GSH, and 7.8 μM to 1 mM Tri (dissolved in acetone; final acetone concentration = 0.5%, v/v), except in the P-450/GST competition experiment shown in Fig. 9. In that case, the final concentration of acetone was only 0.1% and Tri concentration was varied from 1.9 to 125 μM. Incubations were quenched by addition of 0.2 ml of 70% (v/v) perchloric acid, rapidly frozen in liquid nitrogen, and stored at −80°C until analysis. Kidney microsomes were first preincubated with 0.25 mM acivicin to inhibit any GGT activity from contaminating brush-border membranes to minimize further metabolism of DCVG. This concentration of acivicin inhibits GGT activity in rat kidney plasma membranes (Lash and Jones, 1984) and rat kidney proximal tubular cells (Visarius et al., 1996) by >95%. Control experiments with kidney cytosol and liver cytosol and microsomes showed that acivicin had no effect on quantitation of DCVG in those fractions (data not shown).

Assay of DCVG Formation. Perchloric acid extracts of hepatocytes or liver and kidney subcellular fractions were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene as described previously (Lash et al., 1995) and the *N*-dinitrophenyl derivative of DCVG was analyzed by ion-exchange HPLC as described by Fariss and Reed (1987). DCVG content in samples was quantitated with respect to authentic standard and was adjusted according to the content of added L-γ-glutamyl-L-glutamate as HPLC internal standard. The limit of detection was 50 pmol, with a linear detector response being obtained with samples containing from 50 pmol to 10 nmol. The efficiency of derivatization was estimated to be 85 to 110%, with a correction made for this variation by use of an internal standard. Besides comparison of retention time with that of the derivative of authentic DCVG standard, the identity of the DCVG peak was confirmed as done previously (Lash and Jones, 1985) by a 15-min treatment of selected samples with 1 U of a partially purified preparation of GGT that also contains dipeptidase activity (data not shown). As a consequence of this treatment, DCVG was converted to DCVC, whose *N*-dinitrophenyl derivative is also detected by this HPLC method (Lash and Anders, 1989). Amounts of nonenzymatic DCVG formation were approximately 25% of those in the presence of cells or tissues and were subtracted from the latter to obtain enzyme-dependent rates of DCVG formation.

Assay of Chloral Hydrate Formation. Chloral hydrate formation was measured in incubations with pooled human liver microsomes (0.5 mg protein/ml) and pooled liver cytosol (1 mg protein/ml) in the presence of either an NADPH-regenerating system, 5 mM GSH, or both to assess the effect of GST activity on the metabolism of Tri by P-450. Tri was dissolved in acetone (final concentration = 0.1%). This concentration of solvent was shown previously (Lipscomb et al., 1997, 1998b) to have no effect on dimethylnitrosamine demethylase activity, indicating that CYP2E1 was not inhibited under these conditions. Gas chromatographic quantitation of chloral hydrate formation was performed as described by Lipscomb et al. (1997).

Other Assays. GGT activity was determined in cytosol and microsomes from pooled human liver or kidney with L-γ-glutamyl-*p*-nitroanilide and glycylglycine as substrates by measuring formation of *p*-nitroanilide as the increase in absorbance at 410 nm (Orlowski and Meister, 1963). Protein content of samples was measured by the method of Read and Northcote (1981) using bovine serum albumin as standard.

Results

GSH Conjugation of Tri in Human Hepatocytes. Suspensions of human hepatocytes were incubated with 50, 500, or 5000 ppm Tri in the headspace for up to 120 min and DCVG formation was measured (Fig. 1A). These concentrations of Tri in the headspace correspond to 0.022, 0.22, or 2.2 mM Tri, respectively, in the cell suspensions. With 50 ppm Tri, no detectable DCVG was measured until the 120-min time point, when 5.62 nmol of DCVG/10^6 cells was found. In contrast, incubations with both 500 and 5000 ppm Tri exhibited time-dependent increases in DCVG formation, with maximal content of DCVG detected being 21.2 nmol/10^6 cells with 500 ppm Tri and 18.9 nmol/10^6 cells with 5000 ppm Tri. A full concentration dependence profile was obtained by measuring DCVG formation after 120-min incubations with 25 to 10,000 ppm Tri (0.011 to 4.4 mM; Fig. 1B). The 120-min time point was chosen to obtain maximal detectable amounts of DCVG. Amounts of DCVG detected increased with increasing concentration of Tri up to 500 ppm Tri, to a maximum amount of 22.5 nmol DCVG/120 min per 10^6 cells. At concentrations of Tri of 1000 ppm and above, the amount of DCVG detected decreased.

