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In Vitro Metabolism of Methylene Chloride in Human and Animal Tissues: Use in Physiologically Based Pharmacokinetic Models

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In Vitro Metabolism of Methylene Chloride in Human and Animal Tissues: Use in Physiologically Based Pharmacokinetic Models. REITZ, R. H., MENDRALA, A. L., AND GUENGERICH, F. P. (1989). *Toxicol. Appl. Pharmacol.* **97**, 230–246. Physiologically based pharmacokinetic (PB-PK) models describe the dynamic behavior of chemicals and their metabolites in individual tissues of living animals. Because PB-PK models contain specific parameters related to the physiological and biochemical properties of different species as well as the physical chemical characteristics of individual chemicals, they are useful tools for performing high dose/low dose, dose route, and interspecies extrapolations in hazard evaluations. An example of such extrapolation has been presented by M. E. Andersen, H. J. Clewell III, M. L. Gargas, F. A. Smith, and R. H. Reitz (*Toxicol. Appl. Pharmacol.* **87**, 185–205, 1987), who employed a PB-PK model for methylene chloride (CH_2Cl_2) to estimate the chronic toxicity of this material. However, one limitation of this PB-PK model was that the metabolic rate constants for the glutathione-*S*-transferase (GST) pathway in humans were estimated by allometric scaling rather than from experimental data. In this paper we report studies designed to estimate the *in vivo* rates of metabolism of CH_2Cl_2 from *in vitro* incubations of lung and liver tissues from B6C3F1 mice, F344 rats, Syrian Golden hamsters, and humans. A procedure for calculating *in vivo* metabolic rate constants from the *in vitro* studies is presented. This procedure was validated by making extrapolations with mixed function oxidase enzymes (MFO) acting on CH_2Cl_2, where both *in vitro* and *in vivo* rates of metabolism are known. The *in vitro* rate constants for the two enzyme systems are consistent with the hypothesis presented by Andersen *et al.* that metabolism of CH_2Cl_2 occurs *in vivo* by two competing pathways: a high-affinity saturable pathway (identified as MFO) and a low-affinity first-order pathway (identified as GST). The metabolic rate constants for GST obtained from these studies are also consistent with the hypothesis of Andersen *et al.* that production of large quantities of glutathione/CH_2Cl_2 conjugates *in vivo* may increase the frequency with which lung and liver tumors develop in some species of animals (e.g., B6C3F1 mouse). When *in vivo* studies in humans are unavailable, *in vitro* enzyme assays provide a reasonable method for estimating metabolic rate constants.

INTRODUCTION

Methylene chloride (1,2-dichloromethane, CH_2Cl_2) has been reported to increase the incidence of lung and liver tumors in B6C3F1 mice following chronic inhalation exposure to 2000–4000 ppm for 6 hr/day (NTP, 1985). Following this report, several groups have attempted to estimate the probability (based on the NTP report) that human populations exposed to CH_2Cl_2 would experience similar increases in the incidence of lung and/or liver tumors (e.g., Singh *et al.*, 1985). Most of these risk estimations relied heavily upon two untested assumptions: (1) that the "dose" of

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CH_2Cl_2 was a linear function of the nominal exposure concentration, and (2) that the relative sensitivities of different species to a dose of CH_2Cl_2 (in mg/kg/day) could be calculated from an empirical "body surface area" which assumes that humans are always more sensitive to a test chemical than smaller species such as rodents.

Regulatory agencies have used assumptions such as these in estimating risks for chemicals where few or no pertinent toxicological data are available. They do so in the belief that such assumptions are conservative, and will protect the public health. Unfortunately, excessively conservative risk estimates may cause unnecessary removal of commercially important materials from commerce. Hence, the more we can replace empirical assumptions in risk estimations with experimentally validated procedures, the better off we will be in terms of both human health and economic well-being.

Andersen *et al.* (1987) recently suggested a procedure for replacing the empirical assumptions in the CH_2Cl_2 risk assessment of Singh *et al.* (1985) with quantitative measures derived from a well-validated physiologically based pharmacokinetic (PB-PK) model. The PB-PK model incorporated equations describing two metabolic pathways acting on CH_2Cl_2 *in vivo:* one involving oxidation of CH_2Cl_2 to CO by mixed-function oxidases (MFO) (Kubic *et al.*, 1974), and one involving conjugation of CH_2Cl_2 with reduced glutathione by glutathione-*S*-transferases (GST) (Ahmed and Anders, 1976).

A variety of biochemical and toxicological studies suggest that the toxicity of CH_2Cl_2 and other dihalomethanes is associated with the production of reactive metabolites derived from the GST pathway (Andersen *et al.*, 1987; Green, 1983; van Bladeren *et al.*, 1980). Furthermore, it appears that the MFO pathway plays a protective role *in vivo.* The MFO enzymes have a higher affinity for CH_2Cl_2 than the GST enzymes (Gargas *et al.*, 1986; Green *et al.*, 1986) and most of the CH_2Cl_2 is metabolized by MFO at low concentrations. After saturation of the MFO pathway (between 300 and 500 ppm), metabolism by the GST pathway increases disproportionately.

Although this behavior is complex, the PB-PK model has provided quantitative descriptions of the rate of production of GST metabolites in target tissues of different species for a variety of doses and dose routes (Andersen *et al.*, 1987). It was found that the predictions of internal dose obtained from the PB-PK model differed from the predictions of Singh *et al.* (1985) by as much as two orders of magnitude when extrapolating from mice to humans. The USEPA has evaluated this approach and is considering modifying its risk estimates based on this new approach (Blancato *et al.*, 1987).

Since metabolism plays a key role in determining the toxicity of CH_2Cl_2, it is important that the rate constants for each of the metabolic pathways described in the PB-PK model be determined as accurately as possible. The values of the rate constants for the MFO and GST pathways in animals were determined by computerized optimization of *in vivo* gas uptake studies (Andersen *et al.*, 1987). *In vivo* estimates of the rate constants for the MFO pathway in humans were derived from direct measurements of CH_2Cl_2 uptake as well as measurements of carboxyhemoglobin levels in human volunteers exposed to CH_2Cl_2. However, there were no *in vivo* or *in vitro* data available for direct calculation of the rate constants for the GST pathway in humans. Consequently, Andersen *et al.* (1987) estimated the rate constants for the GST pathway by allometric scaling of the *in vivo* rate constants for GST metabolism in animals. Although allometric scaling has been widely used by others in the estimation of metabolic rates (Lindstedt, 1987), it was clear that additional data supporting the validity of such scaling would be valuable.

Additional experiments to estimate the value of the metabolic rate constants for the GST pathway in humans have now been conducted. These experiments involved *in vitro*

measurements of the CH_2Cl_2-dependent GST activity in tissue samples from humans as well as tissue samples from F344 rats, B6C3F1 mice, and Syrian Golden hamsters. The objectives of this work are (1) to describe the collection of the *in vitro* data, and (2) to suggest a mechanism for incorporating these new data into the PB-PK risk assessment for CH_2Cl_2.

