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Genetic Polymorphisms in Assessing Interindividual Variability in Delivered Dose

L. T. Haber,*,[1] A. Maier,* P. R. Gentry,† H. J. Clewell,† and M. L. Dourson*

*Toxicology Excellence for Risk Assessment, 1757 Chase Avenue, Cincinnati, Ohio 45223; and †Environ, 602 East Georgia Avenue, Ruston, Louisiana 71270

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Increasing sophistication in methods used to account for human variability in susceptibility to toxicants has been one of the success stories in the continuing evolution of risk assessment science. Genetic polymorphisms have been suggested as an important contributor to overall human variability. Recently, data on polymorphisms in metabolic enzymes have been integrated with physiologically based pharmacokinetic (PBPK) modeling as an approach to determining the resulting overall variability. We present an analysis of the potential contribution of polymorphisms in enzymes modulating the disposition of four diverse compounds: methylene chloride, warfarin, parathion, and dichloroacetic acid. Through these case studies, we identify key uncertainties likely to be encountered in the use of polymorphism data and highlight potential simplifying assumptions that might be required to test the hypothesis that genetic factors are a substantive source of human variability in susceptibility to environmental toxicants. These uncertainties include (1) the relative contribution of multiple enzyme systems, (2) the extent of induction/inhibition through coexposure, (3) allelic frequencies of major ethnic groups, (4) the absence of chemical-specific data on the kinetic parameters for the different allelic forms of key enzymes, (5) large numbers of low-frequency alleles, and (6) uncertainty regarding differences between *in vitro* and *in vivo* kinetic data. Our effort sets the stage for the acquisition of critical data and further integration of polymorphism data with PBPK modeling as a means to quantitate population variability. © 2002 Elsevier Science (USA)

Key Words: **polymorphism; risk assessment; PBPK model; warfarin; parathion; dichloromethane; methylene chloride; dichloroacetic acid.**

INTRODUCTION

Treatment of human variability (also called intraspecies or interindividual variability) in risk assessment, including protection of sensitive subpopulations, has traditionally been semiquantitative. For noncancer risk assessment, a default uncertainty factor (UF_H) of 10 has been used to account for human variability (Barnes and Dourson, 1988; U.S. EPA, 1994). This factor considers both toxicokinetic and toxicodynamic variability. A number of researchers have evaluated human data on variability in the context of evaluating whether this 10-fold factor accurately accounts for the variability between the average and sensitive human in response to chemicals (Dourson and Stara, 1983; Hattis *et al.*, 1987; Kaplan, 1987; Sheenan and Gaylor, 1990; Calabrese *et al.*, 1992; Calabrese and Gilbert, 1993; Hattis and Silver, 1994; Renwick and Lazarus, 1998; Burin and Saunders, 1999). In general, data from all of these studies indicate that the default value of 10 for intraspecies variability is protective when starting from a median response, or by inference, from a no-observed-adverse-effect level assumed to be from an average group of humans. Although some of these analyses (Calabrese *et al.*, 1992; Hattis *et al.*, 1987; Kaplan, 1987) noted a range of variability greater than 10-fold, it is because these authors evaluated the total range of human variability, rather than considering that the uncertainty factor of 10 is applied to account for the degree of variability from the population average to the sensitive human.

By contrast, variability in the human population has not been addressed explicitly in traditional cancer assessment for genotoxic carcinogens. There are two reasons for this difference. First, a different assumption is used for the origin of the dose–response curve for noncancer endpoints and classical (genotoxic) carcinogens. For noncancer endpoints, the dose–response curve is assumed to be due to differences in sensitivity in the test population. At low doses, only the most sensitive members of the population are expected to respond, if a response is observed. As the dose increases, both the

[1]To whom correspondence and reprint requests should be addressed. Fax: (513) 542-7487. E-mail: haber@tera.org.

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severity of the response and the percentage of the population affected are assumed to increase. As risk assessors characterize the dose response at the lower end of the curve, variability is addressed directly in noncancer assessments. In contrast, according to the simplest version of the cancer paradigm, cancer is assumed to be a stochastic process. Although the stochastic nature of the response generally refers to the probability of the chemical reacting with DNA, it can also be interpreted as meaning that the individuals who get cancer at lower doses are simply unlucky, not more sensitive (e.g., they happened to have a DNA damage event affecting an oncogene, rather than a noncoding DNA region).

In reality, both assumptions are likely to be a bit oversimplified. For example, toxicokinetic variability will still lead to differences in tissue dose for a given exposure, and different people may have different rates of repair of DNA damage on top of the random nature of the cancer process. Nonetheless, the approach for genotoxic carcinogens has generally considered that the models used are sufficiently conservative that sensitive populations are protected (U.S. EPA, 1986, 1996, 1999). Sources of conservatism include (1) dose–response assessments for cancer generally rely on chronic animal bioassays done at high doses (where metabolism may be saturated), so toxicokinetics and other parameters may not be representative of those at low doses; (2) the linear extrapolation is described as a "plausible upper bound estimate of risk at low dose where true risk may be lower, including zero" (U.S. EPA, 1999); and (3) it is assumed that no threshold exists for nongenotoxic carcinogens. Another source of conservatism for both cancer and noncancer risk assessment is that the most sensitive species, strain, and sex is often used, unless there is evidence that the data are not applicable to humans.

The traditional factor of 10 for human variability in noncancer assessment has typically been replaced when adequate data exist to do so, such as when data are found on known sensitive subgroups (e.g., nitrate RfD, U.S. EPA, 2001). Efforts are now under way to formalize this replacement on a more systematic basis with a factor that more accurately represents human variability. "Data-derived" uncertainty factors were developed (IPCS, 1994; Meek, 1994; Renwick and Lazarus, 1998) that divide the intrahuman UF_H into equal factors of 3.16 for toxicokinetics and toxicodynamics, based on the earlier work of Renwick (1991, 1993). When chemical-specific data are available, the toxicokinetic or toxicodynamic components may be replaced with factors derived from the data (e.g., IPCS, 1998; Dourson *et al.*, 1998; Murray and Andersen, 2001). An international effort under the auspices of the International Programme on Chemical Safety has defined the data requirements for development of data-derived uncertainty factors, now referred to as chemical-specific adjustment factors (CSAFs)[2] to replace default uncertainty factors for interspecies differences and human variability (Meek *et al.*, 2001; IPCS, 2001).

Another approach used to account for human variability is to use probability distributions of uncertainty factors to characterize the population and hence UF_H (Baird *et al.*, 1996; Slob and Pieters, 1998; Price *et al.*, 1997; Swartout *et al.*, 1998). One approach to characterize the human distribution (and UF_H) is based on toxicological "first principles," using data on heterogeneity in animals and assumptions about the relationship between animal and human heterogeneity (Baird *et al.*, 1996; Baird, 2001). Another approach is to estimate a distribution of UF_H based on the U.S. EPA definition of the RfD (Swartout *et al.*, 1998; Price *et al.*, 1997). In brief, a log-normal distribution is assumed, with the distribution parameters set such that the median is $10^{0.5}$ and the 95th percentile is 10. Slob and Pieters (1998) used similar assumptions about the shape and width of the distribution to estimate a distribution of UF_H. Hattis and colleagues have been collecting data for a number of years on human variability in parameters representing steps in the pathway from external exposure to production of biological response (e.g., Hattis and Silver, 1994; Hattis and Barlow, 1996; Hattis *et al.*, 1999a,b). Using this database, they estimated that if the population distribution is normal out to the extreme tails, a dose 1/10 that corresponding to a 5% effect level would be associated with an effect incidence ranging from slightly less than 1/10,000 (for a median chemical/response) to an incidence of a few per thousand (for chemicals with high interindividual variability) (Hattis *et al.*, 1999b).

Other authors have used physiologically based pharmacokinetic (PBPK) modeling, sometimes in combination with Monte Carlo analysis, to evaluate the composite effect of variability in a number of physiological parameters. Dankovic and Bailer (1993) used a PBPK model for methylene chloride to evaluate how exercise and variability in metabolism by glutathione *S*-transferases (GST) affects the calculated tissue dose and therefore the cancer risk. Clewell and Andersen (1996) reviewed the use of Monte Carlo analysis with PBPK modeling to determine distributions of risks and effect levels due to parameter variability and uncertainty in PBPK models.

Genetic variability can make an important contribution to human variability, such as in the form of polymorphic genes for metabolism or repair. Although it has long been recognized that genetic polymorphism plays

[2] Abbreviations used: CYP, cytochrome P450; PBPK, physiologically based pharmacokinetic; V_{max}, maximal enzyme velocity; K_m, Michaelis–Menten constant; CSAF, chemical-specific adjustment factors; GST, glutathione *S*-transferase; COHb, carboxyhemoglobin; DPX, DNA–protein cross-links; AChE, acetylcholinesterase; DFP, diisopropylfluorophosphate; DCA, dichloroacetic acid; GSTZ, glutathione transferase ζ; MTBE, methyl tert-butyl ether.

an important role in driving the variability in xenobiotic metabolism, this awareness has typically not translated into the use of these data in a quantitative sense for risk assessment. Instead, CSAFs are based on ratios of a critical metric for the mean of the main group and percentiles for the whole population (Meek *et al.*, 2001; IPCS, 2001). Although few assessments have been developed to date using CSAFs, this approach acknowledges the applicability of incorporating data on key parameters, such as polymorphism data, in the context of a PBPK model to estimate population variability in the dose metric of interest.

The pharmaceutical field has recognized for years the importance of genetic polymorphism of genes that modulate drug kinetics, resulting in the growing field of pharmacogenetics. In the environmental area, a contribution of genetic variability was recognized as early as 1985 (Calabrese, 1985), but researchers have only recently begun to investigate the quantitative effect of genetic variability on tissue dose for individual chemicals. Renwick and Lazarus (1998) examined classical pharmacokinetic parameters (e.g., clearance or area under the curve) for genetically different populations exposed to a number of pharmaceuticals and calculated the fraction of the exposed population that would not be covered by a 3.16-fold factor for toxicokinetic variability. One approach used by several groups (e.g., Heijmans *et al.*, 2000; Katoh *et al.*, 2000; London *et al.*, 2000) has been to conduct epidemiology studies evaluating the association between the presence of polymorphisms and increased risk of an adverse outcome, such as cardiovascular disease or cancer associated with a specific chemical exposure. Another approach is to evaluate how genetic polymorphisms affect the tissue dose of the toxic agent and incorporate that determination into the traditional risk assessment paradigm (Dankovic and Bailer, 1993; El-Masri *et al.*, 1999).

