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Hepatic and Renal Toxicities Associated with Perchloroethylene

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***Abstract*——Metabolism of perchloroethylene (Perc) occurs by cytochrome P450-dependent oxidation and glutathione (GSH) conjugation. The cytochrome P450 pathway generates tri- and dichloroacetate as metabolites of Perc, and these are associated with hepatic toxicity and carcinogenicity. The GSH conjugation pathway is associated with generation of reactive metabolites selectively in the kidneys and with Perc-induced renal toxicity and carcinogenicity. Physiologically based pharmacokinetic models have been developed for Perc in rodents and in humans. We propose the addition of a submodel that incorporates the GSH conjugation pathway and the kidneys as a target organ. Long-term bioassays of Perc exposure in laboratory animals have identified liver tumors in male and female mice, kidney tumors in male rats, and mononuclear cell leukemia in male and female rats. Increases in incidence of non-Hodgkin's lymphoma and of cervical, esophageal, and urinary bladder cancer have been observed for workers exposed to Perc. Limited, and not always consistent, evidence is available concerning the kidneys as a target organ for Perc in humans. Three potential modes of action for Perc-induced liver tumorigenesis are: 1) modification of signaling pathways; 2) cytotoxicity, cell death, and reparative hyperplasia; and 3) direct DNA damage. Four potential modes of action for Perc-induced renal tumorigenesis are: 1) peroxisome proliferation, 2) α-2u-globulin nephropathy, 3) genotoxicity leading to somatic mutation, and 4) acute cytotoxicity and necrosis leading to cell proliferation. Finally, the epidemiological and experimental data are assessed and use of toxicity information in the development of a reference dose and a reference concentration for human Perc exposure are presented.**

I. Introduction

Perchloroethylene (Perc[2]; also known as tetrachloroethylene or tetrachloroethene) is a widely used dry cleaning and metal degreasing solvent. It is a hazardous air pollutant, a common contaminant at Superfund waste sites, and is a surface and ground water pollutant. Over 400 million pounds of Perc are produced annually in the United States. The primary routes of potential human exposure to Perc are inhalation and dermal contact. Approximately 85% of Perc that is used annually is lost to the atmosphere, so that concentrations in air have been reported to range from 30 ppt in rural areas to as high as 4.5 ppb in urban or industrial areas [National Toxicology Program (NTP), 2001]. Perhaps the greatest concern for the general population is contamination of the drinking water with Perc. Current regulations by the U.S. EPA have established a maximum contaminant level of 0.005 mg of Perc/liter (i.e., 5 ppb) in the drinking water (U.S. EPA, 1989).

[2] Abbreviations: Perc, perchloroethylene; EPA, Environmental Protection Agency; NTP, National Toxicology Program; IARC, International Agency for Research on Cancer; P450, cytochrome P450; GSH, glutathione; TRI, trichloroethylene; PBPK, physiologically based pharmacokinetic modeling; RfD, reference dose; RfC, reference concentration; TCA, trichloroacetic acid; DCA, dichloroacetic acid; TCOH, trichloroethanol; TCOG, trichloroethanol glucuronide; CYP, cytochrome P450; GST, GSH *S*-transferase; TCVG, *S*-(1,2,2-trichlorovinyl)glutathione; GGT, γ-glutamyltransferase; TCVC, *S*-(1,2,2-trichlorovinyl)-L-cysteine; FMO, flavin-containing monooxygenase; TCVCSO, *S*-(1,2,2-trichlorovinyl)-L-cysteine sulfoxide; F344, Fischer 344; AOAA, aminooxyacetic acid; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; CCNAT, cysteine conjugate *N*-acetyltransferase; NAcTCVC, *N*-acetyl-TCVC; DCVG, *S*-(1,2-dichlorovinyl)glutathione; HCBD, hexachloro-1,3-butadiene; NAcDCVC, *N*-acetyl-DCVC; NCI, National Cancer Institute; SIR, standardized incidence ratio; SMR, standardized mortality ratio; CI, confidence interval; CDI, chronic dose index; LDH, lactate dehydrogenase; PPARα, peroxisome proliferator-activated receptor-α; TBARS, thiobarbituric acid-reactive substances; NOAEL, no-observable-adverse-effect level; LOAEL, lowest-observable-adverse-effect level; LONEL, lowest-observed-nephrotoxic-effect level; NONEL, no-observed-nephrotoxic-effect level.

Perc has been clearly identified as a carcinogen in experimental animals [International Agency for Research on Cancer (IARC), 1979, 1987; U.S. EPA, 1985, 1991; NTP, 1986] and is considered by the IARC to be *probably carcinogenic to humans* (group 2A) (IARC, 1995). This evaluation was based on the findings of *limited evidence* in humans and *sufficient evidence* in experimental animals of carcinogenicity. IARC also concluded that there is limited evidence in humans for the carcinogenicity of occupational exposures in dry cleaning. Perc is the predominant solvent used in dry cleaning in most areas of the world, including the United States.

The toxic effects of Perc in liver and kidney, which have been observed primarily in experimental animals, are considered to be dependent on its metabolism to reactive metabolites. The pathways for Perc bioactivation and detoxification are rather complex, and several of the enzymes involved exhibit sex- and species-dependent differences. Consequently, it is often difficult to extrapolate results from experimental animals to humans with certainty. Because there is target organ concordance across species for toxicity in general, but not for carcinogenicity, a more complete understanding of the metabolism of Perc in the various target organs and in different species, including humans, is needed to improve predictions for human health risk assessment.

Recent reviews of Perc metabolism and mode of action have been published in a monograph by IARC (1995) and by the U.S. EPA (1991). The IARC monograph contains a concise overview of exposure data, Perc production and use, occupational and environmental occurrence data, and summaries of studies of human cancer that can be attributed to Perc exposure, studies of cancer in experimental animals, Perc metabolism, and target organ toxicity. The EPA document was a response to issues that were raised or left unresolved in the most recent health assessment for Perc (U.S. EPA, 1985) and the addendum that followed (U.S. EPA, 1986). The states of California (California Environmental Protec-

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tion Agency, 2000) and New York (New York State Department of Health, 1997) and the Agency for Toxic Substances and Disease Registry (ATSDR, 1997) have also conducted health assessment reviews of Perc.

This review will focus on Perc metabolism and modes of action for hepatic and renal toxicities. The first section will outline the two principal pathways of Perc metabolism that occur in the liver and kidney, focusing on identification of metabolites that are critical for toxicity and on tissue-, sex-, and species-dependent differences in flux through the various steps. These two pathways are cytochrome P450 (P450)-dependent oxidation and glutathione (GSH) conjugation. The relationship between these two pathways and between metabolism of Perc and that of trichloroethylene (TRI) will also be considered. Physiologically based pharmacokinetic (PBPK) models have become important tools in the evaluation of experimental data and in extrapolation of animal data to humans. PBPK models that have been developed for Perc will be discussed with a focus on their applicability to human health risk assessment. The next two sections will summarize in vivo studies in laboratory animals and occupational and epidemiological studies in humans. The subsequent two sections consider mechanistic data and proposed modes of action for Perc and some of its metabolites in two target organs, the liver and the kidneys. The scientific plausibility and relevance of the mechanistic information and these proposed modes of action for humans will be evaluated. Finally, a reference dose (RfD) and reference concentration (RfC) for Perc exposure will be developed.

Although TRI and Perc have very similar chemical properties, are often used in industry for the same or similar purposes, are often found together as environmental contaminants, are metabolized by essentially all the same enzymes, share many of the same or similar metabolites, and elicit many of the same toxic effects (Green, 1990; IARC, 1995), it would be a serious mistake to assume a priori that risk of hazard from exposure to the two chemicals is the same or that the modes of action and rates of metabolism in target tissues are identical. This is because significant differences are known in the kinetics of metabolism of TRI and Perc by certain enzymes and in the chemical reactivity of certain analogous metabolites. Much more work has been pursued on the metabolism and mode of action of TRI and its metabolites than for Perc and its metabolites. With the caution mentioned above in mind, reference will be made to studies on TRI or its relevant metabolites where appropriate and with proper qualifications. In some cases, the only potentially relevant information that is available comes from such studies on the Perc congener, TRI, or their common metabolites. This review will not repeat detailed discussion of all the studies presented in earlier documents (IARC, 1995; ATSDR, 1997; New York State Department of Health, 1997; U.S. EPA, 1985, 1986, 1991; CAL/EPA, 2000), but will focus on more recent developments in the areas of metabolism and liver and kidney toxicity.

II. Pathways of Perchloroethylene Metabolism

A. Cytochrome P450-Dependent Oxidation and Associated Enzymes

1. Overview of Cytochrome P450-Dependent Pathway. The overall scheme of Perc metabolism by the P450 pathway is shown in Fig. 1. The initial step is catalyzed by P450 and is believed to yield Perc-epoxide (metabolite *2*) as the initial metabolite. There does not appear to be the controversy about the existence of an epoxide intermediate for Perc, unlike that for the related compound TRI (Miller and Guengerich, 1982, 1983; Cai and Guengerich, 1999, 2000). The epoxide (metabolite *2*) generated by the action of P450 on Perc (metabolite *1*) may have several fates, including conversion to oxalate dichloride (metabolite *7*), trichloroacetyl chloride (metabolite *3*), trichloroacetyl aminoethanol (metabolite *8*), and chloral (metabolite *12*). Both chloral and trichloroacetyl chloride may be converted to trichloroacetic acid (TCA; metabolite *4*), which is the predominant metabolite recovered in urine of both humans and rodents (Ohtsuki et al., 1983; Dekant et al., 1987; Birner et al., 1996; Völkel et al., 1998). Additional metabolites are derived from these metabolites, and include dichloroacetic acid (DCA; metabolite *10*), monochloroacetic acid (metabolite *11*), trichloroethanol (TCOH; metabolite *5*), and its glucuronide (TCOG; metabolite *6*), and oxalate (metabolite

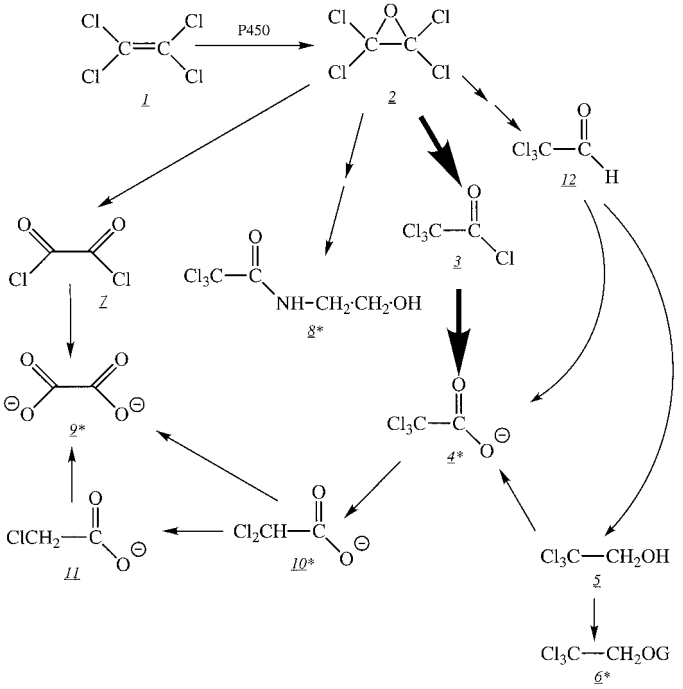

FIG. 1. Metabolism of Perc by the P450 pathway. *Identified urinary metabolites: *1*, Perc; *2*, Perc epoxide; *3*, trichloroacetyl chloride; *4*, trichloroacetate; *5*, trichloroethanol; *6*, trichloroethanol glucuronide; *7*, oxalate dichloride; *8*, trichloroacetyl aminoethanol; *9*, oxalate; *10*, dichloroacetate; *11*, monochloroacetate; *12*, chloral.

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9). After excretion from the liver in the bile, TCOG may undergo significant enterohepatic recirculation (Stenner et al., 1997), being cleaved and thereby regenerating TCOH in the liver. TCOH can also generate TCA. A significant portion of Perc is completely metabolized to CO_2 in a dose-dependent manner (Pegg et al., 1979; Schumann et al., 1980; Frantz and Watanabe, 1983). Because no complete balance study of end metabolites of Perc has been reported in humans, it is possible that all metabolites of Perc have not been identified. Several Perc metabolites are also formed in the oxidative metabolism of TRI (for reviews on TRI metabolism, see Davidson and Beliles, 1991; Goeptar et al., 1995; Lash et al., 2000a). However, the relative importance of individual metabolites varies considerably between the two compounds (Birner et al., 1996). Overall, the data indicate that TCOH, its glucuronide, and their precursor, chloral, are quantitatively less important to Perc metabolism than TCA and its epoxide and trichloroacetyl chloride precursors.

Some authors have described the identification of TCOH in the urine of humans (Ogata et al., 1962; Tanaka and Ikeda, 1968; Ikeda and Ohtsuji, 1972; Ikeda et al., 1972). It should be pointed out, however, that in all of these studies, the method used for TCA metabolite quantitation (the Fujiwara reaction) is indirect and is based on color production before and after oxidation with chromium trioxide and addition of pyridine. The difference between the color production before (=TCA) and after oxidation is considered an estimate of TCOH content. Sakamoto (1976), from comparative urine analysis by gas chromatography and the Fujiwara reaction under different pH and temperature conditions, expressed doubt that the entire fraction detected by the Fujiwara reaction in the oxidation reaction is truly TCOH. Nonetheless, Monster et al. (1983) and Weichard and Lindner (1975) identified small amounts of TCOH (<4 μmol/mmol creatinine) by gas chromatography in the urine of persons exposed to 10 to 30 ppm Perc in air, although others using gas chromatography have not detected TCOH in controlled experimental exposures to pure Perc (Fernandez et al., 1976; Hake and Stewart, 1977; Monster et al., 1979; Völkel et al., 1998). For mice, Yllner (1961) reported that by his chromatographic method, TCOH was not detected in urine as a Perc metabolite. Daniel (1963) reported similar results for the rat, using steam distillation combined with isotopic dilution methodology. Buben and O'Flaherty (1985) were also unable to find evidence of TCOH in the urine of chronically dosed mice as analyzed by gas chromatography; TCA was the only metabolite found. Additionally, Costa and Ivanetich (1980) could not detect TCOH as a product of Perc metabolism by preinduced rat liver microsomal preparations in vitro.

2. Role of Specific Cytochrome P450 Enzymes in Perchloroethylene Metabolism. There is little direct information on the role of specific enzymes in the oxidative metabolism of Perc. Presumably, CYP2E1 plays a significant role in Perc metabolism in rodent liver and kidney and human liver, because this P450 enzyme has a substrate specificity that includes TRI and a variety of other small, halogenated solvents (Guengerich and Shimada, 1991; Guengerich et al., 1991). Although rat kidney expresses CYP2E1 (Ronis et al., 1998; Cummings et al., 1999), human kidney does not appear to express this enzyme (Amet et al., 1997; Cummings et al., 2000b). The liver is quantitatively the predominant site of oxidative metabolism of Perc, although P450s that can metabolize Perc are present to varying degrees in most tissues. Renal oxidative metabolism of Perc by CYP2E1 is, therefore, relevant only for rodents. Other enzymes (including other P450s) may be involved, however, and these can take the place of CYP2E1 in metabolizing Perc. Costa and Ivanetich (1980) showed that hepatic metabolism of Perc in male Long-Evans rats was increased by pretreatment of rats with pregnenolone-16α-carbonitrile or phenobarbital, which induce expression of CYP3A1 and CYP2B1/2, respectively. Pregnenolone-16α-carbonitrile increased Perc P450 metabolism by about 70%, whereas phenobarbital produced a 2.6-fold increase in Perc metabolism by P450. Hence, CYP2B1/2 appears to be the major contributor (presumably in addition to CYP2E1) toward oxidative metabolism of Perc. Interestingly, chlorzoxazone and *p*-nitrophenol were originally considered to be selective CYP2E1 substrates, but recently have been shown to undergo significant metabolism by CYP3A enzymes (Jayyosi et al., 1995; Gorski et al., 1997; Zerilli et al., 1997). Thus, there is precedence for CYP3A enzymes (CYP3A1 or CYP3A2 in the rat and CYP3A4 in humans) metabolizing substrates that are considered to be specific or selective for CYP2E1. The broad range of halogenated hydrocarbons and other small organic molecules that undergo oxidation by CYP2E1 and the existence of several drugs and physiological or pathological conditions that may lead to induction of CYP2E1 suggest that certain conditions or prior or concurrent exposure to other chemicals, such as ethanol or acetaminophen, may alter the metabolism and hence the toxic response to Perc.

3. Role of Genetic Polymorphisms in Cytochrome P450-Dependent Metabolism of Perchloroethylene. Besides alterations in enzyme activity that occur as a consequence of induction or prior or concurrent exposure to cosubstrates, another factor that may influence Perc metabolism by P450 is the existence of genetic polymorphisms. It has become increasingly clear over the past several years that individual susceptibility to many chemicals depends on the genetic makeup of the individual in question.

An increasing number of polymorphisms are being discovered for the human CYP2E1 (McCarver et al., 1998; Hu et al., 1999) and CYP3A4 (Westlind et al., 1999; Sata et al., 2000) genes. Because these enzymes are the ones that are primarily responsible for P450-

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dependent metabolism of Perc, variations in their activities will lead to variations in the amounts of key metabolites that are formed. For those metabolites that are believed to be associated with the cytotoxic and or carcinogenic effects of Perc, these variations will result in altered toxicity. It can be concluded, therefore, that risk will be altered accordingly.