GSH conjugation of Tri in isolated human hepatocytes exhibited a

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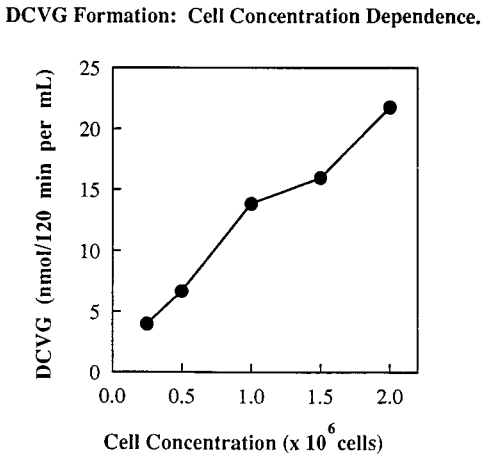

FIG. 2. *Cell concentration dependence of GSH conjugation of Tri in suspensions of isolated human hepatocytes.*

Isolated human hepatocytes at the indicated cell concentrations were incubated at 37°C with 5000 ppm Tri in the headspace for 120 min. DCVG formation was measured after derivatization of acid extracts of samples by HPLC. Results are from a single hepatocyte preparation.

linear dependence on cell concentration between 0.25×10^6 cells/ml and 2.0×10^6 cells/ml (Fig. 2). DCVG formation increased from 3.96 to 21.8 nmol/120 min per ml over the range of cell concentrations used.

Time Course and Kinetics of GSH Conjugation of Tri in Human Liver and Kidney Subcellular Fractions. One of the limitations in the measurement of GSH conjugate formation in tissues that contain significant GGT activity is subsequent metabolism of DCVG by GGT and dipeptidases. GGT activity was measured in kidney and liver subcellular fractions (Table 2). As expected, the kidney microsomal fraction, which contains a significant amount of brush-border membranes, exhibited high GGT activity that was 73-fold higher than that in the kidney cytosolic fraction and 33-fold higher than that in the liver microsomal fraction. Hence, incubations to measure DCVG formation in kidney microsomes are the only ones in which subsequent degradation of DCVG must be considered to obtain accurate quantitation of rates of GSH conjugation of Tri. As described above, this problem was circumvented by pretreatment of kidney microsomes with acivicin.

A time course of GSH conjugation of Tri was measured in human liver cytosol and microsomes in incubations of 0.5, 1, or 2 mg protein/ml subcellular fractions with 5 mM GSH and 2 mM Tri (Fig. 3). Both fractions exhibited time- and protein concentration-dependent increases in DCVG formation, with microsomes exhibiting modestly higher amounts of DCVG formation than cytosol. In liver cytosol (Fig. 3A), the amounts of DCVG detected reached maxima at the 20-min time point and remained unchanged at the 30-min time point. In contrast, the amounts of DCVG detected in liver microsomes (Fig. 3B) reached maxima at the 20-min time point but were lower at the 30-min time point, suggesting that further metabolism of DCVG may have occurred in the liver microsomes.

Human kidney subcellular fractions similarly exhibited time- and protein concentration-dependent increases in DCVG formation (Fig. 4). In the kidney, however, GSH conjugation of Tri in microsomes was approximately 3-fold higher than that in cytosol. Amounts of DCVG formation in kidney cytosol were only 5 to 10% of those in liver cytosol and those in kidney microsomes were approximately 10 to 15% of those in liver microsomes. Several of the samples in both kidney cytosol and microsomes exhibited decreased net formation of DCVG at the 30-min time point as compared with that detected at the 20-min time point, suggesting that additional metabolism of DCVG may have occurred in these samples.

TABLE 2

γ-Glutamyltransferase activity in human kidney and liver subcellular fractions

GGT activity was measured with γ-glutamyl-*p*-nitroanilide and glycylglycine as substrates by measuring formation of *p*-nitroanilide as the increase in absorbance at 410 nm. Results are the means ± S.E. of the indicated number of measurements except for liver microsomes, where the value is the average of two determinations.

Tissue	Fraction	N	Activity
			mU/mg protein
Kidney	Cytosol	3	13.2 ± 1.0
	Microsomes	5	960 ± 77
Liver	Cytosol	9	8.89 ± 3.58
	Microsomes	2	29

GSH conjugation of Tri exhibited approximately linear dependence on GSH concentration in human liver cytosol (Fig. 5), with rates of DCVG formation varying between 16.2 and 78.9 nmol/30 min per mg protein with GSH concentrations of between 1 and 5 mM.

GSH conjugation of Tri also exhibited approximately linear dependence on protein concentration in human kidney cytosol (Fig. 6), with rates of DCVG formation varying between 3.05 and 9.65 nmol/10 min per ml at protein concentrations of 0.5 to 2.0 mg/ml.

Tri concentration dependence of GSH conjugation in the presence of 5 mM GSH in liver cytosol and microsomes (Fig. 7) and kidney cytosol and microsomes (Fig. 8) was determined over a Tri concentration range of 7.8 μM to 1 mM. In all cases, but most prominently in liver subcellular fractions, DCVG formation did not exhibit saturation at 1 mM Tri. Liver cytosol and microsomes exhibited rates of DCVG formation of 7.00 and 2.44 nmol/min per mg protein, respectively, at 1 mM Tri. Kidney cytosol and microsomes exhibited rates of DCVG formation of 0.87 and 5.33 nmol/min per mg protein, respectively, at 1 mM Tri.