In this paper, we use a case study approach to identify critical issues and data needed for the quantitative use of data on polymorphisms in metabolic enzymes in risk assessment. This effort outlines the extent to which existing data can lead to informative application of genetic polymorphism for quantitative risk assessments by identifying areas in which simplifying assumptions are likely to be needed and by identifying minimum data requirements that are likely to be required. An additional purpose of this work is to identify chemicals amenable to a more quantitative analysis. Currently ongoing work, to be described in a follow-up paper, will use data from these case studies, together with appropriate PBPK models, to evaluate how the polymorphisms affect predicted tissue dose, and the implications for UF_H and for cancer risk assessment. In particular, the follow-up work will explore the quantitative relationship between the enzyme variability observed *in vitro* and the resulting variability in tissue dose.

It is also important to note what this paper does not address. Consideration of the effects of polymorphisms on toxicodynamics (defined for these purposes as the aspects that affect the body's response to the chemical) is beyond the scope of this paper. The impact of genetic polymorphisms on toxicodynamics may be more complex than the effects of polymorphisms on toxicokinetics.

METHODS

Potential candidate compounds were identified through a multiple-step screening process. In the first step, recent reviews (Puga *et al.*, 1997; Daly *et al.*, 1998; Eaton and Bammler, 1999; Ingelman-Sundberg *et al.*, 1999; Omiecinski *et al.*, 1999; Tanaka *et al.*, 2000; Wormhoudt *et al.*, 1999) were used to identify a list of metabolizing genes with demonstrated polymorphisms affecting activity. Data from these review articles and studies identified through supplemental literature searches in Medline and Toxline were used to compile an initial list of 17 candidate toxicologically significant chemicals that are substrates for polymorphic enzymes (Table 1).

This initial list of candidate compounds was further culled, based on the strength of the database for each substance, as evaluated by applying the following four criteria. First, the metabolic pathway had to be well-characterized, including identification of the isozyme involved in all major steps. For example, it was not

TABLE 1
Data Availability for Candidate Chemicals

Chemical	Key enzymes identified	Allelic frequency data for key enzyme	Phenotype data	PBPK model
Analine	Yes	Partial[a]	Partial	No
Arsenic	Yes	No	N/A	Yes
Atrazine	Yes	Partial	Yes	Animal only
Benzidine	Yes	Yes	Yes	No
1,3-Butadiene	Yes	Partial	Partial	Yes
Chlorpyrifos	Yes	Yes	Yes	No
Diazinon	Yes	Yes	Yes	No
Dichloroacetic acid	Yes	Yes	Yes	Yes
Methoxychlor	Yes	Partial	Partial	No
MTBE	Yes	Partial	Partial	Yes
Methyl chloride	Yes	Yes	Yes	Animal only
Methylene chloride	Yes	Yes	Yes	Yes
Parathion	Yes	Yes	Yes	Yes
Phenol	Yes	Partial	Partial	Partial
Styrene	Yes	Partial	Partial	Yes
Trichloroethylene	Yes	Yes	Yes	Yes
Warfarin	Yes	Yes	Yes	Partial

[a] Partial indicates that some, but not all, of the key information is available.

sufficient to know that a cytochrome P450 (CYP) catalyzed a specific step; identification of the enzyme as CYP3A4 or CYP2C9, for example, was necessary. Second, allelic frequency data had to be available for all enzymes playing a major role in the metabolism of the compound. Although the initial selection of the chemical was based on a polymorphic enzyme being involved in the chemical's metabolism, this second criterion required that other enzymes involved in the chemical's metabolism not have significant uncharacterized polymorphism. Third, at least some phenotype data (i.e., kinetic parameters such as the V_{max} and K_m) had to be known for the proteins encoded by each major variant allele. At this step, we did not require that these kinetic parameters be known for the chemical of interest, since that was part of the literature review in the case study. Fourth, an existing PBPK model had to be available, or an existing model for a related compound had to be readily adaptable. The list was further culled by eliminating chemicals that are primarily metabolized by CYP2E1, since there is considerable variability in the activity of this enzyme that is not due to polymorphism, and focusing on polymorphism data would ignore a major source of variability. In addition, an attempt was made to avoid chemicals with very complex metabolic pathways involving a number of polymorphic enzymes, although we were unable to completely avoid chemicals metabolized by multiple enzymes. Three of the candidate chemicals (chlorpyrifos, diazinon, and parathion) had similar mechanisms of toxicity. Of these, parathion was chosen, since it is the chemical with the best-characterized metabolism. Based on this screening process, we chose four diverse compounds for this study, methylene chloride (dichloromethane), warfarin, parathion, and dichloroacetic acid. As described in the rest of this paper, the literature on the toxicity and toxicokinetics was reviewed in detail for each chemical, in order to determine the feasibility of conducting a more quantitative analysis using a PBPK model to determine variability in tissue dose.

Overall genotype frequencies were calculated from allele or genotype frequency data provided in the references cited within the respective table. Where allele frequency data were provided, the corresponding genotype frequencies were calculated assuming Hardy–Weinberg equilibrium, i.e., using the equation $1 = (\text{frequency of allele } 1 + \text{frequency of allele } 2 \ldots + \text{frequency of allele } n)^2$. The U.S. average genotype frequency was calculated as the sum of the genotype frequency for each ethnic group multiplied by the percentage of the U.S. population represented by that ethnic group. The percentage of the U.S. population represented by ethnic groups was used as reported in El-Masri *et al.* (1999): Caucasians 72.5%, African Americans 12.2%, Hispanics 11.4%, and Asian Americans 3.9%. If no data were available for a particular group, then the contribution of that group was considered to be equal to the weighted average of the groups for which data were available.

RESULTS

Methylene Chloride (Dichloromethane)

Methylene chloride is used extensively as an industrial solvent and paint stripper. The liver is the primary target of chronic oral and inhalation exposure. Neurotoxicity is the primary effect following acute high-level exposure, although liver toxicity is also observed (reviewed in ATSDR, 1999; IARC, 1999). The production of carboxyhemoglobin (COHb) from methylene chloride metabolism can also result in toxicity, particularly acute effects. Methylene chloride has not been shown to cause tumors in humans, but NTP (1986) concluded that there was "clear evidence" for carcinogenicity in male and female mice, based on increases in alveolar/bronchiolar adenomas and carcinomas and in hepatocellular adenomas and carcinomas. There was also "clear evidence" of carcinogenicity in female rats and "some evidence" for carcinogenicity in male rats, based on increases in benign neoplasms of the mammary gland in both sexes.

As shown in Fig. 1, methylene chloride is metabolized via two major pathways (Gargas *et al.*, 1986). A high-affinity, low-capacity pathway is mediated by CYP2E1, producing carbon monoxide, which forms COHb in the blood. A lower affinity, higher capacity pathway occurs in the cytosol via GST, producing formaldehyde and carbon dioxide. Pharmacokinetic modeling studies have found that tumorigenicity correlates with production of metabolites in the lung and liver via the GST pathway and that the production of metabolites via the CYP pathway does not affect tumorigenicity (Reitz, 1990; Andersen and Krishnan, 1994; Casanova *et al.*, 1996). The lung carcinogenicity of methylene chloride in mice, but not in rats, has been attributed to the greater degree of metabolism occurring via the GST pathway in mice.

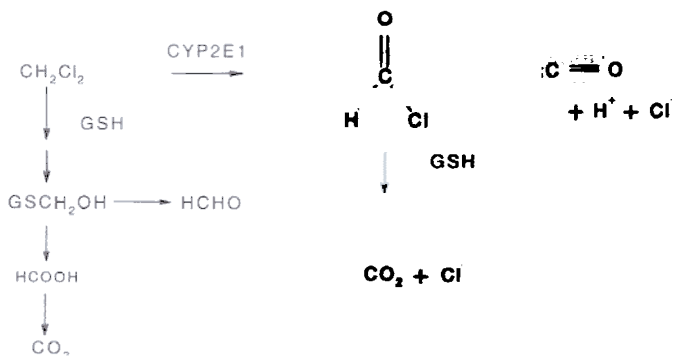

FIG. 1. Summary of methylene chloride metabolism. Adapted from Gargas *et al.* (1986).

TABLE 2
Population Distributions of GSTT Genotypes[a]

Population	Genotype frequency		
	+/+	+/−	−/−
Caucasian	0.31	0.49	0.19
African American	0.28	0.50	0.22
Hispanic	0.47	0.43	0.10
Asian American	0.05	0.33	0.62
U.S. average[b]	0.32	0.48	0.20

[a] Adapted from data presented in El-Masri *et al.* (1999) and Nelson *et al.* (1995) and assuming Hardy–Weinberg equilibrium, where "+" and "−" refer to the wild-type and null alleles, respectively.
[b] U.S. average calculated as for Table 1.

The GST isozyme that metabolizes methylene chloride has been identified as GSTT1-1 (Meyer *et al.*, 1991); other human liver GSTs appear to be unable to substitute for GSTT1-1 if this isozyme is absent (Bogaards *et al.*, 1993). GST is also involved in a secondary reaction of the first pathway (see Fig. 1), resulting in the production of carbon dioxide. There are species-specific differences in lung GSTT1-1 activity, with significant levels seen in the rat, but low expression in the human lung, where activity is approximately 1 order of magnitude lower than that in the human liver (Mainwaring *et al.*, 1996; Sherratt *et al.*, 1997).

A polymorphism in GSTT1 has been well-characterized, and the population distribution of genotypes is shown in Table 2. "Nonconjugators" lack the GSTT1-1 enzyme (Their *et al.*, 1991; Bogaards *et al.*, 1993; Hallier *et al.*, 1994), and the incidence of the polymorphism has been characterized in a number of different ethnic groups (Warholm *et al.*, 1994; Nelson *et al.*, 1995; Bruhn *et al.*, 1998) as shown in Table 2. Heterozygotes, who have one positive allele and one null allele ("low conjugators"), have half the activity of the homozygous "high conjugators" (Wiebel *et al.*, 1999), indicating a significant gene-dosage effect.

