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Maternal-Fetal Pharmacokinetics of Methanol

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate matter), and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 150 projects at institutions in North America and Europe.

Typically, HEI receives half its funds from the U.S. Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the United States. Occasionally, funds from other public or private organizations either support special projects or provide resources for a portion of an HEI study. This study was part of a larger methanol program that received support from the American Petroleum Institute. However, in all cases HEI exercises complete autonomy in setting its research priorities and in reaching its conclusions. An independent Board of Directors governs HEI. The Institute's Research and Review Committees serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded studies are made available as Research Reports, which contain both the Investigators' Report and the Review Committee's evaluation of the work's scientific quality and regulatory relevance.

HEI Statement

Synopsis of Report Number 74

Methanol Distribution in NonPregnant and Pregnant Rodents

BACKGROUND

Many urban locales remain unable to meet federal air quality standards for ozone, in part because motor vehicles produce large amounts of ozone-forming hydrocarbons. The Clean Alternative Fuels Program was initiated in the 1980s to encourage the development of new fuels; in 1990, methanol was designated as a clean alternative fuel by the Clean Air Act Amendments. The proposed use of methanol as an alternative motor vehicle fuel could have remedial effects on pollution by decreasing some hydrocarbon emissions and thereby potentially reducing atmospheric ozone concentrations. As a result, its use could help address the continued inability of many urban areas of the United States to meet air quality standards.

If methanol is used as an alternative fuel, humans will be exposed to increased levels of methanol vapors in evaporative and tailpipe emissions. Although methanol is clearly poisonous when ingested at relatively high levels, projections suggest that humans who inhale the low concentrations of methanol vapors expected to be emitted from motor vehicles will experience little risk of toxicity. However, inhaling low levels of methanol vapors could pose health risks for potentially susceptible populations. Because of the known effects of ethanol on developing fetuses, this population is one that is considered to be potentially susceptible to the neurotoxic effects of methanol. In fact, some animal studies have shown that exposure to high concentrations of methanol (5,000 to 20,000 ppm) can have negative effects on fetal development. In order to evaluate the possible risks of methanol exposure for developing fetuses, the HEI funded a study described in this report that was designed to determine the relationship between methanol exposure and its uptake into and elimination from the blood of nonpregnant and pregnant rodents. Because fetal toxicity cannot be studied directly in humans, the information obtained in this study will be helpful for extrapolating the effects seen in rodents to those anticipated in humans who are exposed to low environmental levels of methanol and for assessing the consequential risks associated with exposure.

APPROACH

Drs. Pollack and Brouwer exposed rats and mice at several different stages of gestation to methanol intravenously or orally (doses ranged from 100 mg/kg of body weight to 2,500 mg/kg) or by inhalation (1,000 to 20,000 ppm for 8 hours). They measured blood, urine, and amniotic fluid concentrations of methanol and used the data to develop a model of methanol distribution in rodents. It should be noted that the lowest inhalation exposure used in this study (1,000 ppm for 8 hours) was significantly higher than those predicted for ambient exposures resulting from the use of methanol fuels (1 to 10 ppm in typical traffic situations, and as high as 200 ppm in a worst-case scenario such as a malfunctioning vehicle in an enclosed garage).

RESULTS AND IMPLICATIONS

During the inhalation exposures, the investigators found that the rate of methanol accumulation in the mouse was two to three times greater than in the rat. This was true in spite of fact that the mouse eliminates methanol from its bloodstream twice as fast as the rat. The investigators hypothesized that this difference between rats and mice was due to more rapid breathing in the mouse, and to the apparently complete absorption of methanol in the nasal cavity of this rodent species. They also found that the uptake and elimination of methanol was virtually unaffected by pregnancy, and that fetal methanol concentrations were approximately the same as in the mother.

Using their results, the investigators constructed a model that describes the uptake and elimination of methanol in rats and mice. This is a critical step in being able to make the interspecies extrapolations necessary for risk assessment. However, the differences seen between these two closely related species indicate that the use of this rodent model to extrapolate to humans could be difficult. As a result, the usefulness of the pharmacokinetic model developed for high-level exposures in rodents may be limited when predicting the effects of the lower ambient exposures on humans. Therefore, validation of this model at lower exposure levels is needed.

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of a research project sponsored by HEI from 1990 to 1994. This study was conducted by Drs. Gary M. Pollack and Kim L. R. Brouwer of the University of North Carolina at Chapel Hill, Chapel Hill, NC. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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ABSTRACT

We undertook the present project to elucidate the physiologic factors that govern methanol delivery to the developing conceptus after maternal methanol exposure, and to develop a physiologically based toxicokinetic model to describe methanol disposition in pregnancy. A multiexperimental approach addressed the goals of this project. Initial experiments characterized the systemic disposition of methanol after intravenous or oral administration to nonpregnant female rats. Methanol absorption from the gastrointestinal tract was rapid (peak concentrations appeared within 1 to 2 hours after administration) and essentially complete (systemic bioavailabilities ranged from approximately 0.6 to 1.0). As anticipated for short-chain aliphatic alcohols, methanol elimination from the systemic circulation was nonlinear due to saturation of the metabolic route or routes responsible for converting methanol to formaldehyde and, ultimately, formic acid. However, a significant parallel linear route of methanol elimination was observed, which accounted for an increasingly significant fraction of total elimination as methanol doses (or systemic concentrations) increased.

The disposition of methanol after oral or intravenous administration was similar in pregnant and nonpregnant female rats, regardless of the gestational stage (day 7, 14, or 20 after conception) at which the toxicokinetics of methanol were examined. This observation indicated that data from nonpregnant subjects could be used in the development of the maternal portion of a comprehensive physiologic model for methanol disposition. Parallel experiments in female mice indicated that methanol elimination was approximately twice as rapid in mice as in rats due to a significantly higher maximal velocity for methanol metabolism in the smaller rodent species. As was the case in the rat, relatively small changes in methanol elimination were observed during the course of gestation in pregnant mice. In both species, the rate of methanol metabolism by fetal liver in vitro was less than 10% that of the metabolic rate in adult liver. The kinetics of methanol delivery into the fetal environment were examined by determining amniotic fluid concentrations of methanol after intravenous administration to pregnant rats. The net rate of methanol translocation from maternal blood to amniotic fluid decreased as methanol concentration increased. Although the mechanism of this anomalous result is unknown, it possibly is due to a methanol-induced decrease in blood flow to the fetus.

A series of experiments was performed to examine the rate and extent of methanol absorption during inhalation exposure at methanol concentrations that have been shown previously to be teratogenic in rats and mice. An apparatus was constructed for whole-body, flow-through exposure of rodents to vapor-phase methanol with simultaneous determination of methanol concentration in air and blood, breathing frequency, and minute volume in individual animals. These experiments revealed that methanol absorption in the rat was mediated entirely in the nose of the animal, and that the extent of methanol absorption decreased with increasing exposure concentration. This unanticipated nonlinear absorption appeared to be a function of the limited aqueous space available for methanol partitioning in the lining of the nose, as well as a concentration-dependent depression of respiratory function by methanol. Moreover, methanol absorption from the airstream was significantly higher in the mouse than in the rat. Despite the approximately twofold more rapid elimination of the alcohol in the mouse, blood methanol concentrations after inhalation exposure were two- to threefold higher in the mouse than in the rat due to differences in inhalation absorption. By incorporating relationships between respiratory parameters, absorption efficiency, and methanol concentration in the airstream, and including the toxicokinetic parameters developed after intravenous administration of the alcohol to both species, we developed a toxicokinetic model capa-

This Investigators' Report is one part of Health Effects Institute Research Report Number 74, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Gary M. Pollack, University of North Carolina at Chapel Hill, School of Pharmacy, Beard Hall CB# 7360, Chapel Hill, NC 27599-7360.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Agreement 816285 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

ble of describing methanol disposition in both the rat and the mouse over a wide range of inhalation exposure conditions. Work continues to determine the degree to which methanol disposition in humans can be predicted by this model.

INTRODUCTION

Incorporation of methanol into fuels for internal combustion engines is likely to increase over the next decade due to the low levels of criteria pollutants produced by methanol-fueled vehicles (Heath 1989), which will raise the risk of significant human exposure to this compound. When neat methanol is used as a fuel, atmospheric methanol concentrations up to 400 parts per million (ppm)* have been estimated in enclosed garages and service stations (Gold and Moulif 1988), which suggests that significant exposure of individuals refueling or operating motor vehicles may occur. Occupational methanol exposures in excess of 200 ppm over an 8-hour shift result in blood methanol concentrations as high as 50 μg/mL (Heinrich and Angerer 1982).

The toxic effects of acute exposure to high methanol doses are well established (Roe 1982; Kavet and Nauss 1990). Systemic accumulation of formate, which occurs in primates but not most other mammalian species, is responsible for the acidosis and amblyopia observed in acute methanol toxicity (Black et al. 1985); the parent compound itself accounts for depression of the central nervous and cardiovascular systems (DeFelice et al. 1976). However, the current interest in methanol toxicity and the development of a rational risk assessment is related primarily to inhalation exposure to relatively low atmospheric concentrations of methanol. Little is known regarding the biologic effects of chronic, low-level methanol exposure, including effects on subpopulations that may be at risk, particularly the developing embryo. The adverse effects of ethanol on embryonic development, including organ malformation and central nervous system dysfunction, have been investigated (Streissguth et al. 1980); limited experimentation indicates that chronic exposure of pregnant rodents to methanol also affects the developing conceptus (Nelson et al. 1985; Rogers et al. 1990, 1993; Bolon et al. 1993, 1994). In addition to structural defects, some data suggest that prenatal methanol exposure may induce behavioral abnormalities early in life unaccompanied by overt structural or biochemical changes (Infurna and Weiss 1986).

Exposure of pregnant rats to very high atmospheric methanol concentrations (20,000 ppm) for 7 hr/day on gestational days 1 through 19 resulted in a significant incidence of congenital abnormalities (Nelson et al. 1985). Mice appeared more sensitive to the developmental effects of methanol, with a significant incidence of severe teratogenic effects (cleft palate and exencephaly) at inhaled concentrations of 5000 ppm (Rogers et al. 1990). The mechanism or mechanisms underlying the apparently greater sensitivity of the mouse conceptus to methanol has not been addressed, but may be related to toxicokinetic differences between species (e.g., different degrees of embryonic exposure at a similar magnitude of maternal exposure, due either to differences in disposition within the maternal circulation or in the kinetics of translocation between the maternal circulation and the embryonic environment). Despite numerous reports concerning methanol-induced toxicity, little information exists regarding the in vivo disposition kinetics of methanol. The available data suggest that methanol elimination from the systemic circulation, like that of ethanol, is capacity-limited (i.e., saturable) in both rats (Tephly et al. 1964) and humans (McMartin et al. 1980). The primary enzymatic pathway that catalyzes methanol metabolism differs between the two species (alcohol dehydrogenase in humans vs. the catalase-peroxidase system in rats (Goodman and Tephly 1970). Although the biochemistry underlying methanol oxidation is well defined, the relationship between methanol exposure and systemic disposition has not been examined. In particular, the accuracy of extrapolating the methanol disposition profile needs to be addressed for assessing the risk associated with chronic exposure to low environmental methanol levels.

A recent study (Dorman et al. 1994) was conducted to examine the disposition of methanol and derived formate in the female cynomolgous monkey. ^{14}C-Labeled methanol was administered via an endotracheal tube and a nonrebreathing valve at 10 to 900 ppm for 2 hours. The 900-ppm methanol exposures were conducted under normal and folate-deficient conditions in a single group of animals. In animals with normal folate status, end-of-exposure blood methanol (0.3 to 300 μM) varied linearly with exposure concentration, consistent with the low systemic methanol concentrations relative to the K_m for methanol metabolism (approximately 1000 μM). Systemic methanol concentrations were elevated approximately twofold in the folate-deficient state. ^{14}C-Methanol–derived folate was 1 to 3 orders of magnitude lower than endogenous blood formate, and suggests that short-term methanol exposures at even moderate concentrations do not result in significant changes in circulating formate.

In general, toxicokinetics deals with the absorption, distribution, and elimination of xenobiotics. Comprehensive characterization of the disposition kinetics of a xenobiotic

* A list of abbreviations appears at the end of the Investigators' Report.

is necessary to relate the administered dose, through the time course of systemic exposure, to exposure of the target organ to the toxicant. Knowledge of this time course (frequently termed molecular dosimetry) for different species is fundamental to the interspecies extrapolation necessary for most toxicologic risk assessments (Andersen et al. 1987; Klaassen and Eaton 1991). For solid- or liquid-phase xenobiotics, the dose administered to experimental subjects is expressed readily as the mass administered via a particular route. For the inhalation of gaseous or vapor-phase xenobiotics, the most common route of exposure for environmentally relevant toxicants, the dose administered is not easily determined, even in controlled laboratory experiments. The ambient concentration of the xenobiotic in an exposure chamber can be measured, and, if the chamber is well stirred, the concentration of inhaled toxicant can be approximated by the ambient chamber concentration. The dose actually administered to the experimental subject, however, is not simply the concentration inhaled, but the difference between the concentration inhaled and the concentration exhaled multiplied by the volume of each breath (the tidal volume, V_T) summed over the experimental period. Both the exhaled concentration and V_T are difficult to measure, particularly in unrestrained subjects.

The model of inhalation toxicokinetics developed for styrene (Ramsey and Andersen 1984) addressed many of the problems associated with comprehensive characterization of xenobiotic disposition during inhalation exposure. The styrene model incorporated the minute ventilation ($\dot{V}$, the product of V_T and the breathing frequency, f) values obtained from published estimates in the physiology literature. In rodents, 70% of $\dot{V}$ is assumed to be the effective portion of V_T that reaches the alveoli per unit of time; the majority of pulmonary absorption of xenobiotic is assumed to occur at this site. The remaining 30% of V_T is dead space in the respiratory tract that does not contribute significantly to absorption. The model also is based on the assumption that equilibration of the inhaled xenobiotic between alveolar air and arterial blood equals the in vitro blood:air partition coefficient, which obviates the need to determine exhaled concentrations. Although the styrene model has proven useful for many nonpolar vapor-phase toxicants, it has not yielded consistent results for polar xenobiotics such as methanol and acetone (Gargas et al. 1993). It predicts that highly water-soluble compounds (i.e., xenobiotics with a high blood:air partition coefficient) would be 100% absorbed into arterial blood. However, complete absorption clearly does not occur for several polar xenobiotics (Gargas et al. 1993), which suggests that the structure of this model (or the underlying assumptions upon which the model was developed) may not be appropriate for such compounds.

We undertook the present project to elucidate the factors that govern methanol delivery to the developing rodent conceptus after maternal exposure to the alcohol, and to develop a physiologically based pharmacokinetic model to describe and predict methanol disposition during pregnancy. Initial experiments sought to define the systemic disposition of methanol in rats and mice after either intravenous or oral administration. We also examined the influence of pregnancy on methanol disposition in rats after intravenous and oral administration. The kinetics of methanol delivery from the maternal circulation to the fetus was examined with intrauterine microdialysis to elucidate the relationship between maternal blood concentrations and the kinetics of methanol penetration into the fetal environment. We constructed an inhalation exposure apparatus to allow characterization of methanol concentrations in the airstream (both inlet and outlet) during exposure experiments in rats and mice, with simultaneous determination of blood methanol concentrations and respiratory function. This apparatus was used to examine several fundamental aspects of methanol disposition during inhalation exposure in rats and mice, including the influence of blood methanol concentration on respiratory function, the relationship between methanol exposure concentration and the efficiency of methanol absorption in the respiratory tract, and the influence of changes in minute volume on methanol absorption. Additional experiments identified the site of methanol absorption within the respiratory tract. We used data generated in these experiments to develop a physiologically based pharmacokinetic model for methanol disposition during inhalation exposure. This model was used to identify potential species-dependent differences in methanol absorption and elimination between the rat and the mouse in an effort to explain differences in the incidence of methanol-induced teratogenicity between the two species.

SPECIFIC AIMS

The long-term goal of this research effort is to predict the potential risk to a human conceptus of maternal exposure to environmental methanol. The specific aims of the present project were to:

1. *Characterize the systemic disposition of methanol in rats and mice.* The predominant component of any physiologically based model of xenobiotic disposition in pregnancy is the maternal system. Thus, it is important to determine the disposition of the agent in question in female animals at various stages of gestation. Both the intravenous and oral routes of administration examined the toxicokinetics of methanol in nonpregnant and pregnant female rats and mice at several gestational stages (7, 14, and 20 days after conception in rats; 9 and 18 days after conception in mice).
2. *Examine the kinetics of methanol delivery to the conceptus.* If methanol-induced teratogenicity is concentration-dependent, predictive models of teratogenic risk must incorporate methanol concentrations at the target site. Microdialysis sampling from amniotic fluid after intravenous administration of methanol to the dam was used to characterize the kinetics of methanol delivery to the fetus.
3. *Elucidate the kinetics of methanol absorption during inhalation exposure.* From an environmental standpoint, the most relevant route of exposure to fuel-based methanol is inhalation. A comprehensive model of methanol disposition must, therefore, incorporate both the site and kinetics of absorption from the airstream, as well as any potential effects of the alcohol on pulmonary function. We examined the uptake of vapor-phase methanol during whole-body exposure of rats and mice in flow-through chambers that allowed estimation of both methanol concentrations and respiratory parameters.
4. *Construct a physiologically based kinetic model capable of predicting methanol disposition in rodents.* Animal-to-human extrapolation of xenobiotic disposition must be performed as part of the risk assessment process. If differences in methanol teratogenicity between species, as have been observed for rats and mice, have a kinetic basis, it is imperative to develop a physiologically relevant model that can describe methanol disposition in both species based upon parameters that can be measured objectively. Data from the experiments performed to address Specific Aims 1 through 3 above were used in the construction of an inhalation model of methanol absorption and disposition.