4. Species Differences in Cytochrome P450-Dependent Metabolism of Perchloroethylene. Species differences exist in the rates of P450-dependent metabolism of many substrates, including Perc. Völkel et al. (1998) compared the metabolism of Perc to either oxidative or GSH-derived metabolites in rats and human volunteers exposed to Perc by inhalation. Humans were exposed to 10, 20, or 40 ppm of Perc for 6 h in an exposure chamber, and rats were similarly exposed to 10, 20, 40, or 400 ppm of Perc for 6 h. TCA was the major urinary metabolite in both species; however, the rate of excretion of TCA was markedly slower in humans than in rats. The elimination half-time of TCA in the urine was approximately 4.1-fold longer in humans than in rats (45.6 versus 11.0 h). Maximal TCA concentrations in blood were 3- to 10-fold lower (depending on dose) in humans than in rats exposed to 10 or 40 ppm of Perc. Humans exposed to 10 or 40 ppm of Perc for 6 h had TCA concentrations in blood after 24 h of 0.45 and 3.04 nmol/ml of plasma, respectively, whereas rats exposed to 10 or 40 ppm of Perc for 6 h had TCA concentrations in blood after 24 h of 4.24 and 9.86 nmol/ml of plasma, respectively. DCA was not detectable in human urine, but was detected in rat urine at cumulative levels that were approximately 10% of those of TCA (approximately 200 nmol and 2 μmol for DCA and TCA, respectively, after 80 h, from a 6-h exposure to 40 ppm Perc).

The results described here are in agreement with a study of urinary trichloro-metabolites in workers exposed to Perc (Ohtsuki et al., 1983), in which the authors concluded that the capacity of humans to metabolize Perc is "rather limited". The authors observed that urinary metabolite levels (presumably, predominantly TCA) increased linearly with exposure concentrations of Perc up to 100 ppm, but then leveled off as exposure concentrations increased, indicating saturation of metabolism at the relatively low dose of 100 ppm. They calculated that workers exposed to a total-weighted average dose of Perc of 50 ppm for 8 h would exhale 38% of the absorbed dose unchanged and would excrete into the urine less than 2% of the absorbed dose. The fraction of Perc metabolized in humans at low, environmental exposures is unknown, but has been estimated in models. In a review of such models, Hattis et al. (1990) presented previous model estimates of the metabolized fraction of a 1 ppm Perc exposure dose ranging from 2% to 86%. More importantly, Bois and his colleagues (1996) combined tools from population pharmacokinetics, Bayesian statistical inference, and physiological modeling to derive a relationship between Perc exposure level and fraction metabolized, using human data from Monster et al. (1979). Their results indicate that the fraction metabolized varies with dose, and the population median fraction metabolized for a 0.001 ppm of Perc exposure is 36%, but only 1.5% at a dose close to the occupational exposure reported in the study by Ohtsuki et al. (1983). Furthermore, differences in half-time and blood levels of TCA in rodents and humans support the conclusion that saturation of Perc metabolism occurs at lower doses in humans, which would thereby lead to a decreased proportion of the total flux through P450 and an increased proportion of the total flux through glutathione *S*-transferase (GST). This difference in relative flux, however, has not been demonstrated directly. These data suggest that the overall kinetics of Perc oxidative metabolism differ significantly between humans and rodents.

B. Glutathione Conjugation Pathway

1. Overview of Glutathione Conjugation Pathway. Besides P450-dependent metabolism, which occurs predominantly in the liver, Perc undergoes conjugation with GSH, which is catalyzed by GSTs, to form *S*-(1,2,2-trichlorovinyl)glutathione (TCVG). This is the initial step in the pathway that leads to formation of a reactive metabolite that is associated with toxic effects in the kidneys (see *Section VII.*). Figure 2 illustrates the pathway leading from GSH conjugation of Perc to generation of reactive metabolites by the cysteine conjugate β-lyase (β-lyase) and other enzymes or to a nontoxic mercapturate excretory product. After formation of TCVG (metabolite *2*), which occurs predominantly in the liver, but is also known to occur in the kidneys (Lash et al., 1998a), TCVG is processed by γ-glutamyltransferase (GGT) and cysteinylglycine dipeptidase to the corresponding cysteine *S*-conjugate *S*-(1,2,2-trichlorovinyl)-L-cysteine (TCVC) (metabolite *3*). The enzymatic activities responsible for the metabolism of TCVG are also present in tissue besides the kidneys, such as the brain, suggesting the possibility that reactive metabolites may be formed in tissues besides the primary target organ.

TCVC can be viewed as a branch point in the pathway, because it serves as a substrate for several enzymes that function in either its detoxification or bioactivation. TCVC is metabolized to reactive species by either the β-lyase (Dekant et al., 1988), to form 1,2,2-trichlorovinylthiol (metabolite *6*), or by a cysteine conjugate *S*-oxidase activity that has been identified as a catalytic function of flavin-containing monooxygenase 3 (FMO3) (Ripp et al., 1997), to form TCVC sulfoxide (TCVCSO) (metabolite *7*). Inhibition of TCVCSO formation by the P450 inhibitor 1-benzylimidazole (Ripp et al., 1997) suggested that P450 can also catalyze the sulfoxidation of TCVC. Both the thiol and the sulfoxide metabolites can rearrange spontaneously to form a thioketene (metabolite *8*) (Ripp et al., 1997; Dekant et al., 1988), which is a reactive and potent acylating agent that can bind to cellular protein or DNA (Birner et al., 1994; Pähler et

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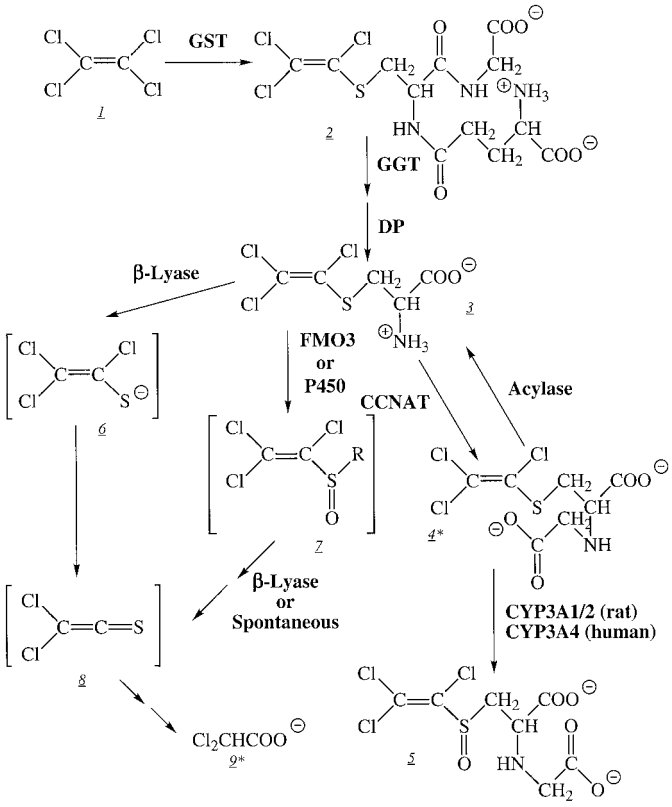

FIG. 2. Metabolism of Perc by the glutathione conjugation pathway. *Identified urinary metabolites: *1*, Perc; *2*, TCVG; *3*, TCVC; *4*, NAcTCVC; *5*, NAcTCVC sulfoxide; *6*, 1,2,2-trichlorovinylthiol; *7*, TCVCSO; *8*, 2,2-dichlorothioketene; *9*, dichloroacetate. Enzymes: GST, GGT, dipeptidase (DP), β-lyase, FMO3, CCNAT, CYP3A1/2, and CYP3A4. Unstable, reactive metabolites are shown in brackets.

al., 1999a,b; Völkel et al., 1999). TCVCSO may also be metabolized by the β-lyase to form a reactive sulfenic acid, although this is likely to be a very minor reaction for the following reasons. First, TCVC-induced nephrotoxicity in F344 rats is actually enhanced by the β-lyase inhibitor aminooxyacetic acid (AOAA) (Elfarra et al., 1999). This suggests that, in the presence of AOAA, TCVC is primarily metabolized to TCVCSO, which then exerts its potent nephrotoxicity by a β-lyase-independent mechanism. Second, although the sulfoxide of *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) (the cysteine conjugate of the Perc congener TRI) was a substrate for a purified preparation of turkey kidney β-lyase, rates of its metabolism to pyruvate were only about 20% of those with DCVC as the substrate (Bhattacharya and Schultze, 1967). By analogy, one would expect TCVCSO to be a poor substrate relative to TCVC for the β-lyase.

The thioketene also decomposes to DCA (metabolite *9*), which is partially recovered in the urine of rats exposed to Perc by inhalation (Dekant et al., 1987; Völkel et al., 1998). Some of the DCA also undergoes further processing to other metabolites. Hence, DCA can be derived from both P450- and GSH-dependent metabolism of Perc; however, most of the urinary excretion product is derived from the thioketene rather than the P450 pathway (Völkel et al., 1998). DCA is also recovered in mouse urine (Yllner, 1961), although the derivation of this metabolite has not been addressed but is likely to derive from both GST and P450 pathways as in the rat. Alternatively, TCVC may be a substrate for the cysteine conjugate *N*-acetyltransferase (CCNAT), which forms the mercapturic acid *N*-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine (NAcTCVC) (metabolite *4*), which is a detoxification product and is readily excreted in the urine (Duffel and Jakoby, 1982; Bartels, 1994; Birner et al., 1996). However, mercapturates such as NAcTCVC can be deacetylated by acylase I to regenerate the cysteine conjugate TCVC (Uttamsingh et al., 1998). An additional bioactivation reaction can occur, whereby the mercapturate is oxidized to the sulfoxide (metabolite *5*) by CYP3A1/2 in the rat or CYP3A4 in humans (Werner et al., 1996). The remainder of this section will discuss the enzymology of each step of the GSH conjugation pathway and, where information is available, describe known tissue-, species-, and sex-dependent differences that may contribute to modulation of the nephrotoxicity or nephrocarcinogenicity of Perc.

It is critical to keep in mind which metabolites are cytotoxic or mutagenic, which are direct precursors of cytotoxic or mutagenic species, and which are detoxication products. As noted above, the cysteine conjugate TCVC (metabolite *3*, Fig. 2) is a precursor to both bioactivation and detoxication products. Metabolites *5*, *6*, *7*, and *8* (the mercapturate sulfoxide, thiol, cysteine conjugate sulfoxide, and thioketene, respectively) are bioactivation products, whereas the mercapturate (metabolite *4*) is the detoxication product that is generally considered to be excreted in the urine. It is important to note, however, that the mercapturate may also be considered a precursor to bioactivation products (see *Discussion*).

2. Glutathione S-Transferases. Because the first step of the GSH-dependent pathway of Perc metabolism is catalyzed by GSTs, sex- or species-dependent differences in this step may play a significant role in determining overall flux and thus generation of reactive and toxic metabolites. There is limited information available on differential expression and activity of the various GST isoenzymes. With specific regard to Perc metabolism, however, no direct information is available on the role of specific isoenzymes in TCVG formation, although some data are suggestive of a function for certain isoenzymes. GSTs are a family of isoenzymes (Mannervik, 1985) that are found in the cytoplasmic compartment of cells in most tissues, with the highest amounts of total GST protein found in the liver. Cytoplasmic GSTs are grouped into seven classes (α, μ, π, κ, σ, θ, and Z, but denoted as A, M, P, T, and Z in humans), based on primary structure, substrate selectivity, sensitivity to inhibitors, and immunological properties. Only GSTα/A, μ/M, π/P, θ/T, and Z/Z are relevant for mammalian liver and kidney. Additionally, a distinct microsomal GST isoenzyme is found in most tissues (Otieno et al., 1997).

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A summary of selected properties of the five mammalian GST isoenzymes in the cytoplasm is presented in Table 1.

From the various immunohistochemical and immunoblot studies of GST isoenzyme expression summarized in Table 1, it is clear that there are species-dependent differences in which isoenzymes are present in liver and kidney. What is unclear from these types of studies, however, is how these qualitative differences can be related to quantitative differences in the ability to metabolize substrates such as Perc, because the isoenzyme specificity and reaction rates for GSH conjugation of Perc by different isoenzymes have not been determined.

Several conclusions, however, can be made from the data summarized in Table 1. In rat kidney, GSTα is the only cytoplasmic GST isoenzyme that has thus far been demonstrated to be present in the proximal tubules (Cummings et al., 2000b). Although the ability of purified rat GSTα to metabolize Perc has not been determined, its congener TRI is an excellent substrate (Cummings et al., 2000b), which suggests that Perc may be as well. In human kidney, besides GSTA (which is the major form), GSTT and GSTP are also present at variable levels. Thus, if Perc is a good substrate for GSTT and/or GSTP, then interindividual variability in expression of these isoenzymes may lead to variation in the ability to form TCVG, thereby altering the risk for nephrotoxicity or nephrocarcinogenicity.

GSTZ is the most recently discovered isoenzyme family. Human GSTZ1–1 is identical to maleylacetoacetate isomerase, which catalyzes the isomerization of maleylacetoacetate to fumarylacetoacetate in the tyrosine degradation pathway, but also catalyzes the oxidative metabolism of DCA to form glyoxylic acid (Board et al., 1997; Tong et al., 1998a,b). A potential role for this newly described isoenzyme in the GSH conjugation of Perc has not been investigated, although a range of small α-haloacids are substrates (Tong, 1998b). Anders and colleagues (Tzeng et al., 2000) recently showed that DCA is a mechanism-based inactivator of GSTZ and that there are four polymorphic variants of the enzyme. Even if Perc is not a substrate for the enzyme, the potent and irreversible inhibition of GSTZ activity by DCA, a metabolite of Perc, suggests that an interaction between Perc and GSTZ occurs at some level. Additional studies are needed to characterize such an interaction.

There has been and continues to be controversy regarding the function of the GSH conjugation pathway in humans with Perc as substrate. Green et al. (1990) reported that TCVG formation, detectable in rodent liver, could not be detected in human liver, whereas Dekant et al. (1987) reported TCVG formation at the limit of detection (i.e., ~0.01 nmol/min/mg of protein) in rat kidney. We reported values in kidney and liver subcellular fractions from rat and mouse (Lash et al., 1998a) that were markedly higher than those reported by either Green or Dekant. As an illustration of the values that we obtained for rates of TCVG formation and of sex- and species-dependent differences in this reaction, Fig. 3 summarizes schematically rates of TCVG formation from incubations of kidney or liver subcellular fractions with 2 mM Perc and 5 mM GSH. The three principal

TABLE 1
Selected properties of GST isoenzymes in the mammalian liver and kidney

GST Class	Presence in the Liver	Presence in the Kidney	Variability in Humans
α/A	Predominant form in human, mouse, and rabbit[a]	Predominant form in rats, rabbits, mice, and humans[b]	Minimal[c]
μ/M	Present in rabbit and mouse[d]	Low levels in rat distal tubules[c] None in human proximal tubules[e] Present at relative high levels in rabbit and mouse[d]	?
π/P	Abundantly present in rat and rabbit cytosol[f] Reported in biliary ducts only[h]	Absent in rat,[g] Clearly but variably present in rabbit and human cytosol[i] Highly variable levels in human cytosol[e] Some expression in loop of Henle, distal convoluted tubules, and collecting duct of humans[j]	Perhaps most variable of all isoenzymes
θ/T	Undetectable in rat[g] Diffuse immunohistochemical staining in mouse pericentral hepatocytes[l] Low levels reported in rat, much higher levels in mouse[m]	Present in human kidney cytosol[e,k] Undetectable in rat[g] Detected in mouse glomerular mesangial cells and collecting duct[l]	Some variability reported in kidney[k] Little apparent variability in kidney[e]
Z/Z	Recently discovered and described in large range of species, including humans[n]	?	Four polymorphic variants[o]

References cited: [a] Campbell et al., 1991; Overby et al., 1994; Mitchell et al., 1997. [b] Campbell et al., 1991; Overby et al., 1994; Mitchell et al., 1997; Rodilla et al., 1998; Cummings et al., 2000b. [c] Cummings et al., 2000b. [d] Overby et al., 1994; Mitchell et al., 1997. [e] Cummings et al., 2000a. [f] Cummings et al., 1999. [g] Cummings et al., 1991. [h] Terrier et al., 1990; Campbell et al., 1991. [i] Rodilla et al., 1998. [j] Terrier et al., 1990. [k] Veitch et al., 1997. [l] Quondamatteo et al., 1998. [m] Mainwaring et al., 1998. [n] Board et al., 1997. [o] Tzeng et al., 2000. ? = unknown.

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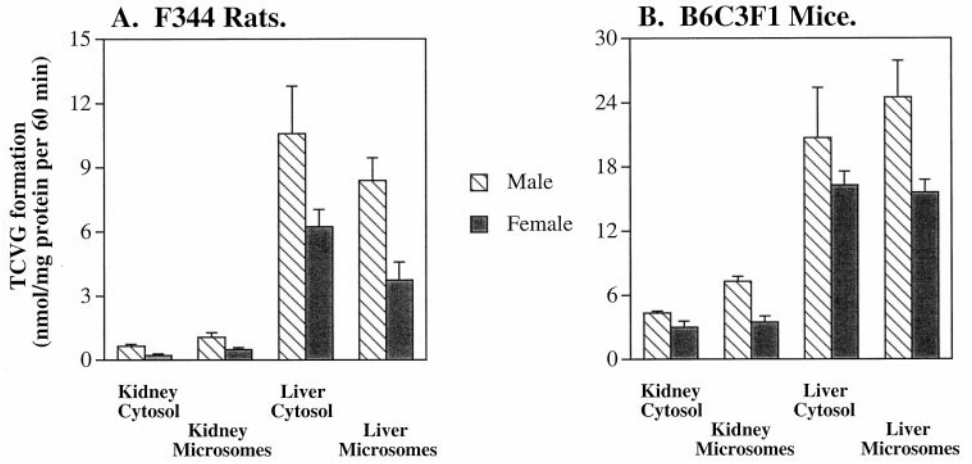

FIG. 3. Perc metabolism to TCVG in the liver and kidney subcellular fractions from male and female F344 rats (A) and B6C3F1 mice (B). Subcellular fractions were obtained by differential centrifugation of homogenates and were incubated for 60 min with 2 mM Perc and 5 mM GSH. TCVG formation was measured after derivatization of acid extracts with iodoacetate and 1-fluoro-2,4-dinitrobenzene, separation by ion-exchange, gradient high-performance liquid chromatography on an amine column using a methanol-acetate mobile phase, and absorbance detection of *N*-dinitrophenyl-TCVG at 365 nm. The limit of detection is ~50 pmol. Results are means ± S.E. of measurements from three separate tissue preparations.

observations from these data are: 1) rates of TCVG formation are higher in males of both species than in females; 2) rates of TCVG formation are markedly higher in mice than in rats, particularly when comparing values in the kidneys; and 3) rates of TCVG formation are 8- to 20-fold higher in rat liver than in corresponding fractions of rat kidney but are only 3- to 5-fold higher in mouse liver than in corresponding fractions of mouse kidney.