Analysis of the kinetics of GSH conjugation of Tri by Eadie-Hofstee plots (Figs. 7C, 7D, 8C, and 8D) resolved metabolism in both liver cytosol and microsomes into two distinct processes, whereas those in kidney cytosol and microsomes were represented by single processes (Table 3). The two processes in liver cytosol and microsomes exhibited similar apparent K_m values but V_{max} values in liver cytosol for the two processes were 2.8- and 3.0-fold higher than the corresponding V_{max} values in liver microsomes. In contrast, the apparent K_m for GSH conjugation in kidney cytosol was similar to the high-affinity systems in both liver fractions but the V_{max} was only 9.2% of that for the low-affinity, high-capacity process in liver cytosol. Kidney microsomes exhibited low-affinity, high-capacity kinetics, with an apparent K_m that was similar to the low-affinity process in liver microsomes and a V_{max} that was 2.0-fold higher than the corresponding process in liver microsomes.

Individual Variation in Rates of GSH Conjugation of Tri in Human Liver. Analysis of rates of GSH conjugation of Tri in 20 individual liver cytosol and microsomal (Table 4) donors revealed considerable variation among individuals. The liver cytosol donors included 9 males and 11 females. Overall, rates of GSH conjugation varied 2.41-fold and exhibited a range of rates of 34.7 to 83.6 nmol DCVG formed/20 min per mg protein. There was no significant difference between males and females, with males exhibiting a rate of DCVG formation of 52.1 ± 5.3 (mean ± S.E.; $n = 9$; range 34.7 to 83.6; 2.41-fold variation) and females exhibiting a rate of DCVG formation of 58.1 ± 4.4 (mean ± S.E.; $n = 11$; range 41.2 to 80.2; 1.95-fold variation) nmol DCVG formed/20 min per mg protein.

The liver microsome donors included five males and 15 females (Table 4). Overall, rates of GSH conjugation varied 6.53-fold and

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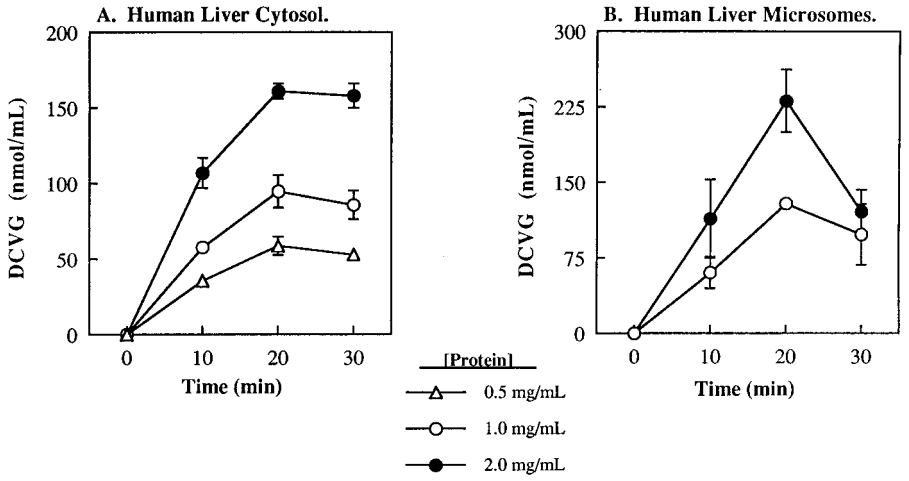

FIG. 3. *Time and protein concentration dependence of GSH conjugation of Tri in human liver cytosol and microsomes.*

Cytosol (A) and microsomes (B) from pooled human liver samples were incubated at 37°C with 2 mM Tri and 5 mM GSH for up to 30 min. DCVG formation was measured after derivatization of acid extracts of samples by HPLC. Results are means ± S.E. of measurements from three separate samples.

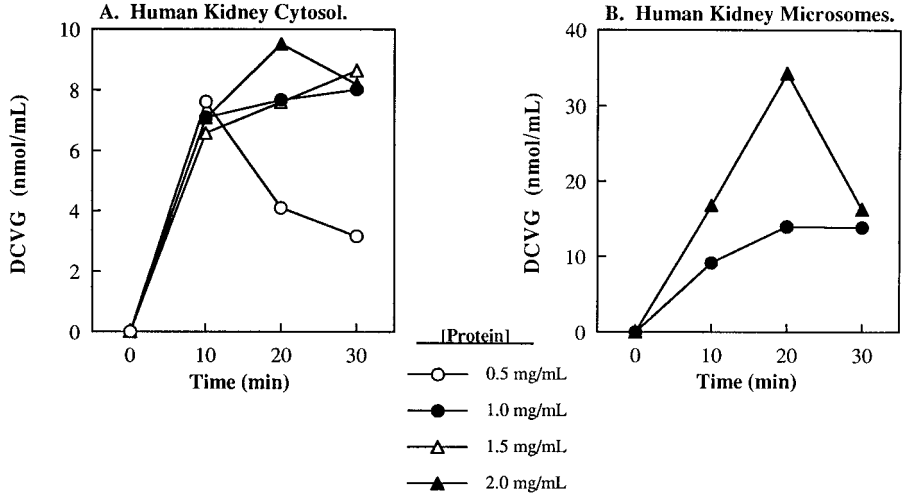

FIG. 4. *Time and protein concentration dependence of GSH conjugation of Tri in human kidney cytosol and microsomes.*