METHODS

Reagents/Test Materials

Spectroscopic-grade CH_2Cl_2 (>99.9% purity) was obtained from Fisher Chemical Company. All other chemicals used in these experiments were reagent-grade or higher materials, obtained from commercial suppliers. Formaldehyde dehydrogenase enzyme (prepared from *Clostridium boidinii*) was obtained from Sigma Chemical Company (St. Louis, MO).

$CH_2{}^{36}Cl_2$ (0.5 mCi/mmol) was synthesized by New England Nuclear (Boston, MA). This material was checked for radiochemical purity by gas chromatography/mass spectrometry prior to use and was found to be 95.0% $CH_2{}^{36}Cl_2$, with small amounts of $CH_3{}^{36}Cl$ (0.6%) and $CH^{36}Cl_3$ (2.8%) identified as minor impurities.

Preparation of Enzymes

Cytosolic and microsomal fractions of lungs and livers were prepared from male F344 rats, B6C3F1 mice, Syrian Golden hamsters, and tissues of otherwise healthy accident victims selected for organ transplantation through the Nashville Regional Organ Procurement Agency (Nashville, TN).

Human samples were screened for HIV and hepatitis A and B viruses by the agency, and samples were diagnosed as being free of debilitating disease. Other metabolic and biochemical studies with some of these and similar samples have been described elsewhere (Beaune *et al.*, 1986; Bocker and Guengerich, 1986; Hall *et al.*, 1987; Knodell *et al.*, 1987; Guengerich *et al.*, 1986a,b).

Tissues were removed from animals or humans and small pieces were rapidly frozen either by immersion in liquid nitrogen (humans) or with dry ice (animals). Samples of frozen tissues were added to 4 vol of cold (4°C) 0.1 M Tris–HCl buffer containing 0.1 M KCl and 0.001 M EDTA (pH 7.4) and homogenized in a Teflon/glass homogenizer. Preliminary experiments with animal tissues verified that enzyme preparations made without the preliminary freezing step did not differ from enzyme preparations from frozen tissue (i.e., freezing of tissues prior to isolation does not affect the enzyme activity).

Samples of human liver from four different individuals were processed individually. Availability of human lung samples was limited, so lung samples from two individuals were pooled (~8 g total tissue weight) for the enzyme preparation. (None of the lung samples were from the same individuals as the liver samples.)

After homogenization, the preparations were centrifuged at 10,000*g* for 30 min, and the pellet was discarded. The supernatant fluid was centrifuged at 100,000*g* for 60 min. The clear, slightly brown supernate from the second centrifugation was carefully decanted, designated "Cytosol," and stored frozen at −80°C until use. The pellet was resuspended in 0.1 M potassium pyrophosphate buffer (pH 7.4) containing 0.001 M EDTA at half the original volume of the first buffer. After recentrifugation at 100,000*g* for 60 min, the supernate of the third centrifugation was discarded, and the pellet was resuspended in 0.01 M Tris–acetate buffer (pH 7.4) containing 1 mM EDTA and 20% (v/v) glycerol at half the original volume of the first buffer. This preparation was stored frozen at −80°C until use, and designated "Microsomes" (Guengerich, 1977).

Assay of GST

Incubation conditions. All incubations were conducted in 1.8-ml glass vials sealed with Teflon-coated rubber septa. The volume of each incubation was ~1.7 ml (giving a free headspace of ~0.1 ml). Each incubation contained potassium phosphate buffer (50–100 mM, pH 7.4) and reduced glutathione (10 mM) unless otherwise noted.

Various concentrations of unlabeled CH_2Cl_2 were dissolved in the buffer mix (containing phosphate, glutathione, and any other components) prior to addition of enzyme by vigorous stirring with a small Teflon-coated stirring bar in a sealed 25-ml volumetric flask for 5–10 min. This buffer/substrate/cofactor mix was made fresh just before each assay. Aliquots of cytosol (0–1.0 ml, 0–15 mg protein) were transferred to the vials and equilibrated briefly at 37°C. Boiled enzyme preparations containing the same amount of protein were used as controls (1 min boiling). The buffer/substrate/cofactor mix and a small amount of labeled substrate dissolved in ethanol (0.003–0.010 ml) were added to each tube at time zero and the vial was immediately sealed with a Teflon septum and incubated for the indicated period of time (usually 30 min).

Extraction of unreacted substrate. At the end of incubation, the contents of the vial were transferred to a glass tube containing 0.5 ml of a solution of 2% Na_2CO_3 and 1% NaCl as well as 3.0 ml of unlabeled CH_2Cl_2 and vortexed vigorously for 10 sec to separate the unreacted substrate from the water-soluble products of the enzyme re-

action (which may include any or all of the following: chloromethyl glutathione, HCHO, HCOOH, CO_3^-, or Cl^-). The aqueous and CH_2Cl_2 phases were separated by centrifugation (2000*g* for 10 min) and an aliquot (1 ml) of the aqueous phase was transferred to a second tube containing 3.0 ml of CH_2Cl_2 for reextraction of unreacted substrate. Following the reextraction, an aliquot of the aqueous phase was assayed by liquid scintillation counting.

Assay of Mixed-Function Oxidase (MFO)

Incubation conditions. All incubations were conducted in 5.0-ml vials sealed with Teflon-coated rubber septa. Each vial contained 50 mmol potassium phosphate buffer (pH 7.4), 10 mmol glucose 6-phosphate, 0.5 mmol $NADP^+$, ~1 unit of glucose-6-phosphate dehydrogenase, $CH_2{}^{36}Cl_2$, and 1–2 mg of microsomal protein in a total volume of 1.0 ml. For microsomal assays, the $CH_2{}^{36}Cl_2$ was dissolved in water instead of ethanol, since even small quantities of ethanol (1% v/v) were found to interfere with the MFO assay. Free headspace in each vial was approximately 4.0 ml. Various concentrations of CH_2Cl_2 were predissolved in the buffer mix prior to incubation as described for the GST assay procedure.

All components except the microsomal protein were added to a sealed vial and allowed to equilibrate for ~3 min at 37°C. At time zero, the microsomal protein was added through the septa with a hypodermic needle and incubation was continued for 30–45 min. Contents of the vials were then transferred to a glass tube containing 0.5 ml of a solution of 2% Na_2CO_3 and 1% NaCl to stop the reaction. Then 3.0 ml of unlabeled CH_2Cl_2 was added and the unreacted substrate was extracted as described for the GST assay.