A critical point to note, that may help explain the discrepancies in reported rates of Perc metabolism by the GSH conjugation pathway, is that the initial product (viz., TCVG) is chemically very unstable and is difficult to synthesize (Lash et al., 1998a). In fact, we were unable to repeat the synthetic method described by Dekant and colleagues (1987). In our hands, we found TCVG to be very susceptible to nonenzymatic degradation and that incubation time for the synthetic reaction between Perc and GSH had to be increased to 3 days to achieve a high enough yield of product. Hence, different assay methods, as well as potential problems with the chemical instability of the product, may have contributed to the discrepancies in the published data. Although we have replicated and performed several validations of our assay procedure, and believe it to provide accurate measurements of TCVG formation, we cannot exclude with absolute certainty that some systematic error in our method may still exist that has led to an overestimation of metabolism. At the present time, therefore, the reason for the large difference in values for TCVG formation obtained by our laboratory and others (Dekant et al., 1987; Green et al., 1990) is not understood.

Little information is available about sex-dependent differences in GSH conjugation of Perc. However, Mitchell et al. (1997) reported distinct gender differences in protein expression of GST isoenzymes for liver, heart, kidney, and gonads in mice, with males expressing 30% to 50% more soluble GST protein than females in liver and kidney. These variations are consistent with the sex-dependent differences in Perc metabolism observed in our laboratory and described above (cf. Fig. 3). These differences likely contribute to the greater susceptibility of males to Perc-induced renal toxicity.

Based on our data with Perc in rodents and also our data with TRI in humans and rodents, showing that rates of GSH conjugate formation were not markedly lower in humans than in rodents, we conclude that the initial step in the GSH conjugation pathway is not likely to be limiting in humans. Limitations in formation of TCVG cannot, therefore, predict any diminished susceptibility of humans relative to that of rodents to the renal effects of Perc. However, interindividual and gender differences, which have only begun to be documented, may have a significant impact on the levels of TCVG formed in humans exposed to Perc and may thus be an important factor for human health risk assessment. Such interindividual differences may also lead to discrepancies under *Results* reported by different labs when samples are taken from only limited numbers of human subjects.

3. γ-Glutamyltransferase. TCVG, like other GSH conjugates, is processed by GGT to the cysteinylglycine conjugate *S*-(1,2,2-trichlorovinyl)-L-cysteinylglycine and then by dipeptidases to form TCVC (Lash et al., 1988). It is these two steps that provide substrate for the actual bioactivation enzymes, which generate the reactive and toxic metabolites. The tissue distribution of GGT activity is a major determinant of the handling of GSH conjugates, including TCVG, and is thus an important factor in the renal specificity of action of nephrotoxic GSH *S*-conjugates. GGT is the only enzyme that can cleave the γ-glutamyl bond found in GSH and GSH *S*-conjugates, and is localized on the luminal, or brush-border,

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plasma membrane of epithelial cells, such as those in the renal proximal tubule, small intestine, and biliary duct. GGT is also an ectoenzyme, with its active site facing outside the cell. Hence, GSH or GSH *S*-conjugates must be in the luminal space of the epithelium to be degraded.

Renal proximal tubular cells have the highest activities of GGT of all tissues. Although GGT activity is also found in the liver, the ratio of renal to hepatic activity is very high but varies among species. For example, in the rat, which is the most common species in which metabolism of GSH and GSH *S*-conjugates has been studied, the kidney/liver ratio of GGT specific activity is 875 (Hinchman and Ballatori, 1990). In contrast, this ratio is only 413 in mice, 100 in rabbits, 15 in guinea pigs, 19 in pigs, and 47 in macaques. When expressed on the basis of total activity in kidneys and liver, the kidney/liver ratio is 142 in rat, 128 in mice, 16 in rabbits, 3 in guinea pigs, 2 in pigs, and 5 in macaques. Although the activity and tissue distribution of GGT activity have not been completely quantitated in humans, GGT activity in human liver is known to be much higher than that in rodent liver. Consequently, the kidney/liver ratio of GGT in humans is likely more similar to that of pigs or macaques than that of rodents. Hence, use of the rat or mouse as a model for the handling of GSH *S*-conjugates in humans will significantly overestimate the contribution of the kidneys and underestimate the contribution of the liver. Nonetheless, two points are critical in considering the handling of GSH and GSH *S*-conjugates: first, the liver is the primary site for formation of GSH and GSH *S*-conjugates in all species and the liver is very efficient at catalyzing efflux of these compounds into the bile or plasma (Lash et al., 1988). Consequently, although the capacity to degrade GSH *S*-conjugates is significantly greater in livers of humans and other primates, compared with that in rodents, these compounds will still be efficiently exported from the liver. Second, the kidneys, as well as other epithelial tissues (e.g., lung type II cells, small intestinal epithelial cells, retinal pigment epithelial cells), but not the liver, have plasma membrane carriers that can transport GSH *S*-conjugates into the cell (Lash and Jones, 1984, 1985). Hence, these conjugates will be directed toward the kidneys by both efficient efflux from the liver and uptake into the kidneys.

Another point that is relevant to a consideration of the importance of GGT in the processing of GSH *S*-conjugates is that this is not a rate-limiting step in the metabolism of these compounds. Thus, although GGT activity in primates is higher in the liver relative to the kidneys than it is in rodents, renal activity is still present at very high levels. Therefore, species-dependent differences in GGT activity are likely to have only a modest, quantitative effect on the overall metabolism of GSH *S*-conjugates and will not be a major factor determining susceptibility to renal toxicity.

4. Cysteine Conjugate β-Lyase. As stated above, formation of the cysteine conjugate TCVC represents a branch point in this metabolic pathway, because TCVC can be both bioactivated and detoxified by different enzymes. The enzyme that has received the most attention and is the primary one responsible for renal bioactivation of nephrotoxic cysteine *S*-conjugates is the β-lyase. The β-lyase catalyzes either a β-elimination reaction, releasing the thiol, pyruvate, and ammonia, or a transamination reaction that produces the corresponding α-keto acid, which subsequently rearranges spontaneously to release the thiol and pyruvate (Elfarra et al., 1987). The β-lyase is a family of pyridoxal phosphate-containing enzymes that are found in several tissues besides the kidneys, including rat and human liver (Tateishi et al., 1978; Dohn and Anders, 1982; Stevens and Jakoby, 1983; Stevens, 1985a; Tomisawa et al., 1986), intestinal microflora (Tomisawa et al., 1984; Larsen, 1985; Larsen and Stevens, 1986), and rat brain (Alberati-Giani et al., 1995; Malherbe et al., 1995). It is important to note, however, that many of these various β-lyase activities are catalyzed by distinct enzymes with varying substrate specificities or are not exposed to cysteine conjugates in their normal processing. Thus, although the liver contains significant β-lyase activity as a catalytic function of kynureninase (Stevens, 1985a), this activity plays little role, if any, in metabolism or toxicity because the liver is not exposed to significant amounts of cysteine conjugates in a way that would lead to intrahepatic bioactivation. No liver pathology is observed after treatment of rats with DCVG or DCVC, the GSH and cysteine conjugates, respectively, of TRI (Dohn and Anders, 1982; Elfarra and Anders, 1984; Elfarra et al., 1986). This presumably would be the case for TCVG or TCVC, although this has not been specifically tested.

The renal β-lyase activity is primarily a catalytic property of glutamine transaminase K (Stevens, 1985b; Lash et al., 1986, 1990; Stevens et al., 1986, 1988; Elfarra et al., 1987; Jones et al., 1988; Abraham and Cooper, 1991; Perry et al., 1993), and this form was initially purified from rat (Stevens et al., 1986) and human (Lash et al., 1990) kidney cytoplasm, with reported molecular weights of 85 to 100 kDa. Multiple β-lyase activities appear to be present in renal cortical mitochondria, a soluble form present in the mitochondrial matrix that is identified with glutamine transaminase K (Stevens et al., 1988) and a membrane-bound form that is distinct from glutamine transaminase K (Lash et al., 1986). Cooper and colleagues (Abraham et al., 1995a,b) subsequently identified a high-molecular-weight form of renal β-lyase with an apparent molecular weight of 330 kDa. This high-molecular-weight form is found in both the cytoplasm and mitochondrial matrix, but is immunologically distinct from glutamine transaminase K and showed no similarities to other, known pyridoxal phosphate-containing enzymes. The relative importance of each form of the β-lyase in the bioactivation of TCVC

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and other nephrotoxic cysteine conjugates is unknown and needs to be studied. The focus of many of these studies on only cytoplasmic β-lyase activity may be misleading because the mitochondrial β-lyase activity may play a key role in cysteine *S*-conjugate-induced toxicity. This is because the mitochondria are prominent and are early subcellular targets in the sequence of events leading to renal cellular injury (Lash and Anders, 1986, 1987).

β-Lyase activity in the kidneys is localized in the proximal tubules, with little or no protein detected in other nephron segments (Jones et al., 1988; MacFarlane et al., 1989, 1993; Kim et al., 1997). This localization agrees with the pattern of tissue injury observed with in vivo exposures of rats to DCVC or DCVG (Elfarra et al., 1986) and with the greater susceptibility to DCVC of isolated proximal tubular cells, compared with distal tubular cells from the rat (Lash et al., 1994).

Kinetic parameters for β-lyase-mediated metabolism of TCVC in kidney cytosol were determined in rat, mouse, and human tissue of both sexes (Green et al., 1990), and these data are summarized in Table 2. From these data, the following conclusions can be made: 1) β-lyase-dependent metabolism of TCVC in kidney cytosol from rats is more efficient and more rapid than that in either mice or humans; 2) although the specific parameters differ, kinetic efficiencies (i.e., V_{max}/K_m) in mouse and human kidney cytosol are similar and lower than in the rat; 3) β-lyase-dependent metabolism of TCVC is significantly more efficient in male than in female rats; and 4) there are no apparent differences in kinetics of TCVC metabolism by the β-lyase in kidney cytosol from male or female humans. The higher rate and efficiency of metabolism in male, compared with female, rats agrees with the greater susceptibility of male rats, compared with female rats to renal toxicity or cancer (IARC, 1979, 1987; NTP, 1986). Because of low sample number and the possibility of significant interindividual variability in human tissues, one cannot conclude at this point that renal β-lyase activity exhibits no sex-dependent variation in humans. Additional studies will be required to fully assess this point. However, the much lower kinetic efficiency in human kidney and the lower overall metabolic rate in humans, compared with rodents, are consistent with other kinetic studies using purified β-lyase from human kidney cytosol with other cysteine conjugate substrates (Lash et al., 1990), and suggest that for the dose normalized to body weight, the generation of nephrotoxic or nephrocarcinogenic metabolites from Perc or TCVG/TCVC may be much less in humans than in rats. However, normalization of dose and metabolic rate to body surface area, a consideration of total area under the curve for the total amount of metabolism in chronic exposures, and the generation of toxic metabolites from Perc in humans may be relatively equivalent to what is produced in rats. McCarthy et al. (1994) measured β-lyase activity in samples of human kidney cortex cytosol, using a variety of halogenated aliphatic and aromatic hydrocarbons as potential substrates. TCVC was a relatively poor substrate, even compared with DCVC, suggesting that human kidney has a limited capacity to generate reactive and toxic metabolites from Perc by the β-lyase pathway. As noted above, insufficient data are available to make any conclusions about sex-dependent differences based on metabolism.

Although little is known about the genetic regulation of the various renal β-lyase activities, potentially important regulation is suggested by two studies. Both MacFarlane et al. (1993), using the mercapturate of hexachloro-1,3-butadiene (HCBD) (*N*-acetyl-*S*-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine), and Kim et al. (1997), using HCBD, showed that pretreatment of rats with low or subtoxic doses produced induction of β-lyase in renal cytosol. Kim et al. (1997) specifically monitored both the high- and low-molecular-weight forms of the β-lyase, and found that only the low-molecular-weight form was induced. The extent of induction, however, was relatively modest, with both protein and activity increasing 1.5- to 3-fold. Nonetheless, these findings have significant implications for susceptibility to renal toxicity from Perc, particularly for workers using Perc over a long period of time or for individuals who might be chronically exposed to Perc as a drinking water or indoor air contaminant. Such individuals may present with higher β-lyase activity than individuals who have no prior exposure to Perc or similar halogenated solvents. This is an intriguing possibility that warrants further investigation.

Direct demonstration of the function of the β-lyase in vivo in either experimental animals or humans has not

TABLE 2
Sex and species-dependent differences in kinetics of renal cytoplasmic cysteine conjugate β-lyase with TCVC as substrate

Species	Parameter	Male	Female
F344 rat	K_m (mM)	0.68 ± 0.06	1.26 ± 0.21
	V_{max} (nmol/min/mg)	4.00 ± 0.11	3.64 ± 0.41
	V_{max}/K_m	5.88	2.88
B6C3F1 mouse	K_m (mM)	5.69 ± 2.22	4.43 ± 1.42
	V_{max} (nmol/min/mg)	1.15 ± 0.31	1.66 ± 0.27
	V_{max}/K_m	0.20	0.37
Human	K_m (mM)	2.53 ± 0.09	2.67 ± 2.11
	V_{max} (nmol/min/mg)	0.49 ± 0.07	0.64 ± 0.54
	V_{max}/K_m	0.21	0.24

Values are means ± S.D. of three to four separate determinations and were reported in Green et al. (1990).

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been possible with substrates such as TCVC or DCVC, because of the reactive nature of the metabolite. However, Iyer et al. (1998) reported the identification of a metabolite of compound A (2-[fluoromethoxy]-1,1,3,3,3-pentafluoro-1-propene) in both rats and humans that could only have arisen by β-lyase-dependent metabolism. Hence, this is the first study to directly show function of the β-lyase in vivo. Although this does not provide a direct measurement of β-lyase activity with Perc, it simply tells us that the activity exists at a quantifiable level in humans and that compound A may be used as an in vivo marker. This is also significant because many investigators have used measurements of urinary mercapturates as an indication of flux through the GSH conjugation pathway. For Perc, excretion of trichloro-metabolites in the urine of humans exposed by inhalation was found to be 100-fold to >1000-fold higher than that of NAcTCVC, depending on time of measurement and exposure dose (Birner et al., 1996; Green et al., 1990; Völkel et al., 1998). These investigators concluded that the flux through the β-lyase pathway in humans is quantitatively insignificant because the amounts of NAcTCVC excreted were so much less than that of P450-derived metabolites. The problem with this type of analysis, however, is that mercapturate excretion only represents a portion of the flux through the overall GSH conjugation pathway because TCVC can have other fates besides conversion to NAcTCVC, and NAcTCVC can have other fates besides urinary excretion (see below). It is the processing of TCVC and NAcTCVC to reactive and toxic products that is important, and neither the total available substrate nor the amount of substrates converted to toxic products has been completely quantified. Hence, without knowledge of precisely what fraction of overall flux urinary mercapturate represents, such conclusions are unjustified. Furthermore, several of the metabolites that are formed in this pathway (i.e., metabolites *5*, *6*, *7*, and *8*; Fig. 2) are highly reactive and unstable. Because of this chemical instability, they are difficult to quantitate. Importantly, much less of a highly reactive metabolite may be required to elicit a toxic response than is necessary for a stable metabolite such as DCA or TCA.

5. Other Reactions of S-(1,2,2-trichlorovinyl)-L-cysteine and Evaluation of Relative Rates of Each Step of the Glutathione Conjugation Pathway. As described in *Section II.B.1.*, TCVC can be viewed as representing a branch point in the overall pathway of GSH *S*-conjugate metabolism (cf. Fig. 2). Besides metabolism by the β-lyase**,** TCVC may be bioactivated by either FMO3 or P450 to form TCVCSO, or is *N*-acetylated to form the mercapturate, which can be readily excreted. TCVCSO may also be a substrate for the β-lyase, although, as noted above, it is likely to be a very poor substrate so that most of the bioactivation occurs by spontaneous decomposition. NAcTCVC can also be deacetylated by an acylase to regenerate TCVC, or can be oxidized to NAcTCVC sulfoxide by CYP3A enzymes. Both TCVCSO and *N*-acetyl-*S*-(1,2,2-trichlorovinyl-L-cysteine sulfoxide may generate highly reactive and cytotoxic alkylating species. Viewing the cysteine conjugate as a branch point brings one readily to the conclusion that the extent of toxicity will be determined largely by two major factors: 1) the chemical reactivity of the product of the β-lyase reaction or the sulfoxide; and 2) the balance between flux through the β-lyase-FMO3 pathways and CCNAT/acylase-CYP3A pathways. It is this balance that determines how much reactive metabolite is formed.

The balance between bioactivation and detoxification steps in the GSH conjugation pathway can be described mathematically and is illustrated in Fig. 4. Relative risk under different situations, such as in specific species, in one sex of a given species, or in cases where one or more enzymes are induced, can then be estimated by the following equation:

$$\text{Toxic metabolite formed} = (a k_{GST} \cdot b k_{\beta\text{-}lyase}\text{-}1 \cdot c k_{FMO}3 \cdot d k_{CYP}3A \cdot e k_{Acylase} \cdot g k_{\beta\text{-}lyase}\text{-}2)/f k_{CCNAT},$$

where *a*–*g* are weighting factors (0–1) that take into account the relative flux of metabolites through each step, and *k* values may be rate constants for each step or rough estimates of rates under specified conditions. The value of this equation can then be compared for different species or individuals to compare risk. One factor that is missing, however, is a susceptibility factor, that would incorporate the likelihood that the formation of a toxic or reactive metabolite leads to a toxic effect as compared with an innocuous effect or to an effect that is repaired. This issue is discussed further in the section below on modes of action in renal toxicity (*Section VII.*). Another missing factor is a conversion factor (or scaling factor) that takes into account physiological and other species differences, including differences in metabolic rate. Such differences can be scaled between species by applying the following equations:

$$\text{Rate of } O_2 \text{ turnover} = k/W^{3/4}$$

$$\text{Rate of } O_2 \text{ turnover per gram of tissue} = k/W^{1/4}.$$

Mean body weights and the calculated conversion factors between species are summarized in Table 3.