Methylene chloride forms an interesting case study because an extensive amount of toxicokinetic data are available, and these data have been used to develop a number of PBPK models of increasing sophistication (e.g., Andersen *et al.*, 1987; Reitz *et al.*, 1988, 1989, 1997; Andersen and Krishnan, 1994; Dankovic and Bailer, 1994; Casanova *et al.*, 1996). Even the earliest model (Andersen *et al.*, 1987) predicted both parent chemical levels and production of metabolites by both pathways. Several authors have used PBPK models to evaluate the effect of the GSTT1 null polymorphism on tissue dose, and on cancer risk, either as point estimates (Dankovic and Bailer, 1994) or as population distributions (El-Masri *et al.*, 1999; Jonsson and Johanson, 2001a).

El-Masri *et al.* (1999) used the model of Casanova *et al.* (1996) to estimate the amount of DNA–protein cross-links (DPX) formed from exposure to methylene chloride. The cancer potency of methylene chloride, using DPX as a dosimeter, was calculated based on the NTP (1996) mouse bioassay. Monte Carlo modeling was used to calculate the distribution of human risk, taking into account the distribution of GSTT1 null in different ethnic groups in the United States, the ethnic distribution of the U.S. population, and variability in a number of physiological parameters. Variability in enzyme activity was included only as the presence or absence of the GSTT1 activity; reduced activity of heterozygotes and variability in activity among those with the GSTT1 activity were not taken into account. Including the data on the polymorphism resulted in a biphasic risk distribution, with a peak at 0 risk (for the GSTT1 null group) and a second, broad peak for the GSTT1 positives, representing the variability in other physiological parameters. Furthermore, at exposure levels of 1–1000 ppm, including the GSTT1 polymorphism resulted in average and median population risk estimates 23–30% lower than when the polymorphism was not included.

Jonsson and Johanson (2001a) enhanced the approach of El-Masri *et al.* (1999) in a number of ways and applied their model to a Swedish population. The PBPK model was enhanced and variability in the metabolic parameters was determined by collecting data on 27 lean and obese males (Jonsson *et al.*, 2001; Jonsson and Johanson, 2001b). A Bayesian technique, Markov-chain Monte Carlo simulation, was used to simultaneously fit a population PBPK model to all of the toxicokinetic data. In addition to the GSTT1 null and high-activity groups, the intermediate activity of heterozygotes was taken into account. Variability in the protective CYP2E1 pathway was also included. Monte Carlo simulations were used to predict the distribution of cancer risks. This study found that, for the Swedish population, inclusion of the polymorphism decreased the mean population risk by 30% or more.

The work of El-Masri *et al.* (1999) and Jonsson and Johanson (2001a) demonstrates that Monte Carlo analysis can be productively combined with PBPK modeling to evaluate the effect of human polymorphisms on risk from environmental chemical exposures. Both studies used cancer risk as the endpoint of concern. Further analysis could evaluate human variability in the production of COHb from acute exposure to methylene chloride. While the GSTT1 null allele decreases cancer risk from methylene chloride exposure, it could increase COHb production, since all methylene chloride metabolism would proceed via the CYP2E1 pathway. However, the magnitude of the increase would be small, since the CYP2E1 pathway predominates at low exposure levels. Instead, variability in CYP2E1 metabolism would be expected to dominate variability in COHb production, and this variability could also be incorporated into the modeling.

Although polymorphisms in CYP2E1 have been identified (Daly *et al.*, 1998; Wormhoudt *et al.*, 1999),

the functional significance of CYP2E1 polymorphisms is unclear (Inoue *et al.*, 2000; Tanaka *et al.*, 2000). Inoue *et al.* (2000) found that three CYP2E1 polymorphisms had no effect within ethnic group (Japanese vs Caucasians) on CYP2E1 protein expression or activity, but significant differences in enzyme activity between Japanese and Caucasians indicated that some polymorphism (as yet unidentified) affecting CYP2E1 expression or activity differs between these two groups. More importantly, CYP2E1 expression is inducible by a variety of chemicals, including ethanol and chlorinated solvents, and individual activity of CYP2E1 is known to vary widely (Snawder and Lipscomb, 2000). Thus, variability in CYP2E1 activity may be dominated by environmental or other factors that regulate CYP2E1, rather than genetic polymorphism of CYP2E1 itself.

Thus, the results with methylene chloride demonstrate that the approach envisioned for the other chemicals is feasible, at least for chemicals with a single well-defined polymorphism. Additional modifications to the approach could extend the analysis from the carcinogenic effects of methylene chloride to its noncancer effects.

Warfarin

Warfarin is a coumarin derivative used clinically as an anticoagulant; its anticoagulant properties also form the basis for its application as a rodenticide. The mechanism of action is well understood and involves interference with vitamin K metabolism (a necessary cofactor for the synthesis of clotting factors) (reviewed in Sutcliffe *et al.*, 1987; Haustein, 1999; Redman, 2001). Warfarin's human toxicity is also related to its anticoagulant activity. The U.S. EPA's warfarin RfD of 0.3 μg/kg-day is based on this endpoint, using a UF of 10 to account for human variability (U.S. EPA, 2001). In addition to hematological effects, warfarin is also a documented human teratogen (Hall *et al.*, 1980; Abbott *et al.*, 1977; Whitfield, 1980). Warfarin can exist as the (*R*) and (*S*) enantiomers (Fig. 2). Although warfarin is administered clinically (and is used as a pesticide) as the racemic mixture, approximately 75% of its activity is attributable to the (*S*) enantiomer (Choonara *et al.*, 1986). The parent compound is the active form of warfarin, and it is inactivated by metabolism.

Warfarin is metabolized in the liver. Each CYP enzyme involved in the metabolism of warfarin acts specifically to hydroxylate certain carbons, with a stereoselective preference for a specific enantiomer, as shown in Fig. 2 (Wang *et al.*, 1983; Rettie *et al.*, 1992; Kunze and Trager, 1996; Kaminsky and Zhang, 1997). More than 80% of (*S*)-warfarin metabolism is catalyzed by CYP2C9 (Black *et al.*, 1996; Rettie *et al.*, 1992), primarily through 7-hydroxlation, but also through 6-hydroxylation (Rettie *et al.*, 1992; He *et al.*, 1999). In an elegant series of experiments, Rettie *et al.* (1992) expressed 11 human CYP genes (but not CYP2C18 or CYP2C19) in human liver HepG2 cells, in order to identify the specific enzymes involved in warfarin metabolism and to identify the products of each enzyme. They found that CYP2C9, CYP1A2, and CYP3A4 all act on (*S*)-warfarin, but that the CYP2C9 accounts for the majority of the *in vitro* activity. CYP2C9 generated the (*S*)-7-hydroxy and (*S*)-6-hydroxy metabolites in a ratio of approximately 3.5:1. The K_m values for 7-hydroxylation of (*S*)-warfarin in HepG2 transfected with human CYP2C9 and in human liver microsomes were very similar, as were the K_m values for 6-hydroxylation of (*S*)-warfarin and the ratio of the V_{max} for (*S*)-7-hydroxylation and (*S*)-6-hydroxylation. Together, these data suggest that CYP2C9 is the principal enzyme involved in metabolizing (*S*)-warfarin *in vivo*.

The metabolism of (*R*)-warfarin is more complex and not quite as well-characterized. (*R*)-Warfarin is metabolized by CYP1A2, 2C18, 2C19, and 3A4, with CYP2C19 identified as a high-affinity 8-hydroxylase (Wienkers *et al.*, 1996). CYP3A4 has a similar affinity for (*R*)-warfarin, but acts as a 10-hydroxylase (Kunze and Trager, 1996; Kaminsky and Zhang, 1997); the affinity of CYP1A1 and 1A2 for (*R*)-warfarin is approximately

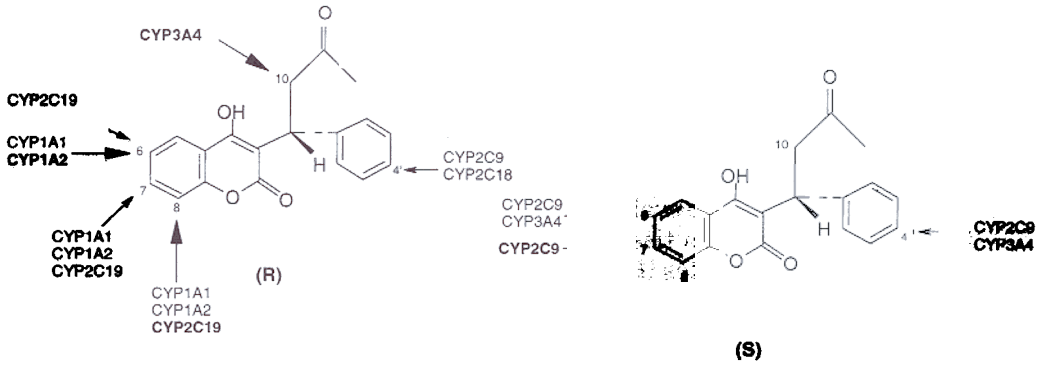

FIG. 2. Enzymes involved in warfarin metabolism and site of oxidation. Adapted from data in Rettie *et al.* (1992); Kaminsky and Zhang 1997); Kunze *et al.* (1996); Sullivan *et al.* (1996); and Wienkers *et al.* (1996). Metabolism occurs primarily via the enzymes shown in bold.