Cytosol (A) and microsomes (B) from pooled human kidney samples were incubated with 2 mM Tri and 5 mM GSH at 37°C for up to 30 min. DCVG formation was measured after derivatization of acid extracts of samples by HPLC. Results are means of measurements from three separate samples for cytosol and are from a single experiment from microsomes. Error bars were omitted for clarity; S.E. values were 5 to 15% of means.

exhibited a range of rates of 9.90 to 64.6 nmol DCVG formed/20 min per mg protein. As with the cytosol donors, there was no significant difference between males and females in microsomes, with males exhibiting a rate of DCVG formation of 36.0 ± 7.9 (mean ± S.E.; n = 5; range 17.9 to 64.6; 3.61-fold variation) and females exhibiting a rate of DCVG formation of 27.6 ± 3.5 (mean ± S.E.; n = 15; range 9.90 to 58.9; 5.95-fold variation) nmol DCVG formed/20 min per mg protein.

Competition between P-450 and GSH Conjugation Pathways. To assess the potential competition between P-450 and GSH conjugation for metabolism of Tri, pooled human liver cytosol and microsomes were coincubated for 20 min with 1.9 to 125 μM Tri and 5 mM GSH in the absence or presence of an NADPH-regenerating system and amounts of DCVG formation were measured (Fig. 9). In the absence of an NADPH-regenerating system, where the P-450 pathway is inactive, amounts of DCVG formation increased with Tri concentration to a maximum value of 145 nmol/20 min per mg protein with 15 μM Tri and decreased with concentrations of Tri above that. In contrast, in the presence of an NADPH-regenerating system, where the P-450 pathway is active, amounts of DCVG formation increased modestly with increasing concentrations of Tri from 30.2 to 63.9 nmol/20 min per mg protein. These results indicate that P-450s efficiently competed with GSTs and deceased GSH conjugation of Tri by as much as 60%.

The ability of GSTs to compete with P-450s for metabolism of Tri was then assessed in incubations of either pooled human liver microsomes alone or pooled human liver cytosol and microsomes with Tri in the presence of either an NADPH-regenerating system, 5 mM GSH, or both (Table 5). Kinetic parameters for Tri for formation of chloral hydrate were determined and showed that unlike the results described above, where P-450s effectively diminished GSH conjugation of Tri, GSH had no effect on P-450-dependent oxidation of Tri.

Discussion

The complexity of the metabolism of Tri makes human health risk assessment difficult. This complexity involves the existence of simultaneous, competing pathways for Tri metabolism and sex-, species-, and tissue-dependent differences in rates and distribution of metabo-

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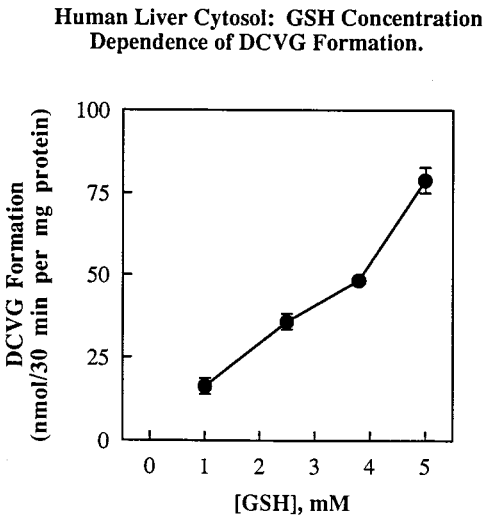

FIG. 5. *GSH concentration dependence of GSH conjugation of Tri in human liver cytosol.*

Pooled human liver cytosol samples (2 mg protein/ml) were incubated at 37°C with the indicated concentrations of GSH and 2 mM Tri for 30 min. DCVG formation was measured after derivatization of acid extracts of samples by HPLC. Results are means ± S.E. of measurements from three separate samples.