The value of "*a*" (the weighting factor for the GST step) can be set to 1.0, because this is the initial step in the pathway and determines overall availability of substrates for each subsequent step. Estimates of weighting factors for the other steps are more difficult to obtain, but we can assume that $(b + f + c - e) = 1$ (fates of TCVC) and $(e + d - f) = 1$ (fates of NAcTCVC). We are also assuming that TCVCSO is a much poorer substrate than TCVC for the β-lyase, as discussed above, and that

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the relative rate of metabolism by the β-lyase for TCVCSO is approximately 20% of that for TCVC, using the values obtained for DCVC and its sulfoxide (Bhattacharya and Schultze, 1967). Hence, $g = 0.2 \cdot b$. For the first set of weighting factors, we estimate values of $b = 0.7$, $f = 0.2$, $c = 0.2$, and $e = 0.1$. From these estimations, we arrive at values for the other factors, namely $d = 1.1$ and $g = 0.14$. The values for the weighting factors "*a*"–"*g*" are rough approximations based on relative activities of the different steps in the pathway, setting the first weighting factor for GST to 1. It is important to incorporate these weighting factors because large variations in a given step should have little influence if that step occurs at a relatively low rate relative to other steps. The next several paragraphs will summarize data for each step in the GSH conjugation pathway and rough estimates of the relative rates in humans, compared with rats.

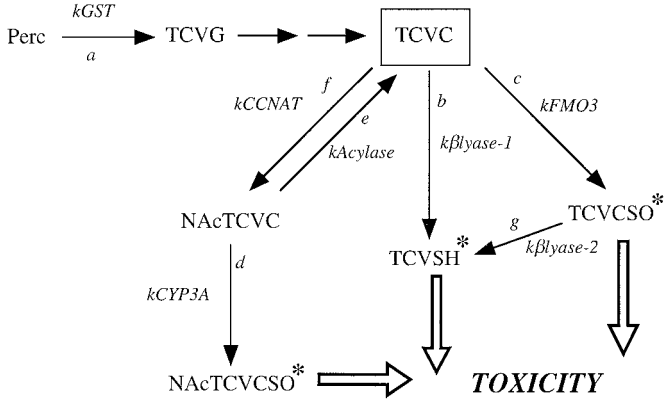

FIG. 4. Analysis of risk or susceptibility to renal injury based on fluxes of cysteine conjugate formation and metabolism. Risk for nephrotoxicity or other toxic effects with the kidneys as a target organ can be calculated from apparent rate constants (*k*) for each enzymatic step involved in the formation and further metabolism of the cysteine conjugate. In this case, (GSH)-derived metabolites of Perc are used, but this approach should apply to other halogenated alkanes or alkenes that form potentially nephrotoxic metabolites by these enzymes. TCVSH, 1,2,2-trichlorovinylthiol; NAcTCVCSO, NAcTCVC sulfoxide. Forward rate constants (*k*) are defined for GST, β-lyase, CCNAT, FMO3, acylase, and CYP3A.

TABLE 3
Calculations for scaling of metabolic rates between species

	Body Weight (kg)		$1/W^{1/4}$	
	Male	Female	Male	Female
Mouse	0.047	0.050	2.147	2.111
Rat	0.471	0.324	1.207	1.326
Human	78.1	65.4	0.336	0.352
Combining Sexes			W	$1/W^{1/4}$
Mouse			0.049	2.128
Rat			0.397	1.260
Human			71.3	0.344
Conversion Factor				
Rat-to-human			0.273	
Human-to-rat			3.663	
Mouse-to-human			0.162	
Human-to-mouse			6.186	
Rat-to-mouse			1.689	
Mouse-to-rat			0.592	

For this type of approach to be useful, of course, data must be available for metabolism by each step in each species and in each organ. In the absence of such data, assumptions can be made to obtain reasonable approximations. As noted previously, more data are available for TRI than for Perc. Lash et al. (1999) reported that the rate of GSH conjugation (*kGST*) of TRI in human kidney and liver is similar to that in rats, although Green et al. (1997) found DCVG formation in humans to be only about 10% of that in rats. Inasmuch as measured rates of TCVG and DCVG formation in subcellular fractions from rat kidney and liver were similar (Lash et al., 1998a,b), we are making the assumption that rates of TCVG and DCVG formation in subcellular fractions from human liver and kidney would be similar to those in rodents as well. A relative value of *kGST* for humans-to-rats for Perc as substrate would, therefore, be between 0.1 and 1.0, based on the range of rates reported in the literature.

A relative value of *kβ-lyase* for humans-to-rats would be 0.05 to 0.1, based on kinetic parameters for TCVC and DCVC metabolism (Green et al., 1990, 1997; Lash et al., 1990).

Little information is available about the relative rates of *N*-acetylation of TCVC in different species. In one study, however, Völkel et al. (1998) reported that the elimination half-time for NAcTCVC in humans was approximately twice that in rats. Based on this, we can assign a relative value of *kCCNAT* for humans-to-rats of 0.5.

Although no information is available on deacetylation of NAcTCVC, Birner et al. (1993) compared rates of deacetylation of *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine (NAcDCVC) in kidneys from humans, Wistar and F344 rats, and NMRI mice. The rates of deacetylation varied by less than 3-fold across species, with the two rat strains exhibiting DCVC formation rates of 0.35 and 0.61 nmol/min/mg of protein, and humans and mice exhibiting rates of 0.41 and 0.94 nmol/min/mg of protein, respectively. Thus, a relative value of *kAcylase* for humans-to-rats would be 0.7 to 1.1.

Ripp et al. (1997, 1999) characterized species-, sex-, and substrate-dependent differences in expression and activity of FMO3 in liver and kidney toward methionine and various cysteine conjugates, including TCVC and DCVC. Rabbit liver was by far the most active and exhibited the highest expression of FMO3. Although human kidney samples were not tested, human liver exhibited comparable activity to that in rat liver, which was 67% of that in rabbit liver. Kinetics of methionine and DCVC sulfoxidation by cDNA-expressed human and rabbit FMO3 were compared, and for both substrates, rates were found to be nearly identical. Rabbit kidney microsomes exhibited about half as much sulfoxidation activity toward methionine as did rat kidney microsomes. Hence, it may be estimated that human kidney exhibits about half as much sulfoxidation activity as

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rat kidney. This would make a relative value for *kFMO3* for human-to-rat of 0.5. Another critical point, however, is that the sulfoxidation activity with different substrates varies considerably (Ripp et al., 1997, 1999). Thus, FMO3-catalyzed TCVCSO formation from TCVC is much slower than FMO3-catalyzed DCVC sulfoxide formation from DCVC, and that this metabolic step contributes very little quantitatively to Perc-induced nephrotoxicity.

There is only one study on sulfoxidation of NAcTCVC and NAcDCVC by CYP3A (Werner et al., 1996), and this was performed in rat liver microsomes. NAcTCVC was actually a better substrate than NAcDCVC for CYP3A, exhibiting a K_m value that was half that for NAcDCVC, although V_{max} values for the two substrates were similar. One important qualification, however, is that the K_m values are in the millimolar range (0.8–2.2 mM), which suggests that at the levels at which NAcTCVC is likely to be found in the renal cell, even at very high exposures to Perc, this pathway will be quantitatively insignificant. Furthermore, the K_m and V_{max} values for deacetylation of NAcTCVC are significantly lower and higher, respectively, than for sulfoxidation of NAcTCVC. In a different study of mercapturate sulfoxidation, Werner et al. (1995) used *N*-acetyl-*S*-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine, the mercapturate of HCBD, as substrate, and found that human CYP3A4 catalyzed the same reaction at comparable rates as did rat CYP3A1. Hence, we can estimate the relative value of *kCYP3A* for human-to-rat as 1.0.

The relative values estimated for each step of the GSH conjugation pathway can be plugged into the equation above to give an overall relative risk for humans as compared with rats of [(1.0)(0.1 to 1.0) · (0.7)(0.05 to 0.1) · (0.2)(0.5) · (1.2)(1.0) · (0.4)(0.7 to 1.1) · (0.14)(0.05 to 0.1)/(0.2)(0.5) = 8.23×10^{-6} to 5.17×10^{-4}]. Hence, we estimated that the relative risk of nephrotoxic effects from Perc exposure for humans can vary by approximately 63.4-fold, from 0.00082% to 0.052%, depending on which data sets are used to estimate species differences in flux at each step. Although this is certainly an oversimplification and a rough estimate of values, it is a starting point. Other factors that need to be considered include flux through the P450 pathway, compared with the GSH conjugation pathway (see *Section II.C.* below), and relative susceptibility of the target cell in rodents and humans to a given amount of reactive metabolite. The analysis presented previously merely considers metabolism differences among species and takes a simplified approach to comparing rates of metabolism and generation of toxic metabolites.

C. Relative Roles of P450 and Glutathione Conjugation Pathways in Perchloroethylene Metabolism

Perc appears to be a much poorer substrate than its congener TRI for P450 (Ohtsuki et al., 1983; Völkel et al., 1998). In vivo, Perc is conjugated with GSH more extensively (1 to 2% of the dose) (Dekant et al., 1986a) than TRI (<0.005% of the dose) (Green et al., 1997). These differences are replicated in in vitro studies, where conjugation of Perc with GSH occurs at faster rates than TRI in mice and rats (Lash et al., 1995, 1998a,b). In experiments to assess the effects of modulation of P450 expression and/or activity on GSH conjugation of Perc, induction of hepatic or renal CYP2E1 by pretreatment of male F344 rats with pyridine significantly diminished rates of TCVG formation (L. H. Lash, W. Qian, P. Huang, A. A. Elfarra, and J. C. Parker, unpublished data). These results suggest that P450 effectively competes with GST for metabolism of Perc. Indeed, Dekant et al. (1987) showed that, in incubations of rat liver microsomes with either Perc or 1-chloro-2,4-dinitrobenzene as substrate, formation of the GSH conjugate was reduced by 70 to 85% by inclusion of NADPH. Although the same type of experiment has not been performed with human tissue, Lash et al. (1999) examined the interaction between GST and P450 in human liver microsomes in the metabolism of TRI: inclusion of NADPH in incubations of human liver microsomes with GSH and TRI significantly reduced formation of DCVG, compared with incubations without NADPH. In contrast, inclusion of GSH in incubations of human liver microsomes with TRI and NADPH had no significant effect on chloral hydrate formation, compared with that in the absence of GSH. Hence, P450 can successfully compete with GST for metabolism of TRI, but GST is ineffective in competing with P450, due to lower affinity and specific activity.

One may conclude that a low-affinity, low-activity pathway (i.e., GSH conjugation) is only of toxicological significance at high doses and/or when the high-affinity pathway (i.e., P450) is saturated. Indeed, this conclusion has been made for both Perc and TRI and particularly for humans, compared with rodents (Green, 1990; Green et al., 1990, 1997; Völkel et al., 1998). This is based in part on kinetics, but also on the relatively low recovery of urinary mercapturates, compared with urinary TCA and related P450-derived metabolites (Green et al., 1990; Birner et al., 1996). Thus, urinary mercapturates comprise from approximately 1% to as little as 0.03% of total recovered urinary metabolites. As far as the kinetic argument is concerned, it is critical to understand that the GSH conjugation pathway yields highly reactive and chemically unstable metabolites, whereas the products of the P450 pathway that are associated with toxicity (e.g., TCA and DCA) are by and large chemically stable, although their relatively unstable precursors (i.e., Perc-epoxide and trichloroacetyl chloride) may also be involved. This is even truer for Perc as substrate than it is for TRI (Lash et al., 1998a). Thus, relatively small amounts of a reactive metabolite may be required to elicit significant biochemical effects in the target cell,

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making arguments based simply on amounts of metabolites formed or isolated without logical foundation.

As far as the argument that mercapturates can be used as a measure of flux through the GSH conjugation pathway, this is also without firm foundation, as discussed above. For the mercapturate to be a valid indicator of the generation of toxic metabolites, one would have to know precisely, over a wide range of relevant substrate concentrations, the quantitative relationship between the various enzymatic steps that metabolize the cysteine and *N*-acetylcysteine conjugates (see Fig. 4). This is exceedingly difficult because many of the metabolites are reactive and are not readily recoverable, either because they form covalent adducts with cellular macromolecules or because they are chemically reactive and not easily quantified. Hence, urinary mercapturates can be validly used as an indicator of exposure, providing evidence of pathway operation to a point, and substrate availability for further processing. Based on the amounts of excretion, however, mercapturates clearly would not be a very sensitive indicator of the amount of toxic products formed. Moreover, no inferences concerning the generation of nephrotoxic and potentially nephrocarcinogenic species should be made from measurements of urinary mercapturates. It is clear, however, that the GST pathway is generally more quantitatively significant in rats than in humans, but that irrespective of species, the relative role of this pathway in Perc metabolism is clearly greater than it is for TRI.

III. Physiologically Based Pharmacokinetic Models for Perchloroethylene

PBPK models have been useful for interspecies extrapolations when, for example, data from humans are lacking. These models can also be useful in determining the influence of changes in specific parameters or physiological functions on the disposition of given chemicals of interest. For example, a PBPK model for Perc developed by Clewell and colleagues (Gearhart et al., 1993) was tested to specifically assess the effect of variations in key parameters on blood and tissue values for Perc and key metabolites. The authors concluded that with the exclusion of metabolism, mechanism, or mode of action, and choice of dose surrogates, parameter uncertainty is not a significant source of variability in the use of this model for risk assessment. Since the time that this paper was published, of course, it has become clear that sex- and species-dependent differences in metabolism and mode of action are significant sources of variability and need to be taken into account.

Two studies by Hattis et al. (1990, 1993) focused on uncertainties in development of PBPK models and corrections that can be made to specific parameters to improve accuracy of risk assessment. In the first paper, Hattis et al. (1990) assessed the role of data set choice and focused on high-dose to low-dose and interspecies extrapolations. They found that indeed, the choice of data set for calibration of metabolic parameters was key and that this factor led to significant variability. In the second study (Hattis et al., 1993), 10 different PBPK models for Perc were compared with actual data on absorption via inhalation and concentrations of Perc in alveolar air and venous blood. Their analysis showed that all models deviate from actual observations and that correction should take into account heterogeneity of the fat compartment with respect to either perfusion and/or partition coefficients and intertissue diffusion of Perc between fat and muscle.

More recently, Bruckner and colleagues developed more detailed PBPK models for Perc, focusing on partition coefficients and tissue distribution (Dallas et al., 1994a), tissue concentration-time data (Dallas et al., 1994b), prediction of systemic uptake and respiratory elimination (Dallas et al., 1994c), and prediction of differences due to species, dose, and exposure route (Dallas et al., 1995). Some of the findings from these studies were that: 1) species-dependent differences in partition coefficients exist in species that are commonly used in toxicity testing; 2) several tissues have very similar elimination half-times for Perc, which is consistent with blood flow limitation for overall Perc metabolism; 3) species differences in blood:air and lung:air partition coefficients can be accounted for to accurately predict systemic absorption of Perc in both rats and humans; and 4) adjustments for differences in species, route of administration, and high-to-low dose can be applied with reasonable success in rats and dogs.

Bois and colleagues (1996) did an analysis to address the question of the fraction of Perc metabolized in humans at low doses. Their primary conclusion was that the proportion of Perc that is metabolized is quite variable and is dependent on dose, making predictions difficult. This highlights the complications inherent in extrapolating to low doses, particularly those that may be most relevant for environmental exposures, and in making predictions of metabolism for a chemical that has multiple fates. These investigators pointed out that the risk of hazard to humans from low-dose Perc exposure would be markedly underestimated if one used standard extrapolation methods from higher doses.

Finally, a more recent PBPK study for Perc was published by Reitz et al. (1996), in which in vivo studies in rats and mice and in vitro studies in rats, mice, and humans were used to refine a "second generation" PBPK model for Perc. The studies clearly establish that the relative ability (i.e., V_{max}/K_m) to metabolize Perc at low, nonsaturating concentrations is much higher in the mouse than in the rat on a per gram of liver basis. Of the various samples of human liver microsomes, some exhibited rates that were similar to those in the rat, whereas others exhibited rates that were significantly lower than those in the rat. The studies indicate, however, that the model still does not account for some

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uncertainty in the dose dependence of Perc metabolism in humans. An additional omission in this model is that of the GSH conjugation pathway. Based on the relatively poor activity of P450 enzymes in metabolizing Perc and the moderately higher activity of GST metabolism of Perc relative to TRI (Lash et al., 1998a), inclusion of estimated parameters for the GSH conjugation pathway, particularly at higher doses of Perc, should improve the ability of the model to predict metabolism.

Another important component of modeling for Perc should include the major metabolite TCA and also DCA. This type of approach has been applied for TRI by Abbas and Fisher (1997), who developed a PBPK model for TRI and several of its P450-derived metabolites in B6C3F1 mice. The model developed for TRI was linked to several submodels for each of the major metabolites. In taking this type of approach for Perc, a much more complete picture of disposition and the generation of potentially toxic metabolites would be obtained. In addition to submodels for oxidative metabolites, a submodel for metabolites of the GSH conjugation pathway should also be incorporated. A scheme for a potential submodel for the GSH conjugation pathway is presented in Fig. 5. The purpose here is not to test and validate a new model, but rather to suggest a concept for further analysis and description of Perc metabolism. The model concept incorporates both intrarenal and interorgan cycles of GSH conjugate metabolism (Lash et al., 1988), with the initial GSH conjugation step occurring in the kidneys or liver, respectively.