RESULTS

Characterization of the Enzyme Activity (GST)

Ahmed and Anders (1976) previously described an assay for the enzyme catalyzing the reaction of CH_2Cl_2 with glutathione (GST). In their procedure, the activity of GST was determined by measuring HCHO production colorimetrically. However, the colorimetric method has limited sensitivity and HCHO may be destroyed by other enzymes present in the crude liver cytosol preparations. Consequently, we believed that intraspecies comparisons based on this assay might be unreliable, and we developed an alternative assay procedure based on the conversion of organic solvent extractable radioactivity (from either $^{14}CH_2Cl_2$ or $CH_2{}^{36}Cl_2$) to a water-soluble form. This assay procedure offers greater sensitivity than the HCHO assay and avoids the potential problems associated with degradation of HCHO in the incubation mixture.

The activity of the GST enzyme as measured by the radioisotope procedure was proportional to time (for 30–45 min of incubation) and the amount of crude protein present in the incubation mixture (up to 10–15 mg protein/incubation). In addition, the conversion of radioactivity was heat sensitive; boiling of the protein solution for 1 min prior to addition of substrate completely abolished the activity. Dialyzed samples of mouse and human cytosol showed an absolute requirement for exogenous glutathione. Optimum activity was obtained after addition of 5–10 mM glutathione to the incubation mixture. Based on these studies, 10 mM exogenous glutathione was added to all subsequent incubation mixtures.

Comparison of Liver Enzymes (GST)

Samples of liver cytosol from humans, F344 rats, B6C3F1 mice, and Syrian Golden hamsters were assayed at 40 mM CH_2Cl_2 concentration. Significant activity was observed in the cytosol prepared from each species with the $^{36}Cl^-$ assay (Table 1). Values in Table 1 are means obtained from several independent experiments. The standard deviations of the means and the number of assays performed are also listed.

The highest levels of GST activity were found in the mouse liver cytosol (25.9 nmol product formed/min/mg protein). GST levels were lower in the rat and hamster cytosols (7.01 and 1.27 nmol product/min/mg protein, respectively). Human cytosol preparations from four different individuals were studied. The activity ranged from 0.0 to 3.03 nmol product formed/min/mg protein. One

TABLE 1

COMPARISON OF $MeCl_2$/GLUTATHIONE-*S*-TRANSFERASE ACTIVITY IN VARIOUS CYTOSOL PREPARATIONS FROM B6C3F1 MICE, F344 RATS, SYRIAN GOLDEN HAMSTERS, AND HUMANS

Species	Enzyme activity (nmol/min/mg)		Ratio
	Liver	Lung	
Mouse	25.9 ± 4.2 (15)	7.3 ± 1.2 (4)	0.28
Rat	7.05 ± 1.7 (6)	1.0 ± 0.1 (4)	0.14
Hamster	1.27 ± 0.21 (6)	0.0 ± 0.2 (4)	—
Human-99	2.62 ± 0.44 (10)	—	
Human-103	−0.01 ± 0.04[a] (6)	—	
Human-105	2.71 ± 0.45 (6)	—	
Human-109	3.03 ± 0.44 (6)	—	
Pooled human	—	0.37 ± 0.25 (2)	0.18[b]

Note. All values are reported as nmol product formed/min/mg protein (mean ± SD) when incubated in a sealed vial containing 0.04 M $MeCl_2$, 0.01 M reduced glutathione, and 0.03 M phosphate buffer (pH 7.4). (*n*) represents the number of independent determinations of enzyme activity (two per experiment) for each preparation.

[a] Microsomes prepared from this sample of human liver exhibited relatively normal activity as judged by the activity with antipyrine and mephenytoin, and 4-aminobiphenyl (Butler *et al.*, 1987).

[b] Ratio to the average activity in four human liver samples.

sample (HL-103) was apparently devoid of activity even though several experiments were run. The other three human liver cytosols all had roughly equivalent GST activity (Table 1).

Comparison of Lung Enzymes (GST)

Samples of cytosol prepared from the lung tissue of these same species were also assayed. The reaction rates (nmol product formed/min/mg protein) are listed in Table 1. The highest rates were observed in the cytosol prepared from mouse lungs (7.3), with lower rates obtained in the cytosol from rat lungs (1.0) and human lungs (0.37). No GST activity was detectable in cytosol prepared from hamster lungs (with a detection limit of ~0.2).

The reaction rates were lower in the lung cytosols than the liver cytosols for each species. The ratio of activities (lung/liver) in the mouse was 0.28, while the ratio of activities in the rat and human were 0.14 and 0.18, respectively. Since no activity was detected in lung cytosol prepared from hamster tissue, the ratio of activities could not be calculated for this species.

Determination of Kinetic Parameters (GST)

The cytosol preparations from the *livers* of B6C3F1 mice, F344 rats, Syrian Golden hamsters, and humans were assayed at various concentrations from 6.7 to 100 mM to conduct an analysis of the kinetic properties of the GST activity in these preparations. Equal quantities of $CH_2{}^{36}Cl_2$ were present in each incubation and the different substrate concentrations were achieved by adding varying amounts of unlabeled CH_2Cl_2 to the individual incubations. The reaction rates observed at the various concentrations (mean of two determinations) are listed in Table 2.

Double-reciprocal plots were constructed for these data according to the method of Lineweaver and Burk (1934). These plots are shown in Figs. 1A (mice), 1B (rats), 1C (hamsters), and 1D (human-99). Examination of these plots revealed that the K_m was quite

TABLE 2

ENZYME ACTIVITIES (GST AND MFO) OBSERVED AT VARIOUS CONCENTRATIONS OF CH_2Cl_2 WITH ENZYMES PREPARED FROM LIVER TISSUE OF MALE B6C3F1 MICE, F344 RATS, SYRIAN GOLDEN HAMSTERS, AND HUMANS

	Reaction rate (nmol/min/mg protein)				
				Human	
Concentration (mM)	Mouse	Rat	Hamster	HL-99	HL-109
		GST assays (liver)			
6.7	4.77	0.75	0.182	—	—
10.0	7.24	1.11	0.307	—	—
12.5	9.92	1.36	0.383	1.53	1.28
16.7	12.3	2.08	0.482	1.95	1.66
25.0	18.5	3.19	0.760	2.58	2.24
50.0	33.2	6.17	1.24	3.95	3.51
100.0	48.6	12.1	2.64	4.74	3.94
		MFO assays (liver)			
1.00	5.87[a]	2.40[a]	7.18[a]	1.49	1.66[a]
1.23	6.52[a]	2.42[a]	8.16[a]	1.85	2.06[a]
1.64	7.18[a]	2.93[a]	8.54[a]	2.09	2.26[a]
2.60	9.02[a]	3.44[a]	11.02[a]	2.28	2.87[a]
5.00	11.40[a]	4.10[a]	14.47[a]	3.34	4.46[a]
10.00	14.40[a]	4.91[a]	18.18[a]	4.58	4.80[a]
		MFO assays (lung)			
5.00	4.62	0.16	0.99	<0.1[b]	—

Note. All values are reported as nmol product formed/min/mg protein.