METHODS AND STUDY DESIGN

A multiexperimental approach addressed the specific aims of this project, which was conducted in six Phases. Many of the methods employed were common to several experiments in different Phases, and are described in the General Methods section. Aspects of study design and conduct specific to particular experiments are described separately.

GENERAL METHODS

Chemicals

Pesticide-grade methanol, for administration to rats and for construction of analytical standards, and acetonitrile, which served as an internal standard for the methanol assay, were obtained from Fisher Chemical Co. (Raleigh, NC). A solution of methanol in normal saline was prepared such that the total volume administered would be 2 mL/kg body weight; the concentration of methanol in saline varied with the dose to be administered. In all experiments, preservative-free heparin (Sigma Chemical Co., St. Louis, MO) was used to maintain cannula patency or inhibit coagulation or both in blood samples. Initial experiments revealed that benzyl alcohol, a preservative used to formulate aqueous solutions of heparin, coeluted with methanol in the gas chromatography assay. All other reagents were obtained commercially, and were of the highest available purity.

Animals

Female Sprague-Dawley rats (Hilltop Laboratory Animals, Scottdale, PA), 250 to 350 g at the start of the experimental period, and female CD-1 mice (Charles River Laboratories, Raleigh, NC), 25 to 35 g at the start of the experimental period, were used in all experiments. All animals were allowed to acclimate to the animal facility in the School of Pharmacy, The University of North Carolina at Chapel Hill, for at least one week prior to experimentation. Mice were housed (4 per cage) on heat-sterilized hardwood-chip bedding (Beta-Chip, Warrington, NY), and were maintained on a 12-hour light/dark cycle with food and water available ad libitum. Rats were housed individually in wire cages under conditions identical to those for mice.

Some animals were used as nonpregnant controls; the remainder were housed with breeder males from the same supplier and were examined daily for vaginal plugs. The presence of a vaginal plug was defined as Gestational Day 0. Methanol disposition in pregnant animals was examined on Gestational Days 7, 14, and 20 (rats) or Gestational Days 9 and 18 (mice) in separate groups.

For experiments designed to examine methanol disposition in rats, animals were anesthetized with ether one day prior to the experiment and a silicone rubber cannula was implanted in the right jugular vein. Additional cannulae were required for some experimental protocols (a carotid artery cannula for determination of transpulmonary extrac-

tion; a femoral vein cannula for continuous methanol infusion). These cannulae were implanted when the jugular vein cannula was placed. Small-bore (0.012" i.d.) silicone rubber cannulae also were inserted into the right jugular veins, of ether-anesthetized mice in some experiments (see individual experimental protocols below). All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of The University of North Carolina at Chapel Hill.

Methanol Analysis

Methanol concentrations in whole blood or urine were determined by gas chromatography with flame ionization detection (Pollack and Kawagoe 1991). Aliquots of rat whole blood (200 μL) were added to 100 μL water containing acetonitrile (0.3 μL/mL) and heparin (200 U/mL) in 0.5-mL polypropylene centrifuge tubes. The tubes were maintained in an ice water bath during the entire preparation procedure to prevent evaporative loss of both methanol and acetonitrile, and were vigorously mixed by vortex immediately following addition of blood to lyse erythrocytes. Proteins in whole blood were precipitated by the addition of zinc sulfate ($ZnSO_4$) (10% in water, 100 μL) with vigorous mixing, followed by addition of sodium hydroxide (NaOH) (0.5 N, 100 μL), again with vigorous mixing. The tubes were centrifuged for 1 minute and cooled on ice. Sample supernatants were collected and transferred to tightly sealed injection vials with Teflon-lined septa. Aliquots (1 μL) of these samples were injected on-column for analysis. Chromatography was conducted on a Shimadzu GC-14A gas chromatograph (Shimadzu Scientific, Norcross, GA) equipped with a flame ionization detector, on-column capillary injector, autosampler, and reporting integrator. Chromatographic separation of methanol and acetonitrile (the internal standard for the assay) from contaminants in prepared whole blood was achieved on a 15 m × 0.54 mm (i.d.) wide-bore fused-silica capillary column, with Carbowax (1.2 μm film thickness) as the bonded stationary phase (Alltech Associates, Deerfield, IL). The carrier gas (helium) was delivered to the column at a rate of 12 mL/min, which produced a back pressure of 0.25 kg/cm^2. Hydrogen and air were delivered to the flame ionization detector at 55 mL/min and 450 mL/min, respectively. Isothermal chromatography was conducted at a column oven temperature of 35°C; injector and detector temperatures were maintained at 120°C. Peak area ratios (methanol to acetonitrile) were determined by the reporting integrator. Following the analysis of each sample (4 minutes after injection) the column oven temperature was elevated rapidly (40°C/min) to a final temperature of 100°C to remove water and endogenous contaminants from the column. The column oven was maintained at the elevated temperature for 3 minutes prior to cooling for subsequent analysis of samples. The assay was linear to 10,000 μg/mL, with a detection limit of 2 μg/mL. The intraday (< 2.5%) and interday (< 5%) coefficients of variation of the assay were independent of concentration.

STUDY DESIGN

Phase I. Systemic Disposition of Methanol in Nonpregnant Rats

Intravenous Administration To characterize the systemic disposition of methanol in the absence of potential influences of pregnancy, methanol was administered as a single intravenous dose to separate groups of nonpregnant rats. Each animal (n = 5 per group) received a single bolus dose (100 or 500 mg/kg) or hand-injected 1.5-minute infusion (2,500 mg/kg) of methanol in saline. All doses were followed immediately by a 0.3-mL saline flush to purge the dosing solution from the cannulae. A separate group of rats, with both jugular vein and carotid artery cannulae, was used to determine the degree of transpulmonary extraction. These rats received a 250-mg/kg dose of methanol injected as an intravenous bolus (n = 4), and blood samples (0.1 mL each) were withdrawn from the jugular vein cannula (or simultaneously from the jugular vein and carotid artery cannulae) at timed intervals for up to 72 hours. Samples were added immediately to tightly capped 0.5-mL polypropylene microcentrifuge tubes containing 0.1 mL ice-cold heparinized (100 U/mL) water to lyse erythrocytes and inhibit blood coagulation. Acetonitrile (50 μg/mL) in the water served as an internal standard for subsequent methanol analysis, as well as a marker of potential evaporative loss during sample storage. All blood samples were stored frozen (–20°C) within 30 minutes of withdrawal, and methanol concentrations were quantitated within 48 hours.

Renal Clearance of Methanol A separate experiment was conducted to assess the contribution of renal excretion to total elimination of methanol. Rats (n = 4) were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (12 mg/kg), and received 50% booster doses as required throughout the course of the experiment. The right jugular vein, urinary bladder, and urethra were cannulated, and rectal temperature was maintained at 37°C with a temperature-controlled heating pad. Each animal received three methanol bolus doses (100, 400, and 2000 mg/kg) through the jugular vein cannula at 1-hour intervals. Blood samples were withdrawn just before, and 20 minutes after, each dose. Urine was obtained immediately prior to each dose by flushing the bladder with 1 mL normal saline followed by 1 mL air, and collecting the effluent from the

urethral cannula. Urine samples were collected in ice-cooled preweighed microcentrifuge tubes covered tightly with parafilm to prevent methanol evaporation. Immediately upon collection, a 100-μL aliquot of urine was treated in a manner identical to that described for blood samples.

Oral Administration Because the oral route represents a significant possible mode of exposure to methanol, additional experiments were conducted to examine the disposition kinetics of methanol after oral administration of a low (100 mg/kg, $n = 6$) or high (2,500 mg/kg, $n = 5$) dose. The intermediate dose used in the intravenous experiment was not included because methanol disposition following administration of either 100 mg/kg or 500 mg/kg could be described with a unique set of toxicokinetic parameters. Methanol was administered by gavage as a solution in saline, and timed blood samples (0.1 mL each) were obtained from the jugular vein cannula for up to 72 hours after administration, depending on the dose level examined.

Phase II. Influence of Pregnancy on Methanol Disposition in the Rat

Intravenous Administration Methanol concentrations in the maternal circulation serve as the driving force for methanol delivery to the developing conceptus. Changes in the maternal disposition of methanol during gestation could alter the rate and/or extent of methanol penetration into the fetal environment, thereby altering methanol concentrations in the target organ in a time-dependent manner. We undertook this experiment to determine whether the systemic kinetics (specifically distribution and elimination processes) of methanol are altered in the course of pregnancy. Because reliable kinetic parameters were obtained in nonpregnant rats in the first phase of this project, only pregnant animals were examined in the present experiment. Methanol disposition in timed pregnant rats was examined at one of three gestational stages: day 7, 14, or 20. Day 0 was the first day a vaginal plug was present. Each animal received either a 100-mg/kg intravenous bolus dose of methanol or a 2500-mg/kg methanol infusion (1.5 minutes) through the jugular vein cannula, followed by a 0.3-mL saline flush, as described above for nonpregnant rats. The 500-mg/kg dose was omitted because the experiment in nonpregnant animals showed that a unique set of toxicokinetic parameters could describe methanol disposition after either the 100-mg/kg or the 500-mg/kg dose. Timed blood samples (0.1 mL) were withdrawn from the cannula for up to 72 hours after methanol administration.

Oral Administration Since ingestion by mouth may represent a significant route of exposure to fuel-based methanol (either from fuel per se or through contamination of ground water), this experiment assessed the influence of pregnancy on methanol disposition after oral administration of the alcohol. This experiment is complementary to the experiment conducted in Phase I of this project that examined the disposition of orally administered methanol in nonpregnant rats, and parallels the intravenous administration experiment in rationale and interpretation. As was the case for intravenous administration, sufficient kinetic data were generated in earlier experiments to obviate the need to examine methanol disposition in nonpregnant animals. Timed pregnant Sprague-Dawley rats were examined at one of three gestational stages (Gestational Day 7, 14, or 20) as described in the preceding experiment. We administered methanol (100 mg/kg or 2500 mg/kg) as a solution in saline by gavage. Timed blood samples were obtained from the jugular vein cannulae for 72 hours after administration.

Phase III. Systemic Disposition of Methanol in the Mouse

Intravenous Administration As discussed in the Introduction, previous work in other laboratories indicates that the developing mouse conceptus may be more susceptible to the teratogenic effects of methanol than the rat conceptus. One possible explanation of this phenomenon is that the systemic disposition of methanol in the mouse differs from that in the rat. To assess the potential species differences in methanol toxicokinetics, the methanol concentration-time profile was examined in mice after intravenous administration. Each mouse ($n = 4$ to 8 mice per time point) received a hand-injected 1-minute infusion (2500 mg/kg; 0.33 g/mL in normal saline) through an indwelling jugular vein cannula. Blood samples (50 μL each) were withdrawn from the cannulae at timed intervals up to 10 hours after infusion.

Oral Administration To examine potential species differences in the oral absorption of methanol, we performed two experiments in which mice received a single dose of the alcohol by gavage. In both experiments, the animals received a 2500-mg/kg dose of methanol (0.33 g/mL in saline). This dose was chosen to correspond with the highest dosage administered to rats in the preceding phases of this project. In the first experiment, mice were killed by decapitation at timed intervals up to 24 hours after methanol administration ($n = 3$ to 7 mice per time point). Following decapitation, we collected trunk blood in 1.5-mL polypropylene tubes, and removed a 100-μL aliquot immediately for methanol analysis. In a subsequent experiment, we withdrew blood samples sequentially through an indwelling jugular vein cannula to avoid potential contamination of the blood sample by methanol reflux from the gastrointestinal tract during decapitation and blood collection. In a parallel experiment, nonpregnant rats received a 2500-mg/kg dose of methanol by gavage, with blood sampling at timed intervals for up to 45 hours after administration.

Influence of Pregnancy To compare the effects of pregnancy on methanol disposition between rodent species, we examined the systemic kinetics of methanol in nonpregnant mice and in pregnant animals at Gestational Day 9 and 18. These gestational stages corresponded to midgestation and near-term pregnancy, respectively. Mice received a single 2500-mg/kg dose of methanol (0.33 g/mL in deionized water) by gavage, and were euthanized by decapitation at timed intervals up to 24 hours after methanol administration (n = 3 to 4 mice per time point per group). After decapitation, we collected trunk blood in 1.5-mL polypropylene tubes, immediately removed a 100-μL aliquot, and added it to a 0.65-mL tube containing ice-cold heparinized water as described above for samples of rat blood.

In Vitro Metabolism of Methanol in Rodent Liver We examined four groups of laboratory animals: adult mice (n = 4 groups of 5 mice each); adult rats (n = 4 rats); near-term (Gestational Day 18) pregnant mice (n = 4 groups of 5 dams each); and near-term (Gestational Day 20) pregnant rats (n = 4 dams). All animals were killed by decapitation and the adult livers were excised rapidly. In each pregnant animal, after the maternal liver was removed the uterus was opened, each fetus was decapitated, and the fetal liver was removed. We rinsed all livers immediately with ice-cold 0.15 M KCl, blotted them dry, and weighed them (adult rat livers were weighed individually; adult mouse livers and all fetal livers were weighed in groups). We prepared a 20% (w:v) homogenate in ice-cold 0.15 M KCl with a loose fitting glass hand homogenizer (Kontes Scientific Glassware, Vineland, NJ), and kept the homogenate on ice until use in metabolism studies (performed within 1 hour). We incubated liver homogenates (7.5 mL) in tightly capped 15-mL polypropylene tubes in a shaking water bath at 37°C with 0.1 M phosphate buffer (pH 7.4, 10 mL total volume), which contained the following as described by Tephly and coworkers (1961): nicotinamide (0.98 mg/mL); semicarbazide (1.67 mg/mL); $MgCl_2$ (0.38 mg/mL). After a 20-minute equilibration, methanol was added to the homogenate to produce initial concentrations of 5, 10, 50, 100, 500, or 1,000 μg/mL (livers from nonpregnant adults), 10, 100, and 1,000 μg/mL (livers from pregnant adults), or 100 or 1000 μg/mL (fetal livers). At 0 and 40 minutes after adding methanol, we removed tubes from the water bath and mixed them by vortex. We then transferred a 100-μL aliquot to a 0.65-mL polypropylene microcentrifuge tube on ice and immediately added 100 μL 10% $ZnSO_4$ and 100 μL 0.1 M NaOH to stop the metabolic reaction and precipitate proteins in the sample. After centrifugation, formaldehyde in the supernatant was quantitated as described below. Preliminary studies indicated that the rate of formation of formaldehyde was linear for at least 40 minutes.

We used a modification of a published procedure to determine the amount of formaldehyde produced by the in vitro preparation (Sawicki et al. 1961). We placed aqueous 3-methyl-2-benzothiazolone hydrazone (0.1%, 150 μL) and 150 μL of the centrifuged supernatant from the incubate in a glass test tube, mixed it by vortex, and allowed it to stand at room temperature for 30 minutes. We added aqueous ferric chloride (0.2%, 750 μL) and the mixture stood at room temperature for 5 minutes prior to dilution to 3 mL with acetone. We determined absorbance of visible light in samples and appropriate blanks at 635 nm. We prepared a standard curve when we added 80 μL of blank liver homogenate to 20 μL of an aqueous formaldehyde solution of varying concentration, and treated these standards identically to the samples. This assay was linear through formaldehyde concentrations of 5000 μg/mL, with a detection limit of 5 μg/mL. The intraday coefficient of variation of the assay was less than 5%, and was independent of formaldehyde concentration.

Phase IV. Methanol Delivery to the Fetal Environment

The effect of a xenobiotic on the developing conceptus is governed by the sensitivity to the agent or biotransformation products (or both), and the degree of exposure to the proximal toxicant. In turn, the magnitude of exposure in utero is determined by the kinetics of maternal exposure to the substrate, the ability of the maternal system to eliminate the substrate, the rate and extent of transfer of the substrate from the maternal circulation to the conceptus and its immediate environment, and the ability of the conceptus to either activate or detoxify the substrate. The present experiment assessed the maternal-fetal translocation of methanol, and obtained parameters required for the construction of a physiologically based pharmacokinetic model describing the disposition of methanol within the rat maternal-fetal unit. We anesthetized pregnant rats (Gestational Day 20) with urethane (1 g/kg by intraperitoneal injection); and maintained maternal body temperature at 37°C with a rectal temperature probe, temperature controller, and heating pad. We implanted silicone rubber cannulae in the right jugular vein (for collection of maternal blood samples) and right femoral vein (for methanol administration), exposed the uterus via a midline incision through the abdominal wall, and selected a single fetus in the middle of the uterine horn for microdialysis probe implantation. We inserted the microdialysis probe (CMA/10, 0.5 mm diameter) through a small puncture wound (made with a 26-gauge needle) in the uterine wall proximal to the head of the fetus, and secured it in place with a drop of cyanoacrylate glue. We replaced the uterus in the peritoneal cavity, closed the incision around the probe, and covered it with moist gauze.

We immediately initiated perfusion of the probe with normal saline (2 μL/min) upon probe implantation. Effluent collected from the probe for 10 minutes allowed equilibration of the system and provided blank microdialysate for analysis.