Jang et al. (1999) assessed the influence of variations in basic pharmacokinetic parameters that are used on the effectiveness of PBPK models to predict observed levels of metabolites. Depending on whether the chemical of interest is slowly or rapidly metabolized, variations in the choice of basic physiological parameters produced more significant differences in either urinary metabolite concentrations or in alveolar and blood metabolite concentrations, respectively. In any case, the major conclusion is that the set of basic physiological parameters that are used must be made carefully to obtain effective PBPK models.

Besides their utility in making interspecies extrapolations, PBPK models can also be used with data from humans to account for gender or ethnic differences. Jang and Droz (1997) measured metabolites of Perc in exhaled air, venous blood, and urine in six male Caucasians (four Swiss, one Yugoslavian, and one Argentine) and six male Asians (four Vietnamese and two Koreans). Observations were compared with predictions, and the models were modified based on ethnic differences in physiological parameters. The differences in the physiological parameters were often as high as 20%, and included differences in average body weight, tissue volumes, and blood flows. The authors demonstrated that the choice of metabolic and physiological parameters are important in determining the accuracy of the PBPK model. Their data on Perc exposures showed that Asians exhibited significantly lower peak TCA concentrations and AUC values in urine but higher Perc concentrations in expired breath and blood than Caucasians, consistent with slower metabolism in the Asians and distributional differences between the two ethnic groups.

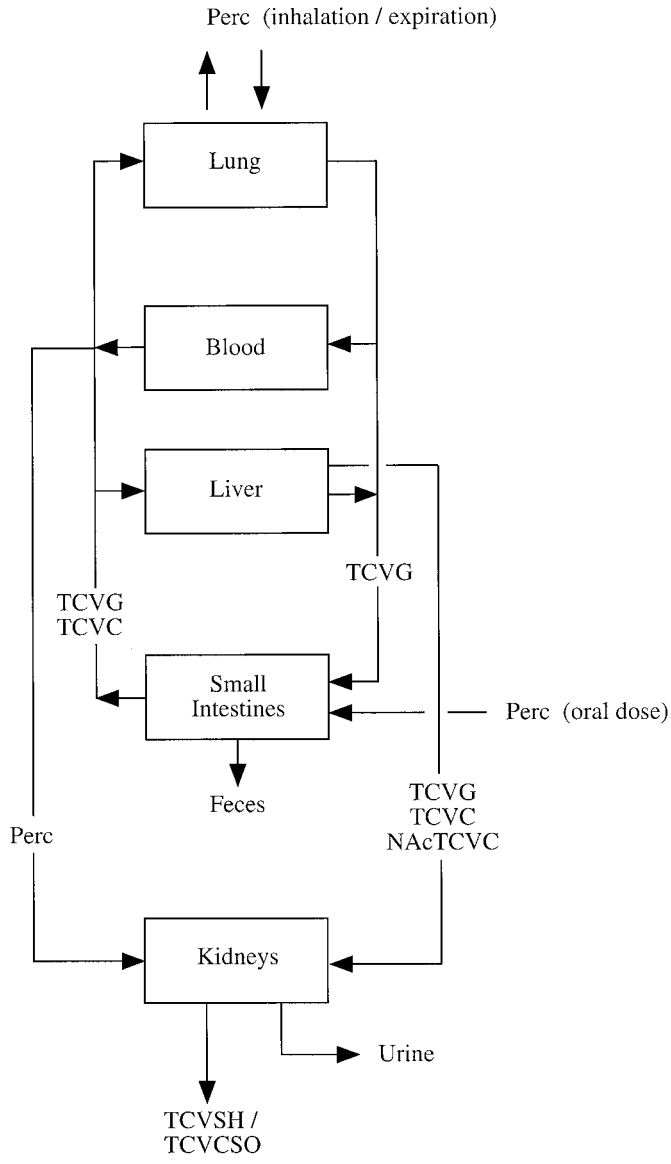

FIG. 5. Structure of proposed submodel for physiologically based pharmacokinetic model of perchloroethylene. The proposed submodel is conceptually based on an approach used by Abbas and Fisher (1997) to incorporate metabolite submodels (in that case, cytochrome P450-derived metabolites) into a physiologically based pharmacokinetic model for trichloroethylene. In this case, a submodel is proposed for metabolites generated by the glutathione conjugation pathway, and includes exposure to Perc by both inhalation and oral routes. Perc is metabolized initially to TCVG in both the liver (primary site) and the kidneys. TCVG is further processed to the cysteine conjugate (TCVC) or the mercapturate NAcTCVC and is then metabolized in the kidneys to either reactive metabolites [1,2,2-trichlorovinylthiol (TCVSH) or TCVCSO] or the mercapturate is excreted in the urine.

IV. Laboratory Animal Studies of Perchloroethylene Toxicity

As noted in the introduction, studies described in the IARC monograph on dry cleaning and various chlori-

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nated solvents (IARC, 1995) and in the U.S. EPA documents (U.S. EPA, 1985, 1986, 1991) present a fairly complete consideration of bioassays for Perc in laboratory animals. In particular and of most relevance for this review, the most recent U.S. EPA document (U.S. EPA, 1991) focused on three responses in laboratory animals: liver tumors in male and female mice; kidney tumors in male rats; and mononuclear cell leukemia in male and female rats. Each of the three tumor responses and the prevailing view of their relevance for human health risk assessment will only be briefly discussed. In addition, gliomas were observed in high-dose male rats in the NTP bioassay (NTP, 1986). Another carcinogenicity study confirmed findings of liver tumors in another strain of mice and leukemia in rats (Nagano, 1993).

Bioassays conducted by the NCI (1977) and the NTP (1986) demonstrated hepatocellular carcinoma in B6C3F1 mice exposed to Perc. The first study involved exposure by intragastric gavage with Perc (males received time-weighted average doses of 536 or 1,072 mg/kg body weight; 450 or 900 mg/kg for 11 weeks, then 550 or 1,100 mg/kg for 67 weeks; females received time-weighted average doses of 386 or 772 mg/kg body weight; 300 or 600 mg/kg for 11 weeks, then 400 or 800 mg/kg for 67 weeks; mice were dosed 5 days per week) in corn oil, and the second involved exposure by inhalation (0, 100, or 200 ppm, 6 h per day, 5 days per week, for 103 weeks). The earlier NCI study was questioned because of the possible effect of contaminating epichlorohydrin in the Perc sample. The Perc that was used in the NCI study was estimated to be 99% pure, with epichlorohydrin concentrations of <500 ppm (NCI, 1977; U.S. EPA, 1985). It was considered unlikely, however, that the tumor response resulted from the low concentration of epichlorohydrin. In the NTP study, pure Perc was used and a dose-related, statistically significant increase in the incidence of hepatocellular carcinoma was observed in both male and female mice.

The 1991 EPA paper noted that there were divergent opinions on the predictive validity of mouse liver tumors in the assessment of carcinogenic risk. The questions about the relevance of mouse liver tumors for human health risk assessment focused on three issues: 1) a high and sometimes variable background incidence of spontaneously occurring tumors in certain mouse strains; 2) the fact that liver cancer is relatively uncommon in the United States (although this is not true in many other parts of the world); and 3) some of the hypothesized mechanisms for mouse liver tumorigenesis may not occur to a significant extent in humans. The overall view of the EPA at the time of the last review of Perc (U.S. EPA, 1991) was that "in the absence of convincing evidence to the contrary, . . . increased incidences of mouse liver tumors in replicate studies is *sufficient* evidence of carcinogenicity". At this point in time, which is 10 years after the last consideration of Perc carcinogenicity, the consensus would still appear to be that one needs to be careful in the extrapolation of evidence of liver tumors in mice to human risk assessment. However, there has been a significant increase in the understanding of mode of action for Perc and its metabolites in the liver (see *Section VI.*), and this should help to improve the precision of risk assessment.

The 1986 NTP study of Perc administration by inhalation to male and female F344/N rats and B6C3F1 mice showed dose-related increases in nephrotoxicity in both sexes of both species and a statistically insignificant increase in the incidence of proliferative lesions of the renal tubular epithelium of male rats. These tubular lesions included tubular cell hyperplasia, renal cell adenomas, and renal cell adenocarcinomas. Despite the lack of statistical significance, the EPA nonetheless viewed these data as providing evidence of a carcinogenic effect in rats. This conclusion was based on three considerations (U.S. EPA, 1991). 1) Renal tubular tumors are exceedingly rare in F344/N rats, with the NTP controls exhibiting renal tubular neoplasms in <0.4% of the more than 1200 controls studied. The overall historical control incidence of renal tubular tumors in male F344/N rats exposed to vehicle controls by gavage is only 0.05%. Hence, the incidence of 8% in the 1986 NTP bioassays is considered significant. 2) There is a complete absence of malignant neoplasms in any control rats tested by the NTP. Hence, the occurrence of two carcinomas in the Perc-exposed rats is viewed as important because the probability that these could occur by chance is estimated to be <0.1%. 3) When the tumor incidence data are analyzed again, but in comparison with historical controls rather than concurrent controls, there was a statistically significant, dose-related positive trend. An important point that complicates analysis of the relevance to humans of Perc-induced kidney tumors in male rats is the putative role of accumulation of α-2u-globulin (α2u) in the mode of tumorigenesis. Although the final conclusion regarding the relevance to humans of the kidneys as a target organ may not be all that different from a decade ago, there is considerably more mechanistic data available to support any conclusions that are made. These issues are discussed in the section on mode of action for renal toxicity (*Section VII.*).

A more recent study by Lock et al. (1996) studied the toxicity in calves of several cysteine conjugates, including both DCVC and TCVC, to both kidney and bone marrow. Although this study confirmed the bone marrow and kidney toxicity of DCVC, no significant pathology in either tissue was observed with TCVC. However, TCVC produced greater inhibition than DCVC of active tubular transport in bovine renal slices. Measurement of TCVC and DCVC metabolism by bovine kidney cytosolic β-lyase showed a 2-fold higher K_m with TCVC as substrate, but a 3-fold higher V_{max} with TCVC as substrate, indicating a higher catalytic efficiency for TCVC than for DCVC. Furthermore, examination of the dosing regimen used in the in vivo treatment of the calves (DCVC was

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administered i.v. as a daily dose of either 1.85 μmol/kg for 10 days or 18.5 μmol/kg for 12 days; TCVC was administered i.v. as either a single 40 μmol/kg dose, followed by a 36 μmol/kg dose on day 25 or a single 80 μmol/kg dose) reveals that the two conjugates were administered much differently so that results may not be directly comparable. Overall, therefore, the data in this paper are consistent with leukemias being a potentially relevant tumor endpoint for DCVC, but possibly not for TCVC. Additional studies will be required to determine the mechanism of the differential response to TCVC and DCVC. These data will not be discussed further in this review, because there is little else available in the literature, and the remaining focus will be on the liver and kidneys.

V. Human Studies of Perchloroethylene Toxicity

A. Occupational Studies

The recent IARC monograph on dry cleaning and chlorinated solvents (IARC, 1995) reviewed most of the studies of human cancers due to occupational exposures up through 1995. Using cohort studies to assess the standardized incidence ratio (SIR) or standardized mortality ratio (SMR), the IARC committee focused on five studies and divided them into two classes: one class containing two studies in which subjects were exposed *predominantly* to Perc and one containing three studies in which subjects had mixed exposure, which included Perc. A recent review by Wartenberg et al. (2000) discusses the epidemiological evidence for dry cleaning workers, and a review by Lynge et al. (1997) discusses the database for other occupations that extensively use Perc, so these will not be discussed here. The discussion here will focus on Perc exposures. In the first pair of studies, where the exposure to Perc was best specified, elevated values of SIR in one study (Anttila et al., 1995) were observed only for the cervix (SIR = 3.2, 95% CI = 0.39–12), kidney (SIR = 1.8, 95% CI = 0.22–6.6), and non-Hodgkin's lymphoma (SIR = 3.8, 95% CI = 0.77–11). The other study from this pair (Ruder et al., 1994) calculated SMR values, and observed increases for the cervix (SMR = 1.6, 95% CI = 0.68–3.1) and esophagus (SMR = 2.6, 95% CI = 0.72–6.8), but not for kidney. It is important to note that renal cancer seldom produces mortality, because it is usually unilateral. Hence, one would not anticipate an increase in mortality. In the three studies with less-defined Perc exposures, one study (Blair et al., 1990) observed increased SMR values for the esophagus (SMR = 2.1, 95% CI = 1.1–3.6), cervix (SMR = 1.7, 95% CI = 1.0–2.0), urinary bladder (SMR = 1.7, 95% CI = 0.7–3.3), and non-Hodgkin's lymphoma (SMR = 1.7, 95% CI = 0.7–3.4), but again not in the kidney. The other two studies with less-defined Perc exposures observed increased SMR values for brain and nervous tissues (SMR = 3.2, 95% CI = 0.67–9.4), for leukemias in general (SMR = 4.9, 95% CI = 1.0–14) (Olsen et al., 1989), and for non-Hodgkin's lymphoma (SMR = 3.2, 95% CI = 0.87–8.1) (Spirtas et al., 1991). The relatively consistent finding of elevated risk for esophageal and cervical cancers in Perc-exposed humans and the absence, to our knowledge, of these cancers in laboratory animals (i.e., rats, mice), suggest that these animals may not completely model the toxic effects of Perc in humans.

One study that was published 3 years prior to, but was not included in, the IARC monograph described isozyme patterns of serum GGT in workers exposed to Perc (Gennari et al., 1992). A sample of 141 workers (124 females and 17 males, aged 20 to 58 years; mean ± S.D. = 43.0 ± 8.0) from 47 small laundries and dry cleaning shops in Bologna, Italy, was studied. The control group consisted of 130 subjects (106 females and 24 males, aged 23 to 56 years; mean ± S.D. = 40.7 ± 9.0) and were students or other university personnel who had no exposure to any chemical agent. None of the workers showed any clinical symptoms of liver disease, and their enzymatic profiles in total blood, including that of GGT, were within the normal reference limits. However, a statistically significant increase in total GGT levels in the serum was found in the exposed subjects. This increase was associated with an increase in one fraction of GGT that is normally present in healthy individuals and with an increase in another fraction of GGT that is considered to be an indicator of hepato-biliary impairment. Although these studies are somewhat preliminary in nature, they suggest that measurement of serum GGT isozyme profile may be a useful biomarker for exposure to solvents such as Perc.

Another study sought to study subclinical hepatotoxicity in dry cleaners exposed to Perc, comparing the sensitivity of hepatic parenchymal ultrasonagraphy with measurements of serum transaminases as biomarkers of liver function (Brodkin et al., 1995). Their studies found mild to moderate changes in hepatic parenchyma more frequently in workers exposed to Perc than in a control population that was not exposed to any chemicals. In contrast, the incidence of increased serum alanine aminotransferase activity in these same workers was much less than that of the changes in ultrasonagraphy. Hence, the serum hepatic transaminase measurements appear to underestimate liver changes that occur as a consequence of exposure to Perc and changes in ultrasonography results appear to be a much more sensitive biomarker. Both this study and the one described previously indicate that the liver is indeed a target organ for Perc in humans.

A recent study (Verplanke et al., 1999) of Dutch dry-cleaning workers exposed to Perc was conducted to test the hypothesis that Perc exposure produces early, adverse effects on the kidneys. The study was conducted on 82 exposed and 19 nonexposed workers. The mean inhaled amount of Perc in the exposed group, which was determined by measurement of Perc concentration in

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alveolar air samples, was 8.4 mg/m^3 (range = 2.2–44.6 mg/m^3), compared with <2.2 mg/m^3 in the nonexposed group. This corresponded to a mean 8-h, time-weighted average exposure of 7.9 mg/m^3 (range = 1–221 mg/m^3) in the exposed group and <1 mg/m^3 in the nonexposed control group. The value for the exposed group is below the Dutch occupational exposure limit, or RfD, of 240 mg/m^3. A chronic dose index (CDI) was also calculated, based on the length of time on the job performing specific tasks, the average exposure dose on the job performing that task, the average duration of the work week while performing the task, and the number of tasks performed. The mean CDI in the exposed group was 400 months × mg/m^3 (range = 12–4,882 months × mg/m^3). This compares with a CDI of 111 months × mg/m^3 (range = 6–1,710 months × mg/m^3) in the nonexposed control group. Effects on renal tubular function were examined by measurement of urinary *N*-acetyl-β-D-glucosaminidase, β-galactosidase, alanine aminopeptidase, and retinol-binding protein. Glomerular function was monitored by measurement of total protein and albumin in the urine. Retinol-binding protein was the only parameter that was increased in the exposed relative to the nonexposed group (75.4 versus 41.6 μg/g of creatinine, respectively). These results suggest that a minor effect on tubular function occurs with chronic exposures to low doses of Perc.

Two new analyses of previous occupational studies of workers exposed to TRI and/or Perc were published after the IARC monograph (McLaughlin and Blot, 1997; Boice et al., 1999) to reevaluate the data. The review by McLaughlin and Blot (1997) focused specifically on the putative association of exposure to TRI and Perc with increased risk of renal cell cancer. They conclude that there is little evidence to support any increased risk, and cite as a basis for their conclusions methodological problems with the few studies that concluded that there was an increased risk of renal cell cancer. Some of the problems cited included design bias in the studies, inappropriate choice of controls, or poor definition of exposure conditions. The study by Boice et al. (1999) reevaluated all of the studies considered by the IARC working group and reported in the monograph (IARC, 1995). The studies included nearly 80,000 workers. There remained problems with classifications of exposures. In all cases, whether exposure was to TRI, Perc, or a mixture of solvents, there was no increased risk of total cancer, and the relative risk for specific cancer sites was close to unity or less. The specific type of cancer that showed some tendency toward increased risk was non-Hodgkin's lymphoma, although the effect was not statistically significant. Because of the limited exposure information, implications specific for Perc can only be tentative.