TABLE 3
Kinetic Data for (*S*)-Warfarin of CYP2C9 Alleles Using 7-Hydroxylation as the Prototype and Selected Data for (*R*)-Warfarin, Using 4′-Hydroxylation

P450 form	Kinetic parameter			
	K_m (μM)	V_{max} (pmol/min/nmol P450)	V_{max}/K_m (pmol/min/nmol P450/μM)	V_{max}/K_m relative to wt in same study (%)
		(*S*)-Warfarin		
CYP2C9*1 (wt)	6.0 ± 0.43[a]	212 ± 7.4	35	100
	2.6 ± 0.3[b]	280 ± 1	108	100
	11.6 ± 0.9[c]	133.3 ± 6.1	11.5	100
	4.1 ± 0.6[d]	421 ± 21	103	100
	3.4 ± 0.0[e]	421 ± 3	124	100
	4.0[f]	0.35 (turnover No.)	N/A	N/A
	4.0[g]	0.097 (turnover No.)	N/A	N/A
CYP2C9*2 (R144C)	12.5 ± 0.7[c]	165.6 ± 5.2	13.2	115
	1.7 ± 0.3[d]	27 ± 1.2	15.9	15.4
	2.1 ± 0.2[e]	47 ± 1.1	22.4	18.1
	6 ± 1[h]	145 ± 4	24.2	N/A
	2.7[f]	0.57 (turnover No.)	N/A	N/A
	3.8[g]	0.060 (turnover No.)	N/A	N/A
CYP2C9*3 (I359L)	30 ± 4.3[a]	41 ± 3.9	1.3	3.7
	10.4 ± 1.7[b]	67 ± 15	6.4	5.9
	92.3 ± 5.5[c]	180.5 ± 8.0	2.0	17.4
CYP2C9*1/CYP2C9*3 (50/50)	6.6 ± 0.4[b]	246 ± 58	37	34.2
		(*R*)-Warfarin		
CYP2C9*1 (wt)	20.2 ± 1.0[c]	212.8 ± 203.2	10.5	100
CYP2C9*2 (R144C)	3.4 ± 0.5[c]	82.6 ± 37.9	24.3	231
CYP2C9*3 (I359L)	10.6 ± 0.6[c]	384 ± 311	36.3	346

Note. N/A, not applicable; wt, wild-type.
[a] Haining *et al.* (1996), baculovirus/insect cell system, purified enzyme.
[b] Takahashi *et al.* (1998b), yeast expression, microsomes.
[c] Sullivan-Klose *et al.* (1996), yeast expression, microsomes.
[d] Rettie *et al.* (1994), HepG2 cells, cell lysate.
[e] Rettie *et al.* (1994), HepG2 cells, particulate preparation.
[f] Crespi and Miller (1997), baculovirus/insect cell microsomes.
[g] Crespi and Miller (1997), lymphoblast microsomes.
[h] Rettie *et al.* (1999), expressed in insect cells, purified enzyme.

fivefold lower (Zhang *et al.*, 1995). (*R*)-Warfarin also binds to CYP2C9 with an affinity similar to that of (*S*)-warfarin (Kunze *et al.*, 1991), but it is poorly metabolized by this enzyme, with a K_m more than 3 orders of magnitude higher than the K_m for (*S*)-warfarin (Sullivan-Klose *et al.*, 1996). The resulting K_i for the inhibition of (*S*)-warfarin metabolism by (*R*)-warfarin, as measured in this system was 6.0–6.9 μM (Kunze *et al.*, 1991). Downstream metabolism of warfarin includes reductive metabolism of the acetonyl side chain, glucuronidation, and sulfation (reviewed by Kaminsky and Zhang, 1997; Redman, 2001), but the rate-limiting step is inactivation of warfarin by the CYPs.

Polymorphisms have been identified for several of the enzymes in warfarin metabolism, and the clinical relevance of the polymorphisms has been investigated (Aithal *et al.*, 1999; Furuya *et al.*, 1995; Steward *et al.*, 1997; Takahashi *et al.*, 1998a,b; Taube *et al.*, 2000). These studies have shown that the polymorphisms discussed below have clinical significance in identifying safe and efficacious doses of warfarin. Table 3 presents the kinetic differences in the metabolism of (*S*)-warfarin and (*R*)-warfarin by the three human CYP2C9 alleles, as characterized from cDNA expression systems. In order to characterize the warfarin kinetics of the individual isozymes, the cloned human genes have been expressed in a baculovirus/insect cell system (Haining *et al.*, 1996; Rettie *et al.*, 1999) or in recombinant yeast microsomes (Sullivan-Klose *et al.*, 1996). In the study that identified the greatest effect of the variant protein (Haining *et al.*, 1996), the K_m for the CYP2C9*3 variant is about five times the wild-type value (lower affinity), and the V_{max} is about 5-fold lower, resulting in a 26- to 27-fold reduction in the V_{max}/K_m ratio, a measure of the catalytic efficiency (also termed the substrate-limited intrinsic clearance) of that allele.

Table 4 presents allelic frequencies for CYP2C9. The two variant alleles are CYP2C9*2, in which a cysteine

TABLE 4
Population Distributions of CYP2C9 Genotypes[a]

Population	Genotype frequency[b]					
	*1/*1	*1/*2	*1/*3	*2/*2	*2/*3	*3/*3
Caucasian	0.74	0.14	0.10	0.01	0.01	
African American	0.97	0.02	0.01			
Hispanic	ND	ND	ND	ND	ND	ND
Asian American	0.96		0.05			
U.S. average[d]	0.78	0.12	0.09	0.01	0.01	

Note. ND, no data available.

[a] Data adapted from allelic frequency data reported in Wormhoudt *et al.* (1999). Allele *1 is the wild-type encoding arginine at position 144; *2 encodes cysteine at position 144; and *3 encodes leucine instead of isoleucine at position 359.

[b] Calculated from allelic frequencies assuming Hardy–Weinberg equilibrium.

[c] Frequency less than 0.01.

[d] U.S. average genotype frequency was calculated as the sum of the individual products of the genotype for each ethnic group and the percentage of that ethnic group in the U.S. population. Contributions of each group to the total U.S. population were done as reported in El Masri *et al.* (1999).

replaces an arginine at position 144, and CYP2C9*3, in which a leucine replaces the isoleucine residue at position 359. There is general agreement in the literature that the CYP2C9*3 allele is associated with the poor metabolizer phenotype (Aithal *et al.*, 1999; Steward *et al.*, 1997; Takahashi *et al.*, 1998a,b; Taube *et al.*, 2000), and these *in vivo* data are supported by *in vitro* enzyme kinetic data (Haining *et al.*, 1996; Sullivan-Klose *et al.*, 1996).

Although the data for the CYP2C9*2 allele are less clear, this allele appears to result in a smaller decrease in warfarin metabolism. Furuya *et al.* (1995) found that patients with one allele of the CYP2C9*2 variant tended to require a lower maintenance dose of warfarin than patients with two wild-type alleles, but the difference was relatively small and there was considerable overlap in the distribution of required doses. Taube *et al.* (2000) found that patients heterozygous for CYP2C9*2 tended to require a lower maintenance dose of warfarin, and the few patients homozygous for CYP2C9*3 required even lower doses. The *in vitro* data are mixed. Using cloned genes in a HepG2 expression system, Rettie *et al.* (1994) found that both the K_m and the V_{max} were decreased compared to wild type, resulting in a 5.5-fold decrease in the catalytic efficiency (Table 3). A later study by the same group (Rettie *et al.*, 1999) found no effect on K_m and a marginal decrease in V_{max} for the purified CYP2C9*2 enzyme expressed in insect cells, in comparison with the results of Haining *et al.* (1996) for the wild-type allele in the same expression system. Using a yeast expression system, Kaminsky *et al.* (1993) found that the rate of metabolite formation for the variant was approximately twice that of the wild type, but the variant did not affect regioselectivity or stereoselectivity. Together, the *in vivo* and *in vitro* data indicate that clearance of (*S*)-warfarin would be markedly slower in people with the CYP2C9*3 allele and that clearance in people with the CYP2C9*2 allele would be slightly slower, if at all affected at environmental exposure levels. A minimal effect of the CYP2C9*2 allele makes biological sense, since the affected amino acid (number 144) is not in any known substrate recognition site, while residue 359 (affected in the CYP2C9*3 variant) is located close to the proposed substrate recognition site 5 (Gotoh, 1992). Crespi and Miller (1997) investigated the differences between the wild-type and CYP2C9*2 variant in greater detail by comparing kinetics of cDNA-expressed microsomes in a baculovirus/insect cell system and in human lymphoblasts (Table 3). They found that the K_m of the variant was similar to that of the wild type, but slightly lower, and that the V_{max} of the wild type was higher. They also found that the ratio of activity between the wild type and variant varied with the expression system and that the activity of the wild type was strongly dependent on the amount of NADPH-cytochrome P450 oxidoreductase present, while the activity of the CYP2C9*2 allele was essentially unaffected by the amount of oxidoreductase.

Although functional polymorphisms have been identified for CYP2C19 (e.g., Roh *et al.*, 1996), the limited available data indicate that these polymorphisms are not clinically significant for warfarin metabolism. Takahashi *et al.* (1998a) determined the CYP2C19 genotype (wild type or containing one or both variant alleles) for 76 Japanese patients receiving warfarin and measured the unbound oral warfarin clearance. No significant difference in (*R*)-warfarin clearance was found between the 56 patients with at least one wild-type allele and the 10 patients with only variant alleles. The study authors concluded that the CYP2C19 polymorphism plays an insignificant role in variability in (*R*)-warfarin metabolism *in vivo*. This conclusion is limited by the small sample size. In addition, sensitivity to polymorphic differences is reduced, since the authors pooled heterozygotes containing one wild-type and one variant allele together with the wild-type homozygotes. In support of this idea, data for the CYP2C9*3 allele show that clearance in the heterozygote is intermediate between that of the wild type and the homozygous variant (Takahashi *et al.*, 1998b). Another reason that the CYP2C19 polymorphism is likely to have a relatively small impact on (*R*)-warfarin metabolism is that the CYP 1A1 and CYP1A2 isozymes also act at the same sites; polymorphisms in CYP1A1 have been reported to have a minimal effect on (*R*)-warfarin metabolism (reviewed in Redman, 2001).

Although a fully developed PBPK model for warfarin that includes metabolism has not been published, it appears that the data are available to develop such a model. Black *et al.* (1996) evaluated the toxicokinetics of

warfarin in six human subjects and calculated the half-life, clearance, AUC, and volume of distribution for (*R*)- and (*S*)-warfarin. Luecke *et al.* (1994) developed a PBPK model for human pregnancy that took into account how body parameters change with time and used warfarin as a test case. Warfarin metabolism in the liver was modeled as a single compartment, using rat metabolism parameters from Luecke *et al.* (1980). The human model has not been validated, and it appears that no allometric scaling was done for the metabolic parameters. A rat PBPK model for intravenous dosing has been developed, validated, and used to model the decrease of warfarin elimination by bromosulfophthalein (Luecke and Wosilait, 1979). Mungall *et al.* (1985) also developed an empirical toxicokinetic model to describe warfarin metabolism, and Chan *et al.* (1994) described an empirical toxicokinetic/toxicodynamic model for warfarin. However, these models were not based on physiological parameters and so cannot be adapted for the current project. Kunze and Trager (1996) determined the kinetic parameters (V_{max} and K_m) for the formation in human liver microsomes of (*S*)-7-hydroxywarfarin from (*S*)-warfarin and for the formation of (*R*)-6, (*R*)-7, (*R*)-8, and (*R*)-10-hydroxywarfarin from (*R*)-warfarin.