After the equilibration period, we injected methanol as a bolus into the femoral vein cannula (100 mg/kg or 500 mg/kg doses, $n = 3$ animals per dose level) or infused it continuously through the femoral vein cannula (100 mg/kg/hr or 1000 mg/kg/hr, 1 mL/hr total volume, $n = 3$ animals per infusion rate) for 5 hours. We obtained blood samples (200 μL) from the jugular vein cannula at 15-minute intervals and collected microdialysate quantitatively at 10-minute intervals for the duration of the experiment. At the end of the experiment, a single amniotic fluid sample was collected by aspiration to quantitate directly methanol in amniotic fluid. Whole blood and amniotic fluid samples were processed for analysis as described previously; microdialysate samples (20 μL) were added to 100 μL water containing acetonitrile (0.075 μg/mL as an internal standard) in autosampler vials. After mixing by vortex, we injected 1 μL on-column for analysis. We calculated the concentration of methanol in the fetal compartment from the methanol concentration in the microdialysate corrected for the efficiency with which the microdialysis probe recovered methanol. We estimated in vivo probe recovery efficiency as the ratio of methanol concentration in the final microdialysate sample to the methanol concentration in amniotic fluid at the end of the experiment. In vivo probe recoveries (approximately 35%) were in good agreement with the recovery of methanol from a normal saline solution in vitro at 37°C.

Phase V. Disposition of Methanol During Inhalation Exposure in Rats

Determination of Respiratory Parameters This experiment developed a method to expose unrestrained laboratory rodents to vapor-phase methanol while simultaneously measuring ventilatory parameters (breathing frequency and the minute volume, $\dot{V}$), determining methanol concentrations in the airstream, and collecting blood samples for subsequent quantitation of methanol. This experiment also allowed assessment of variation in ventilation uptake with both duration of exposure and exposure concentration.

The experiment used a rectangular 6.25-L exposure chamber with glass sides and bottom (Figure 1), and a removable polystyrene chamber lid with a one-piece, 0.25"-thick rubber gasket affixed to the outer perimeter. The lid had holes for gas, electrical, and instrument connections, and was clamped to the tank with four removable straps. The chimney for the cannula tether was made from a 30-cc syringe barrel attached in the center of the lid and capped with a rubber cork; the cannula was threaded through a small hole in the cork and sealed with stopcock grease. Copper screening protected the fan (Archer, providing air circulation at 3.5 ft^3/min) which was attached to the lid to direct the circulated air toward the center of the chamber. We trimmed and clamped a pressure transducer (Archer piezoelectric speaker element 273-091) into a 2" PVC pipe fitting that was threaded for 1/4" plastic tubing. The leads from the transducer were connected to a strip chart recorder (Fisher Recordall 5000) that was run at 0.01 volts full scale and 25 cm/min nominal chart speed. The actual paper speed was calibrated for each experiment.

$\dot{V}$ was calculated from the tidal volume (V_T) and the breathing frequency (f) by the method of Drorbaugh and Fenn (1955) as modified by Epstein and Epstein (1978) and confirmed by Jacky (1980). This method is based on the assumption that the expired breath returns to end-nasal conditions (28.8°C and 100% humidity), and therefore requires measurement of both chamber air temperature and humidity. We passed the effluent from the chamber over the sensing head of a thermo-hygrometer (Hanna HI 8564) to determine both temperature and humidity. Pilot studies with this instrument and a standard mercury-in-glass thermometer indicated no difference (± 0.1°C) between inlet, internal chamber, and outlet temperatures. The chamber was calibrated for measurement of V_T with a variable speed calibration pump after each experiment. For calibration, the subject animal was killed in situ with a urethane overdose via the jugular vein cannula, and all gaskets, lines, and valves remained undisturbed. The calibration device was a 1-cc syringe with the plunger driven in a reciprocating

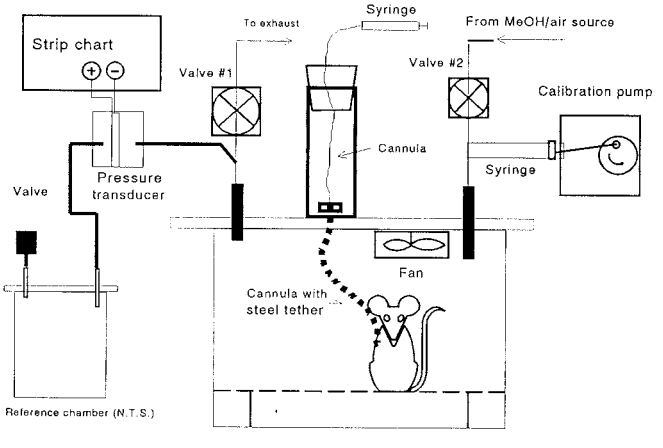

Figure 1. Schematic representation of the exposure chamber allowing measurement of methanol concentrations in inlet air, outlet air, and blood, as well as continuous monitoring of respiratory parameters in ambulatory rats and mice.

fashion by a variable-speed motor (Figure 1). Both the amplitude and frequency of the calibration syringe plunger were varied to bracket the range of f and V_T observed in the laboratory animal. Calibration curves typically were flat between $f = 70$ to 120 breaths/minute. At higher frequencies the amplitude of the recorder trace decreased due to inertia, although breathing at rates exceeding 120 per minute were uncommon.

A compressor supplied source air to the chamber. The flow (1.1 ± 0.1 L/min) was split before entry into the chamber, with one portion passed through neat methanol maintained at 0°C. Gas flow was controlled with rotameter valves, and was measured precisely with a 2-L bubble meter (SKC, Soap Film Flowmeter) while the methanol concentrations in the air stream were observed via the gas chromatograph. Chambers and gaskets were allowed to equilibrate overnight prior to each experiment.

In pilot studies the chamber was modified to permit a probe (an extension of one of the stainless steel tubes used in the gas chromatograph connections) to be positioned inside the chamber. We programmed the chromatograph to obtain samples from the influent (just prior to the chamber), the effluent (just after the chamber), and in the chamber itself. During exposure of a rat to 5,000 ppm methanol, we positioned the probe as close to the animal as possible. After equilibration, the methanol concentration in the chamber effluent was approximately 90% of the inlet concentration; methanol concentrations in the chamber itself were statistically indistinguishable from the effluent concentration. The equality between chamber and effluent concentrations indicates that the chamber behaved as a well mixed compartment, and was treated in this manner in all subsequent calculations and modeling.

We observed a small degree of nonspecific loss of methanol when exposures were conducted in chambers containing dead animals. The degree of loss was the same whether the exposure was run in an empty chamber or one with a dead animal, which suggests that adsorption to skin and fur was negligible. The nonspecific loss of methanol was due to some leakage of air from the chamber, which was corrected by measuring the inlet and outlet air flow, as well as to adsorption of methanol to various components of the device. Adsorption was compensated for by preequilibrating the chamber with methanol before each experiment. In all cases, nonspecific methanol loss was less than 5% of methanol uptake by the animal.

The experiment exposed rats to 0, 1,000, 5,000, 10,000, 15,000 or 20,000 ppm methanol for 8 hours. We measured methanol in the inflow and effluent air at timed intervals throughout the experiment, and measured $\dot{V}$ at least once per hour, which required discontinuing the methanol supply for 30 seconds to obtain each measurement. We measured effluent air temperature and humidity just before each $\dot{V}$ determination.

Absorption of Methanol During Inhalation Exposure After constructing and validating the experimental apparatus required for toxicokinetic studies of methanol during inhalation exposure, the present experiment examined the kinetics of methanol absorption from ambient air. In addition, the relationship between methanol absorption and exposure concentration, as well as that between methanol absorption and exposure time, was evaluated. Rats were exposed to methanol for 8 hours as described in the preceding experiment at 1,000, 5,000, 10,000, 15,000, or 20,000 ppm. We measured methanol in the inflow and effluent chamber air at timed intervals, as well as the temperature and humidity of the effluent air. Respiratory parameters (f and V_T) were determined at hourly intervals as described above. We obtained blood samples through an indwelling jugular vein cannula (exteriorized from the exposure chamber) at timed intervals throughout the experiment. Urine was collected at the end of the experiment for methanol determination. This determination corrected for losses of methanol from chamber air into urine that had pooled in the chamber during the exposure period.

Construction of a Toxicokinetic Model for Methanol Inhalation in Rats The preceding experiments indicated that methanol absorption was dependent on both the exposure concentration (decreasing absorption with increasing concentration, which suggests that systemic methanol may inhibit further absorption from ambient air) and respiratory parameters. Respiration, in turn, was dependent on methanol concentrations in the systemic circulation. The present experiment established the relationship between ventilation rate and methanol absorption, assessed the proximal site of methanol absorption during inhalation exposure, and developed a mathematical model capable of predicting systemic methanol concentrations under specific exposure conditions.

To determine the influence of systemic methanol concentration on methanol absorption, we exposed rats ($n = 4$) to methanol (5,000 ppm) as described above. After 4 hours, a 2-g/kg bolus dose of methanol (to achieve blood concentrations of approximately 5,000 mg/L, i.e., equivalent to the blood concentration observed at a 20,000 ppm exposure) was administered via the jugular vein cannula. Examination of methanol uptake and disposition continued for an additional 4 hours.

To explore the relationship between respiration rate and methanol absorption, we exposed rats to methanol (5,000 ppm) for 4 hours as described in the preceding experiment.

At the end of the 4-hour exposure, the animals received either pentobarbital (40 mg/kg via the jugular vein cannula, $n = 4$) to induce deep anesthesia and decrease respiratory function, or CO_2 via the inlet air (final concentration ranging from 2.5% to 5%, $n = 4$) to stimulate respiration. Methanol exposure continued for an additional 4 hours. Throughout the experiment, we monitored methanol concentrations and respiratory function as described above.

The fact that the fractional absorption of methanol decreased with increased exposure concentration suggests that methanol absorption may be saturable. In view of the high water- (or blood)-to-air partition coefficient for methanol, we hypothesized that such saturable absorption could occur only if methanol was being absorbed in the limited aqueous space associated with the nasal cavity. To test this hypothesis, we anesthetized each rat with urethane, and inserted a polyethylene (PE-60) cannula into the trachea just caudal to the larynx. Cyanoacrylate glue fixed the PE-60 tubing to the trachea and the surrounding tissue. The mouth of the animal also was glued shut to prevent any inhalation via the oral cavity. We placed the rat in the exposure chamber and externalized the jugular vein and tracheal cannulae. We then exposed the animals to methanol (12,500 ppm) for 8 hours, and withdrew blood samples at timed intervals through the tracheal cannula as well as the standard sampling sites. A 25-μL bolus of dichloromethane added to the chamber at hourly intervals served as a marker for poor absorption from the upper respiratory tract. Detection of dichloromethane in the trachea confirmed that appropriate samples of tracheal air were being obtained; the absence of methanol in the presence of dichloromethane supported the hypothesis that methanol is absorbed predominantly in the nose.

Phase VI. Disposition of Methanol During Inhalation Exposure in Mice

In previous experiments with either intravenous or oral administration, methanol disposition in the mouse differed significantly from that in the rat. In particular, the apparent metabolic capacity (V_{max}) for methanol elimination was approximately twofold higher in the mouse than in the rat. The present experiment compared methanol disposition during inhalation exposure between the two species. In particular, the goals of this experiment were to (1) determine the ventilation parameters for mice during methanol exposure, (2) describe methanol absorption from the airstream during inhalation exposure of mice, and (3) compare methanol absorption during inhalation exposure between the mouse and the rat.

Two exposure chamber configurations were used for this experiment. Groups of 8 or 9 mice were exposed simultaneously in a 6.25-L chamber to determine methanol blood concentrations during inhalation exposure. The nominal methanol exposure concentrations for these group-exposed mice were 2,500, 5,000, and 10,000 ppm. A glass block was used to reduce the volume of the chamber to 1.5L to determine respiratory parameters in individual mice. We measured the tidal volume and breathing frequency in individual animals as described in the previous experiment with rats. Measurements were taken at least once per hour, with 10 measurements typically obtained in an eight–hour exposure. For the individually exposed mice, nominal methanol exposure concentrations of 2,500, 5,000, 10,000, and 15,000 ppm were used. In all cases, the actual exposure concentration was determined and used in all subsequent calculations.

During group exposure, we removed 3 to 4 mice from the chambers at timed intervals (only one removal per chamber) and obtained trunk blood samples. These animals defined the cumulation of systemic methanol during exposure. The remaining mice in each chamber were removed after 8 hours. Loss of methanol from the chamber during removal was quantitated and subsequent calculations reflect appropriate corrections in methanol delivery. Groups of 3 to 4 mice in the 8-hour exposure cohort were killed at timed intervals up to 20 hours after exposure, and trunk blood was collected to assess the rate of methanol elimination. Two semiindependent methods determined elimination of methanol: (1) for animals killed during exposure, the blood concentration at each time point should, in theory, equal the methanol extracted from the airstream minus the mass eliminated (corrected for the apparent volume of methanol distribution); and (2) after termination of methanol exposure, elimination of methanol from blood should be predictable by the systemic kinetic parameters estimated in mice receiving intravenous or oral methanol. Both of these assumptions were tested.

DATA ANALYSIS

Methanol Disposition After Intravenous Administration

Pharmacokinetic Analysis Concentration-time data obtained after intravenous doses of 100 or 500 mg/kg to nonpregnant rats were well described by a two-compartment model with saturable elimination from the central compartment (Gibaldi and Perrier 1982). The equation describing methanol flux in the central compartment was:

$$\frac{dC_1}{dt} = k_{21} \bullet C_2 - k_{12} \bullet C_1 - \frac{V_{max} \bullet C_1}{K_m + C_1} \quad (1)$$

where C_1 and C_2 are methanol concentrations in the central and peripheral compartments, k_{12} and k_{21} are first-order

rate constants for intercompartmental transfer, and V_{max} and K_m govern methanol elimination. For nonpregnant rats that received the 2,500-mg/kg dose, Equation 1 failed to describe the concentration-time data, and a model incorporating parallel saturable and linear routes of clearance was used (Equation 2):

$$\frac{dC_1}{dt} = k_{21} \bullet C_2 - k_{12} \bullet C_1 - \frac{V_{max} \bullet C_1}{K_m + C_1} - \lambda \bullet C_1 \quad (2)$$

where λ is the first-order rate constant for methanol elimination by the parallel linear process (presumably representing methanol elimination in urine, expired air, and possibly other nonsaturable routes). In subsequent experiments, the more comprehensive model (Equation 2) was fit to the data from all dose levels. During the 1.5-minute injection period in the 2,500 mg/kg group, methanol input (k_0/V_c) was added to the right side of Equation 2, where k_0 is the methanol infusion rate (1,667 mg/kg/min) and V_c is the apparent volume of the central compartment. We fit both equations to the concentration-time data from individual rats with the nonlinear regression program PCNONLIN (Statistical Consultants, Inc., Apex, NC) to obtain estimates of each kinetic parameter. The ability of each equation to describe the data was assessed based upon Akaike's information criterion (AIC; Yamaoka et al. 1978). The program also determined the initial concentration in the central compartment ($C_1 0$) in the case of Equation 1. V_c was calculated as the ratio of dose to $C_1 0$, and the steady-state volume of distribution (V_{ss}) was estimated as:

$$V_{ss} = \left[\frac{k_{12} + k_{21}}{k_{21}}\right] \bullet V_c \quad (3)$$

We calculated methanol extraction (E) across the lung at each time point as

$$E = \frac{C_{arterial} - C_{venous}}{C_{arterial}}. \quad (4)$$

The mean transpulmonary extraction in each animal was taken as the average of E at each time point following attainment of distribution equilibrium between arterial and venous blood.

We assessed the linearity of renal methanol clearance by plotting urinary excretion rate [mass of methanol recovered in each urine collection (ΔX_u) divided by the collection interval (Δt)] versus the blood methanol concentration (C_B) at the midpoint of the collection interval. Renal clearance (Cl_R) was calculated at each methanol dose level in each animal as:

$$Cl_R = \frac{\Delta X_u/\Delta t}{C_B} \quad (5)$$

Statistical Comparisons We assessed the statistical significance of dose-dependent differences in kinetic parameters by one-way analysis of variance (ANOVA). When the ANOVA was significant, paired comparisons between groups, with adjustment of the criterion for statistical significance for multiple comparisons when appropriate, were performed. The influence of gestational stage on methanol disposition after intravenous administration also was assessed by ANOVA. We examined the significance of differences in methanol concentrations between arterial and venous blood with the paired Student's t test. In all cases, a probability level of $p < 0.05$ was considered statistically significant.

Methanol Disposition After Oral Administration

Kinetic Analysis Concentrations of methanol in whole blood were determined by gas chromatography. We fit the methanol concentration-time data with a one-compartment model that incorporated first-order absorption from the gastrointestinal tract and either Michaelis-Menten elimination (100 mg/kg) or parallel Michaelis-Menten and linear (2,500 mg/kg) elimination, by nonlinear least-squares regression as described in the preceding experiment. A one-compartment kinetic model, as opposed to the two-compartment system used to analyze methanol concentration-time data after intravenous administration, was used because the absorption phase following oral administration masked the distributive phase. In the mouse, methanol absorption from the gastrointestinal tract could not be modeled as a single, first-order process. The data obtained from these experiments were consistent, however, with a dual absorption process consisting of a rapid phase and a slow phase. The kinetic model applied to these data incorporated two first-order absorption rate constants, as well as a fraction of the bioavailable dose that was absorbed by each process, and is shown in Equation 6.

$$\frac{dC}{dt} = F \bullet \left(k_{AF} \bullet \frac{X_F}{V} + k_{AS} \bullet \frac{X_S}{V}\right) - \frac{V_{max} \bullet C}{K_m + C} \quad (6)$$

where k_{AF} and k_{AS} are the first-order rate constants associated with the rapid and slow absorption processes, respectively, and X_F and X_S are the amounts of methanol absorbed by each of the processes. In cases in which only one absorption process was apparent, a single first-order absorption rate constant (k_a) was used.