[a] Mean value from two independent experiments.

[b] Pooled human lung samples; no activity observed at detection limit of 0.05–0.1 nmol/min/mg protein.

high in each species (40 mM or higher). Since CH_2Cl_2 has limited water solubility, it was not possible to employ concentrations of CH_2Cl_2 higher than 100 mM in these experiments. Consequently, it was difficult to establish an accurate value for K_m and V_{max} in some of the liver cytosol preparations (e.g., rat and hamster) where the K_m is apparently significantly higher than 100 mM.

The values of K_m and V_{max} in human and mouse cytosol were estimated by computer optimization using the Simusolv computer program with relative least-squares weighting (i.e., each data point was given equal importance in estimating the kinetic constants, regardless of the absolute magnitude of that rate). This type of weighting is appropriate because each incubation contained the same amount of radioactivity, giving approximately equal precision to the determination of enzyme rates throughout the concentration range studied.

Reasonable "solutions" for the computer optimizations (i.e., solutions where the variability of the estimate was small relative to the magnitude of the estimate itself) were obtained for the mouse cytosol and the two human cytosols studied (Table 3). However, the computer was unable to find a reasonable "solution" for the data generated with the rat and hamster cytosols. With these data the ratio of V_{max} and K_m was critical, but the abso-

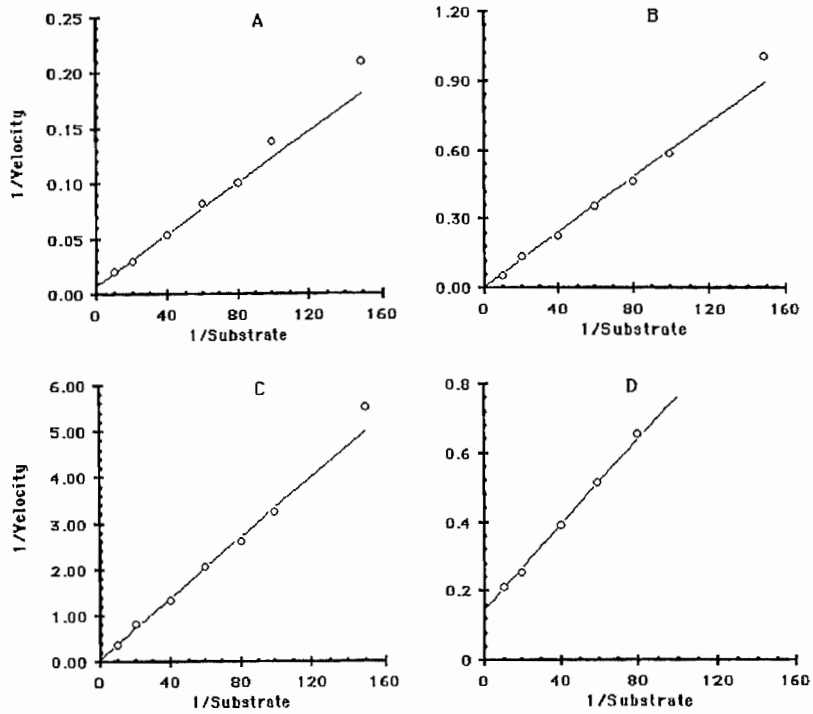

FIG. 1. Lineweaver–Burk plots (1/velocity versus 1/substrate) of the concentration dependence of GST activity in cytosols prepared from various species. Concentrations of CH_2Cl_2 in the incubation mixture varied from 6.7 to 100 mM, and the incubations also contained 8 mM glutathione and 60 mM phosphate buffer (pH 7.4). Plots are shown for (A) mouse cytosol, (B) rat cytosol, (C) hamster cytosol, and (D) human cytosol prepared from liver 99.

lute values of the constants were not. From examination of the Lineweaver–Burk plots (Fig. 1) it was clear, however, that the K_m in all the animal cytosols was significantly higher than the K_m observed in the samples of human cytosol studied.

Characterization of the Enzyme Activity (MFO)

The microsomes obtained from the various animal species were assayed in a sealed vial containing ~4 ml of room air as headspace and an NADPH regenerating system (glucose 6-phosphate and glucose-6-phosphate dehydrogenase). Under these conditions, the rate of conversion of $CH_2{}^{36}Cl_2$ to $^{36}Cl^-$ was proportional to time (for 20–30 min) and proportional to protein concentration (up to 1–2 mg protein/incubation). Boiling the enzyme prior to incubation completely inhibited the reaction.

Comparison of Liver Enzymes (MFO)

The highest activity was obtained in preparations of microsomes from the hamsters. Microsomes from the other species were less active in the order hamster > mouse > rat ~ human. For example, the activities (nmol product formed/min/mg protein) measured at a substrate concentration of 5 mM CH_2Cl_2 in hamster, mouse, rat, and human were 14.5, 11.4, 4.1, and 3.3–4.5, respectively (Table 2).

TABLE 3

KINETIC CONSTANTS OBTAINED FROM *IN VITRO* EXPERIMENTS WITH GST AND MFO ASSAYS IN ENZYME PREPARATIONS FROM THE LIVERS OF MALE B6C3F1 MICE, F344 RATS, SYRIAN GOLDEN HAMSTERS, AND HUMANS

Species	*N*	K_m (mM)	V_{max} (nmol product formed/min/mg prot.)
		GST assays	
Mouse	5	137 ± 21	118.2 ± 14.4
Rat	5	N.S.[a]	N.S.[a]
Hamster	5	N.S.[a]	N.S.[a]
Human-99	5	43.8 ± 4.5	7.05 ± 0.44
Human-109	5	44.1 ± 8.1	6.04 ± 0.67
		MFO assays	
Mouse	12	1.84 ± 0.33	15.90 ± 1.10
Rat	12	1.42 ± 0.74	5.39 ± 0.94
Hamster	12	2.07 ± 0.30	20.80 ± 1.15
Human-99	6	2.57 ± 2.17	5.27 ± 1.85
Human-103	6	1.95 ± 5.24	1.53 ± 1.56
Human-105	6	0.92 ± 0.29	13.00 ± 1.13
Human-109	12	2.82 ± 1.47	6.24 ± 1.39

Note. Constants were obtained by relative least-squares weighting using the SimuSolv computer program. Values reported are the best estimates ± SD of the estimate as calculated by the computer program.

[a] Data collected but no acceptable solution obtained during computer optimization.

Comparison of Lung Enzymes (MFO)

Microsomes obtained from the lungs of the various species were less active (per mg protein) than preparations from the liver. For instance, the activities of MFO enzyme at 5 mM CH_2Cl_2 in hamster, mouse, rat, and humans were 0.99, 4.62, 0.16, and <0.1 (limit of detection) nmol/min/mg protein, respectively (Table 2). The ratios of MFO activity in lung tissue to MFO in liver tissue were 0.068, 0.405, 0.039 in hamsters, mice, and rats, respectively. Since no activity was observed in the pooled sample of human lung tissue, it was not possible to calculate the ratio of activities in the two tissues for humans.