An interesting aspect of warfarin metabolism is the difference in activity of the (*R*) and (*S*) enantiomers and their differences in metabolism. This means that exposure to a racemic mixture of warfarin can be considered quantitatively as exposure to two different chemicals, which are metabolized by different enzymes and which have the same effect, but with potency differing by threefold. The inhibition of the metabolism of the (*S*) enantiomer by (*R*)-warfarin will also need to be addressed, since the enzyme kinetics were determined with single enantiomers, but exposure occurs to the racemic mixture. This can be done quantitatively using the K_i calculated by Kunze *et al.* (1991).

A second aspect to consider is that the 2C9 enzyme has three major polymorphic forms. Theoretically, this increases the possible allelic combinations from three, for a gene with a wild-type and a single variant allele, to six. Although this makes the resulting mathematical calculations of overall variability more complex, the principle of the evaluation is unaffected. The presence of multiple alleles with differing activities is also likely to drive the population distribution toward being one broad distribution, rather than a bimodal distribution with clearly separated peaks. For this case study, it may be possible to reduce the analysis to an evaluation of the CYP2C9*1 and CYP2C9*3 alleles. The CYP2C9*2 allele has a K_m in the same range as that of the wild type, although the V_{max} is somewhat reduced (Table 3). This suggests that at environmental exposure levels, the CYP2C9*2 allele will behave very similarly to the wild-type allele. Quantitatively predicting the metabolic parameters for different genotypes is also likely subject to some imprecision in the heterozygotes. Takahashi *et al.* (1998b) measured the enzyme kinetics of purified wild-type and variant CYP2C9*3, as well as a 1:1 mixture of the two proteins, and found that the K_m of the mixture was the average of the two pure enzymes, but the V_{max} of the mixture was close to that of the wild type.

A third aspect of the analysis is that both CYP2C19 and CYP3A4 play significant roles in the metabolism of (*R*)-warfarin. Since warfarin metabolism is an inactivating step, metabolism by either enzyme is functionally equivalent, suggesting that one enzyme can compensate for low levels of the other. This is consistent with the finding of Takahashi *et al.* (1998a) that the CYP2C19 variants did not affect the average unbound oral clearance. In this case, functional polymorphisms in CYP3A4 have not been sufficiently well-characterized to use in a quantitative manner (see the parathion case study, below) and metabolism by CYP3A4 would be expected to compensate for low activity of CYP2C19. Overall, the effect of multiple enzymes catalyzing the same reaction is to dilute the potential impact of any single gene polymorphism.

Finally, warfarin kinetics are altered by a number of drugs that induce enzymes that metabolize warfarin or that are direct inhibitors of warfarin metabolism. For example, CYP2C9 is inducible by rifampicin, ethanol, and phenobarbital, while a number of drugs, including fluoxetine, phenytoin, and tolbutamide, inhibit (*S*)-warfarin metabolism (reviewed in Miners and Birkett, 1998). The inhibition kinetics for some of these chemicals have been characterized *in vitro* (Kunze and Trager, 1996) and *in vivo* (Choonara *et al.*, 1986; Chan *et al.*, 1994; Black *et al.*, 1996). Competitive inhibition would not affect the V_{max} for a chemical, but would affect the apparent K_m. This means that long-term pharmaceutical exposure (which would generally be at much higher daily doses than environmental exposure to chemicals) could have a substantial effect on the kinetics for metabolism of environmental chemicals. While this issue is not directly related to the effects of polymorphisms, enzyme induction and inhibition both contribute to overall human variability in enzyme activity and would need to be considered in a complete description of human variability.

In summary, warfarin would be a useful case study to characterize the contribution of genetic polymorphism to variability in tissue dose. The mechanism of action is well understood. Phenotype data and allelic frequencies are available for alleles of the key metabolic enzyme, CYP2C9, although other enzymes playing less significant roles have not been as well-characterized. It also appears that a PBPK model can be developed to integrate the polymorphism data on warfarin with other data on human variability. Several issues will need to be addressed (either quantitatively or by considering alternative assumptions) in developing a more

quantitative approach using a PBPK model. These include consideration of the different activities of the (*R*) and (*S*) enantiomers, taking into account the three major allelic forms of CYP2C9, accounting for the contributions of CYP2C9 and CYP3A4 in the metabolism of (*R*)-warfarin, and consideration of the effect on metabolism of other coexposures that can alter warfarin kinetics.

Parathion

Parathion is a prototypical member of the class of organophosphate pesticides. Parathion and other prominent members of this class, such as chlorpyrifos and diazinon, exert their toxicity primarily through the inhibition of acetylcholinesterase (AChE) in the central and peripheral nervous system. Chronic syndromes associated with acute high-dose exposures to organophosphates might also be due to inhibition of the neuronal carboxylesterase, neuropathic target esterase. As shown in Fig. 3, parathion is oxidized by CYP to phosphooxythiran, a reactive intermediate. This reactive intermediate can spontaneously degrade, either to nontoxic compounds (*p*-nitrophenol and either diethyl phosphate or diethyl thiophosphate) via dearylation or to paraoxon via oxidative desulfuration (Mutch *et al.*, 1999). Essentially all the AChE inhibition seen following parathion exposure is ascribed to its metabolite paraoxon (reviewed in Ecobichon, 1996). Thus, the balance of these alternative end products of CYP-mediated oxidation reactions can be an important determinant of parathion toxicity.

The liver is an important site for the oxidative metabolism of parathion. Mutch *et al.* (1999) determined paraoxon formation rates in incubations with microsomes prepared from human liver microsomes from three individuals. Kinetic parameters for paraoxon formation for the individual livers were V_{max} values of 143.1, 167.8, and 313.5 pmol/min/mg protein and corresponding K_m values of 13.7, 9.0, and 15.9 μM. Kinetic parameters for *p*-nitrophenol formation were not calculated, but following treatment with 20 and 200 μM parathion, the median ratios of paraoxon to *p*-nitrophenol formation were 0.143 and 0.247, respectively. Butler and Murray (1997) reported that median rates of paraoxon and *p*-nitrophenol formation were 3.03 and 2.26 nmol/min/mg, respectively, in human liver microsomes incubated with 250 μM parathion. Thus, the relative importance of oxidative desulfuration and dearylation reactions remains uncertain.

The identity of CYP isoforms contributing to oxidative metabolism of parathion has been investigated in several recent studies (Butler and Murray, 1997; Mutch *et al.*, 1999; Sams *et al.*, 2000). Experiments using human liver microsomes cotreated with CYP inhibitors, and parathion metabolism experiments in human cell lines expressing specific CYP isoforms, demonstrate that CYP3A4 activity accounts for the greatest extent of parathion metabolism (Butler and Murray, 1997; Mutch *et al.*, 1999). Other CYP isoforms thought to play a lesser role include CYP1A, CYP2B6, CYP2D6, and CYP2C. Sams *et al.* (2000) measured the ability of CYP inhibitors to block AChE inhibition, as a measure of paraoxon formation, and reported that both CYP2D6 and CYP3A4 play a major role in the bioactivation of parathion.

CYP isoforms capable of metabolizing parathion may not have identical tendencies to result in desulfuration versus dearylation of parathion. For example, Butler and Murray (1997) measured the proportion of

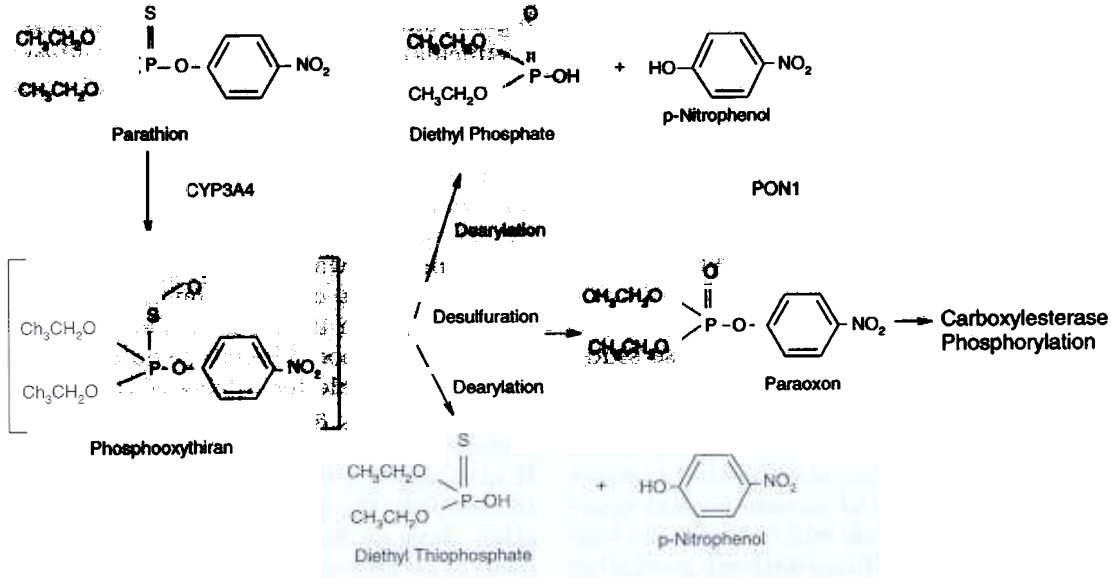

FIG. 3. Parathion metabolism. Adapted from Mutch *et al.* (1999)

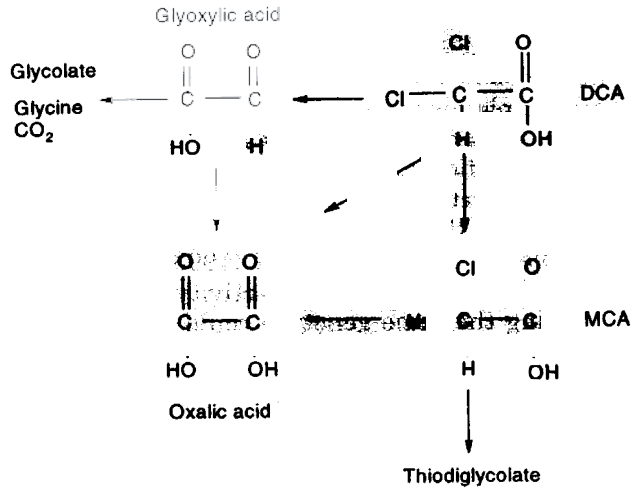

FIG. 4. Summary of dichloroacetic acid metabolism. Adapted from Bull (2000) and Lash *et al.* (2000). Reproduced with permission from *Environ. Health Perspect.*

paraoxon and *p*-nitrophenol in microsomes from human cells expressing various human CYP450 isoforms. Based on these data, CYP2D6 and 2B6 appeared to favor paraoxon formation over *p*-nitrophenol formation as a percentage of total oxidative metabolites formed, while CYP3A4 and CYP1A2 formed nearly equal amounts of each metabolite.