In addition to the model-dependent analyses described above, the total area under the curve (AUC) of the blood methanol concentration-time profile was calculated with the linear trapezoidal approach through the last sample obtained (time t), and was extrapolated to time infinity by

$$AUC\Big|_t^\infty = \frac{C_t}{\lambda_z} \quad (7)$$

in which C_t is the methanol blood concentration at time t and λ_z is the terminal elimination rate constant as estimated by least-squares regression of the log-transformed concentration-time data in the terminal (log-linear) disposition phase. We compared these AUC values to those obtained from animals receiving intravenous methanol to determine the systemic bioavailability of the alcohol following oral administration.

Statistical Comparisons We assessed the statistical significance of differences in kinetic parameters between the two oral dose levels with the unpaired Student's t test. The influence of gestational stage on methanol disposition after intravenous administration also was assessed by ANOVA. When the ANOVA was significant, we performed paired comparisons between groups, with adjustment of the criterion for statistical significance for multiple comparisons when appropriate. We examined differences in methanol disposition between the rat and the mouse by the unpaired Student's t test. In all cases, a probability level of $p < 0.05$ was considered statistically significant.

Kinetics of Methanol Metabolism in Vitro

Kinetic Analysis To model the concentration-time data obtained in the in vitro metabolism experiments, data were fit with an equation describing Michaelis-Menten–type formation of formaldehyde in the reaction vessel:

$$\frac{dX}{dt} = \frac{V_{max} \bullet C}{K_m + C} \tag{8}$$

where dX/dt is the rate of formaldehyde formation at a given methanol concentration C.

Statistical Comparisons We assessed the significance of differences in kinetic parameters between liver homogenates from nonpregnant and pregnant animals, and between homogenates from rat versus mouse liver, with an unpaired Student's t test. A probability level of $p < 0.05$ was the criterion of significance.

Kinetics of Methanol Delivery to the Fetal Environment

Kinetic Analysis We analyzed maternal whole blood methanol concentration-time data, and estimated amniotic fluid methanol concentration-time data (i.e., methanol concentrations in microdialysate corrected for the in vivo efficiency of methanol recovery by the microdialysis probe) according to standard model-independent techniques. The maternal whole blood or amniotic fluid AUC from time 0 through the end of the sampling period were calculated according to the linear trapezoidal method. Because data in the terminal elimination phase were unavailable, truncated AUC values were employed. Average concentrations in maternal blood and the fetal environment were calculated as the appropriate AUC divided by the duration of sampling. Fetal-to-maternal partition coefficients for methanol were expressed as the corresponding concentration ratios either at fixed time points or as time-averaged concentrations. We estimated the initial rate of methanol permeation into the fetal compartment by regressing the fetal-to-maternal concentration ratio versus time during the first 1 to 2 hours of the experiment, when net translocation of methanol from the maternal to fetal compartment appeared to be a linear function of time.

Statistical Comparisons The statistical significance of differences in methanol permeation rate or fetal-to-maternal partitioning was assessed by one-way ANOVA across administration regimen (low and high bolus doses, low and high rates of continuous infusion). When the ANOVA was significant, we performed paired comparisons between groups, with adjustment of the criterion for statistical significance for multiple comparisons when appropriate. The strength of the relationship between the reciprocal of maternal blood methanol concentrations and fetal permeation rate was determined by orthogonal linear least-squares regression. A probability level of $p < 0.05$ was considered statistically significant.

Methanol Disposition During Inhalation Exposure

Kinetic Analysis The rate of methanol extraction from the air stream in the exposure chamber (dX_E/dt) was calculated as:

$$dX_E/dt = Q \bullet (C_{in} - C_{out}) \tag{9}$$

where Q is the rate of air flow through the chamber, and C_{in} and C_{out} represent the inflow and effluent methanol concentrations, respectively. The total mass of methanol extracted from the air stream through time t was the integral of Equation 9 from time 0 (the beginning of the exposure period) through time t. Q was measured at the beginning and the end of each experiment, as well as at several intermediate times; Q did not vary significantly during the 8-hour exposure, and the average of all measurements was used in the kinetic calculations.

The rate of methanol uptake (dX_A/dt) from the airstream into the animal was calculated as:

$$dX_A/dt = V \bullet C_{out} \bullet \Phi \tag{10}$$

where Φ is the fraction of the methanol extracted from the air stream that was actually absorbed into the systemic circulation. The theoretical maximum mass of methanol absorbed through any particular time t (assuming absorption was 100% efficient) then could be calculated as the integral from 0 to t of $V \bullet C_{out} \bullet dt$. The actual fraction

absorbed then could be calculated as the ratio of X_E to the theoretical maximum mass absorbed at any point in time during the exposure experiment. The fraction absorbed also was calculated as the ratio of the total body load plus the cumulative mass eliminated through each time point (X_B) to the theoretical maximal mass absorbed at each time point, where X_B was calculated as described below. In theory, X_B and X_E should be equivalent.

Throughout this investigation, the value of $\dot{V}$ was based on the volume of completely humidified and heated air in the lungs at full expansion (also referred to as $\dot{V}_E$ in order to distinguish this parameter from the volume of air that actually passes the nares, $\dot{V}_i$, which is slightly smaller than $\dot{V}_E$). In addition, a range of ventilation rates was observed in each animal during inhalation exposure, in part due to variability over time in both the activity of the animal and measurement of respiratory parameters, and in part due to changing respiratory function during methanol accumulation in the systemic circulation. The term summation of ventilation (Σ_{vent}) is used to indicate the summation of $\dot{V}$ over the course of an exposure experiment, in order to compare ventilatory status across exposure conditions.

Elimination of methanol from the systemic circulation during inhalation exposure was computed segmentally based on the kinetic parameters derived from methanol administration by the intravenous and oral routes. The methanol concentration that served as the driving force for systemic elimination was assumed to be the average of observed blood concentrations at the beginning and the end of the period to be computed. To perform a mass balance on methanol extraction and uptake, the mass eliminated from the animal through any point in time t ($X_{E,t}$; calculated as the integral of Equation 9) is added to the total body load present in the animal at that point in time. The total body load at time t ($X_{body,t}$) was estimated as:

$$X_{body,t} = C_t \bullet V_{ss} \qquad (11)$$

where C_t is the blood methanol concentration at time t and V_{ss}, which was estimated in the intravenous administration experiments, is the apparent distributional volume for methanol after distribution equilibrium was achieved.

Statistical Comparisons We used least-squares regression analysis to relate blood methanol concentrations to time during and after inhalation exposure. Linear least-squares regression analysis was employed to relate total methanol body load to time or exposure concentration. The statistical significance of differences between exposure groups was assessed by ANOVA. When the ANOVA was significant, we performed paired comparisons between groups, with adjustment of the criterion for statistical significance for multiple comparisons when appropriate. In all cases, a probability level of $p < 0.05$ was considered statistically significant.

Development of an Inhalation Model for Methanol Toxicokinetics

The model is based on a simple mass-balance equation with the chamber and the rat as control volumes, and solves for the mass delivered to the rat:

$$Q \bullet C_{in} = Q \bullet C_{out} + dX_L/dt + dX_A/dt \qquad (12)$$

where dX_L/dt represents the rate of nonspecific loss of methanol from the exposure chamber, dX_A/dt is the rate of absorption into the animal (Equation 10), and all other parameters are as defined above. Of the parameters appearing in Equation 12, C_{in}, Q, and dX_L/dt are a function of the experimental exposure conditions and the apparatus, and are assumed known without error. The results of previous experiments in this project indicated that $\dot{V}$ is a function of methanol blood concentration; Φ is a function of C_{in} and $\dot{V}$, and methanol blood concentration (the dependent variable in the kinetic model) is a function of methanol elimination kinetic parameters, Φ, C_{in}, and the duration of exposure (or time after exposure has ceased). It should be noted that results of experiments in this project in which intravenous methanol was administered to rats during inhalation exposure indicated that Φ is not a function of, or is only weakly related to, the methanol concentration in blood. Therefore, to evaluate Φ as a function of C_{in} and $\dot{V}$, the data from all the previous experiments was pooled and fit with the equation:

$$\Phi = \left(\frac{C_{in}}{1000_{ppm}}\right)^a \bullet (\dot{V})^b \qquad (13)$$

where a and b serve as allometric exponents. The results of nonlinear regression analysis revealed that $a = -0.130 \pm 0.041$ and $b = -0.026 \pm 0.020$, which indicates that Φ is strongly dependent upon C_{in} but only a weak function of $\dot{V}$.

We also used nonlinear regression analysis to relate $\dot{V}$ at each hour during exposure to the blood methanol concentration, under the assumption that systemic methanol can perturb respiratory function. The standard log-linear pharmacodynamic equation (Equation 14) was fit to the $\dot{V}$ versus blood methanol concentration (C):

$$\frac{\dot{V}_0 - \dot{V}}{\dot{V}_0} = m \bullet \ln(C) + b \qquad (14)$$

where $\dot{V}_0$ is the preexposure value of $\dot{V}$, $\dot{V}$ is the measured parameter at each point in time, m is the slope of the

log-transformed concentration-response relationship, and b is the intercept of the concentration-response profile. Nonlinear regression analysis of the change in ventilation from baseline with increasing blood methanol concentrations yielded $m = 0.124 \pm 0.030$ and $b = -0.287 \pm 0.080$. Although Equation 14 is of limited theoretical utility, it does allow prediction of respiration during methanol exposure over a wide range of exposure conditions.

The final model equation defining the relationship between methanol blood concentration (at any given time C_t) and time during inhalation exposure is:

$$C_t = \sum_{0}^{n} \left(\frac{1}{V_{ss}} \bullet \frac{(Q \bullet C_{in} - dX_L/dt)}{\left(\frac{Q}{\Phi \bullet \bar{V}} + 1\right)} - V_{max} \bullet \left(\frac{C_{t-1}}{K_m + C_{t-1}}\right) \right) \bullet \Delta t \quad (15)$$

where n is the number of concentrations to be simulated. Equation 15 was solved with a spreadsheet program with finite time increments (0.1 hour) for an 8-hour exposure. All parameter values were taken as the average from the previous experiments in this project. Equation 15 was not fit to the concentration-time data, but we used it to simulate the observed data by incorporation of relevant parameter estimates from previous experiments. Methanol concentrations used in calculating $\bar{V}$ and the rate of elimination via the Michaelis-Menten equation were assumed to be the average of C_{t-1} and C_t.

RESULTS

PHASE I. SYSTEMIC DISPOSITION OF METHANOL IN NONPREGNANT RATS

Intravenous Administration

For the two lower doses examined (100 and 500 mg/kg), similar sets of kinetic parameter values, consistent with a single saturable elimination pathway, described the blood methanol concentration-time data (Table 1). The mean whole blood methanol concentration-time data for all three doses are displayed in Figure 2. The solid lines indicate the predicted methanol concentrations for each dose based upon the average kinetic parameters obtained from the animals receiving 100 mg/kg and 500 mg/kg. It is apparent that these average parameters were consistent with the methanol concentration-time data in these two groups. However, the kinetic model significantly overestimated methanol concentrations in rats receiving the 2500-mg/kg dose (Figure 2C).

The fit of the kinetic model incorporating parallel linear and nonlinear routes of methanol clearance to the data obtained from the 2500-mg/kg group is shown in Figure 2D. Inclusion of a second, concentration-independent route of clearance was required to fit the data from these animals with Michaelis-Menten parameters (V_{max} and K_m) similar to those estimated from animals that received 100 mg/kg or 500 mg/kg. Incorporation of the parallel linear pathway in the kinetic model improved the fit for the 2500 mg/kg group

Table 1. Kinetic Parameters Defining Systemic Methanol Disposition After Single Intravenous Doses to Nonpregnant Female Rats

	Dose (mg/kg)		
Parameter[a]	100	500	2500
V_{max} (μg/mL/hour)	74.0 ± 54.9	93.6 ± 23.6	150 ± 84
K_m (μg/mL)	29.0 ± 13.1	39.8 ± 7.5	48.7 ± 16.8
V_c (mL/kg)[b]	476 ± 131[c]	286 ± 55	273 ± 160
V_{ss} (mL/kg)[b]	872 ± 106	840 ± 21	635 ± 135[c]
k_{12} (hour^{-1})	8.11 ± 7.56	13.3 ± 3.7	13.9 ± 6.2
k_{21} (hour^{-1})	7.79 ± 3.78	6.62 ± 0.70	9.25 ± 4.01
λ (hour^{-1})	NA[d]	NA	0.0689 ± 0.0320

[a] Data expressed as mean ± SD for 5 animals per group.

[b] Statistically different across dose levels, $p < 0.05$.

[c] Statistically different from other experimental groups, $p < 0.05$.

[d] Kinetic model that describes methanol disposition after administration of the two lowest doses did not incorporate a parallel linear route of elimination.

as determined by the AIC value. Use of the parallel linear and nonlinear elimination model did not improve the description of the data, compared to the simple Michaelis-Menten elimination model, for the two lower doses.

The values for V_{max} and K_m estimated from the experimental data were approximately 100-μg/mL/hr and 40 μg/mL, respectively (Table 1). Although the mean values of both V_{max} and K_m tended to rise with increasing dose, these parameters did not vary statistically among the three dose levels. The apparent volume of distribution for the central compartment (V_c) was significantly larger ($p < 0.05$) for the 100-mg/kg dose, and V_{ss} was smaller ($p < 0.05$) for the 2500-mg/kg dose, as assessed by analysis of variance. We observed no significant differences between groups for the intercompartmental transfer rate constants. The first-order rate constant for the linear methanol elimination pathway in the 2500 mg/kg dose group ($0.0689 \pm 0.0320\ hr^{-1}$) multiplied by the estimated volume of distribution for the central compartment (273 ± 160 mL/kg) yielded a clearance of 16.9 ± 4.6 mL/hr/kg.

Renal Clearance of Methanol

In an effort to establish the mechanism underlying the apparent linear component of methanol elimination, we examined both transpulmonary extraction and renal excretion of methanol. Methanol extraction by the lungs was evaluated by quantitating the arterial-venous methanol concentration gradient. We determined renal clearance by relating the rate of appearance of methanol in urine to the circulating concentrations of the alcohol. Transpulmonary methanol extraction was minimal (3.24 ± 2.32%) after a 250-mg/kg dose administered intravenously (data not shown), indicating that pulmonary elimination (via exhalation and/or biotransformation) was not a significant contributor to the systemic kinetics of methanol elimination. In contrast, the urinary excretion rate of methanol was linearly related to whole blood methanol concentrations (Figure 3), suggesting that renal clearance was a concentration-independent (linear) process. The estimated renal clearance (9.16 ± 4.27 mL/hr/kg) accounted for approximately 50% of the apparent linear component of methanol elimination.

To determine the contribution of the parallel linear pathway to systemic methanol elimination, the steady-state methanol elimination rate by each pathway was simulated over a wide range of blood methanol concentrations. The average values of V_{max}, K_m, as well as λ determined in the 2500-mg/kg group, were used in these simulations, and linear methanol elimination was expressed as a fraction of total elimination (Figure 4). At the high blood methanol concentrations (approximately 10,000 μg/mL) observed after a dose of 2500 mg/kg, simulations predicted that elimination would be mediated predominantly (approaching 90% of total elimination) by the linear pathway. At lower concentrations produced by the 100- or 500-mg/kg doses (< 1000 μg/mL), the linear pathway contributed less than 30% to total systemic methanol elimination.

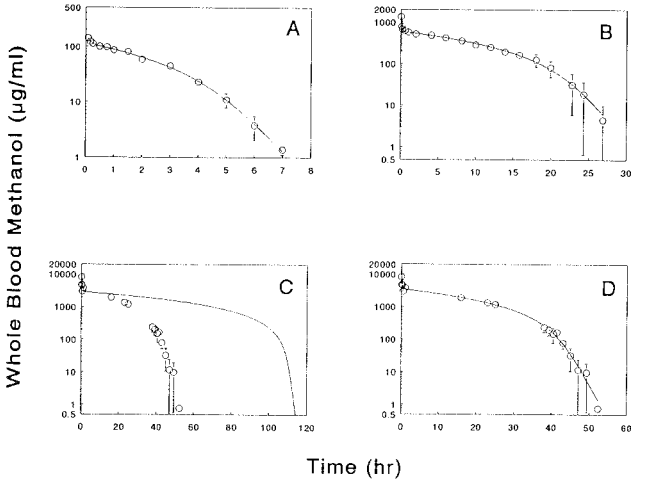

Figure 2. Methanol concentration-time profiles after intravenous administration of 100-mg/kg (A), 500-mg/kg (B), or 2500-mg/kg (C and D) doses in nonpregnant rats. Circles and bars indicate means ± SD; solid lines indicate the best fit of a two-compartment model with saturable elimination (A, B) or parallel linear and saturable elimination (D) to the data. The line in panel C represents the simulated concentration-time profile for the 2500-mg/kg dose based upon the Michaelis-Menten parameters recovered from the 100- and 500-mg/kg groups.