Determination of Kinetic Parameters (MFO)

The microsome preparations (MFO) from the livers of the various species were also subjected to kinetic analysis. A series of incubations with concentrations of CH_2Cl_2 varying from 1 to 10 mM were conducted, and the reaction rates observed (nmol/min/mg protein) are listed in Table 2. Double-reciprocal plots (Lineweaver and Burk, 1934) of the data are presented in Figs. 2A (B6C3F1 mice), 2B (F344 rats), 2C (Syrian Golden hamsters), and 2D (human-99).

Values of K_m and V_{max} were determined by computer optimization, and the results (±SD) are listed in Table 3. Reasonable solutions were obtained for each of the data sets analyzed. The Michaelis constants (K_m's) were very similar in the seven sets of liver microsomes analyzed; the values ranged from 0.92 mM (human-105) to 2.8 mM (human-109).

It is noteworthy that the values of K_m obtained from *in vitro* studies differ significantly from those observed *in vivo* by Andersen *et al.* (1987). For example, the *in vitro* K_m reported for the rat is 1.42 mM, while the *in vivo*

K_m determined for rats by Andersen *et al.* was 0.0098 mM (0.771 mg/liter).

Before making direct comparisons of these parameters, it is necessary to correct the *in vivo* K_m's for the fact that the K_m used by Andersen *et al.* refers to the concentration of CH_2Cl_2 in blood at equilibrium with the liver rather than the concentration of CH_2Cl_2 in liver itself (i.e., the K_m's need to be multiplied by the liver/blood partition coefficient). Furthermore, the K_m determined *in vitro* is based on a nominal concentration of CH_2Cl_2 in the sealed 5-ml vial (calculated by dividing the amount added to the vial by the volume of the liquid phase). However, since CH_2Cl_2 is quite volatile, approximately 40% of it is present in the headspace instead of the liquid phase at equilibrium. However, even after correction for these two factors, the difference between the *in vitro* and *in vivo* values of K_m is still almost two orders of magnitude.

The highest rates of oxidation were observed in microsomes from hamster liver (V_{max} = 20.8), with lower rates in mice, rats, and humans. The microsomes prepared from the liver of human-103 exhibited much lower activity than the microsomes from the other three human samples, and it is noteworthy that the cytosol prepared from this individual also exhibited extremely low GST activity (Table 2). However, in other studies conducted by one of us (F.P.G.) the liver microsomes prepared from HL-103 had one of the higher rates of 4-aminobiphenyl oxidation (Butler *et al.*, 1987).

DISCUSSION

Limitations of the GST Assay

Development of a radioisotopic assay for MFO was relatively straightforward, and the procedures used here were similar to those reported by others. However, the development of a sensitive assay procedure for GST presented a number of technical difficulties. Because of the low water solubility and high volatility of CH_2Cl_2, scrupulous attention must be given to ensuring that CH_2Cl_2 has fully dissolved but not evaporated from the incubation mixture. For this reason, unlabeled CH_2Cl_2 was dissolved in the enzyme buffer by vigorous stirring for 10 min in a tightly sealed flask prior to addition of the cytosol and labeled substrate. All incubations were conducted in glass vials sealed with Teflon-coated septa. Incubation volumes were adjusted to leave little or no headspace (no more than 0.1 ml over a 1.7-ml incubation volume). Even so, it was not possible to conduct GST assays at substrate concentrations higher than about 100 mM CH_2Cl_2. Since the K_m values for CH_2Cl_2 were all 40 mM or higher, this made it difficult to get an accurate estimate of K_m. However, it should be pointed out that the linear nature of velocity versus substrate curves led to accurate V_{max}/K_m ratios, which were utilized in the modeling experiments.

Consistency with Other Studies

Kubic and Anders (1975) studied the *in vitro* metabolism of dihalomethanes (including CH_2Cl_2) to CO and inorganic halide by subcellular fractions from rat liver. They reported that metabolism was heat sensitive, NADPH dependent, and localized in the microsomal fraction. These results are consistent with the data we obtained with the MFO preparations in all species.

Ahmed and Anders (1975) studied the conversion of dihalomethanes to formaldehyde and inorganic halide by cytosolic preparations from various tissues of the rat. The data we have reported for GST are consistent with their studies in that (1) an absolute requirement for glutathione was established in dialyzed enzyme preparations, (2) the Michaelis constant (K_m) for CH_2Cl_2 was very high in liver cytosol preparations (Ahmed and Anders reported a K_m of 122 mM for rat liver), and (3) cytosol prepared from lung tissue contained much less GST activity than cytosol prepared from liver tissue.

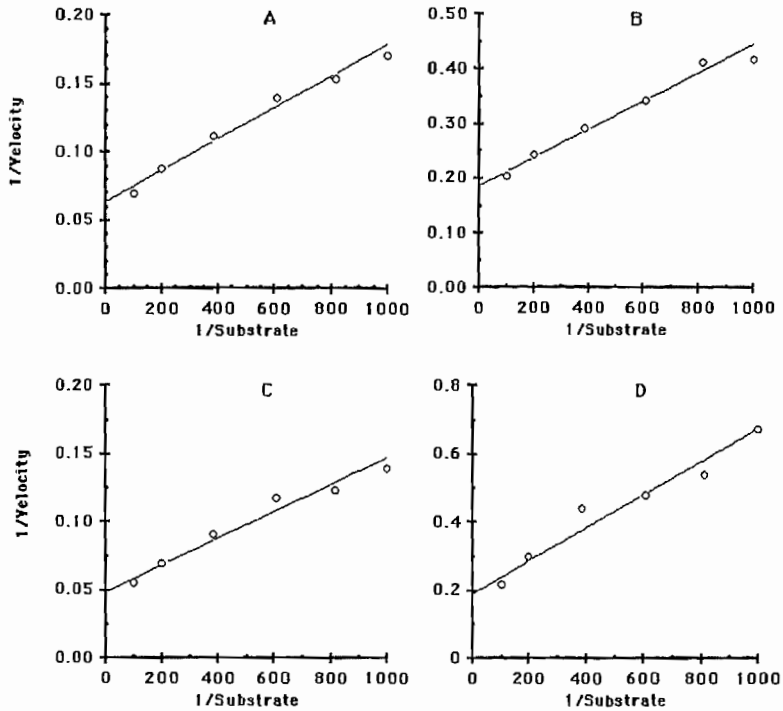

FIG. 2. Lineweaver–Burk plots (1/velocity versus 1/substrate) of the concentration dependence of MFO activity in microsomes prepared from various species. Concentrations of CH_2Cl_2 in the incubation mixture varied from 1 to 10 mM, and the incubations also contained 50 mmol potassium phosphate buffer (pH 7.4), 10 mmol glucose 6-phosphate, 0.5 mmol $NADP^+$, ~1 unit of glucose-6-phosphate dehydrogenase, $CH_2{}^{36}Cl_2$, and 1–2 mg of microsomal protein in a total volume of 1.0 ml. Plots are shown for (A) mouse microsomes, (B) rat microsomes, (C) hamster microsomes, and (D) human microsomes prepared from liver 99.