A high degree of variability in CYP3A4 activity has been well-documented, but only recently have data on a potential genetic basis for this variability begun to be characterized. Although evidence is building for functional polymorphism in CYP3A4, the evidence remains too preliminary to predict with confidence the degree to which polymorphism might impact parathion metabolism.

The first variant allele to be characterized (*CYP3A4*1B*) results in a single nucleotide change in the *CYP3A4* gene promoter. The functional significance of this variant allele remains an area of debate. Ball *et al.* (1999) reported no difference in CYP3A4-dependent enzymatic activities, including erythromycin demethylation or nifedipine kinetics in individuals homozygous for the wild-type and variant alleles. Westlind *et al.* (1999) reported interindividual variation of CYP3A4 activity of 31-fold in 46 human livers, but two individuals heterozygous for the variant allele did not have responses different from the median value for the whole group. von Moltke *et al.* (2000) reported low clearance values of two CYP3A4 substrates, alprazolam and trazodone, in an individual homozygous for the wild-type allele. Since low clearance could be observed even in individuals homozygous for the wild-type allele, these studies suggest that mechanisms other than *CYP3A4*1B* are responsible for variability in CYP3A4 activity.

On the other hand, Wandel *et al.* (2000) tested the functional effect of the *CYP3A4*1B* allele in African American and European American men by measuring plasma concentrations of midazolam. The clearance of midazolam following intravenous dosing was 15% lower in African Americans (4 heterozygous; 10 homozygous for variant) compared to European Americans (all wild-type homozygous). The individuals homozygous for the variant allele had 30% lower systemic midazolam clearance than subjects homozygous for the wild-type allele. However, no difference in oral clearance was observed, suggesting that variability in systemic metabolism due to the polymorphism was overshadowed by other factors affecting oral clearance (e.g., intestinal absorption and metabolism).

Recent papers have identified additional variant alleles for *CYP3A4*. Sata *et al.* (2000) identified a serine to proline change at codon 222 (referred to as *CYP3A4*2*), at a frequency of 2.7% in a Caucasian population, but absent in African or Chinese subjects. *In vitro* analysis revealed a six- to nine-fold lower intrinsic clearance (V_{max}/K_m) of nifedipine for this variant, but no kinetic differences in testosterone 6β-hydroxylase activity, demonstrating a clear substrate dependence for functional differences.

Several additional variant alleles have been reported at low frequency in Chinese populations. Sata *et al.* (2000) reported a methionine to threonine change at codon 445 (*CYP3A4*3*) in a single Chinese subject. Hsieh *et al.* (2001) identified three variant alleles in a group of 102 Chinese subjects: *CYP3A4*4* (Ile118Val), *CYP3A4*5* (Pro218Arg), and *CYP3A4*6* (A17776 insertion). The CYP3A4 activity in these individuals was measured by 6β-hydroxycortisol/cortisol ratio and was compared to the general Chinese population. Individuals harboring variant alleles (all heterozygous) had lower metabolic ratios, providing preliminary evidence for a functional effect of these variants. Further studies will be needed to confirm the presence of these variant alleles in larger populations.

Taken together, evidence is increasing for functional polymorphisms in CYP3A4. However, the data are not sufficient for evaluation of their impact on parathion metabolism for two reasons. First, in all cases except *CYP3A4*1B*, which probably has limited if any functional significance, allele frequencies are low, and thus the population frequencies of the variants need additional study. In addition, functional implications of variants are substrate-dependent, limiting the appropriateness of generalizing to untested substrates such as parathion.

Paraoxon can be detoxified through competing mechanisms, enzymatic hydrolysis by paraoxonase (PON1) and phosphorylation of carboxylesterases lacking hydrolysis activity. Like, the carboxylesterase, the classic AChE inhibition by paraoxon occurs via its phosphorylation by paraoxon. Early observations of bimodal and trimodal distributions of paraoxonase activity, as well as pedigree studies, suggested a genetic basis for

TABLE 5
Population Distributions of PON1 Genotypes

Population	Genotype frequency[a] Q/Q	Q/R	R/R
Caucasian[b]	0.47	0.43	0.10
African American[c]	0.35	0.48	0.17
Hispanic[d]	0.15	0.48	0.37
Asian American[e]	0.17	0.48	0.35
U.S. average[f]	0.41	0.45	0.15

[a] Genotype frequency was calculated for codon 192 variant, where Q refers to glutamine at this position and R refers to arginine. Frequencies were based on population or allele frequency data presented in the cited references and assuming Hardy–Weinberg equilibrium.
[b] Adapted from Eckerson *et al.* (1983); Mueller *et al.* (1983).
[c] Adapted from Diepgen and Geldmacher-von Mallinckrodt (1986).
[d] Adapted from Davies *et al.* (1996).
[e] Adapted from Sanghera *et al.* (1998).
[f] U.S. average calculated as for Table 1.

this variability. Adkins *et al.* (1993) and Humbert *et al.* (1993) determined that a glutamine (Q192) at position 192 of paraoxonase confers a low-activity phenotype, while arginine (R192) results in high paraoxonase activity. Heterozygotes have an intermediate activity phenotype. The distribution of PON1 genotypes in the U.S. population is shown in Table 5.

Several studies have provided quantitative estimates of human paraoxonase activity. Mueller *et al.* (1983) determined the rate of formation of *p*-nitrophenol from paraoxon in the serum of high and low paraoxonase activity individuals. They found that the V_{max} was 200 μmol/min for the low-activity phenotype and 420 μmol/min for the high-activity phenotype. The K_m values were not significantly different for the two groups (0.46 mM in the low-activity group and 0.42 mM in the high-activity group). Smolen *et al.* (1991) characterized enzymatic activities of the purified isozymes. K_m values for low-activity and high-activity variants were 0.503 and 0.265 mM, respectively. Paraoxonase turnover numbers at V_{max} concentrations were 344 and 659 min^{-1} for low- and high-activity variants, respectively. In this same paper, the activity in serum was 0.001 μmol/min/mg for low-activity individuals versus 0.005 μmol/min/mg for high-activity individuals. Davies *et al.* (1996) reported levels of paraoxonase activity in serum from individuals of varying genotype. The mean paraoxonase activity was 328 ± 79 U/L (μmol/min/L) for low-activity homozygous, 977 ± 171 U/L in heterozygous, and 1769 ± 354 U/L in high-activity homozygous individuals. In a recent paper, Li *et al.* (2000) reported kinetic parameters for purified human PON1 proteins. The K_m was 0.81 mM and the V_{max} was 0.57 μmol/min/mg for the low-activity isoform, while the K_m was 0.52 mM and the V_{max} was 3.26 μmol/min/mg for the high-activity allele. Together these data suggest that both changes in K_m and V_{max} can contribute to differences in the catalytic efficiency of PON1.

The amino acid change at position 192 does not appear to completely account for the degree of human variability in human PON1 activity. Although earlier studies had identified as much as a 40-fold variability in paraoxonase activity, remaining variability among individuals with the same low-metabolism genotype still ranged over 13-fold. Furlong *et al.* (1993) tested whether this variation in paraoxonase activity among individuals having the same genotype could be explained by differences in protein levels. Based on Western blots of serum from five homozygous low-activity individuals, the level of activity correlated well with the amount of PON1 present in the serum, providing strong evidence that both genotype and protein level will likely affect *in vivo* levels of PON1 activity. Another possible explanation for variability in PON1 activity is the possibility that uncharacterized genetic polymorphisms might also play a role. Recently two human genes, *PON2* and *PON3*, have been characterized that are structurally related to *PON1* (Mochizuki *et al.*, 1998; Primo-Parmo *et al.*, 1996). No data on the ability of these genes to metabolize organophosphates were identified.

Human sensitivity to organophosphates depends on interactions with their metabolites and multiple proteins. For example, Mutch *et al.* (1992) measured variability in several enzyme activities related to paraoxon kinetics and dynamics. PON1 activity ranged over approximately 6-fold, but effects on AChE and cholinesterase activities were less variable, on the order of 2-fold. Variability in neuropathy target esterase (a potential marker for delayed neuropathy) was 6.5-fold.

The potentially important role of enzymes other than PON1 as a determinant of paraoxon toxicity was well demonstrated by Li *et al.* (2000). *Pon1* gene knockout mice were not more sensitive to paraoxon than wild-type mice, suggesting that *in vivo*, PON1 is not a major detoxification enzyme. This finding was supported by the absence of a protective effect from injections of rabbit PON1 or human PON1 and by observations of paraoxon-treated transgenic mice overexpressing the human PON1 gene. In contrast to the results for paraoxon, PON1 was important in modulating the toxicity of the chlorpyrifos-oxon and diazoxon in mice, perhaps due to the greater catalytic efficiency of PON1 for these organophosphate metabolites than for paraoxon. This finding underscores the need for substrate-specific metabolism data when assessing the role of gene polymorphisms on target dose or effect. Similar results were not observed in rats, in which PON1 injections did protect against parathion toxicity (Costa *et al.*, 1990). Thus, there may be additional species-dependent factors that modulate the relative importance of PON1 in parathion toxicity.

The lack of an effect of PON1 status in mice suggests that other pathways play an important part in

modulating parathion toxicity. Similarly, it has been suggested that paraoxonase may play a limited role in parathion metabolism in humans at low exposure levels, due to its low catalytic efficiency toward parathion compared to activity toward other organophosphate oxons (Li *et al.*, 2000). In support of this idea, Mutch *et al.* (1999) reported that EDTA-mediated inhibition of PON1 in human liver microsomes had no effect on the formation of paraoxon or *p*-nitrophenol from parathion and only a limited effect on *p*-nitrophenol formation when microsomes were treated with saturating (1 mM) concentrations of paraoxon.