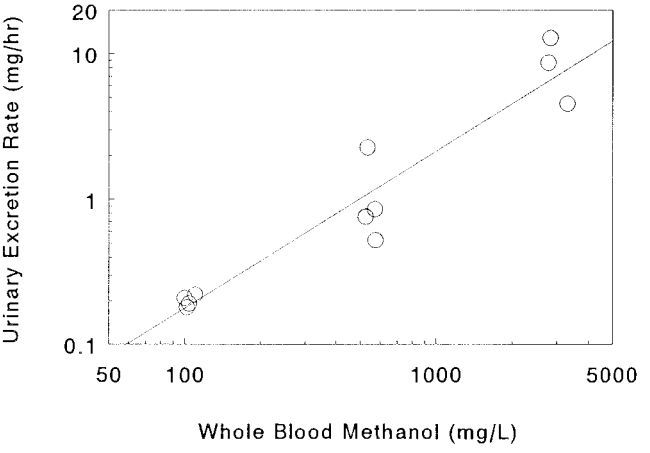

Figure 3. Relationship between urinary excretion rate of methanol and whole blood methanol concentration in nonpregnant rats after intravenous administration of 100, 500, or 2,500 mg/kg methanol. Line indicates the results of orthogonal linear least-squares regression analysis.

Oral Administration

Representative methanol concentration-time profiles from animals receiving either the 100-mg/kg or the 2500-mg/kg oral dose are displayed in Figure 5. The data from both dose levels are well described by a one-compartment model with Michaelis-Menten elimination. The calculated pharmacokinetic parameters for methanol disposition following oral administration are compiled in Table 2. Methanol was absorbed rapidly from the gastrointestinal tract. Based on the mean first-order rate constant for absorption, the absorption half-life was approximately 1.5 minutes (100-mg/kg dose) to 7.6 minutes (2500-mg/kg dose). As was the case for intravenous administration, the estimated V_{max} for methanol elimination was somewhat lower after oral administration of 100 mg/kg methanol as compared to the 2500-mg/kg oral dose group. The K_m for methanol elimination was comparable between oral and intravenous administration; K_m was statistically higher in the 2500-mg/kg oral dose group as compared to the 100-mg/kg oral dose. Estimates of systemic bioavailability (F), which were taken as the ratio of oral to intravenous AUC, indicated that essentially 100% of the oral dose was absorbed, and that first-pass loss during gastrointestinal absorption was minimal.

PHASE II. INFLUENCE OF PREGNANCY ON METHANOL DISPOSITION IN THE RAT

Intravenous Administration

Representative concentration-time profiles for pregnant rats that received a 100-mg/kg intravenous dose of metha-

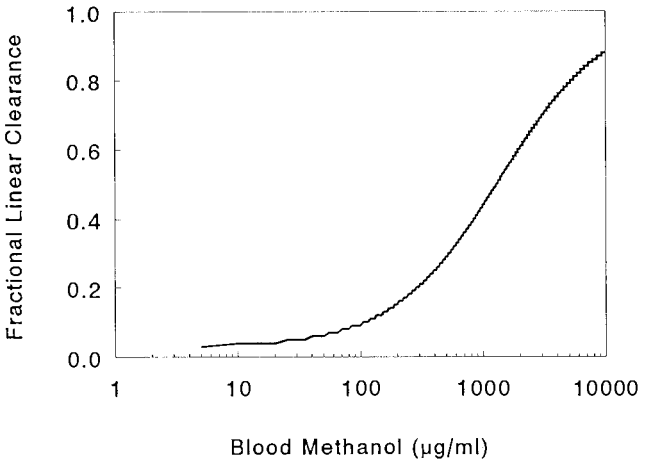

Figure 4. Simulation of a relationship between the fraction of total methanol elimination mediated by the linear pathway and methanol concentration using data from nonpregnant rats exposed to 100, 500, or 2,500 mg/kg methanol by intravenous administration. Simulation was based on average kinetic parameters from the model incorporating both linear and nonlinear routes of clearance; see text for discussion.

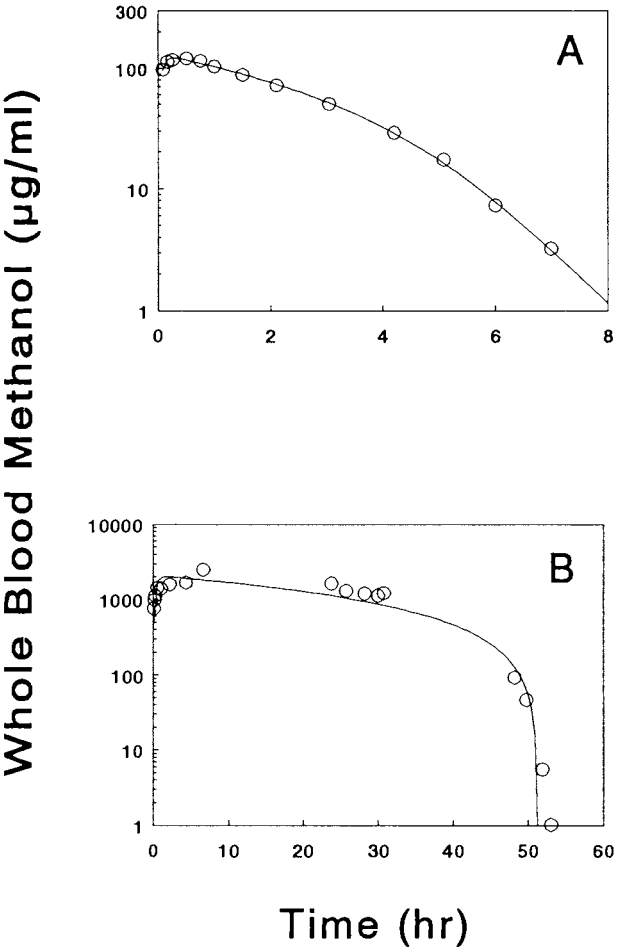

Figure 5. Methanol disposition after a 100-mg/kg (A) or 2500-mg/kg (B) oral dose to representative nonpregnant rats. Symbols represent observed data; lines indicate the fit of a one-compartment model with first-order absorption to the data. See text for details of model structure.

Table 2. Methanol Disposition After Oral Administration to Nonpregnant Rats

	Dose (mg/kg)	
Parameter[a]	100 (n = 6)	2500 (n = 5)
V_{max} (µg/mL/hour)	25.5 ± 11.0	46.3 ± 4.4[b]
K_m (µg/mL)	20.4 ± 13.2	36.3 ± 7.3[b]
V/F (mL/kg)	1018 ± 183	1253 ± 144[b]
k_a (hour^{-1})	26.0 ± 13.9	5.42 ± 1.10[b]
AUC (mg • hour/L)	296 ± 60	62666 ± 6163
F (%)	102	77.7

[a] Parameters expressed as mean ± SD for n animals per group.

[b] Statistically different from 100-mg/kg dose, $p < 0.05$.

nol are displayed in Figure 6. The relevant kinetic parameters are compiled in Tables 3 and 4 for the 100-mg/kg and 2500-mg/kg doses, respectively. In general, methanol disposition was similar in pregnant and nonpregnant rats. At both dose levels, the V_{max} tended to decrease in the latter stages of pregnancy, although the trend was not statistically significant; no trend in the apparent K_m was noted with increased duration of gestation. As would be anticipated from the trend observed for V_{max}, the total AUC, reflective of the time- (or concentration)-averaged instantaneous methanol clearance over the entire concentration-time profile, tended to increase during the course of gestation for the low-dose group, although this trend also failed to achieve statistical significance ($p > 0.06$). Both V_c and V_{ss} were unaffected by pregnancy ($p > 0.1$), although V_{ss} tended to increase during pregnancy in the high-dose group. In contrast, both distributional rate constants decreased statistically across gestational stage ($p < 0.05$) in the low-dose group but not in the high-dose group. The rate constant governing the parallel linear methanol elimination pathway appeared to be lower in pregnant rats as compared to nonpregnant animals, and this difference approached statistical significance ($p < 0.06$).

Two animals that each received a 2500-mg/kg methanol dose on Gestational Day 20 delivered pups during the course of the experiment. At the end of the sampling period, the blood methanol concentrations in these two animals were an order of magnitude higher than in the animals that did not deliver during the experiment; blood methanol concentrations in the pups were indistinguishable from those in the dam (data not shown). This preliminary observation suggests that the systemic accumulation of methanol may be altered in the immediate prenatal period and that, at least at the end of pregnancy, circulating concentrations in the fetus are similar to those in maternal blood. Similar observations of altered systemic clearance of therapeutic agents in the immediate perinatal period were made previously (Nation 1983).

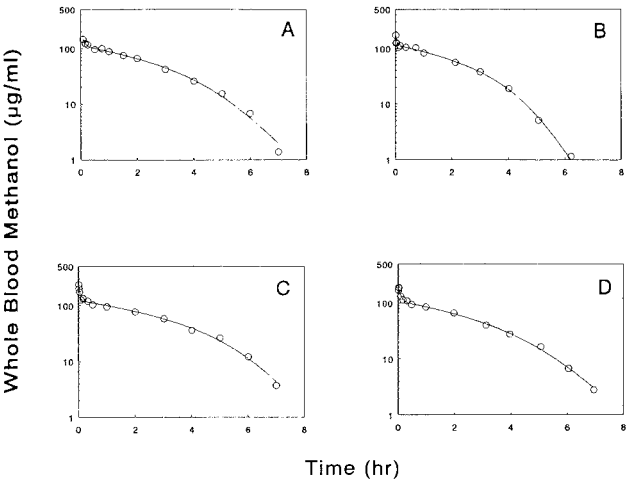

Figure 6. Methanol disposition in representative rats after a 100-mg/kg intravenous dose on gestational day 0 (A), 7 (B), 14 (C), or 20 (D). Symbols represent observed data; lines indicate the fit of a two-compartment model to the data. See text for details of model structure.

Oral Administration

Representative concentration-time profiles after oral administration of 100-mg/kg methanol doses to rats at the three target gestational stages are presented in Figure 7. The kinetic parameters associated with these disposition pro-

Table 3. Influence of Gestational Stage on the Disposition of Methanol After Maternal Administration of a 100-mg/kg Intravenous Dose

	Gestational Stage			
Parameter[a]	Nonpregnant ($n = 5$)	Day 7 ($n = 4$)	Day 14 ($n = 4$)	Day 20 ($n = 4$)
V_{max} (µg/mL/hour)	74.0 ± 54.9	64.7 ± 17.1	69.3 ± 31.7	47.0 ± 15.9
K_m (µg/mL)	29.0 ± 13.1	25.0 ± 9.2	47.0 ± 15.9	26.3 ± 16.0
V_c (mL/kg)	476 ± 131	430 ± 90	350 ± 98	525 ± 82
V_{ss} (mL/kg)	872 ± 106	877 ± 60	883 ± 89	851 ± 54
k_{12} (hour^{-1})[b]	8.11 ± 7.56	30.6 ± 22.8	18.2 ± 5.1	3.79 ± 2.68
k_{21} (hour^{-1})[b]	7.79 ± 3.78	25.5 ± 13.1	11.6 ± 2.5	5.42 ± 3.01
AUC (mg • hour/L)	290 ± 14	313 ± 44	337 ± 34	353 ± 38

[a] Parameters expressed as mean ± SD for n determinations.

[b] Statistically different across gestational stages, $p < 0.05$.

files are compiled in Table 5. Table 6 provides the same data for a 2500-mg/kg dose. At the 100-mg/kg dose level, the pattern of apparent gestational effects was similar after oral administration of methanol as compared to intravenous administration. V_{max} tended to decrease during gestation, although the trend was not statistically significant; K_m fluctuated between experimental groups but evidenced no discernible trend with gestational stage ($p > 0.4$). Total systemic exposure to methanol, as expressed by the AUC extrapolated through infinite time, was not affected by pregnancy. Both the apparent volume of distribution (V) (contaminated by an indeterminate systemic bioavailability [F], therefore expressed as V/F) and the first-order rate constant governing absorption evidenced statistically significant changes during the course of pregnancy in the low-dose group, although these changes are of doubtful biologic significance. No such differences were observed at the 2500-mg/kg dose level. It is interesting to note that a modest decrease in oral bioavailability was observed in pregnant rats as compared to nonpregnant animals, consistent with reports of decreased oral bioavailability of therapeutic agents in pregnant women. This decrease in F undoubtedly accounts for the observed increase in V/F. In the high-dose group, F did not decrease in pregnant animals. Examining the bioavailability data as a whole, the systemic availability of orally administered methanol in pregnant and nonpregnant rats was dose-independent (86.3 ± 11.4% at 100 mg/kg; 85.5 ± 8.9% at 2500 mg/kg).

Table 4. Influence of Gestational Stage on the Disposition of Methanol After Maternal Administration of a 2500-mg/kg Intravenous Dose

	Gestational Stage			
Parameter[a]	Nonpregnant ($n = 5$)	Day 7 ($n = 5$)	Day 14 ($n = 5$)	Day 20 ($n = 3$)
V_{max} (μg/mL/hour)	150 ± 84	154 ± 48	132 ± 44	110 ± 35
K_m (μg/mL)	48.7 ± 16.8	68.0 ± 24.7	40.8 ± 20.2	61.0 ± 31.7
V_c (mL/kg)	273 ± 160	318 ± 81	403 ± 148	336 ± 67
V_{ss} (mL/kg)	635 ± 135	700 ± 160	810 ± 50	797 ± 58
k_{12} (hour^{-1})	13.9 ± 6.2	8.30 ± 4.54	9.28 ± 10.3	11.9 ± 3.1
k_{21} (hour^{-1})	9.25 ± 4.01	7.20 ± 4.91	6.03 ± 4.10	8.65 ± 2.55
λ (hour^{-1})	0.0689 ± 0.032	0.0249 ± 0.034	0.0270 ± 0.0190	0.0439 ± 0.014
AUC (mg • hour/L)	71,195 ± 9,668	72,002 ± 10,970	59,229 ± 4,468	62,901 ± 4,904

[a] Parameters expressed as mean ± SD for n determinations.

Table 5. Influence of Gestational Stage on the Disposition of Methanol After Maternal Administration of a 100-mg/kg Oral Dose

	Gestational Stage			
Parameter[a]	Nonpregnant ($n = 6$)	Day 7 ($n = 5$)	Day 14 ($n = 5$)	Day 20 ($n = 5$)
V_{max} (μg/mL/hour)	25.5 ± 11.0	21.1 ± 2.4	21.6 ± 6.0	13.8 ± 2.6
K_m (μg/mL)	20.4 ± 13.2	14.9 ± 5.1	22.8 ± 9.8	12.8 ± 13.5
V/F (mL/kg)[b]	1018 ± 183	1095 ± 88	1205 ± 90	1288 ± 118
k_a (hour^{-1})[b]	26.0 ± 13.9	14.4 ± 3.8	14.7 ± 5.3	35.5 ± 14.4
AUC (mg • hour/L)	296 ± 60	262 ± 26	252 ± 43	299 ± 61
F (%)	102	83.7	74.8	84.8

[a] Parameters expressed as mean ± SD for n determinations.

[b] Statistically different ($p < 0.05$) across gestational stages.

PHASE III. SYSTEMIC DISPOSITION OF METHANOL IN THE MOUSE

The concentration-time data obtained after oral administration of a 2500-mg/kg dose of methanol to nonpregnant mice are displayed in Figure 8. The kinetic profile was associated with a relatively large degree of interanimal variability in blood methanol concentrations 0 to 2 hours after gavage (Figure 9), and a good fit of a kinetic model to these data could not be obtained. We hypothesized that this early variability was an artifact of the decapitation method used to collect the blood samples. To examine this possibility, and to attempt to obtain more accurate estimates of the kinetic parameters that govern methanol disposition in this animal species, we performed experiments that used oral administration and an implanted jugular vein cannula to sample blood. These data are included in Figure 9, superimposed on the original data (after oral administration to mice not equipped with indwelling cannulae). To assess whether these data were different from those obtained after decapitation, we averaged the blood methanol concentrations from each experiment over the period from 0.5 to 1.5 hours to yield a single mean value for the blood concentration over that time period. When these mean values from cannulated mice were compared to those from decapitated animals with the unpaired Student's t test, they were not statistically different ($p > 0.05$). Because the data did not differ, and because sampling by decapitation may have resulted in some contaminated samples during the early period after gavage, we used the intravenous sampling data for the first 2 hours after gavage to fit the toxicokinetic model to the oral data. The concentration-time data ob-

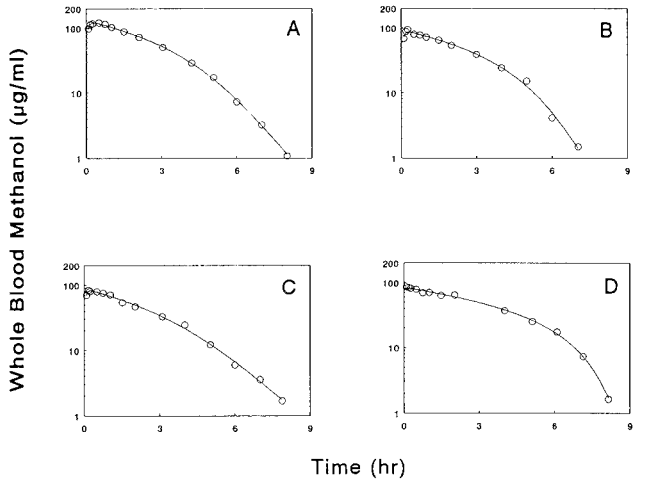

Figure 7. Methanol disposition in representative rats after a 100-mg/kg oral dose on gestational day 0 (A), 7 (B), 14 (C), or 20 (D). Symbols represent observed data; lines indicate the fit of a one-compartment model to the data. See text for details of the model structure.