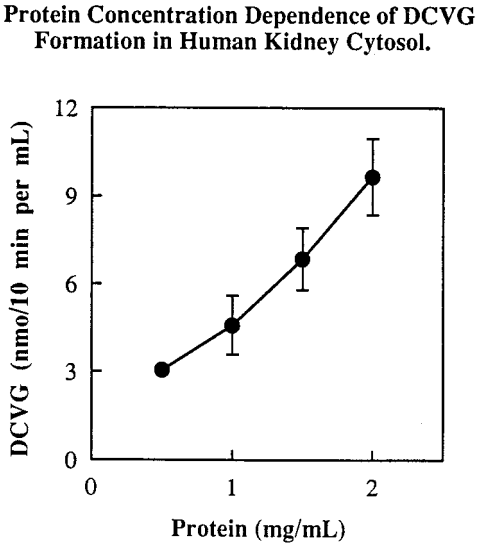

FIG. 6. *Protein concentration dependence of GSH conjugation of Tri in human kidney cytosol.*

Pooled human kidney cytosol samples at the indicated protein concentrations were incubated at 37°C with 1 mM Tri and 5 mM GSH for 10 min. DCVG formation was measured after derivatization of acid extracts of samples by HPLC. Results are means ± S.E. of measurements from three separate samples.

lites. The U. S. Environmental Protection Agency is currently reevaluating Tri (Maull and Lash, 1998). A central point of contention involves the biological significance of the GSH conjugation pathway in the nephrotoxicity of Tri in humans exposed either occupationally or environmentally to Tri. To help address this question, the goal of this study was to determine kinetic parameters of GSH conjugation of Tri in human liver and kidney. If indeed this pathway does not contribute significantly to overall Tri metabolism in humans, then it would be expected that rates of DCVG formation in human liver and kidney should be low relative to P-450-catalyzed oxidation of Tri. Additionally, rates of DCVG formation in human tissue should be low relative to those observed in corresponding tissues of male rats, in whom the kidney is an established target organ for Tri (Anders et al., 1988; Davidson and Beliles, 1991; Goeptar et al., 1995).

The present results show that human hepatocytes and human liver and kidney cytosol and microsomes catalyze GSH conjugation of Tri at rates that are significantly higher than those measured previously in rats and are comparable to those measured in mice (Lash et al., 1995, 1998). One difference in incubation conditions with the isolated hepatocytes between the present study and the previous ones that may contribute to the observed differences is that Tri was added to the headspace in the present study, whereas it was dissolved in acetone and added directly into the liquid phase in the previous studies. Nonetheless, the present results confirm that although the liver is the primary site of GSH conjugation, the entire GSH conjugation pathway can also occur within the kidneys. The kinetics of GSH conjugation in the two tissues were different, however, in that only one process was found in kidney subcellular fractions, whereas two distinct processes were identified in liver subcellular fractions. Furthermore, total capacity for GSH conjugation of Tri is markedly higher in human liver than in human kidney, based on the markedly lower V_{max} in human kidney cytosol and the greater tissue mass for liver than for kidney.

Comment is necessary concerning some of the time courses and concentration dependence profiles. For example, the formation of DCVG in isolated hepatocytes incubated with 7.8 μM to 1 mM Tri for 120 min (cf. Fig. 1B) reached a maximum at 500 ppm Tri and was decreased at higher concentrations of Tri. There are two possibilities that may explain this behavior; either the enzyme(s) involved in DCVG formation may be inhibited by higher amounts of product or other enzymes may catalyze further metabolism of DCVG only at higher substrate concentrations. It should be noted that these same cells showed similar behavior at higher Tri concentrations in measurements of P-450 activity (Lipscomb et al., 1998a). In some of the time courses of DCVG formation, [e.g., DCVG formation in liver microsomes (cf. Fig. 3B) or kidney cytosol or microsomes (cf. Fig. 4)], the amount of DCVG measured reached a maximum at 10 to 20 min and declined thereafter. Possible explanations for this may be substrate or product inhibition or additional metabolism of the formed DCVG. Indeed, substrate-based inhibition of P-450 activity by Tri was observed in recent studies with human hepatocytes (Lipscomb et al., 1998a) and human liver microsomes (Lipscomb et al., 1998b) and the concentration of Tri at which inhibition began to be observed was the same in those studies and the present study.

There was also some degree of variation in the absolute activity of various pooled samples that were used in some of the assays. This should not be unexpected because different individual samples comprised the various pooled samples and these individual samples can exhibit severalfold variation in rates (cf. Table 4). It was necessary to use pooled samples for most of the studies (except, of course, those specifically assessing individual variability) because of limitations in the amount of the human tissue available. Additionally, use of pooled samples is preferable and makes more sense from the point of view of experimental design because the pooled samples will reflect general or average rates of metabolism in the population.

The position has been taken that the flux of Tri through the GSH conjugation pathway is significantly smaller than that through the P-450 pathway (Green et al., 1997). Consequently, the conclusion is made that the amount of reactive metabolites of Tri generated in human kidney is not high enough to produce the biochemical effects that have been described in male rats. The following observations support this conclusion: 1) Ratios of oxidative metabolites to the mercapturate in the urine of exposed humans of 100:1 to as high as 3000:1 have been reported (Birner et al., 1993; Bernauer et al., 1996), 2) nephrotoxicity or kidney tumors have rarely been observed in