B. Epidemiological Studies of the General Population Exposed to Perchloroethylene

The problem evaluating epidemiology studies of people exposed to contaminated drinking water was addressed in the recent IARC monograph (1995). One major issue with these studies, as with many similar studies, is the inability to precisely define the composition of the chemical exposures. Hence, associations between risks and exposures are sometimes difficult to assign. Significant increases in cancer incidence, as reported by odds ratio values were calculated in one study for urinary bladder cancer and leukemia. Another study in which groundwater levels of Perc were considerably lower than in the first study found no differences in incidence rates for numerous types of cancers. Two other studies of populations from cities with groundwater contaminated with Perc found slight increases in leukemias or non-Hodgkin's lymphoma.

A recent population-based, case-control study was conducted to evaluate the relationship between various cancers and exposure to Perc in the drinking water (Paulu et al., 1999). The study is a new analysis of the same population that was discussed in the 1995 IARC monograph and concerns drinking water supply in a portion of Massachusetts that was contaminated due to the use of Perc in the preparation of vinyl liners for water pipes. Depending on latency period, which refers to the time period of exposure, significant increases in odds ratio values were observed for lung and colon-rectum cancer.

VI. Modes of Action for Perchloroethylene in Hepatic Toxicity

A. Overall Patterns and Metabolites Associated with Hepatic Toxicity

Hepatotoxicity and hepatocarcinogenesis due to Perc exposure are believed to be a consequence of Perc metabolism by P450. Mice are the species that appear to be most susceptible to liver carcinogenesis from Perc exposure. In particular, cytotoxicity and carcinogenesis have been associated primarily with the metabolite TCA. It is possible that DCA may also play a role in Perc-induced liver toxicity and carcinogenesis. Although there is a commonality between Perc and TRI, because both chemicals generate TCA and DCA as oxidative metabolites, some of the mechanistic information that has been obtained for TRI may not apply to Perc. What differs, however, is the kinetics of metabolism for the two solvents, the precursors to TCA, and the existence of potential, multiple routes to DCA in the case of TRI but not Perc. Therefore, dose dependence will play a major role in determining the type of toxic responses obtained. As noted previously in *Section II.A.*, Perc is a poorer substrate than TRI for P450s. Hence, the various P450-derived metabolites from Perc and TRI will be produced at different rates. Some of the differences in responses

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that are observed with exposures to Perc or TRI, however, suggest that other factors besides differences in kinetics of metabolism play a role in the mode of action for Perc-induced hepatic toxicity.

Several potential modes of action have been suggested for Perc-induced liver toxicity and carcinogenesis. Because these modes of action are dependent on effects derived from TCA and possibly also DCA, the concepts summarized later will borrow from those of Bull (2000), who has written a review on mode of action of liver tumor induction by TRI. In addition, Perc-epoxide and trichloroacetyl chloride may also contribute to Perc-induced hepatotoxicity. An hypothesized scheme of events that leads to liver toxicity from Perc exposure is presented in Fig. 6. The proposed mode of action focuses on consequences of TCA and DCA formation. The sequence of events in the liver may be grouped conceptually into three potential modes of action for Perc (i.e., TCA and DCA), according to Bull (2000):

1. Modification of signaling pathways
2. Cell death and reparative hyperplasia
3. Somatic mutation.

There is evidence that supports each of these modes of action. Key factors as to whether or not these modes of action are important or actually lead to tumor formation are both quantitative and qualitative. On the quantitative side, rates of TCA and DCA formation differ between sexes and species, and many of the proposed effects are highly dose-dependent. On the qualitative side, there are species-dependent differences in responsiveness of systems to TCA and DCA, such that a specific mode of action may not occur in one species, whereas it may be quite prominent in another species.

Although the scheme in Fig. 6 shows TCA and DCA eliciting some distinct effects, but essentially merging in the sequence of events that are elicited by exposure to Perc or either of these two metabolites, there is significant evidence that TCA and DCA act by distinct mechanisms and that the liver tumors caused by each have significantly different properties (Pereira, 1996; Latendresse and Pereira, 1997; Pereira et al., 1997). For example, foci of altered hepatocytes and tumors from mice treated with DCA were eosinophilic and contained GSTπ, TGF-α, *c-jun*, *c-myc*, CYP2E1, and CYP4A1. In contrast, those from mice treated with TCA were predominantly basophilic and lacked GSTπ and most of the other markers (Pereira, 1996; Latendresse and Pereira, 1997; Pereira et al., 1997). Additionally, dose-response curves, progression to cancer, and postexposure regression of lesions differed markedly for TCA and DCA (Latendresse and Pereira, 1997). In that study, Latendresse and Pereira suggested that the differences in dose-response behavior were further evidence of a distinction in modes of action between TCA and DCA. However, these dose-response and time course differences may simply be indicative of differences in the kinetics and disposition of TCA and DCA, which are metabolized at markedly different rates in the liver (Larson and Bull, 1992; Toxopeus and Frazier, 1998) and do not necessarily indicate distinct modes of action. Administration of mixtures of TCA and DCA to mice produced liver tumors that exhibited properties like DCA and not TCA (Pereira et al., 1997), providing further evidence that the two chemicals act by different modes of action. Moreover, the *ras* mutation frequency of TCA-induced liver tumors in mice differs from that of typical peroxisome proliferators (e.g., clofibrate), suggesting a distinct mode of action (Maronpot et al., 1995).

In general terms, neither Perc nor its P450-derived metabolites, TCA or DCA, are particularly potent, acute cytotoxic agents. In fact, in incubations of isolated hepatocytes from either male or female F344 rats with 1 or 10

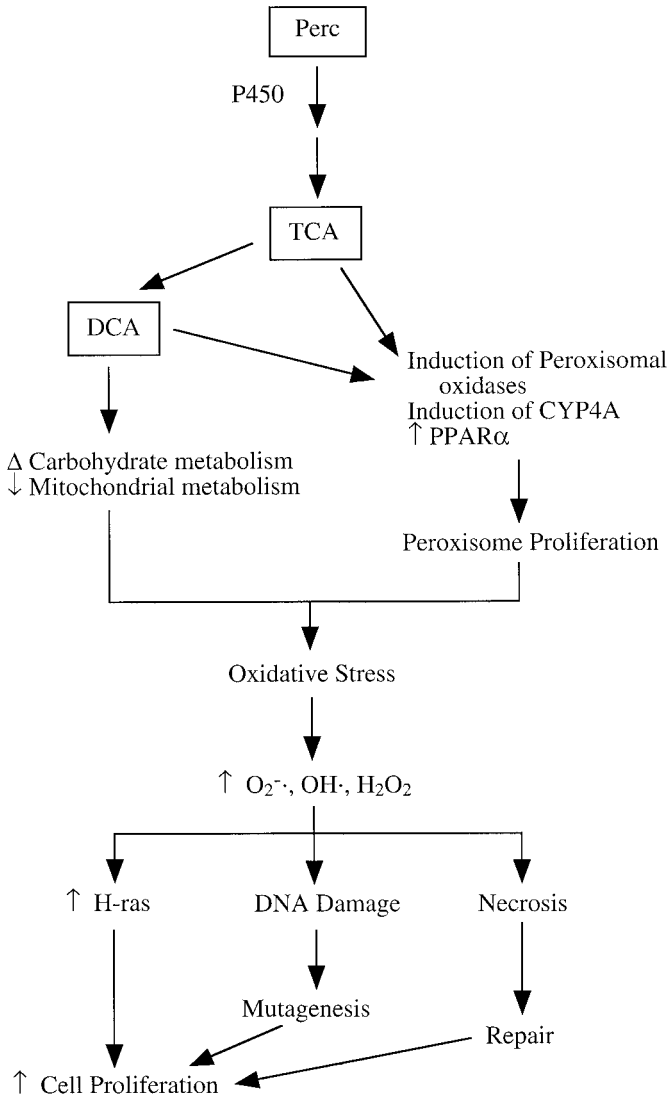

FIG. 6. Summary scheme of the postulated modes of action of perchloroethylene via the cytochrome P450 pathway for hepatotoxicity and hepatocarcinogenicity. The scheme summarizes suggests modes of action that lead to cytotoxicity and tumorigenesis in the liver due to Perc exposure. TCA and DCA are believed to play critical roles that produce changes in the liver cell.

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mM Perc, no significant increases in release of lactate dehydrogenase (LDH) are observed (Table 4). LDH release is an index of cell death, and its measurement is a convenient means of screening or comparing cytotoxic potencies of numerous chemicals in isolated cell or cell culture preparations. Additionally, TCVG is not acutely cytotoxic in isolated hepatocytes, but this to be expected because hepatocytes cannot transport GSH *S*-conjugates into the cell (Lash et al., 1988). Mitochondrial function was only marginally affected by Perc in incubations with isolated mitochondria from rat or mouse liver. In contrast to these studies with the parent chemical, TCA and DCA significantly inhibit mitochondrial respiration when they are incubated with isolated liver mitochondria from rats or mice (L. H. Lash et al., unpublished data).

Bruschi and Bull (1993) also found that neither DCA nor TCA were particularly potent in causing acute cellular necrosis in isolated hepatocytes from B6C3F1 mice. Moreover, their data suggest that the mechanism of cytotoxicity involves conversion to glyoxylate, which is then oxidized to glycolate. Glycolate then serves as a substrate for the peroxisomal glycolate oxidase, which generates reactive oxygen species and causes depletion of intracellular GSH. According to their data, the conversion of monochloroacetic acid, DCA, and TCA to glyoxylate occurs at too slow a rate to produce enough oxidative injury, so cytotoxicity occurs only at the very highest doses of the chloroacetates.

The overall conclusion is that liver toxicity and/or carcinogenesis, when they do occur after exposure to Perc, are attributable to TCA and/or DCA formation. The kinetics of metabolism and the sensitivity of target molecules to these species are likely the keys to understanding species differences in responsiveness to Perc and its metabolites. Formation of TCA and DCA, however, cannot explain the overall greater potency of Perc, compared with TRI in tumorigenicity because Perc is less extensively metabolized. The difference in the TCA precursors may help to explain the greater potency of Perc. Additionally, chloral hydrate is another metabolite of both Perc and TRI that produces liver tumors in male B6C3F1 mice (Rijhsinghani et al., 1986). The quantitative significance of chloral hydrate, compared with TCA and DCA with regard to liver toxicity, however, is likely very small. This is particularly true for Perc because, although chloral hydrate is the predominant intermediate in P450-dependent metabolism of TRI, it is a minor intermediate in Perc metabolism.

TABLE 4
Sex and tissue dependence of acute cytotoxicity of perchloroethylene and its glutathione conjugate[a]

	LDH Release (%)	
	Male	Female
Hepatocytes		
Control	18.9 ± 1.4	24.0 ± 3.1
Perc		
1 mM	22.6 ± 2.5	24.6 ± 4.4
10 mM	23.0 ± 2.6	23.3 ± 0.8
TCVG		
1 mM	17.3 ± 1.5	27.6 ± 4.4
10 mM	22.9 ± 2.1	31.0 ± 3.8
Kidney cells		
Control	25.3 ± 4.2	22.1 ± 3.8
Perc		
1 mM	47.9 ± 6.5*	23.7 ± 6.2
10 mM	64.6 ± 8.2*	22.2 ± 5.5
TCVG		
1 mM	44.6 ± 3.8*	23.6 ± 4.2
10 mM	64.5 ± 2.8*	25.5 ± 4.2

[a] Isolated hepatocytes (1×10^6 cells/ml) and isolated kidney cells (1×10^6 cells/ml) were prepared from male or female F344 rats by collagenase perfusion. Cells were incubated for up to 4 h in hepatocytes or up to 3 h in kidney cells on a Dubnoff shaking metabolic incubator (60 cycles/min) at 37°C under an atmosphere of 95% O_2/5% CO_2 with either Krebs-Henseleit buffer containing 25 mM HEPES, 25 mM $NaHCO_3$, and 2% (w/v) bovine serum albumin (= control) or buffer containing 1 or 10 mM of either Perc or TCVG. At various times, aliquots of cells were removed for determination of release of LDH by measurement of NADH oxidation as a decrease in A_{340} in the presence of pyruvate. Results are the means ± S.E. of measurements from three to six separate cell preparations.

* Significantly different ($P < 0.05$) from the corresponding control. (From L. H. Lash, W. Qian, A. A. Elfarra, R. J. Krause, and J. C. Parker, unpublished data).

B. Peroxisome Proliferation and Enzyme Induction

Peroxisome proliferating agents exert their effects on the liver and certain other tissues by activation of the nuclear receptor protein PPARα. This protein then stimulates the synthesis of several peroxisomal enzymes and selected P450 enzymes (e.g., CYP4A forms) that are involved in lipid metabolism, which in turn increases the number and size of peroxisomes in the liver and selected other tissues. As shown in Fig. 6, both TCA and DCA are believed to induce peroxisomal and microsomal enzymes, such as fatty acid β-oxidation and CYP4A, and activate the PPARα receptor (Odum et al., 1988; Zanelli et al., 1996; Maloney and Waxman, 1999). Although both haloacids activate PPARα, however, the amount of activation due to either TCA or DCA is considerably less than a much lower concentration of Wy-14,643, which was used as a positive control (Maloney and Waxman, 1999).

Peroxisome proliferation can produce an oxidative stress because of the increased generation of reactive oxygen species. These reactive oxygen species may, in turn, lead to activation of oncogenes, including H-*ras* (see *Section VI.C.*), DNA damage (see *Section VI.D.*), or cellular necrosis. Each of these three effects can produce increased cell proliferation, which ultimately can lead to tumorigenesis. Besides potential effects on peroxisomes, DCA is also well established in its ability to alter carbohydrate and mitochondrial metabolism. In fact, a recent study (Ebrahim et al., 1996) of Perc-induced alterations in hepatic and renal glucose metabolism showed that oral administration of Perc (3 g/kg body weight per day × 15 days) in sesame oil to mice caused a significant increase in liver weight, degeneration and necrosis of hepatocytes, and a significant decrease in blood glucose levels. Along with the decrease in blood glucose, activities of glycogenic enzymes were elevated, whereas those of gluconeogenic enzymes were decreased. Interestingly, concomitant administration of either 2-deoxy-D-glucose

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or vitamin E almost completely prevented the pathological and biochemical alterations. Whether or not these effects of Perc were due to generation of DCA was not characterized, although it is likely to be the case.

The principal issue at hand regarding the effects on oxidative metabolism in the cell, and specifically in the peroxisomes, is whether the early changes induced by TCA and/or DCA are of a sufficient magnitude to produce the later effects in humans, as well as in rodents. The entire issue of species differences in sensitivity to peroxisome proliferators has been controversial, both in general for many classes of chemicals (Kluwe, 1994; DePierre et al., 1995; Lake, 1995; Roberts, 1999) and specifically for Perc (Odum et al., 1988). In the study by Odum and colleagues, male and female F344 rats and B6C3F1 mice were administered Perc to study the role of TCA generation in tumorigenesis and peroxisome proliferation in liver and kidney. Due to the pharmacokinetics of Perc, male mice were exposed to 6.7-fold higher amounts of TCA than male rats, and peroxisome proliferation was only observed in male mouse liver. The authors concluded that the process of peroxisome proliferation does not play a significant role for the hepatic effects of Perc in the rat. Just based on the differences in metabolic rates and pharmacokinetics, any potential role for peroxisome proliferation would be even less in humans than in the rat. Another factor that may come into play is species differences in sensitivity of the PPARα receptor to stimulation. Maloney and Waxman (1999) found that some peroxisome proliferators, but not TCA or DCA, produced less activation of human PPARα than they did of mouse PPARα. Hence, it would appear that this aspect of the peroxisome proliferation process is not less sensitive to chemicals than it is in rodents. The response that is generated by stimulation of the PPARα receptor may still be significantly less in human liver, contributing to the diminished overall response in humans, compared with rodents, but this mode of action may still be relevant to humans at some dose level.

C. Oncogene Activation

As discussed previously (see *Section VI.A.*), liver tumors or foci of altered hepatocytes from mice treated with TCA or DCA differ markedly in their pathology and in the expression of several proteins (Latendresse and Pereira, 1997; Stauber and Bull, 1997; Stauber et al., 1998). This is particularly true with respect to the presence of growth factors and oncogenes in these altered cells. DCA-induced tumors or foci uniformly expressed *c-jun*, *c-myc*, and TGF-α, and did not (in contrast with normal hepatocytes) express TGF-β. TCA-induced tumors or foci stained very variably for these and other markers, and at least half of the cells examined in this study were negative for virtually all the markers. This prominent difference in expression of regulatory genes and growth factors is consistent with the two acids affecting distinct signaling mechanisms. Hence, the scheme of initial effects of TCA and DCA in the liver illustrated in Fig. 6 is certainly a vast oversimplification.

Another proto-oncogene that has received considerable attention with respect to its expression in several types of liver tumors is H-*ras*. Anna et al. (1994) reported on H-*ras* codon 61 mutations induced by either DCA, TRI, or Perc in livers of B6C3F1 mice. From the frequency and pattern of mutations that are observed, one can often make conclusions about the mechanism by which the mutation occurred. For example, a chemical-specific pattern of mutations that is distinct from that which occurs in spontaneous tumors is consistent with the chemical acting by a genotoxic mechanism. In contrast, a mutational spectrum that is not chemical-specific, but is similar to that which occurs in spontaneous mutations, is consistent with the chemical acting by a nongenotoxic or secondary mechanism for tumor induction. The mutational spectra and frequency for DCA, TRI, and Perc in H-*ras* codon 61 were all very similar, which would be expected based on TRI and Perc metabolism leading to DCA and DCA being more directly linked to the tumor induction. A selective growth advantage to certain spontaneously initiated hepatic neoplasms was also suggested by the H-*ras* mutation data. These data suggest further that nongenotoxic modes of action play an important role in liver tumorigenesis in B6C3F1 mice induced by DCA, TRI, and Perc. Mutational spectra of human liver tissue after exposure to Perc, DCA, or TCA have not been done yet. Hence, it is unclear whether the same effects will be seen in human liver, and if the spectra and frequency are consistent with a genotoxic or a nongenotoxic mode of action. Additional studies will be required to elucidate this issue.