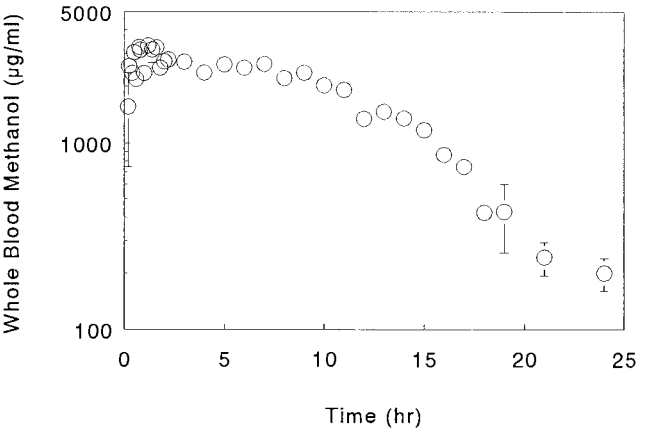

Figure 8. Methanol disposition after administration of a 2500-mg/kg oral dose to nonpregnant mice. Circles and bars indicate means ± SD for $n = 3$ to 4 animals per time point; absence of error bars indicates SD smaller than the size of the symbol.

Table 6. Influence of Gestational Stage on the Disposition of Methanol After Maternal Administration of a 2500-mg/kg Oral Dose

	Gestational Stage			
Parameter[a]	Nonpregnant ($n = 5$)	Day 7 ($n = 5$)	Day 14 ($n = 4$)	Day 20 ($n = 3$)
V_{max} (µg/mL/hour)	46.3 ± 4.4	51.9 ± 11.0	55.0 ± 7.4	41.6 ± 6.8
K_m (µg/mL)	36.3 ± 7.3	42.6 ± 23.4	46.2 ± 11.8	46.6 ± 8.1
V/F (mL/kg)	1,253 ± 144	1,129 ± 204	1,078 ± 137	1,207 ± 73
k_a (hour^{-1})	5.42 ± 1.10	11.0 ± 4.3	5.37 ± 1.16	6.03 ± 0.70
AUC (mg•hour/L)	54,872 ± 7,928	56,537 ± 4,983	53,794 ± 7,595	59,975 ± 5,323
F (%)	77.7	78.3	90.8	95.3

[a] Parameters expressed as mean ± SD for n determinations.

tained after either oral or intravenous methanol administration in the mouse are displayed in Figure 10. For the intravenous route, data were obtained only over the first 10 hours after exposure. During this time period, blood methanol concentrations did not differ significantly between the oral and intravenous routes of administration ($p > 0.05$). To fit these data, a precise value for K_m was difficult to determine because background (predose) methanol concentrations approached the K_m. Thus, in fitting the data, the K_m was fixed at 48.7 mg/L (i.e., the experiment-wise average value determined in the previous studies in rat). The K_m in rats was determined more precisely than in mice because of the use of data from a range of methanol doses in the larger species. The concentration-time data obtained from the experiments in the nonpregnant rat, for comparison with parallel experiments in mice, are displayed in Figure 11. The data that depict methanol disposition after intravenous administration were taken from the previous experiments in nonpregnant rats; the data after oral methanol administration were generated in the present experiment. To compare the disposition kinetics appropriately between the two species, we fixed the value of K_m in the rat also at 48.7 mg/L. Estimates of the kinetic parameters recovered from the present experimental data are displayed in Table 7. When normalized for body weight, the maximal elimination rate (V_{max}) was significantly higher ($p < 0.05$) in the mouse (117 ± 3 mg/hr/kg) than in the rat (60.7 ± 1.4 mg/hr/kg). The only other parameter that differed between the two species was V_c. However, because the rat required a two-compartment model to describe the concentration-time data adequately in this study, a more appropriate comparison is that of the V_c in the mouse to the steady-state volume of distribution (V_{ss}) in the rat. These values did not differ substantially (0.764 vs. 0.854 in the mouse and rat, respectively). Near-term pregnant mice appeared to absorb methanol at a somewhat slower rate than nonpregnant mice (Figure 12). Gestation also clearly affected the amount of time required to eliminate methanol from the maternal circulation in mice, similar to observations made in the previous experiment in rats.

The kinetic parameters associated with the disposition of methanol in pregnant mice as compared to nonpregnant female mice are compiled in Table 8. As for the rat, V_{max}

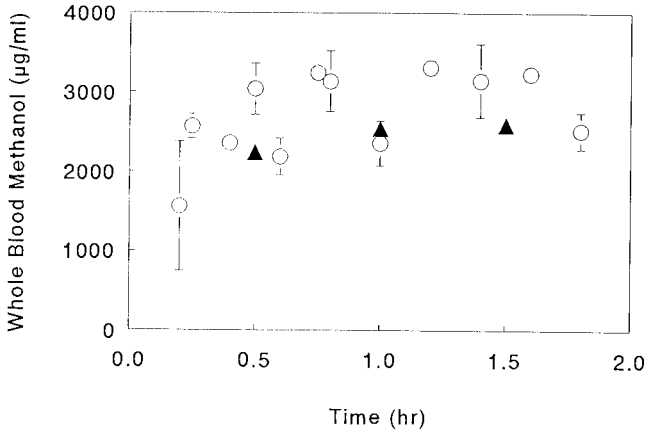

Figure 9. Methanol disposition during the first 2 hours after a 2500-mg/kg oral dose to nonpregnant mice. Open circles indicate blood samples obtained by decapitation; solid symbols represent samples obtained via an indwelling jugular vein cannula. Circles and bars indicate means ± SD.

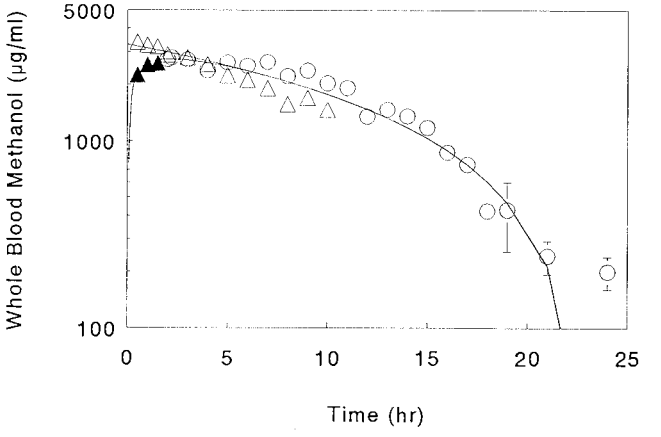

Figure 10. Methanol disposition in nonpregnant mice after intravenous (open triangles) or oral (open circles) administration of 2500 mg/kg. Solid triangles represent samples obtained from mice with indwelling jugular vein cannulae (see Figure 9). Lines indicate simultaneous fit of a one-compartment model to the data from both routes of administration. See text for details of model structure. Circles and bars indicate means ± SD.

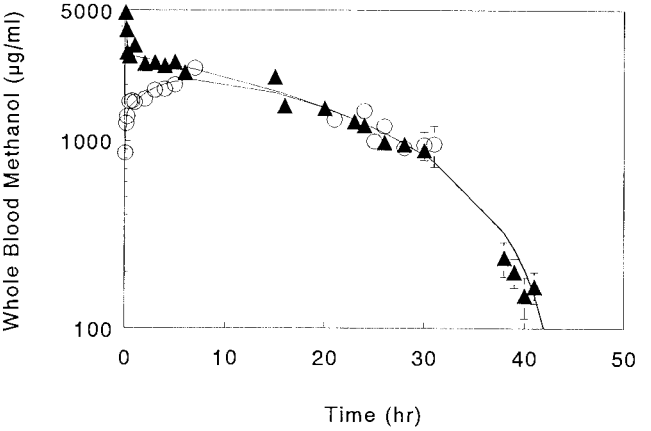

Figure 11. Methanol disposition in nonpregnant rats after intravenous (triangles) or oral (circles) administration of 2500 mg/kg. Lines indicate simultaneous fit of a two-compartment model to the data from both routes of administration. See text for details of model structure.

for methanol elimination in the mouse decreased with gestation (ANOVA, $p < 0.05$), although differences between either of the pregnant groups and nonpregnant mice were not statistically significant by pairwise comparison. V_{max} for methanol elimination in the mouse was approximately twice that in the rat, regardless of gestational stage. In addition to the changes in V_{max}, pregnancy was associated with a significant decrease in X_F and a significant increase in k_{AF} after the 2500-mg/kg oral dose.

Representative concentration-time profiles for production of formaldehyde in nonpregnant and near-term pregnant adult rat and mouse liver homogenates are presented in Figure 13; similar profiles were obtained in all homogenates. Consistent with the in vivo data, the rate of formaldehyde production from methanol in mouse liver homogenates was approximately twice that in rat liver homogenates (Table 9). Pregnancy also was associated with a statistically significant, albeit relatively minor, decrease in the V_{max} for formaldehyde formation in both rat and

Table 7. Kinetic Parameters That Define Systemic Methanol Disposition in Female CD-1 Mice and Female Sprague-Dawley Rats After Oral Administration of 2500 mg/kg Methanol[a]

Parameter[a]	Mouse	Rat
V_{max} (mg/hour/kg)[b]	117 ± 3	60.7 ± 1.4
K_m (mg/L)[c]	48.7	48.7
V_C/F (L/kg)[b]	0.764 ± 0.020	0.302 ± 0.04
k_{AF} (hour^{-1})	5.28 ± 5.95	4.00 ± 1.08
k_{AS} (hour^{-1})	0.69 ± 0.57	NA[d]
X_F (mg/kg)[e]	1782 ± 15	NA[d]
V_{ss}/F (L/kg)	NA[f]	0.854
k_{12} (hour^{-1})	NA[f]	6.39 ± 1.7
k_{21} (hour^{-1})	NA[f]	3.47 ± 0.31

[a] Data expressed as means ± SE (returned from nonlinear regression analysis) for at least 4 animals per group.

[b] Statistically different between species, $p < 0.05$.

[c] Value fixed during model fitting; see text for details.

[d] Only one absorption process was apparent for the rat.

[e] X_F is the total amount of the dose that was available for absorption via the rapid process.

[f] A one-compartment model was used that did not incorporate a K_{AS}, X_F, V_{ss}, k_{12}, or k_{21}.

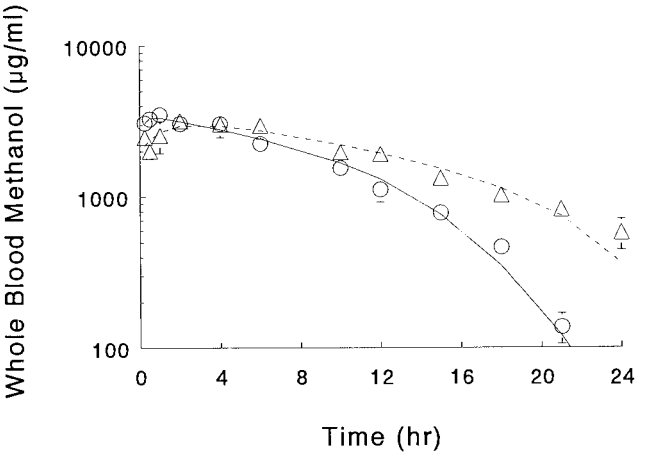

Figure 12. Methanol disposition in nonpregnant (open circles) and near-term pregnant (open triangles) mice after a 2500-mg/kg oral dose. Symbols and bars indicate means ± SD for $n = 4$ mice per time point; lines indicate the fit of a one-compartment model to the data.

Table 8. Kinetic Parameters That Define Systemic Methanol Disposition in the CD-1 Mouse After Oral Administration of 2500 mg/kg Methanol to Nonpregnant and Pregnant Animals

	Gestational Stage		
Parameter[a]	Nonpregnant	Day 9	Day 18
X_F (mg/kg)[b,c]	1700 ± 10	1830 ± 90	1340± 40
k_{AF} (hour^{-1})	16.8 ± 3.7	7.34 ± 1.12	29.9 ± 1.8
k_{AS} (hour^{-1})	4.53 ± 3.76	0.394 ± 0.180	0.787 ± 0.591
V_{max} (mg/kg/hour)[c]	134 ± 6	131 ± 3	96.8 ± 6.2
K_m (mg/L)	48.7[d]	48.7[d]	48.7[d]
V/F (mL/kg)[c]	0.708 ± 0.028	0.564 ± 0.313	0.702 ± 0.528

[a] Parameters are expressed as mean ± SE (returned from nonlinear regression analysis), $n = 4$ mice per time point.

[b] X_F is the total amount of the dose that was absorbed via the fast process.

[c] Significantly different across gestational stages, $p < 0.05$.

[d] Value fixed during model fitting; see text for details.

mouse liver homogenates; V_{max} at Gestational Day 20 was approximately 85% of that in livers from nonpregnant rats and mice, again consistent with the data obtained from the in vivo experiments. Formaldehyde production was significantly lower in fetal rat and mouse liver homogenates in comparison to the adult liver; fetal mouse liver homogenates converted methanol to formaldehyde at a significantly higher (approximately 40%) rate than fetal rat liver homogenates.

PHASE IV. METHANOL DELIVERY TO THE FETAL ENVIRONMENT

The in vitro recovery of methanol from an unstirred solution of normal saline by the microdialysis probe was 45%. When the microdialysis probe is implanted in tissue, it is assumed that the fluid layer surrounding the tip of the probe is unstirred. Conversely, when the probe is implanted in a blood vessel (recovery = 62%), the fluid that surrounds the probe tip is assumed to be well stirred. The present in vitro experiment demonstrated that methanol recovery was lower in an unstirred vessel than in a stirred vessel, although the vigor with which the vessel was stirred did not seem to influence recovery. In consideration of the fact that some physical movement of the fetuses is present in the anesthetized dams, it is unclear whether amniotic fluid represents a stirred or unstirred compartment. Thus, we calibrated probe recovery when we obtained a single amniotic fluid sample at the end of the experiment, instead of using the more classic in vitro approach.

The methanol concentration-time profile in maternal blood and amniotic fluid microdialysate from a representative pregnant rat that received the low bolus dose is presented in Figure 14. The lines represent the best fit of a two-compartment model with Michaelis-Menten elimination from the blood (maternal) compartment to the data. The flux of methanol through maternal blood and amniotic fluid is well described by the model. The initial rate of methanol penetration into the fetal compartment was estimated from the slope of the rising portion of the amniotic fluid microdialysate concentration-time profile. The mean initial permeation rate was significantly different across the four experimental groups (Figure 15). Permeation rate was approximately one order of magnitude higher in the low infusion group than in the other three treatment groups.

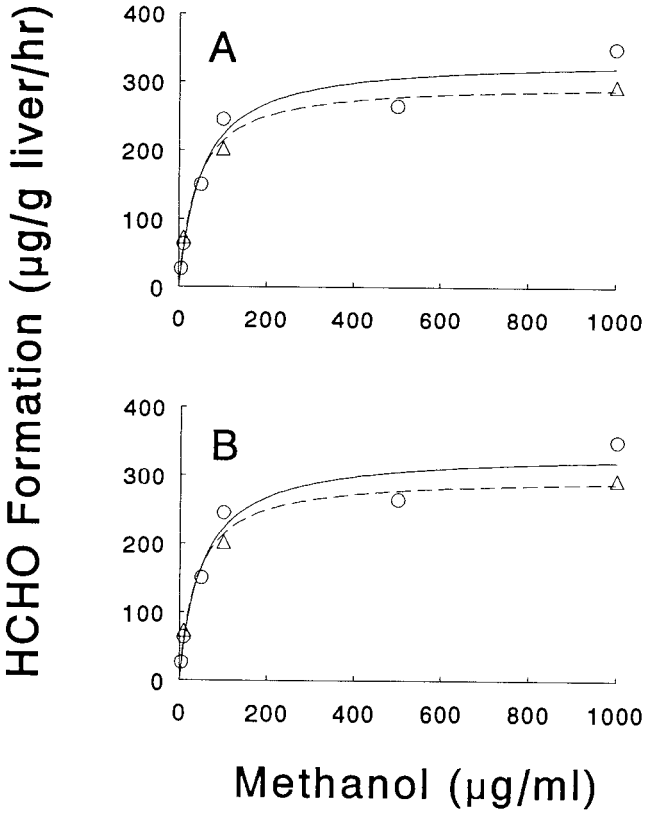

Figure 13. Rate of in vitro formaldehyde formation from methanol in representative liver homogenates from adult nonpregnant (circles and solid lines) and near-term pregnant (triangles and dashed lines) rats (A) and mice (B). Symbols represent observed data; lines indicate the fit of the Michaelis-Menten model to the data.