Differences between in Vivo and in Vitro Conditions

It was noted under Results that the K_m values (for the MFO enzyme) obtained from *in vitro* studies were much higher than the values obtained from *in vivo* experiments conducted by Andersen *et al.* (1987). There are several possible explanations for this. First, homogenization of the tissue and purification of the "microsome fraction" undoubtedly induce significant structural changes in the microenvironment of the enzymes (e.g., lipophilicity of membranes). Furthermore, the ionic strength, degree of oxygenation, and concentrations of cofactors present in *in vitro* incubations undoubtedly differ from *in vivo* conditions. Consequently, it is not surprising to see differences between the kinetic parameters obtained from *in vivo* and *in vitro* experiments. However, since the enzymes were prepared with the same techniques from each species, it seems likely that the relative affinities and velocities observed in *in vitro* preparations from different species will reflect the relative affinities and velocities occurring *in vivo*.

Calculation of Constants for Use in the PB-PK Model

MFO constants. In vivo V_{max}'s for MFO activity in B6C3F1 mice, F344 rats, and ham-

TABLE 4

COMPARISON OF MFO ACTIVITY CALCULATED FROM *IN VIVO* EXPERIMENTS AND *IN VITRO* ENZYME ASSAYS

	Mouse	Rat	Hamster	Human
Body weight (g)	34.5	233.0	140.0	70,000
Liver weight (g)	1.84	5.41	4.34	2,198
$V_{max\ 1}$ (nmol/g/hr)	6700	3260	5560	637
K_m (μM)	4.66	9.08	7.64	6.83
Calc (nmol/g/hr)	1180	324	644	81.4
Obs (nmol/mg/hr)	352	144	431	31–433
Ratio (Calc/Obs)	3.36	2.25	1.49	0.2–2.6
Mean ratio		2.37 ± 0.94		1.13 ± 1.04

Note. Rates of *in vivo* metabolism are calculated according to Andersen *et al.* (1987) by converting V_{max} (mg/hr/animal) to $V_{max\ 1}$ (nmol/g liver/hr), K_m from mg/liter to μmol/liter, and solving the Michaelis–Menten equation with a concentration of 1 μM. *In vitro* rates are given as nmol converted/hr/mg microsomal protein at a nominal substrate concentration of 1 mM.

sters were obtained by computer optimization in the studies of Andersen *et al.* (1987) and are listed in Table 4. To compare these numbers with *in vitro* rates, several steps are necessary. First the overall V_{max}'s (rate of metabolism in the whole animal) are converted to rates per gram of liver tissue by dividing by the grams of liver per animal. This assumes that all MFO metabolism in the animal occurs in the liver. Since the liver has a much larger volume and higher specific enzyme activity than the lung, this is probably a good approximation.

Then the K_m's and adjusted V_{max}'s from the *in vivo* experiments are used to calculate the rate of metabolism for a concentration in the linear portion of the velocity versus concentration curve (1 μM or 10^{-6} M) according to the Michaelis–Menten equation:

$$\text{rate} = \frac{V_{max} * \text{concn}}{K_m + \text{concn}}. \qquad (1)$$

Finally, the calculated rate of *in vivo* metabolism is compared to the rates observed in the *in vitro* experiments at a concentration in the linear portion of the velocity versus concentration curve (nominal concentration = 1 mM in this case). The ratios of the calculated *in vivo* rates and the observed *in vitro* rates are listed in Table 4.

Since the units of the *in vivo* rate (nmol/hr/gram liver) and the *in vitro* rate (nmol/min/mg microsomal protein) and the incubation concentrations are different, the ratio of activities is not expected to be unity. However, the *ratio* of activities is fairly consistent across the three animal species (mouse = 3.36, rat = 2.25, hamster = 1.49), suggesting that the *in vitro* and *in vivo* results are proportional to each other in spite of the obvious variations in *in vivo* and *in vitro* conditions.

In vitro measurements of MFO activity in microsomes prepared from human livers revealed considerably more variation than was present in microsomes prepared from the animal species: the activity ratios observed in liver preparations from four individuals were 0.91, 2.6, 0.19, and 0.82 (mean activity ratio = 1.13 ± 1.01, Table 4). Examination of the data revealed that one individual (HL-105) had an unusually high level of *in vitro* MFO activity (producing the low activity ratio of 0.19). Without this individual the mean activity ratio would have been 1.45.

We have since carried out additional studies of oxidative metabolism using a related substrate ($CHCl_3$) with nine samples of human liver, including the four discussed here. These studies also suggest that the MFO activity in the liver of HL-105 is abnormally

high. Results with the other eight samples are more consistent, although the range of high to low activities is still about fivefold (Reitz *et al.*, unpublished data). Similar variability in tissues from human accident victims have been observed by others. This emphasizes the need for analysis of samples from as many different humans as practical when conducting these types of extrapolations.

It must also be noted that the Andersen *et al.* estimations of *in vivo* rates of MFO metabolism in humans are less precise (because of the nature of the *in vivo* experiments) than the *in vivo* estimations of MFO metabolism in animals. Nevertheless, it is encouraging that the mean ratio of *in vivo* to *in vitro* MFO activities in humans (1.13 ± 1.04) is within a factor of 2 of that observed in the animal experiments (2.37 ± 0.94).

GST constants. In the original presentation of their PB-PK model for CH_2Cl_2 disposition, Andersen *et al.* (1987) described the metabolism of CH_2Cl_2 by the GST pathway as a pseudo-first-order process; that is, the rate of reaction (dAM2/dt) was linearly related to the concentration in the liver tissue times the first-order rate constant K_F and the volume (vol) of the tissue:

$$\frac{d\text{AM2}}{dt} = (K_F)(\text{concn})(\text{vol}). \qquad (2)$$

This equation is equivalent to a simplified form of the Michaelis–Menten equation:

$$\frac{d\text{AM2}}{dt} = \frac{(V_{max})(\text{concn})(\text{vol})}{(K_m + \text{concn})} \sim \frac{(V_{max})}{K_m}(\text{concn})(\text{vol}), \qquad (3)$$

where V_{max} has units of mg/hr/liter of tissue and concn is small relative to K_m. Under these conditions, K_m + concn is approximately equal to K_m so that V_{max}/K_m is equivalent to the constant K_F.