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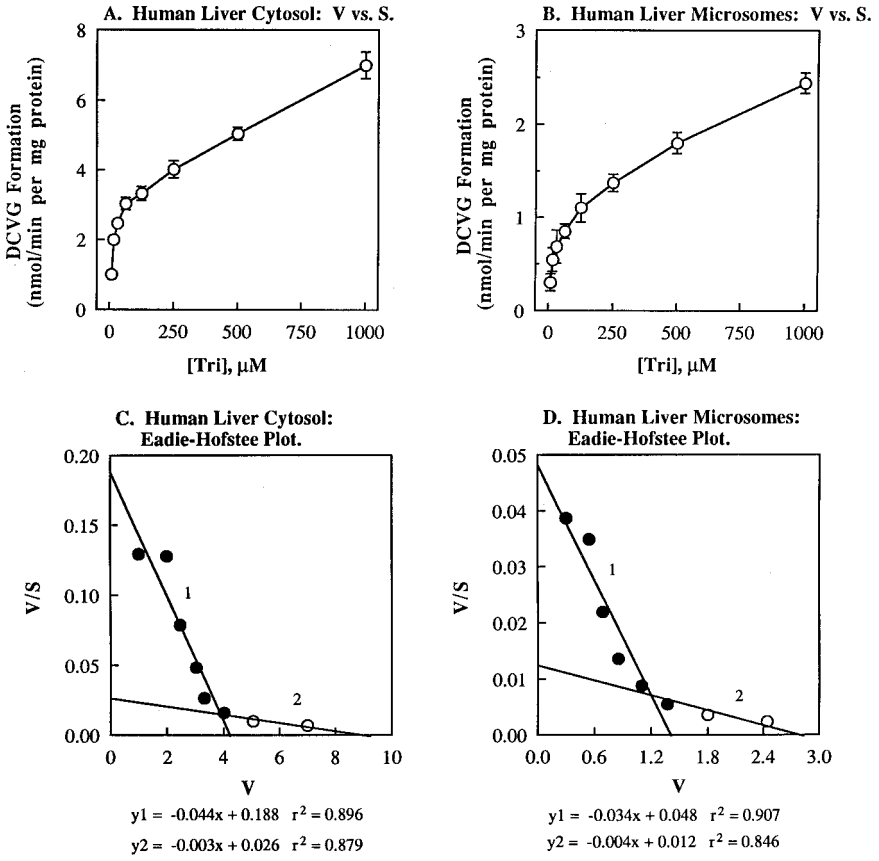

FIG. 7. *Kinetics of GSH conjugation of Tri in human liver cytosol and microsomes.*

Pooled human liver cytosol (A, C) or microsomes (B, D) (1 mg protein/ml) were incubated at 37°C with the indicated concentrations of Tri and 5 mM GSH for 20 min. DCVG formation was measured after derivatization of acid extracts of samples by HPLC. In C and D (Eadie-Hofstee plots), V = DCVG formation in nmol/min per mg protein and S = [Tri] in mM. For liver cytosol (C), two Tri concentration ranges were used for linear curve-fitting (line 1 [6 data points]: 7.8 to 250 μM; line 2 [3 data points]: 250 to 1000 μM). For liver microsomes (D), two Tri concentration ranges were used for linear curve-fitting (line 1 [6 data points]: 7.8 to 250 μM; line 2 [4 data points]: 125 to 1000 μM). Results are means ± S.E. of measurements from three separate samples.

humans (McLaughlin and Blot, 1997), and 3) activity of the β-lyase in human kidney cytosol is only about 10% of that in rat kidney cytosol (Lash et al., 1990).

Rates of GSH conjugation of Tri in human liver and kidney subcellular fractions reported in the present study are up to an order of magnitude greater than those reported by Green et al. (1997). In incubations with 1.9 mM Tri and 5 mM GSH, Green et al. (1997) found rates of DCVG formation of 2.5, 1.6, and 0.19 pmol/min per mg protein in liver cytosols of male mice, rats, and humans, respectively. In contrast, in incubations with 2 mM Tri and 5 mM GSH, we previously reported rates of DCVG formation of 408 and 122 pmol/min per mg protein in liver cytosols of male mice and rats, respectively (Lash et al., 1995, 1998) and in the current work, we report a rate of DCVG formation of 5.77 nmol/min per mg protein in pooled human liver cytosol. Similarly, Green et al. (1997) were unable to detect net enzymatic formation of DCVG in incubations with rat kidney subcellular fractions. In contrast, in incubations with 2 mM Tri and 5 mM GSH we previously reported a rate of DCVG formation in male rat kidney cytosol of 7.5 pmol/min per mg protein (Lash et al., 1995, 1998) and in the current work, we report a rate of 0.7 nmol/min per mg protein in pooled human kidney cytosol.

The controversy between our present and previous results and those of Green et al. (1997) has not been resolved. Differences in analytical methods (radiolabeled substrate with HPLC separation versus derivatization and HPLC separation) may contribute to the discrepancies in measured rates. Beyond this, no other explanations have been found to explain the discrepancies. Corrections were made in the present studies for nonenzymatic DCVG formation and our analytical method involved measurement of the *N*-dinitrophenyl derivative of DCVG and quantitation with respect to authentic standard. Hence, we were certain of the identity of the measured compound.