One mechanism for removal of paraoxon is through paraoxon phosphorylating carboxylesterases, rather than the phosphorylating target for paraoxon toxicity, AChE. In this way, carboxylesterases serve as a nonenzymatic sink for the paraoxon, serving to reduce the chance for interactions of paraoxon with the target enzyme. The capacity of the carboxylesterases to remove paraoxon is therefore a function of the amount of protein available. Tang and Chambers (1999) investigated the role of this pathway by measuring *p*-nitrophenol activity in rat liver homogenate or rat serum, with or without addition of esterase. At low concentrations or short incubation times, paraoxon removal via carboxylesterase phosphorylation was greater than by PON1-mediated hydrolysis, although this nonspecific activity was rapidly saturable. The importance of carboxylesterases was confirmed *in vivo*, based on the finding that brain AChE levels were significantly inhibited after pretreatment with a carboxylesterase inhibitor. The relative contribution of nonspecific carboxylesterases and enzymatic hydrolysis by paraoxonase is an important consideration in evaluating the potential role of *PON1* polymorphism on susceptibility to parathion toxicity. Sweeney and Maxwell (1999) recently developed a quantitative model to estimate the relative roles of hydrolase and carboxylesterase activities in protection against organophosphates, which might be useful for further determinations of the relative importance of these two pathways for paraoxon detoxification.

Gearhart *et al.* (1990) developed a PBPK model for the organophosphate diisopropylfluorophosphate (DFP), which was chosen as a model compound to represent the class of highly toxic organophosphate compounds, including parathion. In addition to data for DFP, the model was designed to incorporate disposition of parathion as well. Metabolic parameters derived from data in the rat were used to develop the model and included parameters to describe the metabolism of parathion to paraoxon (liver and kidney compartments), paraoxon hydrolysis by paraoxonase (blood, brain, liver, kidney, rapidly perfused tissue), and activity of esterases for nonhydrolytic binding of paraoxon (all tissue compartments except fat). Model simulations for AChE inhibiton in humans were not reported for parathion, although the authors noted that preliminary simulations resulted in general agreement with human kinetic data from the literature, after modification of some of the enzyme parameters. The model predictions generally agreed with tissue concentrations of parathion and paraoxon observed experimentally for the rat. Opportunities for further optimization of the model that were discussed included modeling the effects of prolonged exposure on AChE synthesis rates and assumptions regarding cross-species scaling of enzyme parameters. This latter issue may be of particular importance, as significant interspecies differences to parathion have been reported (Johnson and Wallace, 1987; Veronesi and Ehrich, 1993).

In summary, parathion provides another example of a compound for which integration of polymorphism data with PBPK modeling would provide insight into the relative contribution of genetic factors to overall variability. Parathion is particularly useful as an example of a chemical for which there are multiple detoxification routes. Although the toxic mechanism and metabolism of parathion are well-studied, new information on functional *CYP3A4* polymorphisms and any genetic factors affecting *PON1* expression that become available will allow even greater opportunity to apply this case study. Parathion was chosen as a well-studied member of the family of organophosphate pesticides. However, the relative contributions of organophosphate detoxification mechanisms to the disposition of related compounds, such as chlorpyrifos and diazinon, are not the same as for parathion. Therefore, it will also be of interest to expand this work to look at other members of this class of compounds.

Dichloroacetic Acid

Dichloroacetic acid (DCA) is a common drinking water disinfectant by-product, resulting in low-level exposure of a large proportion of the population (Boorman, 1999). DCA has been employed as a therapeutic agent to treat lactic acidosis, diabetes, and familial hyperlipidemia in humans for many years, based on its apparent ability to enhance the activity of pyruvate dehydrogenase; reported adverse effects are limited to mild sedation and mild peripheral neuropathy (Stacpoole *et al.*, 1998). In animals, targets for the noncancer toxicity of DCA include metabolic alterations, liver toxicity, testicular effects, neurotoxicity, and, at higher doses, developmental toxicity (Cicmanec *et al.*, 1991; Linder *et al.*, 1997; Moser *et al.*, 1999; Smith *et al.*, 1992; Epstein *et al.*, 1992). In animal studies, DCA treatment induces liver tumor formation at high doses (reviewed in IPCS, 2000). Although epidemiological data support a link between ingestion of chlorinated drinking water and risk of rectal, colon, and bladder cancer, chlorinated water is a complex mixture, and an association with DCA has not been established (Mills *et al.*, 1998; Boorman, 1999).

Studies on the toxicokinetics of DCA (Crabb *et al.*, 1981; Evans and Stacpoole, 1982; Larson and Bull, 1992; Lin *et al.*, 1993) have shown that DCA is metabolized by oxidative dechlorination to glyoxylate, which is in turn metabolized to oxalate (Fig. 4). This metabolic pathway also occurs in humans, as evidenced by the appearance of oxalic acid in the urine of DCA-treated humans. There has been no evidence of thiodiacetic acid or monochloracetic acid excretion in DCA-treated humans (Stacpoole, 1989; Stacpoole *et al.*, 1998; Stacpoole and Greene, 1992), suggesting that the reductive dechlorination pathway is not likely to be important.

Lipscomb *et al.* (1995) found that DCA metabolism results from GSH-dependent pathways, rather than oxidation by CYP. Tong *et al.* (1998a) determined that glutathione transferase ζ (GSTZ) is responsible for DCA metabolism in both humans and rodents. Based on species differences in metabolic capacity (V_{max}/K_m), GSTZ-dependent metabolism of DCA is highest in the mouse, with intermediate rates in rats, and the slowest metabolism of DCA occurring in humans.

Genetic polymorphism of *GSTZ* may engender differences in DCA susceptibility within human populations. Blackburn *et al.* (2000) reported on polymorphism of *GSTZ* in a population of 141 Caucasians. Three alleles were identified, *GSTZ*1A*, *GSTZ*1B*, and *GSTZ*1C*. Based on *in vitro* experiments with purified proteins, GSTZ1A had a 3.6-fold higher activity toward DCA than the other two allelic variants. The activities for the different allelic variants were 1610 ± 20 nmol/min/mg for GSTZ1A, 450 ± 20 nmol/min/mg for GSTZ1B, and 450 ± 30 nmol/min/mg for GSTZ1C. The K_m values were not reported. The effect of this polymorphism on *in vivo* DCA metabolism in humans has not been reported. Blackburn *et al.* (2000) calculated allelic frequencies of 0.09, 0.28, and 0.63 for *GSTZ*1A*, *GSTZ*1B*, and *GSTZ*1C*, respectively. Thus, assuming Hardy–Weinberg equilibrium, the segment of the population homozygous for the high-activity allele might be small (i.e., 0.8%). No data were identified on allelic frequencies in other ethnic groups.

Unlike many other chemicals, DCA can inhibit its own metabolism. Several studies in rodents have demonstrated that pretreatment with DCA inhibits the metabolism of subsequent doses (Gonzalez-Leon *et al.*, 1997; James *et al.*, 1997). The plasma elimination half-life is also increased in humans following repeated dosing with DCA (Curry *et al.*, 1991), suggesting that autoinhibition also occurs in humans, but the supporting *in vitro* data are mixed. Cornett *et al.* (1999) found in *in vitro* studies that DCA pretreatment markedly inhibited its own metabolism in rat cytosol, but they did not observe any inhibition in human cytosol. In contrast to this result, Tzeng *et al.* (2000) observed a dose-dependent inhibition of GSTZ1 using human liver cytosol. Species differences in DCA-induced inhibition were noted, with relative rates of DCA-induced inactivation of GSTZ reported as rat > mouse > human. Tzeng *et al.* (2000) also found differences in the rate of inactivation of the different allelic forms of GSTZ, suggesting that these differences may be as important, or more important, than the differences in catalytic efficiency. The rate constant for inactivation of GSTZ1A was 3.0 ± 0.1 min^{-1}, a rate approximately half that of the proteins encoded by the other variant alleles.

Several PBPK models have been developed for DCA or have incorporated elements of DCA metabolism as part of larger models designed to evaluate the disposition of trichloroethylene, of which DCA is a minor metabolite. Clewell *et al.* (2000) included a submodel for DCA in their human trichloroethylene model. Metabolism and excretion of DCA were considered, but autoinhibition of metabolism was not included in this model. Mouse models of DCA metabolism also exist (Abbas and Fisher, 1997; Greenberg *et al.*, 1999; Barton *et al.*, 1999), all developed as part of larger trichloroethylene models. Chen (2000) proposed a biologically based dose–response model for liver tumors induced by TCE and DCA.

Thus, DCA is a chemical to which many people are exposed and for which there is a polymorphism in the key metabolic enzyme. The implications of this polymorphism are likely to be complex and depend on whether the key toxic agent is DCA or its metabolite(s) (or both). Comparing different PBPK dose metrics for tumorigenicity and DCA tumor formation, Barton *et al.* (1999) suggested that the prevalence of DCA-induced tumors was correlated with the amount metabolized, while the tumor multiplicity appeared to correlate better with area under the curve of DCA in the liver. These results suggest that there may be different roles in the tumorigenic process for the parent compound and DCA metabolites. The toxic agent for the noncancer effects of DCA is also unknown. Cornett *et al.* (1999) suggested that DCA-mediated inhibition of GSTZ might be an important factor in the toxicity of DCA. GSTZ is a key enzyme for tyrosine metabolism, and inhibition of tyrosine metabolism may result in increased levels of reactive tyrosine metabolites that may adversely affect the liver and nerves, targets of DCA toxicity. Data from Moser *et al.* (1999) suggest that DCA-induced neurotoxicity (at low doses, evidenced by gait abnormalities and impaired righting reflex) may be due to the parent compound, rather than a DCA metabolite. Overall, these data suggest that noncancer effects of DCA at low doses may be attributed primarily to the parent compound, while DCA tumorigenicity at low doses is primarily due to metabolites, and tumorigenicity at higher doses may be enhanced by the parent. However, these data, particularly the data addressing the toxic moiety for noncancer effects, are preliminary.

An issue to consider in evaluating the implications of the GSTZ polymorphism is how to account for DCA inhibiting its own metabolism. The activity of purified

GSTZ1A is about 3.5-fold that of the other forms, and GSTZ1 is inactivated by DCA at a rate about half that of the other forms. Both the differences in activity and the differences in the rate of inactivation work in the same direction, so that people with the GSTZ1A form would have higher production of DCA metabolites than people with other forms (all other things being equal). This means that the GSTZ1A form would increase the risk of effects resulting from DCA metabolite(s), but decrease the risk of effects resulting from DCA itself. Determination of the quantitative implications of the increased activity of GSTZ1A form would require a validated PBPK model that accounts for autoinhibition from long-term exposure to DCA.