Using the PB-PK model developed by Andersen *et al.* (1987), it can be calculated that the maximum concentrations of CH_2Cl_2 in lung or liver tissue during the rodent bioassays (with concentrations up to 4000 ppm CH_2Cl_2) would be no more than ~2 mM. Since the K_m for GST in cytosolic preparations from rodents was always >137 mM (Table 3), the description of the GST pathway as a first-order pathway in the PB-PK model appears consistent with the *in vitro* kinetic constants reported in this paper.

If we make the assumption that a liter of liver from each species contains roughly equal amounts of cytosolic protein, we can rearrange the first equation to solve for K_F in terms of dAM2/dt (the velocity of the enzyme reaction),

$$K_F = (K)\frac{\text{velocity}}{\text{concn}}, \qquad (4)$$

so that K_F is equal to a constant K times the velocity measured *in vitro* (nmol product formed/min/mg protein) divided by the *in vitro* substrate concentration (velocity/substrate, V/S ratio).

There are many differences between *in vitro* enzyme assays and *in vivo* conditions in the tissues of a living animal, so it is not possible to calculate the value of the constant K from *in vitro* assays alone. However, if the *in vivo* K_F and the *in vitro* V/S ratio are both determined in a single species (e.g., B6C3F1 mice), then the value of K for that species can be calculated as K_F divided by the velocity/substrate (V/S) ratio observed *in vitro*. Once the value of K is known in a single species, K_F in the other species may be estimated by multiplying K times V/S as shown in Eq. (4).

This procedure involves the assumption that the proportionality constant K is identical in all species (i.e., that the changes in enzyme activity introduced by dissecting the tissues out of the animal, homogenizing them, and then incubating them in a synthetic incubation medium are similar in each preparation). Since all the enzymes were carefully prepared under identical conditions from mammalian species, this assumption seems reasonable. Furthermore this assumption is supported by the consistency of the *in vivo/in vitro* MFO activity ratios discussed earlier.

TABLE 5

RECALCULATION OF THE METABOLIC RATE CONSTANT FOR THE GST PATHWAY (K_F) FROM THE *IN VITRO* STUDIES OF GST

	V/S	Calculated K_F	Original K_F
Mouse	741.4 ± 31.1	4.01[a]	4.01[a]
Rat	116.9 ± 8.6	0.63	2.21
Hamster	29.6 ± 1.5	0.16	1.51
Human	105.6 ± 12.0	0.43[b]	0.53

Note. Column 1 contains the velocity to substrate ratio (*V/S*). Column 2 contains the value of K_F calculated from the *in vitro* studies as outlined in the text. Column 3 lists the values of K_F cited by Andersen *et al.* (1987) for their PB-PK model. The value of K_F cited by Andersen *et al.* for the mouse is used as a reference point for calculating the *in vivo* rate constants in the rat, hamster, and human.

[a] Used as a reference point for the other values.

[b] Value multiplied by 0.75 to correct for the fact that only three of the four human liver samples had detectable activity.

V/S ratios for GST activity in tissues from B6C3F1 mice, F344 rats, Syrian Golden hamsters, and two different human livers were determined by dividing the *in vitro* reaction velocities in Table 2 (columns 2–6) by the substrate concentration (column 1, Table 2). The *V/S* ratio was essentially constant up to 25 mM in all species, but declined at concentrations 50 mM or higher in the two human liver samples examined (calculations not shown). Consequently, the average *V/S* ratio for all concentrations less than 50 mM in a given species (Table 5, column 1) was used in these calculations. Values of K_F calculated for the other species, using the mouse data as a reference point, are listed in column 2 of Table 5. Since one of the four human liver samples tested had no detectable activity, the estimated mean value of K_F in humans was calculated by multiplying the average *V/S* ratio measured in HL-99 and HL-109 by 0.75.

The estimated value of K_F for humans obtained by this procedure (0.43) was very close to that which Andersen *et al.* obtained by allometric scaling of the mouse data (0.53; Table 5, column 3). The value of K_F for rats (0.63) was somewhat lower than that estimated by Andersen *et al.* in gas uptake experiments (2.21), and the value of K_F in hamsters (0.16) was almost an order of magnitude less than the value which Andersen *et al.* derived from gas uptake studies (1.51). It seems likely that the discrepancy in the hamster constants is related to an inherent limitation of the gas uptake procedures.

Since the gas uptake technique depends upon measuring overall rates of loss of CH_2Cl_2 from the chamber (Gargas and Andersen, 1988), uncertainty in the values of the estimated rate constants increases when one of the two pathways is much more active than the other. In the mouse, the ratio of GST to MFO activity, measured at substrate concentrations of 6.7 and 5.0 mM CH_2Cl_2, respectively, was 0.420 (Table 2). In contrast, the ratio of GST and MFO activities in rats dropped to 0.183 and the ratio in hamsters was only 0.013. Clearly the values for K_F estimated from gas uptake studies in mice will be the most reliable, the values estimated from gas uptake studies in rats somewhat less reliable, and the values for K_F in the hamster studies will contain the highest levels of uncertainty. Based on these considerations, it seems clear that the B6C3F1 mouse data provide the most logical reference point for *in vivo* KF calculations.

On the other hand, since MFO plays a significant role in total metabolism of CH_2Cl_2 in all the species, the gas uptake studies would be expected to give a reasonably accurate approximation of the *in vivo* activity of this pathway in all the species studied: B6C3F1 mice, F344 rats, and Syrian Golden hamsters.

Distribution of GST Metabolism between Lung and Liver

Andersen *et al.* (1987) estimated the total rates of MFO and GST metabolism (in all tis-

sues) from their gas uptake studies. To estimate the proportion of this metabolism which occurred in the two target tissues (lung and liver), they used data generated by Lorenz *et al.* (1984). Lorenz *et al.* measured GST and MFO activity in lung and liver tissues of mice, rats, hamsters, and humans with model substrates (2,5-dinitrochlorobenzene for GST and 7-ethoxycoumarin for MFO). Andersen *et al.* assumed that the relative enzyme activity in each tissue with CH_2Cl_2 as a substrate would be the same as the relative activity reported by Lorenz *et al.* with model substrates when they constructed their PB-PK model.

The *in vitro* data which we have collected with CH_2Cl_2 as a substrate in lung and liver preparations may now be used to calculate the relative activities of GST in the two tissues directly. The ratios of specific activities for GST in lung/liver are 0.28 (mice), 0.14 (rats), and 0.18 (humans) (Table 1, column 4). These numbers are approximately twice as high as the values reported by Lorenz *et al.* (1984) for mice (0.14), rats (0.056), hamsters (0.077), and humans (0.047).