Hence, we report rates of GSH conjugation of Tri in human liver and kidney tissue that are significantly higher than those in corresponding tissue fractions from the rat. These results indicate that the initial step in the GSH conjugation pathway is not limiting in humans and is not responsible for the lower susceptibility of humans (as compared with rats) to Tri-induced renal injury. Based on previous results (Lash et al., 1990; Green et al., 1997), the β-lyase step and not the *N*-acetylation reaction, is likely to be the primary determinant of how much reactive and toxic species is formed. Results from a recent study (Lash et al., 1999), in which human volunteers were exposed by inhalation for 4 h to 50 or 100 ppm Tri, support the conclusions of this study that GSH conjugation of Tri occurs at significant rates. In that study, micromolar concentrations of DCVG were detected in the blood of human volunteers, indicating significant activity, presumably predominantly in the liver, of the GSH conjugation pathway with Tri as substrate.

Individual variation in rates of GSH conjugation of Tri were also found. They are similar in magnitude to those previously reported by Green et al. (1997) and are similar to the degree of variation exhibited in the P-450-dependent metabolism of Tri (Lipscomb et al., 1997). Thus, interindividual variability must be taken into account when considering the role of GSH conjugation in the overall metabolism

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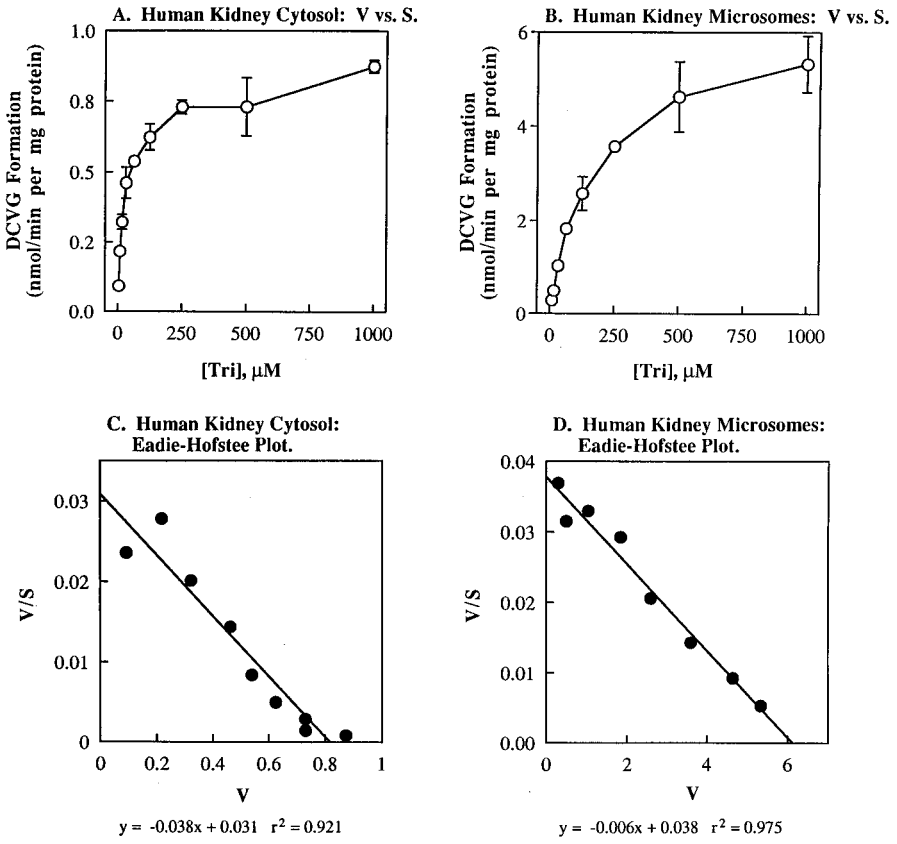

FIG. 8. *Kinetics of GSH conjugation of Tri in human kidney cytosol and microsomes.*

Pooled human kidney cytosol (A, C) or microsomes (B, D) (1 mg protein/ml) were incubated at 37°C with the indicated concentrations of Tri and 5 mM GSH for 10 or 20 min, respectively. DCVG formation was measured after derivatization of acid extracts of samples by HPLC. In C and D (Eadie-Hofstee plots), V = DCVG formation in nmol/min per mg protein and S = [Tri] in mM. Results, expressed as rates of DCVG formation per min, are means ± S.E. of measurements from three separate samples.

TABLE 3

Kinetics of GSH conjugation of Tri in human kidney and liver subcellular fractions

Activity of GSH conjugation of Tri was measured by incubating human liver or kidney subcellular fractions (1 mg protein/ml) at 37°C with 5 mM GSH and 7.8 μM to 1 mM Tri for 20 min (liver cytosol and microsomes, kidney microsomes) or 10 min (kidney cytosol). DCVG formation was measured by HPLC after derivatization of acid extracts of samples. Kinetic parameters were calculated from Eadie-Hofstee plots from the data presented in Figs. 7 and 8, which were obtained from measurements from three separate sets of determinations.