Table 9. In Vitro Metabolism of Methanol in Liver Homogenates from Nonpregnant Adults, Near-Term Adults, and Near-Term Fetuses[a]

Homogenate	V_{max} (µg HCHO/ g liver/hour)	K_m (µg/mL)
Nonpregnant adult rat	364 ± 22	39.3 ± 13.0
Gestational day 20 adult rat	314 ± 13[b]	35.5 ± 5.0
Gestational day 20 fetal rat	19.2 ± 5.4[b,c]	–[d]
Nonpregnant adult mouse	678 ± 38	45.6 ±16.2
Gestational day 18 adult mouse	576 ± 28[b]	41.3 ± 8.3
Gestational day 20 fetal mouse	27.4 ± 3.8[b,c,e]	–[d]

[a] Parameters are expressed as means ± SD for $n = 4$ determinations per group.

[b] Statistically different from nonpregnant adult animal, $p < 0.05$.

[c] Model fitting was not performed for fetal livers; value given is an average rate of formaldehyde formation for all determinations.

[d] K_m was not determined for fetal livers.

[e] Significantly different from fetal rat liver, $p < 0.05$.

Since the low infusion group would be expected to have the lowest blood methanol concentrations during the initial portion of the profile, this observation suggests that permeation rate may be inversely proportional to maternal methanol concentration. Indeed, a plot of permeation rate versus the reciprocal of maternal blood methanol (Figure 16) yielded a linear relationship, which indicates that penetration of the alcohol into the fetal environment was inversely proportional to the systemic methanol concentration in the dam.

The apparent dependence of methanol penetration into the fetal environment on maternal blood methanol was not limited to the initial distributive phase. As displayed in Figure 17, an inverse relationship was observed between

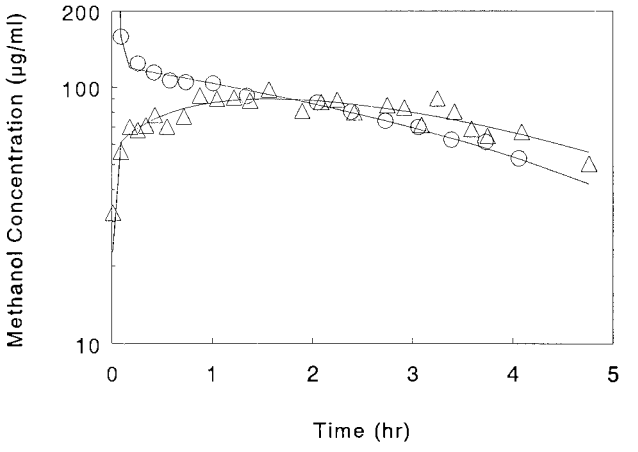

Figure 14. Methanol disposition in maternal blood (circles) and amniotic fluid (triangles) in a representative pregnant rat on Gestational Day 20. Symbols represent observed data; lines indicate the fit of a two-compartment model to the data. See text for details of model structure.

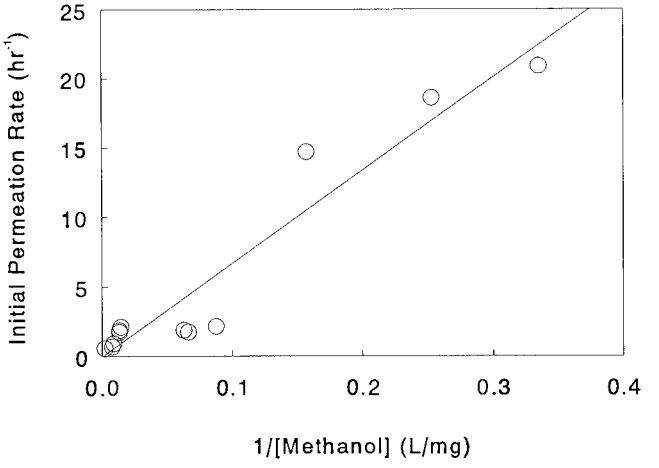

Figure 16. Relationship between permeation rate and the reciprocal of maternal whole blood methanol concentration in the rat. Methanol was administered intravenously at doses of 100, 500, or 2500 mg/kg. The line indicates the result of orthogonal linear least-squares regression of the data.

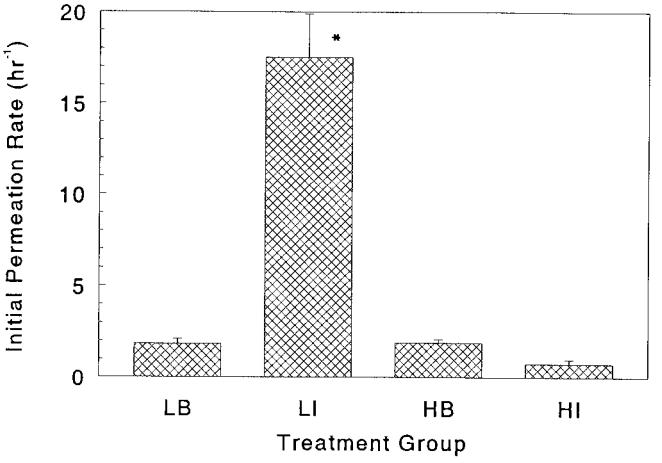

Figure 15. Mean (± SD) rate of methanol permeation from the rat maternal circulation to amniotic fluid in the low infusion (LI), high infusion (HI), low bolus (LS), and high bolus (HB) groups. Asterisk indicates statistically significant differences between the low infusion group and each other group.

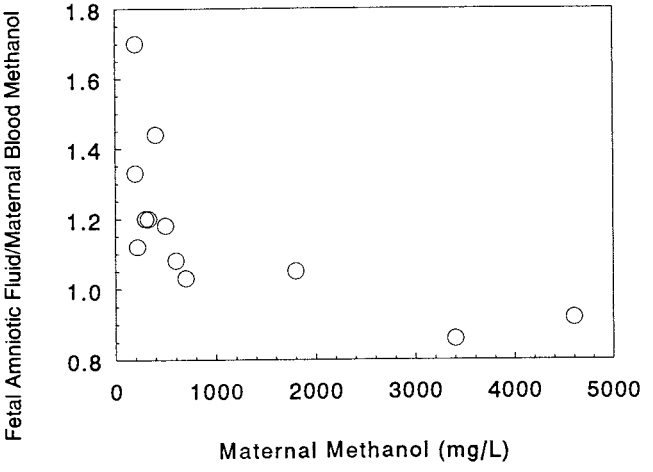

Figure 17. Decrease in methanol partitioning between the rat fetal environment and the maternal circulation with increasing maternal methanol concentrations. Methanol was administered intravenously at doses of 100, 500, or 2500 mg/kg. The ratio of amniotic fluid methanol to maternal blood methanol concentration was plotted against maternal blood methanol. Symbols indicate the results obtained from individual rats.

the time-averaged fetal:maternal methanol ratio (as expressed by the ratio of amniotic fluid to maternal blood AUC calculated over the entire duration of the experiment) versus the time-averaged maternal blood methanol concentration. The behavior of methanol partitioning between maternal blood and the fetal environment may have some bearing on teratogenic potential.

PHASE V. DISPOSITION OF METHANOL DURING INHALATION EXPOSURE IN RATS

Determination of Respiratory Parameters

The approach we used to quantitate methanol concentrations in air and blood, and to simultaneously determine respiratory parameters, provided a feasible method with which to characterize ventilation volume and rate, extraction of methanol from ambient air, and uptake of methanol into the systemic circulation. We evaluated the method at methanol exposure concentrations from 0 to 20,000 ppm, and for exposure durations of up to 8 hours. Even after we preexposed rats to the inhalation chamber, animals evidenced a brief period (typically < 15 minutes) of initial activity. Most animals slept, or were inactive for the majority (6 to 7 hours) of the 8-hour exposure. A few rats, however, were active sporadically throughout the experiment. Because we monitored ventilation parameters continuously, changes in respiration associated with changes in physical activity did not confound analysis of the methanol uptake data. Consistent with the foregoing experiments that used intravenous or oral administration of methanol, we observed no apparent central nervous system depression or ataxia, even at the highest exposure concentration. An average of 2 to 4 mL of urine was recovered from the chamber at the end of the exposure period. The methanol concentration in these urine samples was high, varied in proportion to the exposure concentration, and was consistent with a water:air partition coefficient of approximately 7000 (Perry 1973).

Total ventilation over the exposure period (Σ_{vent}, i.e., $\dot{V}$ summed over 8 hours and normalized for body weight) for each group is displayed in Table 10. Regardless of normalization, the within-group variance in this ventilation parameter was high, which suggests significant interanimal variability in respiratory parameters. This observation highlights the importance of monitoring the respiratory status of individual animals, whenever feasible, throughout the exposure period.

Total ventilation over the exposure period is displayed versus exposure concentration in Figure 18. A slight downward trend in Σ_{vent} with increasing atmospheric methanol concentration was apparent graphically, although this trend was not statistically significant. However, ventilation is not likely to be a function of atmospheric methanol per se, but rather related to the systemic concentration of methanol in the animal during exposure (e.g., if methanol depresses respiratory function in a concentration-dependent manner). To address this point, we examined ventilation rate at hourly intervals during methanol exposure, and pooled data across all exposure groups. These data are plotted against the corresponding blood methanol concentration in Figure 19. Examining respiratory function in this manner indicates that ventilation may be dependent upon circulating concentrations of methanol, particularly when concentrations exceed 2000 μg/mL. Thus, any toxicokinetic

Table 10. Influence of Inhaled Methanol Exposure on Total Ventilation over the Exposure Period

Dose[a] (ppm)	*n*	Total Ventilation over the Exposure Period	± SD	CV (%)
0	4	227.8	45.3	20
1,000	4	287.3	36.8	13
5,000	3	255.6	30.3	12
10,000	3	256.7	25.7	10
15,000	3	254.3	28.7	11
20,000	4	232.6	16.9	7
Pooled	21	251.9	31.0	12

[a] Inlet airstream concentration.

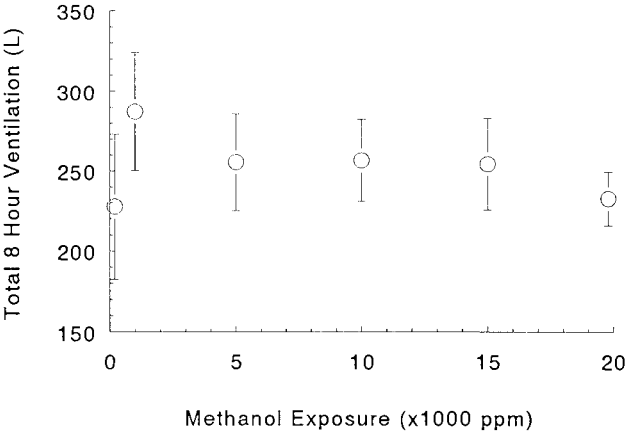

Figure 18. Relationship between total ventilation over an 8-hour exposure (Σ_{vent}) and methanol exposure concentration in nonpregnant rats. Circles and bars indicate means ± SD of 3 to 4 rats per group.

model developed to describe and predict systemic methanol concentrations during inhalation exposure should incorporate potential time- (and exposure concentration)–dependent changes in respiration due to accumulation of the alcohol in the systemic circulation.

A comparison of the ventilatory parameters recovered in this experiment to commonly cited values in the literature is provided in Table 11. The values taken from the literature for $\dot{V}$ and V_T were normalized per kilogram of body weight, and the ventilation was summed over 8 hours (Σ_{vent}). Compared to rats exposed via a nose-only protocol, in which the animal is restrained in a tube, Σ_{vent} measured by the present method was significantly lower due to a lower breathing frequency (f) in the chamber-exposed animals. The comparatively high values of both Σ_{vent} and f during nose-only exposure are similar to measurements taken in the first few minutes of exposure in the chambers used in the present project, consistent with heightened physical activity of the ambulatory rats during this time period.

Representative profiles for the time course of cumulative methanol extracted from the airstream ($X_{E,t}$) and the calculated cumulative mass taken up into the animal ($X_{B,t}$, i.e., total body load at time t plus the amount eliminated from the systemic circulation through time t) are presented in Figure 20 for rats exposed to methanol at 5000 ppm or 15,000 ppm. In both cases, $X_{E,t}$ and $X_{B,t}$ were approximately equal, which suggests that either parameter (or an average of the two) would serve as a useful estimate of the total mass of methanol taken into the animal through any point in time. Uptake into the animal ($X_{A,t}$), computed with a theoretical fraction absorbed of 1.0, differed from $X_{E,t}$ and $X_{B,t}$. At 5000 ppm, $X_{A,t}$ was slightly larger than $X_{E,t}$ and $X_{B,t}$, consistent with a fraction absorbed of 0.9. At 15,000 ppm, $X_{A,t}$ was much larger than $X_{E,t}$ and $X_{B,t}$; the degree to which

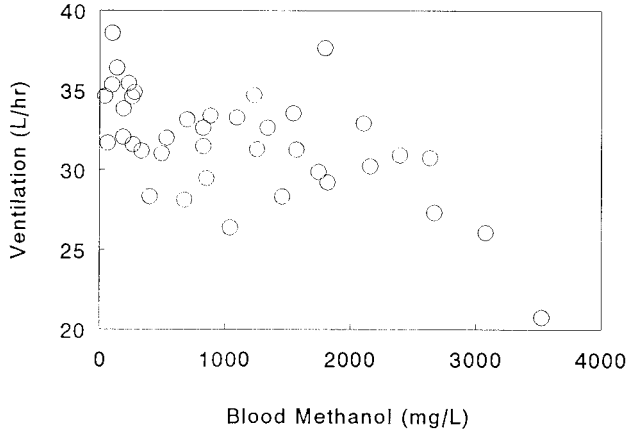

Figure 19. Relationship between ventilation rate and systemic methanol concentration. Data represent results from all exposed rats at hourly intervals during the 8-hour inhalation exposure period at each dose.

$X_{A,t}$ exceeded $X_{E,t}$ and $X_{B,t}$ suggested a fraction absorbed of 0.6. Although the relationship between methanol absorption and exposure concentration was the subject of a subsequent experiment, the method developed to evaluate methanol disposition during inhalation exposure can yield valuable information about not only the influence of methanol on respiratory function, but also the absorption of methanol from ambient air over an 8-hour exposure.

Absorption of Methanol During Inhalation Exposure

Based on the observations made during development and validation of the inhalation exposure apparatus, the present

Table 11. Ventilation Parameters in Rats Compared with Findings in the Published Literature

Reference	Total Ventilation over the Exposure Period (L/8 hours)	Breathing Frequency (minute^{-1})	Tidal Volume (mL/kg)
Whole-Body Exposures			
Andersen et al. 1987	250	NR[a]	NR
Ramsey and Andersen 1984	171	NR	NR
Horton 1988	174	NR	NR
Kleinman and Radford 1964	132	110	2.5
Costa and Tepper 1988	412	120	7.1
Lai et al. 1978	336	103	6.8
Present study			
0 ppm	228	89	5.3
1,000 ppm	287.3	74	8.0
10,000 ppm	256.7	88	6.1
20,000 ppm	232.6	80	6.0
Pooled	252		
Nose-Only Exposures			
Sabourin et al. 1992	568	170	7.0
Mauderly 1986	311	131	4.9
	478	142	7.1
	307	129	4.9
	340	116	5.9
Dahl et al. 1987	336	NR	NR
	440	NR	NR
Present study (first 12 minutes at 5,000 ppm)	327	123	5.5

[a] NR = not reported; presumably these parameters were not measured in the indicated studies.

experiment was designed to evaluate methanol absorption under a variety of exposure conditions. Typical profiles for the mass of methanol extracted from the air ($X_{E,t}$), mass in the blood plus mass eliminated ($X_{B,t}$), and estimated mass absorbed at a fractional absorption of 1.0 during methanol exposure at concentrations of 5,000 and 15,000 ppm are shown in Figure 20, and at concentrations of 10,000 and 20,000 ppm are displayed in Figure 21. The minute ventilation (V) at each time point during exposure also is shown in Figure 21. Ventilation for each rat remained fairly constant during exposure. The animal exposed to the 5,000-ppm concentration was relatively active throughout the exposure period, and ventilation in this animal is reflective of that level of physical activity. As was observed in the previous experiment, $X_{E,t}$ and $X_{B,t}$ were, on balance, approximately equal during the course of the experiment regardless of concentration. In some animals, these parameters diverged to a certain extent (e.g., the 20,000-ppm exposure in Figure 21), undoubtedly due in part to experimental error. The method used to calculate $X_{B,t}$ (i.e., application of mean kinetic parameters to estimate the mass eliminated through time, t) also may have contributed to differences between two parameters that, in theory, should be identical. However, the differences observed were small in comparison to the total mass of methanol absorbed by the animal, and did not complicate further kinetic analysis. Although the total mass of methanol taken up by the animal increased as exposure concentration increased, the relationship was not proportional, which suggests that the fraction absorbed (Φ) differed across exposure concentrations.

Mean $X_{E,t}$, $X_{B,t}$, and the theoretical maximum amount absorbed through time t for each exposure condition is displayed in Figure 22. We used summations of $X_{E,t}$, $X_{B,t}$, and $X_{A,t}$ over 8 hours to calculate Φ (Table 12). The 8-hour Φ, based on the average of $X_{E,t}$ and $X_{B,t}$ versus the theoretical maximal amount absorbed, is displayed in Figure 23 for each of the exposure groups. The fraction absorbed decreased as exposure concentration increased. One-way ANOVA revealed that differences in absorption between the groups were significant ($p = 0.0044$).