D. Oxidative Stress and Genotoxicity

Several studies have shown lipid peroxidation in liver to be induced by treatment of either rats or mice in vivo or isolated hepatocytes in vitro with either Perc or its metabolites TCA or DCA (Larson and Bull, 1992; Suzuki et al., 1994; Austin et al., 1996; Ni et al., 1996). Larson and Bull (1992) administered TCA or DCA at doses of 100, 300, 1,000, and 2,000 mg/kg to rats and mice, and measured thiobarbituric acid-reactive substances (TBARS) in liver homogenates. Both compounds enhanced the formation of TBARS in a dose-dependent manner, with DCA being the more potent of the two chemicals. For example, the ability of TCA and DCA to induce TBARS formation was compared with that of CCl_4, which is a well established hepatotoxicant that acts via a free-radical mechanism. At doses of 2 g of TCA/kg, 1 g of DCA/kg, or 1.6 g of CCl_4/kg and 6 h after administration, DCA and CCl_4 both produced nearly 400 nmol of TBARS/g of liver, whereas TCA produced only about 100 nmol of TBARS/g liver. These results were interpreted to indicate more extensive metabolism of DCA than TCA and that the metabolic pathway occurs

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by a free radical-generating, reductive dechlorination mechanism.

Ni et al. (1996) similarly demonstrated lipid peroxidation in the livers of B6C3F1 mice treated with TCA. These authors also showed that induction of P450 with pyrazole and inhibition of P450 with a general P450 inhibitor, 2,4-dichloro-6-phenylphenoxyethylamine, increased and decreased, respectively, the production of lipid peroxidation products. This study actually identified lipid peroxidation products by chromatographic analysis rather than assessing lipid peroxidation by use of the somewhat controversial TBARS as a lipid peroxidation marker. This validation of lipid peroxidation as a biochemical mode of action in chloroacetate-induced hepatotoxicity adds support to the overall scheme proposed in Fig. 6.

A problem with measurements of lipid peroxidation is that the process often occurs as a consequence of cell injury, rather than as an early event that plays a central role in the mode of action. One way of demonstrating that lipid peroxidation plays a role and is not merely an epiphenomenon is to show that it occurs in the proper sequence of events and that it can be causally associated with another biochemical event. This was done in two recent studies (Austin et al., 1996; Toraason et al., 1999), in which the investigators showed that hepatic lipid peroxidation in mice or rats due to exposure to either Perc or TCA and DCA was associated with formation of 8-hydroxydeoxyguanosine. Formation of 8-hydroxydeoxyguanosine can produce mutations, specifically G-to-T and A-to-C transversions (Austin et al., 1996). This finding provides evidence that a genotoxic mechanism may be involved in the mode of action of Perc- or TCA-/DCA-induced liver tumorigenesis.

A nongenotoxic mode of action involving alterations in DNA methylation was shown in a study by Tao et al. (1998): a 38-week administration to female B6C3F1 mice of 25 mmol of DCA or TCA/l in the drinking water produced a significant decrease in the levels of 5-methylcytosine in the DNA from liver tumors. Hypomethylation of DNA is a proposed nongenotoxic mechanism involved in carcinogenesis and tumor promotion. Hence, both DCA and TCA were shown to alter DNA methylation, which may then result in increases in the transcription of certain genes. These authors also made the interesting observation that termination of DCA, but not TCA, exposure resulted in a rebound of 5-methylcytosine levels in hepatic DNA, suggesting that there are differences in the specific mechanisms by which these two chloroacetates induce liver carcinogenesis.

A compilation of genotoxicity tests for Perc and some of its key metabolites published up to 1989 is given in the 1991 EPA Response document. Tests for genotoxicity included gene mutation tests, such as the Ames assay with *Salmonella* strains, a yeast reverse mutation test, a *Drosophila* mutation test, chromosomal aberration tests, various measurements of DNA damaging activity, and quantitation of DNA binding. Basically, the results for Perc as the test agent were mostly negative. Where positive results were observed, they were considered to be very weak. Tests for genotoxicity of P450-derived metabolites of Perc (chloral hydrate, TCA, DCA) were mixed, with some weak-to-moderate-positive results and some negatives. One complication, particularly with Perc, is that cytotoxicity was often observed, preventing attainment of a positive test response.

Hence, there is evidence for both a genotoxic and a nongenotoxic mode of action in liver tumorigenesis induced by either Perc or its chloroacetate metabolites. The findings of oxidized or otherwise altered DNA bases due to Perc exposure are most likely due to the two metabolites, TCA and DCA, and not the parent compound itself, although this has not been directly demonstrated. The weight of evidence is overall supportive of the predominance of nongenotoxic mechanisms of action for TCA and DCA. Whether these processes also occur in human liver at the doses of TCA or DCA that can occur in actual exposures has not yet been tested. Certainly, differences in DNA repair and cytosine methyltransferase activities will also play a key role in determining whether any chemically induced changes in the DNA are retained. Studies are, therefore, suggested to quantitate the activities of these repair processes in human liver and to assess their relationship to chemically induced DNA damage.

E. Cell Proliferation

The ultimate effect of the various proposed modes of action is increased cell proliferation. Stauber and Bull (1997) and Stauber et al. (1998) found that both TCA and DCA increased cell replication in liver tumors and in cultured hepatocytes. Both TCA and DCA appear to promote the survival and growth of initiated cells, presumably through a combination of one or more events, including alterations in DNA replication, DNA damage and mutagenesis, cycles of cellular necrosis and repair, and oncogene activation. Stauber and Bull (1997) also suggested that DCA may act in part by inhibition of kinases, which are important enzymes that modulate cell growth and differentiation.

VII. Modes of Action for Perchloroethylene in Renal Toxicity

A. Overall Patterns and Metabolites Associated with Renal Toxicity

As discussed previously in the section on metabolism (see *Section II.B.*), renal toxicity associated with Perc is thought to be associated with metabolism by the GSH conjugation pathway. The one possible exception to this

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relates to the α2u accumulation seen in male rats, which is not likely to be dependent on metabolism, but is due to an effect of the parent compound (see *Section VII.C.*). Besides the α2u issue, very few mechanistic studies of nephrotoxicity have been conducted with Perc or its GSH-derived metabolites. There are several likely reasons for this, including the generally greater interest of investigators in TRI and the relative chemical instability of TCVG and TCVC, compared with DCVG and DCVC. This latter point has both practical and mechanistic implications. On the practical side, TCVG and TCVC are much more difficult to synthesize in reasonable quantities and with high purity than the corresponding conjugates of TRI, owing to a higher chemical reactivity of the conjugates of Perc (Lash et al., 1998a). The mechanistic implications of this are that dose responses and molecular targets for reactive metabolites of TCVC are not likely to be the same as those of DCVC. Certainly, however, many of the biochemical effects that have been demonstrated for DCVC are the same as those for TCVC, so that some insight on mode of action in the kidneys for Perc and TCVC may be gleaned from studies with TRI and DCVC, respectively. As noted previously, one must be cautious in assuming that whatever effects have been observed for DCVC will also be observed for TCVC.

As shown in Table 4, unlike incubation of isolated hepatocytes with Perc or TCVG, incubation of isolated kidney cells from male F344 rats with either Perc or TCVG produced significant time- and concentration-dependent increases in acute cytotoxicity, as indicated by LDH release. Two significant points should be noted. First, the degree of cytotoxicity in isolated kidney cells from male rats of Perc (48% and 65% LDH release with 1 and 10 mM, respectively) and TCVG (45% and 65% LDH release with 1 and 10 mM, respectively) are significantly higher than those reported for TRI (29% LDH release with 1 mM) and DCVG (38% LDH release with 1 mM), respectively (Lash et al., 2001). Second, whereas both Perc and TCVG produced marked cell death in male rat kidney cells, both were without effect in female rat kidney cells. Hence, the in vitro data showing a marked sex dependence in susceptibility mirrors the sex dependence of in vivo susceptibility to Perc.

Four major mechanisms by which Perc may cause renal tumors will be discussed. These include peroxisome proliferation, α2u accumulation, genotoxicity, and acute cytotoxicity leading to cell proliferation. A suggested scheme for Perc-induced renal injury and tumorigenesis is shown in Fig. 7 and is based largely on a similar scheme proposed for TRI (Lash et al., 2000b). The scheme shows the reactive metabolite of TCVC that is generated by the β-lyase having four initial effects: 1) mitochondrial dysfunction, 2) protein alkylation, 3) DNA alkylation, and 4) induction of an oxidative stress. This may be viewed as the first biochemical level in the mechanism of toxicity. Progression to subsequent levels depends on the balance between the first-level effects and repair processes. Although this scheme has largely been derived from studies with DCVC, several of the effects have also been observed with TCVC. A major difference between DCVC and TCVC is likely dose dependence, because the reactivity of the thiolate (RS^-) species and the thioketenes derived from the action of the β-lyase on the two cysteine conjugates differs.

B. Peroxisome Proliferation

Just as with peroxisome proliferation in the liver, any positive response from Perc would be associated with formation of one or both of the two chloroacetate metabolites, TCA and DCA. A key question in analyzing whether peroxisome proliferation has any role in Perc-induced renal tumorigenesis is whether there is sufficient formation of TCA or DCA within the kidneys or whether there is sufficient TCA or DCA that forms in the liver and is translocated to the kidneys. As discussed above (*Section II.*), Perc is a relatively poor substrate for P450 and hence, the likelihood that sufficient TCA or DCA will either form in the kidneys or be translocated to the kidneys from the liver is very small in rodents and

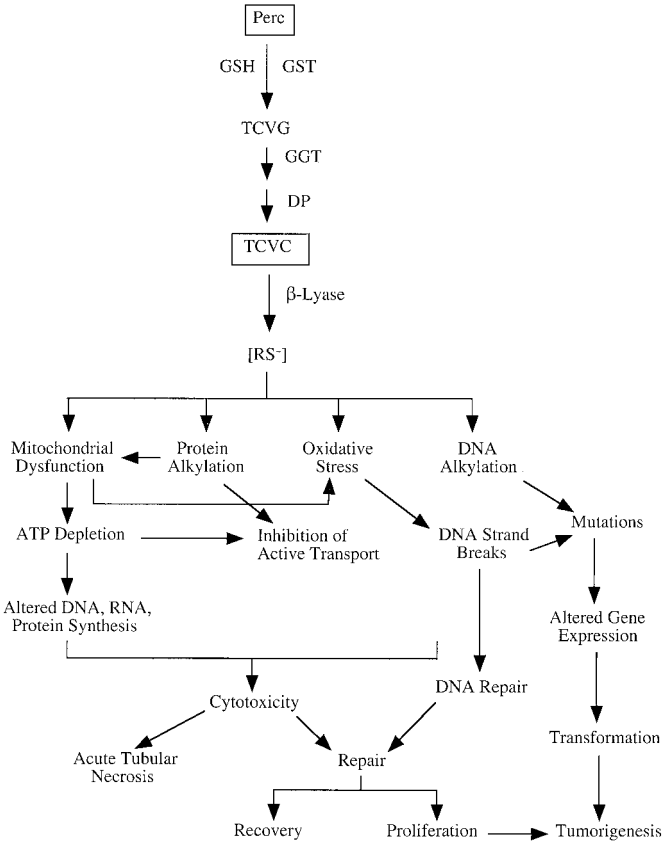

FIG. 7. Summary scheme of the postulated modes of action of perchloroethylene via the glutathione conjugation pathway for nephrotoxicity and nephrocarcinogenicity. The scheme summarizes demonstrated and hypothesized modes of action of Perc in mammalian kidney, showing the various intracellular targets and the interplay between them in ultimately causing nephrotoxicity or nephrocarcinogenicity. DP, dipeptidase; RS^-, reactive thiol and subsequent species generated from β-lyase-catalyzed metabolism of TCVC.

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even smaller in humans. Although P450-dependent metabolism of Perc in the kidneys has not been reported, P450-catalyzed oxidation of TRI in male rat kidneys is markedly slower than that reported for male rat liver (Cummings et al., 2001). Based on these measurements and those of P450-dependent Perc metabolism in liver, we previously concluded that GSH conjugation of Perc plays a markedly greater role in renal and overall metabolism of Perc than it does for TRI (Lash et al., 1998a). This provides further support for the suggestion that amounts of renal TCA and/or DCA formed during Perc exposures will be insufficient to produce peroxisome proliferation in the kidneys. Furthermore, peroxisomes appear to be differentially regulated in the liver and kidney (De Craemer et al., 1994; Van den Branden et al., 1995), with the kidneys generally being far less responsive than the liver.

Goldsworthy and Popp (1987) directly tested whether Perc could induce peroxisome proliferation in the liver and kidney of rats and mice, using increases in cyanide-insensitive palmitoyl-CoA oxidation activity as a marker enzyme. Perc elevated enzyme activity only in the mouse liver and kidney. This suggested that peroxisome proliferation does not correlate with Perc-induced renal carcinogenicity in the rat. In a study in rats and mice on tumorigenesis and peroxisome proliferation induced by Perc, Odum et al. (1988) observed a small, but statistically significant increase in cyanide-insensitive palmitoyl-CoA oxidation in male rat kidney at the lower dose of Perc (200 ppm by inhalation), but not at the higher dose of Perc (400 ppm by inhalation). Surprisingly, somewhat larger, statistically significant increases were observed in female rats at both doses of Perc, which does not correspond with the sex dependence of sensitivity to Perc-induced renal injury. Hence, our overall view is that peroxisome proliferation is not relevant for the mode of action of Perc in the kidneys of either rats or humans.

C. α2u-Globulin Nephropathy

α2u globulin is the major component of the urinary protein load in male rats and is unique to male rats, although homologous proteins exist in other species, including humans. Renal proximal tubules reabsorb protein from the glomerular filtrate and toxicants or pathological conditions that interfere with this process may cause an excessive accumulation of α2u in lysosomes of renal proximal tubular cells. However, a similar phenomenon has not been observed in female rats or in other species. A number of chemicals, many of them halogenated organic solvents, have been shown to cause the so-called "hyaline (protein) droplet nephropathy" in male rats.

Goldsworthy et al. (1988) examined the ability of Perc to induce α2u accumulation, protein droplet nephropathy, and cellular proliferation in the kidneys of male and female Fischer 344 rats. Perc produced accumulation of α2u in male but not female rats, and this correlated with both protein droplet nephropathy and increases in cellular proliferation. Green et al. (1990) also exposed male F344 rats to Perc by either oral gavage or inhalation and observed a marked accumulation of α2u only in male rats exposed to high doses of Perc (1,500 mg/kg for up to 42 days) by oral gavage. In contrast, male rats exposed to 400 ppm Perc by inhalation for 28 days did not exhibit the α2u protein droplet nephropathy. Bergamaschi et al. (1992) also demonstrated α2u accumulation in S2 segments of rat proximal tubules due to a daily exposure of rats to 500 mg of Perc/kg b.wt. in corn oil for 4 weeks. These studies are consistent with α2u accumulation being a high-dose phenomenon that is restricted to male rats. Additionally, the α2u accumulation response occurs at higher doses than those required to induce renal tumors, suggesting that there is no relationship between the two processes.

The prevailing view with respect to the α2u hypothesis is that the phenomenon is male rat-specific and that this mode of action is not relevant to humans (National Research Council, 1995). The α2u found in male rats is structurally related to a group of transport proteins, many of which are found in humans. The proteins of this family of about 20 proteins, called lipocalins, are similar in molecular weight, have some sequence homology, and some are known to have similar tertiary structure to α2u. The only protein with a known physiological function is retinol-binding protein, although all the proteins of the family are thought to be carriers of lipophilic molecules. Since concentrations of these homologous proteins in human urine are well below those of α2u that are found in male rats, it is highly unlikely that enough protein could accumulate in human kidney to produce the same sort of hyaline droplet nephropathy that is seen in the male rat (Flamm and Lehman-McKeeman, 1991). In the case of Perc, however, evidence for α2u accumulation occurred only at doses above those required for tumorigenesis. In addition, the observance of nephrotoxicity in female rats and in both sexes of mice is inconsistent with the α2u hypothesis. Despite the decision of the EPA and the report of the National Research Council, considerable controversy still exists in the scientific community regarding both the mechanism of renal carcinogenesis induced by chemicals that produce α2u and the relevance of this to human health risk assessment.

It is important to note that the Perc-induced α2u response is likely due to the parent chemical rather than to metabolites, as it is based on charge and lipid solubility of the inducing chemical more than on specific interactions with reactive metabolites.

D. Genotoxicity

The 1991 EPA Response document on Perc (U.S. EPA, 1991) summarizes the studies through 1989 on the genotoxicity testing of Perc and some of its metabolites, and

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is discussed in *Section VI.D*. There have been no additional studies since 1989 that have focused on the metabolites of interest for the kidneys (i.e., TCVG or TCVC). TCVG and TCVC are generally found to be relatively potent mutagens, according to in vitro tests, such as the *Salmonella*/Ames assay or unscheduled DNA synthesis in a renal cell line. From the available literature, it is not clear whether the mutagenicity occurs by a direct interaction with cellular DNA or if some indirect effect occurs. Modified DNA bases or adducts have been reported in the urine of humans or the urine and tissue of rats exposed to Perc (Völkel et al., 1999). Another study by the same group (Völkel and Dekant, 1998), however, determined that the formation of the cytosine adduct with chlorothioketene, which is the reactive metabolite generated from DCVC by the β-lyase, is not very efficient under physiological conditions. The authors concluded that formation of DNA adducts may not represent a useful biomarker for effects of TRI. One can readily extrapolate this to Perc, because the reactive metabolite generated by the action of the β-lyase on TCVC should be even more unstable than the one for TRI.

In one study in which the mutagenicity of TCVC was compared with that of two other cysteine conjugates (DCVC and PCBC), the authors found TCVC to be significantly more potent than the other two conjugates (Dekant et al., 1986b). Mutagenic activity was also inhibitable by AOAA, consistent with a requirement for metabolism by the β-lyase.

Recent studies (Brauch et al., 1999) of TRI exposure in humans with renal cell carcinoma provided additional evidence for a specific genotoxic mechanism involving mutations in the VHL gene. Although VHL mutations have not been defined in cases of Perc exposure, it is reasonable to assume that they would be present as well.