Tissue/Subcellular Fraction	V_{max}	K_m
	nmol DCVG/min per mg protein	*μM Tri*
Kidney		
Cytosol	0.81	26.3
Microsomes	6.29	167
Liver		
Cytosol-1	8.77	333
Cytosol-2	4.27	22.7
Microsomes-1	3.10	250
Microsomes-2	1.42	29.4

and nephrotoxicity of Tri. It should also be noted that among the separate, duplicate measurements for each individual tissue sample (cf. Table 4), some amount of variation was observed. Although at least half of the duplicate samples for either cytosol or microsomes were within 10% of each other, many exhibited significantly more variation. The reason for this variability is unknown but should not relate to any defect in the assay method, based on validation of sample recovery, derivatization efficiency, and corrections that were made for background or nonenzymatic product formation (present study and Lash and Jones, 1985; Lash et al., 1995, 1998).

TABLE 4

Individual variation in GSH conjugation of Tri in samples of human liver cytosol and microsomes

GSH conjugation of 1 mM Tri was measured by incubating samples of human liver cytosol or microsomes from individual donors (1 mg protein/ml) at 37°C with 5 mM GSH for 20 min. DCVG formation was measured by HPLC after derivatization of acid extracts of samples. Results are the averages of duplicate measurements from single samples. The variation between duplicates was less than 20% for most of the samples, although approximately 25% of the duplicates differed by as much as 40% from each other.

Liver Cytosol		Liver Microsomes	
Sample Number	DCVG Formation	Sample Number	DCVG Formation
	nmol/mg protein/20 min		*nmol/mg protein/20 min*
10	83.6	53	11.4
11	50.2	59	21.1
17	72.7	60	39.0
19	80.2	65	30.7
29	67.7	67	28.0
31	41.7	78	64.6
32	46.4	79	26.1
33	70.0	84	9.90
34	71.0	98	16.9
37	37.9	100	38.4
39	42.2	102	21.2
40	34.7	126	33.2
41	43.4	127	30.3
42	60.6	138	42.8
43	45.8	142	17.9
44	41.2	155	19.1
45	47.5	177	20.0
46	64.9	207	22.5
47	45.3	211	58.9
48	60.7	212	42.3

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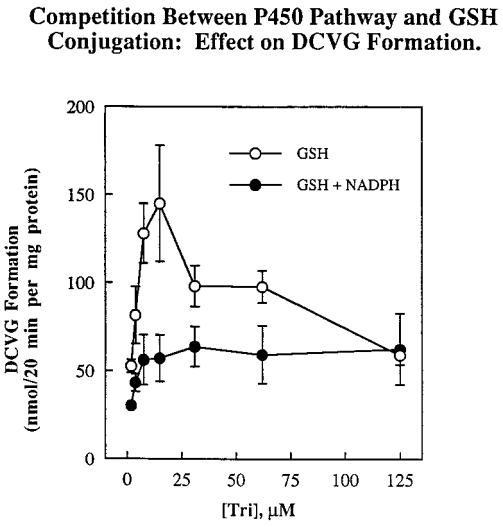

FIG. 9. *Assessment of competition between P-450 and GSH conjugation for Tri metabolism: GSH conjugation activity.*

Aliquots of pooled liver cytosol and microsomes (1 and 0.5 mg protein/ml, respectively) were incubated at 37°C with the indicated concentrations of Tri and 5 mM GSH for 20 min. DCVG formation was measured after derivatization of acid extracts of samples by HPLC. Results are means ± S.E. of measurements from three separate samples.

TABLE 5

Assessment of competition between P-450 and GSH conjugation for Tri metabolism in human liver: P-450 activity

Liver microsomes (0.5 mg protein/ml) were incubated at 37°C singly or with liver cytosol (1.0 mg protein/ml) in the presence of either an NADPH-regenerating system, 5 mM GSH, or both. Gas chromatographic quantitation of chloral hydrate formation was performed as described by Lipscomb et al. (1997). Data are presented as means ± S.E. from three experiments.

Incubation	V_{max}	K_m
	pmol/min per mg protein	*μM Tri*
Microsomes + NADPH[a]	1441 ± 110	24.6 ± 1.8
Microsomes/Cytosol + NADPH	1684 ± 36	21.6 ± 1.6
Microsomes/Cytosol + NADPH + GHS	1634 ± 53	22.8 ± 2.9
Microsomes/Cytosol + GSH	29.9 ± 3.1	5.6 ± 2.4

[a] Data are from Lipscomb et al. (1998b).

The ability of P-450-dependent metabolism, which becomes active on addition of an NADPH-regenerating system, to effectively compete with GSH conjugation and the inability of GSH to diminish rates of chloral hydrate formation indicate that P-450-dependent oxidation of Tri is the kinetically favored pathway for Tri metabolism, in agreement with previous work (reviewed in Davidson and Beliles, 1991; Goeptar et al., 1995). Nonetheless, it is clear that GSH conjugation occurs at measurable rates in human liver and kidney.

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