In summary, it appears that proceeding with the DCA case study is feasible. Although there are several outstanding issues, these issues can be addressed by considering the implications of multiple alternatives. For example, since it is not known whether DCA or one of its metabolites is responsible for the observed toxic effects, the implications of polymorphisms can be determined under both the scenario that the parent and that a metabolite is the toxic moiety. The overall metabolic pathway for DCA is well-characterized, and this case study provides an opportunity to study the effects of genetic polymorphism on autoinhibition as well as altered catalytic efficiency of the key enzyme. A PBPK model can be developed that describes DCA metabolism sufficiently to evaluate the implication of the GSTZ polymorphism, although developing a PBPK model that fully describes DCA metabolism might not currently be feasible, due to the complexity of the total metabolic process.

DISCUSSION

In this paper we present a summary of the toxicokinetics and genetic polymorphism data for methylene chloride, warfarin, parathion, and DCA to highlight data requirements for the meaningful application of genetic polymorphism data in human health risk assessments. We show that meaningful application of polymorphism data requires that several key issues be addressed (Table 6).

Several key points affect whether information on variability due to genetic polymorphism is likely to be informative for a given chemical. The spectrum of currently identified polymorphisms constitutes only one of the many sources of human variability. For example, the degree of variability in CYP2E1 and PON1 activities is not adequately explained by known polymorphisms. Additional variability may result from as-yet-unidentified alleles in these genes, as well as from the contribution of polymorphism in other genes that encode similar enzymes or that regulate their activity. Clearly, there are also environmental contributions to the degree of variability that is observed. Perhaps one of the best examples is the ability of coexposures to induce or inhibit these metabolizing genes, as seen with drug–drug interactions for CYP2C metabolism of warfarin (Miners and Birkett, 1998). Finally, the metabolism of a chemical at low doses may be determined by nonenzymatic factors, such as blood flow to the liver. Because flow-limited metabolism would mean that a large difference in enzyme activity would result in a minimal difference in tissue dose at low exposure levels, PBPK modeling to determine the effect on tissue dose is an important tool in evaluating the effect of enzyme polymorphisms.

The relative contribution of known genetic polymorphisms and environmental factors to the total variability in tissue dose from a given external dose is an important consideration that highlights the advantages and disadvantages of alternative approaches for using human variability data in risk assessment. A thorough evaluation of variability (and the contribution of the polymorphism to the overall variability) needs to account for variability in multiple parameters. PBPK models combined with Monte Carlo modeling provide a convenient approach for evaluating the implications of multiple sources of variability, as was done by El-Masri *et al.* (1999) and Jonsson and Johanson (2001a) for methylene chloride. Other authors have directly evaluated variability in parameters affecting tissue dose, such as variability in enzyme kinetics (Lipscomb *et al.*, 1999) or variability in physiologic parameters (Hattis *et al.*, 1999b).

The genetic approach (consideration of allelic frequencies of identified polymorphisms) may have the advantage of more easily providing a broad picture of the population distribution. The enzyme activity of different alleles can be obtained relatively easily from cloned genes, and allelic frequencies can be determined using relatively noninvasive sampling methods (i.e., from blood samples). This means that information on allelic frequencies in different ethnic groups can be used to develop an overall population distribution of allelic frequencies, without having to measure enzyme activity from a large number of human samples. By contrast, obtaining a sample of liver tissue requires invasive methods, making it more difficult to obtain a sufficiently large number of samples to be representative of the diversity in the population.

TABLE 6
Common Data Gaps Increasing Uncertainty

Extent of induction/inhibition through coexposure
Relative contribution of multiple enzyme systems
Allelic frequencies for major ethnic groups
Large numbers of low-frequency alleles
Absence of chemical-specific phenotype data
Uncertainty regarding differences in *in vitro* and *in vivo* kinetic data

On the other hand, measuring enzyme activity using cloned genes cannot take into account other factors unrelated to the polymorphism, such as variability in transcriptional regulation, enzyme synthesis, and degradation or other factors associated with the cellular milieu that regulate the amount or activity of the enzyme. In this way, measuring activity using liver biopsy tissue provides a more accurate measure of the actual variability in expression of the enzymes. For example, evaluating variability in actual enzyme activity is preferable when most of the variability is due to environmental, rather than genetic sources, such as for CYP2E1. In addition, while *in vitro* analysis can assess the catalytic efficiency of the polymorphic gene product, its importance relative to other enzymes is not easily weighed. As highlighted for warfarin (Wang *et al.*, 1983; Rettie *et al.*, 1992; Kunze and Trager, 1996; Kaminsky and Zhang, 1997) and parathion (Butler and Murray, 1997; Mutch *et al.*, 1999), there are multiple CYPs that catalyze the oxidation of these chemicals, and the overall variability in activity due to the combination of these enzymes is perhaps best estimated using the human liver samples. In the event that data from purified variant proteins are used, the choice of which of the minor pathways to include in the analysis requires a balance between model accuracy and overparameterization. As experience in application of polymorphism data in the PBPK approach increases, simplifying assumptions such as the degree to which minor pathways can be ignored will be needed. Overall, the two approaches have a complementary utility, with the genetic approach providing a broader picture of variability in the general population, but the liver biopsy approach perhaps providing a more accurate determination of the variability in enzyme activities.

Regardless of the approach used, appropriately evaluating the impact of polymorphisms on variability in tissue dose requires the integration of the variability in multiple parameters (or at least in the rate-limiting parameter), a task which is conveniently approached using PBPK modeling combined with Monte Carlo analysis. It is also important to recognize the potential for differences between the kinetics at pharmacologic doses (comparatively high doses designed to consistently elicit an effect) and at lower doses more characteristic of environmental exposures. High doses are more likely to result in saturation of metabolic capacity, with metabolism limited by the V_{max} of key enzymes. By contrast, metabolism at lower exposures may be limited by the K_m of key enzymes or even by blood flow rate to the liver. PBPK models are well-suited to simulate the kinetics at a variety of exposure levels, so that the impact of variability at environmentally relevant exposures can be quantitatively evaluated.

One goal of our analysis of the case studies was to identify the extent to which the data on a series of well-studied chemicals would support an analysis using the PBPK approach. Our results highlight a number of critical data needs and simplifying assumptions that are likely to be required. One area in need of development is to define the minimal set of polymorphism data for implementation of the approach. One of the criteria for selection of the case study chemicals was the availability of allelic frequency data for the key enzymes involved in its metabolism. While each chemical met this criterion, the data were not always optimal for studying the contribution of genotype frequencies in a diverse U.S. population. For example, PON1 genotype frequencies in Asian Americans were calculated from a study of Chinese subjects (Sanghera *et al.*, 1998), and estimates for African Americans were based on African populations (Diegpen and Geldmacher-von Mallinckrodt, 1986). In some cases, no data were available, even for the most populous ethnic groups. For CYP2C9, no estimate of genotype frequency was available for Hispanics (Wormhoudt *et al.*, 1999), and for GSTZ, data were available only for Caucasians (Blackburn *et al.*, 2000). Thus, an important decision for the analysis is the degree to which reliable data on each ethnic group is available for making a reasonable estimate of total human variability.

We also found that simplifying assumptions may need to be made in the common situation where multiple enzymes are involved in a chemical's metabolism. In such cases, it may be appropriate to focus on the enzyme for the rate-limiting step. It may also be appropriate to downplay the impact of enzymes that carry out metabolic steps for which other, higher efficiency enzymes also play a role.

Another criterion for inclusion in the case studies was the availability of phenotype data for each allelic variant. Our cases were of minimal complexity. However, for substrates metabolized by *N*-acetyltransferase or CYP2D6, two genes having a large number of variant alleles (Wormhoudt *et al.*, 1999), the task of including all variant alleles would be overwhelming. Four specific issues regarding the source of the phenotype data became apparent in our review of the data. First, it became clear that general phenotyping data are not likely to be sufficient for chemical-specific risk assessments. The importance of phenotyping data using the specific chemical of interest is clearly demonstrated by the substrate specificity of CYP2C9 for warfarin isomers and differences in catalytic efficiency of PON1 for related organophosphate pesticides.

Second, the implications of using enzyme kinetic data from purified variant proteins versus tissues from individuals having known genotypes need to be considered. As described above for the genetic approach versus the overall variability approach, data from purified proteins (or cloned proteins) allow activity to be attributed to a single enzyme but remove from consideration enzyme regulation and other factors affecting enzyme activity.

Third, the suitability of general phenotyping tests for evaluating disparity in allelic variants must be considered. Phenotyping tests are often developed to maximize the difference between the variant proteins, and therefore the degree of variability observed in an *in vitro* assay may overestimate *in vivo* differences. In addition, for data-rich chemicals, a choice might need to be made regarding which of multiple inconsistent values should be used for kinetic parameters. For example, for warfarin, not only did the values differ considerably between studies, but the interstudy differences were often larger than the differences between alleles. The different studies were not even consistent in ratios of key parameters of the different alleles. These observed differences are likely to be due to differences in experimental conditions. For example, Crespi and Miller (1997) noted the role of salt concentration and of oxidoreductase levels in determining the activity of CYP2C9. Other differences may be related to varying levels of protein expression in different systems. One alternative for addressing the inconsistent kinetic parameters is to determine which values are the best predictors of the *in vivo* data. Another alternative would be to choose the set of biologically meaningful parameters that gives the most conservative results.

CONCLUSIONS

The case studies presented in this paper are a first step toward developing an approach for quantitatively incorporating polymorphism data into development of toxicity values for environmental chemicals. Based on the data presented in this paper, it is apparent that enough quantitative data exist to productively evaluate the degree to which variability in enzyme kinetics for these chemicals affects the delivered tissue dose. Although several issues have been identified for each chemical, addressing the implications of the issues requires working through the case studies, applying the appropriate PBPK model, and quantitatively evaluating the implications of different assumptions.

Taken together, these data provide guidance regarding the critical uncertainties that are likely to be faced in the application of polymorphism data for quantitative risk assessments. There are important advantages in coupling genetic polymorphism data and PBPK modeling, particularly for beginning to estimate the range in tissue dose variability for large populations. We evaluated the data sets for a series of diverse chemicals to identify key areas of uncertainty. There is a clear need to put this method to use, as a means to better describe the overall human variability in dose of toxic agent resulting from genetic variability. The development of standard guidelines on assumptions for integrating genetic data with PBPK models will lead to improved incorporation of data on human variability, and ultimately improved risk assessments, although for most chemicals this is a long-term project before such an approach would be ready for use in the regulatory arena.

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