Similarly, the distribution of MFO activities between lung and liver tissues may be estimated from activities observed at 5 mM CH_2Cl_2 (Table 2). The ratios calculated for the three animal species were 0.405 (mouse), 0.039 (rat), and 0.068 (hamster). Distribution of activity in humans could not be estimated because activity was not observed in human lung, but the ratio can safely be assumed to be very low. These results are in good agreement with the distribution estimated from the data of Lorenz *et al.* for mouse (0.416), hamster (0.064), and human (0.001), but are considerably lower than the ratio Lorenz reported for the rat (0.136). The reason for the discrepancy in the rat data is not clear.

Effect of Changing KF on MFO Parameters

The gas uptake experiments of Andersen *et al.* (1987) measure total metabolism in the animal (from both the GST and MFO pathways). To maintain mass balance, a decrease in the rate of GST metabolism needs to be balanced by a corresponding increase in MFO metabolism. However, since GST is a small fraction of total metabolism in the rat and hamster (Table 2), the changes in MFO parameters should be minimal. To verify this, the gas uptake data of Andersen *et al.* (1987) were reanalyzed. The value of K_F was fixed at that calculated from the *in vitro* data, and the values of V_{max} and K_m were adjusted to give the best possible fit of the gas uptake data. Optimizations were conducted with the integrated software package SimuSolv.[2] After optimization, the value of V_{max} had risen from 1.50 to 1.73 in the rat, and had changed from 2.05 to 2.16 in the hamster. Similarly, the values of K_m changed from 0.77 to 0.89 in the rat, and from 0.65 to 0.82 in the hamster.

Calculation of Internal Dose with the New Constants

The new values of K_F, V_{max}, and K_m and the new ratios of GST and MFO activity in lung versus liver (except for human MFO activity, where the ratio calculated from Lorenz's data was used) were inserted into the PB-PK model of Andersen *et al.* (1987) and the "tissue doses" of reactive metabolites (defined as the average rate of production of GST metabolites during a 24-hr period divided by the volume of the tissue) associated with selected exposures to CH_2Cl_2 in B6C3F1 mice, F344 rats, Syrian Golden hamsters, and humans were calculated. These tissue doses are listed in Table 6, along with the values of the tissue doses originally calculated by Andersen *et al.* (1987).

The calculated values of the tissue doses (using the new constants) in the various species correlate well with the observed sensitiv-

[2] Registered trademark of the Dow Chemical Company, SimuSolv is available from Mitchell and Gauthier Associates, 73 Junction Square Drive, Concord, MA.

TABLE 6

CALCULATION OF THE IMPACT OF THE NEW PARAMETERS OBTAINED FROM *IN VITRO* STUDIES (K_F, THE FIRST-ORDER RATE CONSTANT FOR THE GST PATHWAY, AND A_1 AND A_2, THE RATIOS OF ENZYME ACTIVITY IN LUNG AND LIVER TISSUE) UPON CALCULATION OF INTERNAL DOSE WITH THE PB-PK MODEL OF ANDERSEN *ET AL.* (1987)

	Concentration (ppm)	GST	
		Lung	Liver
	New constants		
Mouse	4000	5.22×10^{2}	1.81×10^{3}
Rat	4000	9.59×10^{1}	6.76×10^{2}
Hamster	3500	2.73×10^{1}	1.67×10^{2}
Human[a]	1	4.06×10^{-3}	6.53×10^{-3}
	Original constants		
Mouse	4000	2.55×10^{2}	1.81×10^{3}
Rat	4000	1.28×10^{2}	2.24×10^{3}
Hamster	3500	1.20×10^{2}	1.50×10^{3}
Human	1	1.31×10^{-3}	8.04×10^{-3}

Note. Concentrations are in ppm, and dose surrogates for GST in the lung and liver are in mg metabolite/liter tissue/day (adjusted for 5 days of exposure/week).

[a] MFO activity could not be detected in human lung, so the distribution of MFO activity between lung and liver was based on the A1 constant calculated from the data of Lorenz *et al.* (1984).

ity of those species in long-term bioassays. Mice developed high incidences of malignant tumors of the lung and liver when exposed to CH_2Cl_2 (NTP, 1985) but hamsters exposed to equivalent concentrations of CH_2Cl_2 failed to show a tumorigenic response at any site (Burek *et al.*, 1984). Rats were intermediate between these two species in the levels of GST metabolites produced in lung and liver, and rats exposed to equivalent levels of CH_2Cl_2 did not have increased incidences of tumors in the lung or liver, although increases in the rate of development of nonmalignant mammary tumors were observed in two studies (Burek *et al.*, 1984; NTP, 1985).

The predicted average daily production of GST metabolites in the target tissues (the "tissue dose") of humans exposed to 1 ppm of CH_2Cl_2 for 6 hr/day, 5 days/week, was 129,000- to 280,000-fold lower than observed in the target tissues of B6C3F1 mice exposed to 4000 ppm (Table 6). Two factors are involved in the more than proportional drop in internal liver dose when extrapolating from mice (at 4000 ppm) to humans (at 1 ppm): (1) At 1 ppm most of the CH_2Cl_2 is metabolized by MFO because this enzyme has a higher affinity for CH_2Cl_2 than GST. At 4000 ppm, MFO has "saturated" so that disproportionate amounts of CH_2Cl_2 are available for metabolism by the lower-affinity GST enzyme. (2) Levels of GST enzymes are much lower in human tissues than in mice.

In the case of lung tissue, competition between the two enzymes for CH_2Cl_2 is less important than absolute levels of GST enzymes. In fact, since MFO activity in human lung is so low, setting human lung MFO activity to zero in the PB-PK model only increased the predicted dose of GST metabolites at 1 ppm by 1%.

As pointed out earlier by Andersen *et al.* (1987), failure to consider these factors when performing carcinogenic risk estimations can lead to serious overestimations of risk. For example, the internal dose in humans exposed to 1 ppm CH_2Cl_2 for 6 hr/day, 5 days/week, calculated by the PB-PK model with the original constants of Andersen *et al.* was 144- to 167-fold lower for lung and liver, respectively, than estimated by Singh *et al.* (1985). Substituting the new model parameters and making the same type of comparison, the difference between the procedure of Singh *et al.* and Andersen *et al.* was nearly the same: 95-fold lower for lung tissue and 204-fold lower for liver tissue.

SUMMARY

The new *in vitro* data generated in these experiments provide independent support for the PB-PK model for CH_2Cl_2 formulated by Andersen *et al.* (1987). The data are consis-

tent with the hypothesis that the rate of activation of CH_2Cl_2 to toxic metabolites by the GST pathway occurs much more slowly in humans than in the B6C3F1 mouse, and suggests that conventional risk estimation procedures, such as that of Singh *et al.* (1985), may greatly overestimate the likelihood that CH_2Cl_2 will cause tumors in humans.

Although there are undoubtedly many other crucial factors that must be considered in chemical carcinogenesis, quantitative analysis of the delivery of parent chemical and its metabolites to target tissues should provide a more accurate basis for risk assessment than the empirical procedures now used in conventional risk analysis.

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