Thus, it appears that reactive metabolites derived from TCVC are likely to be genotoxic. A limitation to the importance of this genotoxicity in Perc-induced kidney tumorigenesis is that cell death may occur by either necrosis or apoptosis at a high enough dose, thus eliminating potentially transformed cells that could serve as foci for a tumor.

E. Acute Cytotoxicity and Cell Proliferation

There have been very few mechanistic studies of Perc-induced nephrotoxicity. In terms of dose response of acute cytotoxicity in renal cell preparations and prevention of cytotoxicity by the β-lyase inhibitor AOAA, these have been demonstrated for TCVC and TCVG (Vamvakas et al., 1989). In the few studies where TCVC and DCVC have been directly compared (Werner et al., 1996; Birner et al., 1997) in terms of their cytotoxicity, TCVC has been found to be slightly to markedly more potent than DCVC. This agrees with previous comments about the chemical instability of the TCVC molecule and its thioketene relative to DCVC and its thioketene. The only exception to this is a study of cysteine conjugate-induced renal and bone marrow toxicity in calves (Lock et al., 1996). In this study, DCVC produced potent toxicity in both target organs, whereas the cysteine conjugates of both Perc and HCBD were not toxic. This contrasting finding may be species-specific and its relevance to humans is unclear at present.

As far as the sequence of biochemical events that lead from exposure to recovery or some toxic response, the processes that are illustrated in Fig. 7 likely occur as they do for DCVC (see Lash et al., 2000b). As noted several other times throughout this paper, the two major differences between TRI and Perc will be in the rate of formation of reactive metabolite from the cysteine conjugate and the chemical reactivity of the reactive metabolite generated by the action of the β-lyase. One biochemical mechanism that has received a fair amount of attention by Dekant's group (Birner et al., 1994; Pähler et al., 1998, 1999a,b) is the observed formation of di- and trichloroacetyl protein adducts, which have been detected by both chromatographic and immunochemical methods.

As far as the ability of Perc, through metabolites of the GSH conjugation pathway, to cause the activation of repair and proliferation processes in the kidneys, as has been suggested for TRI, there are no data directly on this point. Presumably, some of the same oncogenes (e.g., GADD53, GADD145, hsp60) may be activated by TCVC as occurs during exposure to DCVC. It is conceivable, therefore, that a nongenotoxic mechanism for tumorigenesis may be operative, involving repeated cycles of cell injury and repair. The studies cited above on genotoxicity that referred to limitations in the ability to detect Perc-induced mutations because of cytotoxicity raise the possibility that the same may occur for repair and proliferation. Hence, if a given concentration of TCVC is too cytotoxic, a cell population from an exposed kidney may not be of high enough viability to produce a significant degree of proliferation.

An additional mechanism for renal injury has been proposed for the related chemical TRI that may have some relevance for Perc. Green and colleagues (Green et al., 1998; Dow and Green, 2000) have observed that rats administered TRI by either gavage or inhalation excrete large amounts of formic acid (which is not derived from TRI) in their urine. They suggest that specifically two metabolites of TRI, TCA and TCOH, inhibit one-carbon metabolism, leading to folate deficiency, which in turn leads to excretion of excess formic acid. The authors suggest that this formic acid contributes to the kidney damage observed in long-term studies with TRI. As discussed in our recent review on the mode of action of TRI in kidney tumorigenesis (Lash et al., 2000b), whereas this proposed mechanism may play some role in the response to TRI, there are much data that support the involvement of metabolites from the GSH conjugation and β-lyase pathways, and that are inconsistent with

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formate excretion being a major mode of renal damage. As for the plausibility of this mechanism with Perc as the toxicant, further investigation is needed to provide an assessment.

VIII. Development of Reference Dose and Reference Concentration for Perchloroethylene Exposure

This section presents a derivation of health benchmarks useful for evaluating Perc exposure levels that may pose a hazard to humans. The quantitative estimation of health benchmarks for noncancer toxicity from exposure to Perc is made by consideration of the available database for the two target organs under consideration in this review (i.e., the liver and kidney), definition of a no-observable-adverse-effect level (NOAEL) as a starting point, and the quantitative and qualitative nature of correction factors that are needed to extrapolate from rodents to humans. These items are integrated into an inhalation RfC and an oral RfD. An RfC is an estimate (with uncertainty spanning perhaps an order of magnitude) of a continuous inhalation exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious (i.e., noncancer) effects during a lifetime (U.S. EPA, 1994). An RfD is an estimate (with uncertainty spanning perhaps and order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious (i.e., noncancer) effects during a lifetime (Barnes and Dourson, 1988). The RfD is derived through a process of considering available studies and selecting critical effects that occur at the lowest oral or i.p. dose, then selecting an oral or i.p. dose or point of departure at which the critical effect is not observed or would occur at a relatively low incidence (e.g., 10%), and then reducing this dose by uncertainty factors to reflect differences between study conditions and the conditions of concern for human exposure.

Two other terms are also used in the derivation of RfC and RfD values: a NOAEL, or no-observed-effect-level, is the highest experimental dose without a statistically or biologically significant effect. There may be effects at the NOAEL, but they are judged not to be adverse. Definition of the NOAEL is markedly affected by experimental design and sample size, such that a given dose may satisfy the criteria for being a NOAEL in one study, but may produce an adverse effect in another study. A lifetime NOAEL is preferred. However, if a subchronic NOAEL is used as a surrogate for a lifetime NOAEL, then a factor of up to 10 is applied to estimate a lifetime NOAEL. If a lowest-observable-adverse-effect level (LOAEL), is used instead of a NOAEL, a correction factor of up to 10 may also be applied to estimate the NOAEL.

The paragraphs that follow present selected studies that we use to calculate RfD and RfC values.

Data for RfD based on hepatotoxicity. A NOAEL for Perc-induced hepatotoxicity in male F344 rats of 100 mg/kg can be defined from data in Toraason et al. (1999). This study involved the i.p. administration of 100, 500, or 1,000 mg/kg of Perc and collection of blood, liver, kidney, and brain samples 24 h after treatment. For all parameters measured, the 100 mg of Perc/kg b.wt. dose was without effect. Buben and O'Flaherty (1985) also examined liver toxicity after oral dosing of mice with Perc and reported a LOAEL of 100 mg/kg/day. In this study, mice were treated with Perc doses of 0, 20, 100, 200, 500, 1,000, 1,500, or 2,000 mg/kg/day 5 days a week for 6 weeks.

Data for RfC based on hepatotoxicity. Mice appear to be more sensitive than rats to the liver toxicity induced by Perc inhalation. The lowest reported effect level for liver effects in laboratory animals is in mice exposed to 9 ppm (= 63 mg/m^3) of Perc for 30 days (Kjellstrand et al., 1984). This value can thus be used as the LOAEL for hepatotoxicity from Perc inhalation.

Data for RfD based on nephrotoxicity. For the kidneys as target organ, Hayes et al. (1986) reported a LOAEL of 400 mg/kg/day for oral exposure of rats to Perc for 90 days in their drinking water. Jonker et al. (1996) studied Perc-induced nephrotoxicity in female Wistar rats by oral gavage administration of Perc in corn oil, given once daily for 32 consecutive days. They defined a lowest-observed-nephrotoxic-effect level (LONEL) dose of 2400 mg/kg. They then arbitrarily defined the no-observed-nephrotoxic-effect level (NONEL) as the LONEL/4, or 600 mg/kg. If we take the arbitrarily defined NONEL as a NOAEL, using the kidneys as target organ, we can then calculate an RfD value.

Data for RfC based on nephrotoxicity. A LOAEL for Perc-induced renal toxicity has been reported as 100 ppm (700 mg/m^3) for inhalation exposure in mice in the NTP bioassay (NTP, 1986). B6C3F1 mice were exposed to Perc for 6 h/day, 5 days/week, for up to 2 years.

Calculation of reference doses and reference concentrations. Data described above, which will be used in the calculation of RfD and RfC values, are summarized in Table 5. RfDs and RfCs apply to lifetime human exposures, including those individuals that may be hypersensitive because of genetic polymorphisms in metabolizing enzymes, simultaneous exposures to other chemicals, pre-existing conditions or diseases, or a variety of other reasons. Inasmuch as the conditions under which NOAELs are determined cannot account for all possible scenarios and are usually determined in animal species and not in humans, a series of correction factors are used to try and account for all these variables.

The following correction or uncertainty factors may be applied.

1. Animal-to-human uncertainty. Cross-species uncertainty is accounted for by application of uncertainty factors. Based on differences in metabolism that were discussed in *Sections II.A.* and *II.B.5.*, we can apply

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TABLE 5
Summary of studies used in the calculation of RfD and RfC values based on hepatotoxicity and nephrotoxicity

		Value	Species	Study Type	Reference
Hepatotoxicity					
RfD	1. NOAEL	100 mg/kg/day	Rat	Acute	Toraason et al., 1999
	2. LOAEL	100 mg/kg/day	Mouse	Subchronic	Buben and O'Flaherty, 1985
RfC	3. LOAEL	9 ppm	Mouse	Subchronic	Kjellstrand et al., 1984
Nephrotoxicity					
RfD	4. LOAEL	400 mg/kg/day	Rat	Subchronic	Hayes et al., 1986
	5. NOAEL	600 mg/kg/day	Rat	Subchronic	Jonker et al., 1996
RfC	6. LOAEL	100 ppm	Mouse	Chronic	NTP, 1986

an uncertainty factor of 10 for the liver as target organ and 250 for the kidneys as target organ.

2. Sensitive human subpopulation correction. An uncertainty factor of 10 is applied to both target organs to account for factors such as genetic polymorphisms, pre-existing diseases or conditions, or concurrent exposures to chemicals that alter responsiveness to Perc.

3. Subchronic-to-chronic uncertainty. Inasmuch as the NOAEL for the liver RfD was determined in an acute exposure study and the two LOAELs for the liver RfD and RfC were determined in subchronic exposure studies, an uncertainty factor of 10 is applied to account for potentially increased toxicity from chronic exposures. For the kidney, both the LOAEL and the NOAEL for the RfD were determined in subchronic studies, an uncertainty factor of 10 is applied for potentially increased toxicity from chronic exposures. As the LOAEL for the kidney RfC was determined in a chronic study, no extra uncertainty factor is needed for this calculation.

4. LOAEL-to-NOAEL correction. In those cases where a LOAEL is calculated, an extra correction factor of 10 is applied to estimate a NOAEL.

The calculation of uncertainty factors for the six sets of values (three each for liver and kidneys as target organs) and the RfD and RfC values determined for each study are summarized in Table 6. The RfD or RfC value is then calculated by dividing the NOAEL or LOAEL by the composite uncertainty factor. The calculated RfD values vary considerably, depending on which study is used to provide the NOAEL or LOAEL and on which target organ is used. A lower value is generally obtained with nephrotoxicity as the guiding response, with values varying 10- and 15-fold for the two pairs of studies for liver and kidneys as target organs, respectively. The current RfD value is 10 μg/kg/day. The RfD values calculated in the present review span a range of from 15% of the current value to 10-fold greater than the current value. If we use the kidneys as the driving target organ and take a conservative approach, then the RfD should probably be lowered to 5 μg/mg/day.

For the RfC value, a 4-fold higher value was obtained using nephrotoxicity as the guiding response. The RfC value using the kidney data does not differ significantly from the currently established 5 ppb. However, if we use the liver as the more sensitive target organ and take a conservative approach, then the RfC should probably be lowered to 1 ppb.

IX. Summary and Research Needs

Perc is metabolized by two main pathways, P450 and GST. Available data indicate that, in human liver, CYP2E1, CYP2B1/2, and CYP3A4 are primarily responsible for oxidative metabolism. Genetic polymorphisms in P450 enzymes may have a profound effect on Perc metabolism, and these must be taken into account in an assessment of human health risk. P450-dependent metabolism of Perc is generally slower than that of TRI, and rates of metabolism in humans are considerably less than those in rodents. Metabolites derived from P450 metabolism, specifically TCA and DCA, are linked to hepatotoxicity and liver tumorigenesis.

Although the initial step in the GSH conjugation pathway occurs primarily in the liver, metabolites are di-

TABLE 6
Summary of uncertainty factors and calculation of RfD and RfC values for liver and kidney as target organs[a]

Study No.	Uncertainty Factors					RfD or RfC
	Animal-to-Human	Sensitive Humans	Subchronic-to-Chronic	LOAEL-to-NOAEL	Composite	
Hepatotoxicity						
1	10	10	10	1	1,000	100 μg/kg/day
2	10	10	10	10	10,000	10 μg/kg/day
3	10	10	10	10	10,000	0.9 ppb
Nephrotoxicity						
4	250	10	10	10	250,000	1.6μg/kg/day
5	250	10	10	1	25,000	24 μg/kg/day
6	250	10	1	10	25,000	4 ppb

[a] Study numbers correspond to those in Table 5. Uncertainty factors are used to correct for extrapolations from animals to humans, potentially greater toxicity in chronic exposures as compared with subchronic or acute exposures, and use of a LOAEL rather than a NOAEL value. RfD and RfC values are then based on application of the composite uncertainty factors (product of all uncertainty factors) to the LOAEL or NOAEL values given in Table 5, dividing the LOAEL or NOAEL dose by the composite uncertainty factor.

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rected to the kidneys by way of interorgan translocation pathways. After processing to the cysteine conjugate, renal β-lyase is the enzyme primarily responsible for conversion to a reactive metabolite that can covalently bind to protein and/or DNA. Sex- and species-dependent differences have been documented in many of the steps of this pathway, and such differences need to be taken into account for human health risk assessment. Based on calculations of differences in relative flux at each step in Perc metabolism by the GSH conjugation pathway, it is estimated that metabolic flux to generate a reactive metabolite in humans is 0.00082% to 0.052% of that in rats. The reason for the 63.4-fold variation is the disagreements in the literature on metabolic rates for several of the steps for this pathway. This analysis suggests that humans should be exposed to far less reactive metabolite per a given dose of Perc than rats and would thus likely exhibit much less sensitivity to Perc-induced toxicity.

In vitro studies have demonstrated that P450 effectively competes with GST for metabolism of Perc, owing to a significantly lower K_m. One might naturally assume from this that the low-affinity/low-activity pathway (namely GST) is of little toxicological importance. However, this would be a mistake because one must consider that the GST pathway generates reactive metabolites, whereas the two key metabolites generated by the P450 pathway are chemically stable.

PBPK models are becoming increasingly sophisticated and have been adapted to Perc. Abbas and Fisher (1997) took their PBPK model for Perc a step further by creating submodels for some of the key metabolites. A new, additional submodel is proposed here for the GST pathway. The concept of this submodel is presented to account for a metabolic pathway and target organ that has traditionally not been considered in PBPK models of Perc and similar chemicals.

Animal bioassays were used by IARC to conclude that Perc is "a probable human carcinogen", because there is sufficient evidence in laboratory animals. Primary target organs are the liver in mice, the kidneys in male rats, and the immune system in male and female rats. Occupational studies of Perc exposure reveal significantly elevated risks for cancer of the cervix, esophagus, non-Hodgkin's lymphoma, and in some studies, the kidneys and/or urinary tract.

The mode of action of Perc in the liver is seen as involving three potential mechanisms: 1) modification of signaling pathways, 2) cell death and reparative hyperplasia, and 3) somatic mutation. Liver toxicity is believed to be associated with two P450-derived metabolites, TCA and DCA. The types of alterations in signaling pathways include enzyme induction, oncogene activation, peroxisome proliferation, and alterations in intermediary metabolism. The relevance of peroxisome proliferation for humans is in question. There is some evidence in favor of a genotoxic mechanism, but at best, DCA and TCA are only weakly mutagenic.

The mode of action of Perc in the kidneys is seen as involving mitochondrial dysfunction, protein alkylation, DNA alkylation, and oxidative stress as initial responses. Although nongenotoxic mechanisms are clearly envisioned, TCVC is also a fairly strong mutagen, suggesting that genotoxic mechanisms may also be significant. Peroxisome proliferation likely plays even less of a role in the kidneys for humans than it does in the liver. α2u accumulation is observed in kidneys of rats exposed to doses of Perc that exceed those used in the carcinogenicity bioassays. If the nephropathy in rats that is induced by Perc is due in part to the α2u accumulation, then the findings of nephrotoxicity and nephrocarcinogenicity in rats will be difficult to extrapolate quantitatively to humans. However, other modes of action are likely to be responsible for the renal effects in rats, and these do have relevance for humans.

RfD and RfC values are calculated based on using either the liver or the kidneys as the target organ. Two studies each for an RfD and one each for an RfC are used for the analysis. If a conservative approach is applied in both cases, then our recommendation is that nephrotoxicity be used as a driving force for calculation of the RfD, and a value of 5 μg/kg/day, which is half of the current RfD, be used, and hepatotoxicity be used as a driving force for calculation of the RfC, and a value of 1 ppb, which is one-fifth of the current RfC, be used.

Additional research is needed in several aspects of Perc metabolism and mode of action. For example, metabolism data for the P450 and GST pathways, particularly in human tissue, would be useful in improving PBPK models and in decreasing uncertainty factors that are used to extrapolate from animals to humans. Inasmuch as TCVC is believed to be the penultimate nephrotoxic metabolite for Perc-induced nephrotoxicity and nephrocarcinogenicity, whole animal exposure studies and bioassays with this metabolite would be very useful in providing further validation of this pathway and in determining dose-response and time course relationships. Such studies, in particular chronic exposure studies, would also help provide more accurate RfD and RfC values. As noted in *Section VII.*, much of the information on mode of action for Perc-induced renal toxicity derives from studies with TRI and its cysteine conjugate DCVC. Additional studies with TCVC would provide more precise information on how the reactivity of and responses to the reactive species derived from TCVC and DCVC differ.

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Note Added in Proof. Please note that the presentation of estimates for RfD and RfC values are based on a limited data set from liver and kidney toxicity and represent only one method of estimating these values. This approach is not necessarily the one that would be used by the official risk assessment by the U.S. EPA and simply represents a suggested approach by the authors.

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