The time course for changes in fractional absorption over the 8-hour exposure for each group is displayed in Figure 24. Each hourly point was based on the summation to that

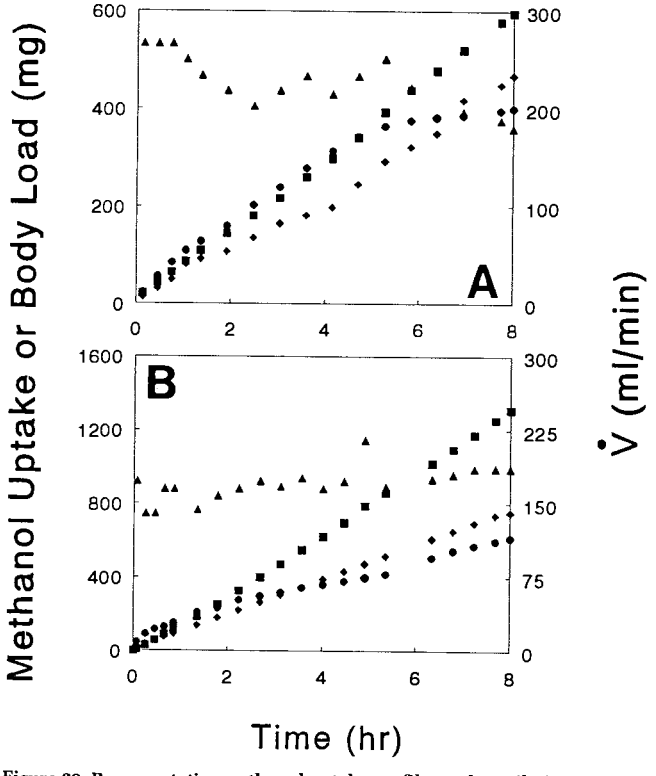

Figure 20. Representative methanol uptake profiles and ventilation rates in rats during exposure to 5000 ppm (A) or 15,000 ppm (B) methanol by inhalation. Triangles indicate ventilation rate determined over timed intervals; squares indicate the calculated $X_{A,t}$ assuming $\Phi = 1.0$; diamonds indicate calculated $X_{E,t}$; circles indicate calculated $X_{B,t}$. See text for explanation of symbols and relevant equations.

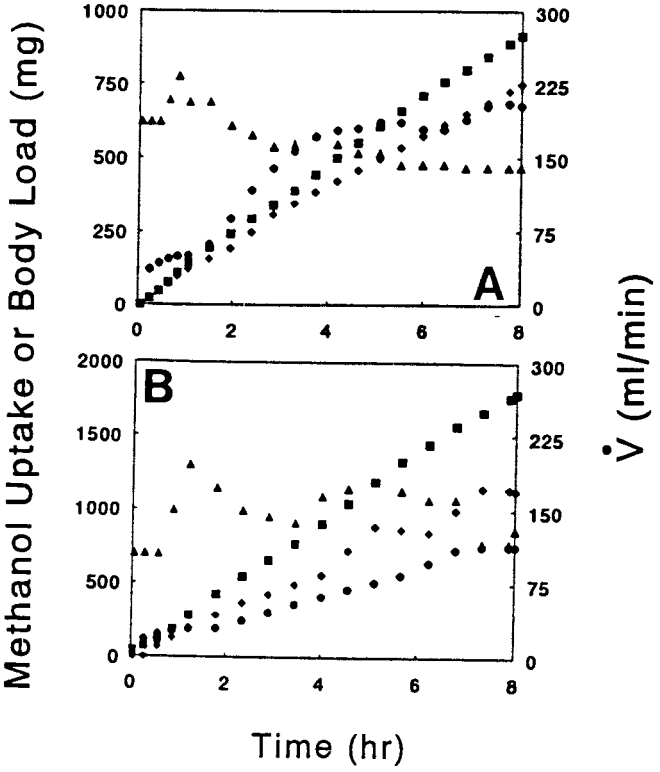

Figure 21. Representative methanol uptake and ventilation profiles for rats exposed to 10,000 ppm (A) or 20,000 ppm (B) metbanol by inhalation. Triangles indicate ventilation rate determined over timed intervals; squares indicate the calculated $X_{A,t}$ assuming $\Phi = 1.0$; diamonds indicate calculated $X_{E,t}$; circles indicate calculated $X_{B,t}$. See text for explanation of symbols and relevant equations.

time of the average of $X_{E,t}$ and $X_{B,t}$, divided by $X_{A,t}$. In general, Φ tended to decrease with time in all exposure groups, which suggests that fractional absorption may be influenced by methanol blood concentration. However, the nature of the data presented (i.e., summed across time of exposure) may have concealed a more pronounced relationship between Φ and time. To overcome this limitation, we examined the average values of $X_{E,t}$, $X_{B,t}$, and $X_{A,t}$ for each time interval during exposure (Figure 25). A trend of decreasing Φ with time was apparent in this analysis as well, although it was not as striking as that in Figure 24. These observations suggest that Φ may be a function of methanol blood concentration.

Results of the preceding experiment suggest that systemic methanol may alter respiratory function in rats. The relationship between Φ and Σ_{vent} was examined because the fractional absorption of methanol also appeared to be a function of methanol blood concentration. Figure 26 shows Φ for individual rats under all exposure conditions. For this analysis, we categorized animals by Σ_{vent} in each exposure group. In all five exposure groups, the animal with the lowest Σ_{vent} had the highest Φ, and in three of the five groups the rat with the highest Σ_{vent} had the lowest Φ. These results are in contrast to the time-dependent nature of methanol absorption. At long exposure times, when blood methanol concentrations are high, respiration should be depressed and absorption should be high. However, the fractional absorption of methanol actually decreased with time during exposure, which suggests that factors other than ventilation rate control the fraction of inhaled methanol absorbed into the systemic circulation. Total methanol absorption, however, increased as ventilation rate increased, as described in the preceding experiment.

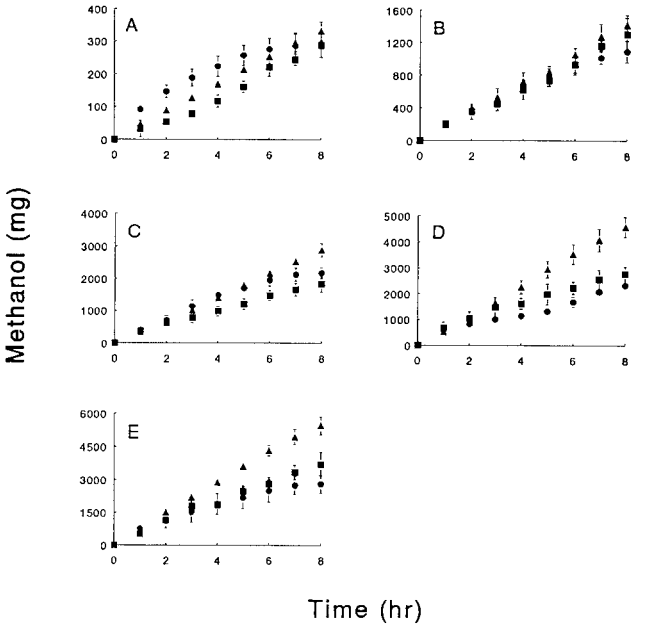

Figure 22. Mean ± SD theoretical maximal absorbed methanol (squares), $X_{E,t}$ (triangles), and $X_{B,t}$ (circles) during inhalation exposure to methanol at 1000 ppm (A), 5000 ppm (B), 10,000 ppm (C), 15,000 ppm (D), or 20,000 ppm (E). The difference between the theoretical maximal absorbed amount and $X_{E,t}$ or $X_{B,t}$ indicates the relative magnitude of Φ; increasing difference between these parameters is associated with decreasing fractional methanol absorption (Φ). See text for details.

Construction of a Toxicokinetic Model for Methanol Inhalation in Rats

The results of the preceding experiment suggest that the absorption of methanol is concentration-dependent. Such nonlinear absorption could be due to the influence of methanol on respiratory parameters in the rat. Alternatively, methanol absorption may be mediated entirely in the nasal cavity. Despite the large water-to-air partition coefficient for this compound, nonlinear (apparently saturable) absorption could occur if methanol concentrations in the limited aqueous space reached the level of saturation. To address this hypothesis, we obtained samples of inhaled air directly from the trachea of each rat during inhalation exposure to methanol via an indwelling tracheal cannula. Figure 27 depicts the methanol concentration as it enters and leaves the exposure chamber compared to the methanol concentration in tracheal air over an 8-hour exposure. These data indicate that only a very small portion of the inhaled methanol reaches the upper portion of the trachea; no detectable methanol was present early in the experiment, and only 1.5% of C_{in} during the latter stages of exposure. We obtained similar results at a 5000-ppm exposure with a cannula pushed to the back of the nasal cavity

Table 12. Fractional Absorption of Inhaled Methanol During an Eight-Hour Exposure

Exposure Concentration	*n*	Fractional Absorption from Airstream (Φ)[a]	Fractional Absorption from Airstream (Φ)[b]	Average[c]
1,000	4	0.88	0.86	0.87
5,000	3	0.76	0.92	0.84
10,000	3	0.75	0.63	0.69
15,000	3	0.51	0.61	0.56
20,000	4	0.52	0.68	0.60
Mean		0.68	0.74	0.71

[a] Calculated as the ratio of mass extracted from the airstream (X_E) to theoretical mass absorbed over 8 hours as described in the text.

[b] Calculated as the ratio of total body load (X_B) to theoretical mass absorbed over 8 hours as described in the text.

[c] Average of the two methods for calculating Φ.

(data not shown), which suggests that only limited quantities of inhaled methanol escape this region of the upper respiratory tract. In fact, the majority of the methanol detected in tracheal air may originate from the systemic circulation rather than from the airstream per se. Methanol concentrations in tracheal air were similar to the ratio of whole blood methanol concentrations divided by the approximate partition coefficient of methanol in blood (K_p = 3000). It should be noted that the ventilation rate in animals anesthetized with urethane was very low, with an average Σ_{vent} of 54 L/kg compared to approximately 250 L/kg for

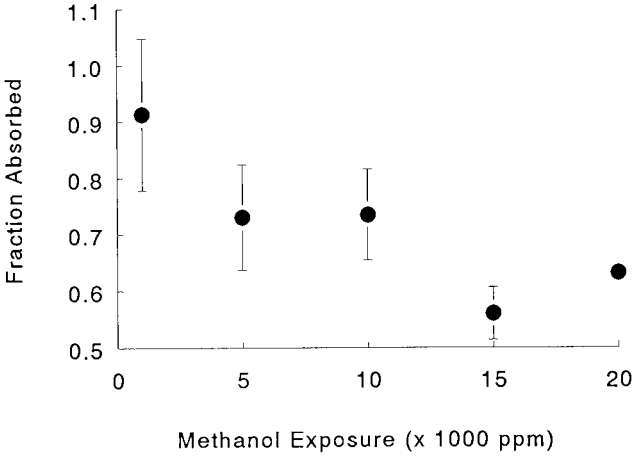

Figure 23. Relationship between the fraction of methanol absorbed (Φ) and methanol inhalation exposure concentration in nonpregnant rats. Circles and bars indicate means ± SD for n = 3 to 4 rats per group. The difference across concentration was statistically significant (ANOVA, $p < 0.05$).

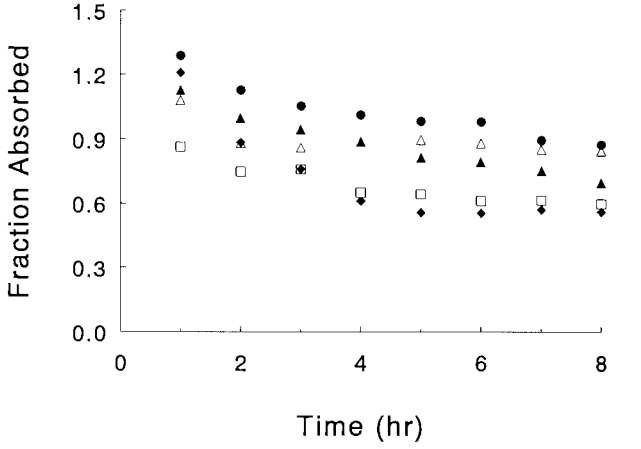

Figure 24. Fractional methanol absorption (Φ) in rats versus time based on cumulative methanol extraction data at various inhalation exposure concentrations (solid circle = 1000 ppm; open triangle = 5000 ppm; closed triangle = 10,000 ppm; closed diamond = 15,000 ppm; open square = 20,000 ppm). Symbols indicate mean data for n = 3 to 4 rats per group; error bars have been omitted for clarity.

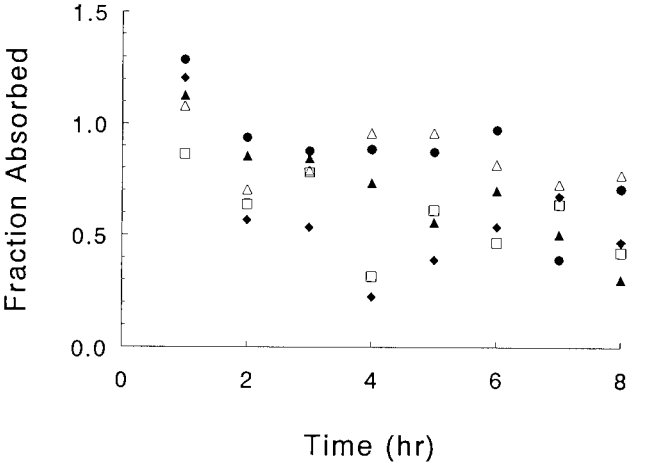

Figure 25. Fractional methanol absorption in rats versus time based on interval determination methanol extraction data at various inhalation exposure concentrations (solid circle = 1000 ppm; open triangle = 5000 ppm; closed triangle = 10,000 ppm; closed diamond = 15,000 ppm; open square = 20,000 ppm). Symbols indicate mean data for n = 3 to 4 rats per group; error bars have been omitted for clarity.

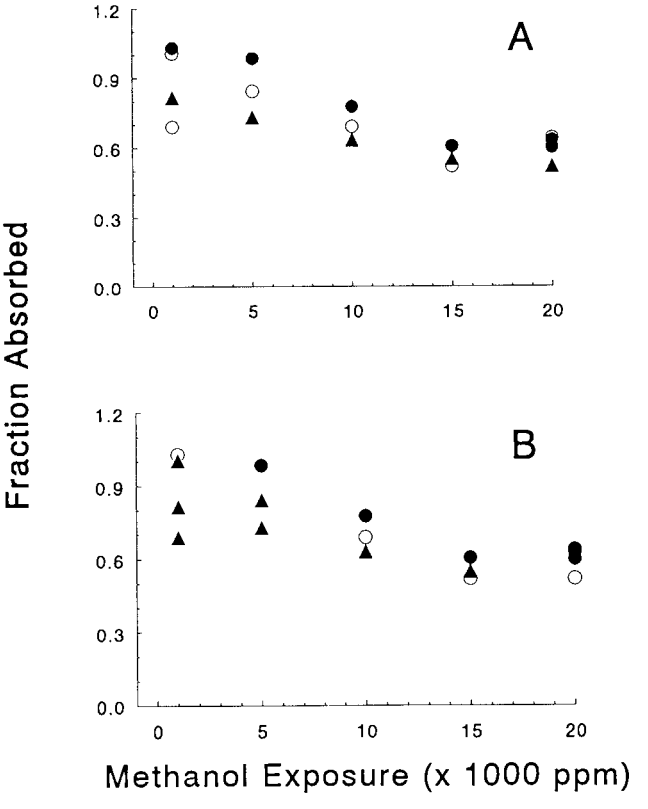

Figure 26. Fractional methanol absorption (Φ) versus exposure concentration for rats with low (closed circle), intermediate (open circle), or high (closed triangle) total ventilation (Σ_{vent}) based on cumulative (A) or interval (B) data. Data indicate that, on balance, animals with low ventilation rates evidence a higher fractional absorption of methanol than rats with high ventilation rates.

unanesthetized animals. Experiments in Phase V of this project indicated that the fraction of inhaled methanol absorbed into the systemic circulation increased as ventilation rate decreased. Thus, the data from the present experiment in anesthetized rats may underestimate the amount of methanol reaching the trachea through the nose in unanesthetized rats, because the anesthetized animals breathed at a slower rate. Despite this limitation, it is likely that the nonlinear fractional absorption of methanol in the rat is due to predominant absorption in the upper respiratory tract, where the available aqueous volume is limited, as opposed to absorption from the alveoli in which partitioning between air and the biologic matrix (lung tissue and alveolar blood) appears to conform to virtual sink conditions.

An alternative mechanism for the apparent nonlinear absorption of methanol during inhalation exposure is that uptake from the airstream is limited by the concentration of methanol in blood. This might occur if washout of methanol from blood into alveolar air is significant. To examine this possibility, we administered an intravenous bolus dose of methanol to rats during inhalation exposure at 5000 ppm. The intravenous dose selected was calculated to produce circulating methanol concentrations equal to those associated with inhalation exposure of 15,000 to 20,000 ppm methanol. The results of this experiment are displayed in Figure 28. These data indicate that the mass apparently absorbed by the animal (with an assumed fractional absorption of 1.0) approached the mass extracted from the airstream, which suggests that the actual fractional absorption approached 1.0. Despite the change in ventilation that occurred after administration of the bolus dose, we observed no deviation in the relationship between calculated absorption and mass extracted from the airstream after the intravenous dose was administered. The cumulative fractional absorption of methanol in the animals that received the superimposed bolus dose is compared to that in animals exposed to 5000 ppm methanol without the additional intravenous administration (data obtained in the previous experiment) in Figure 29. No obvious effect of the intravenous methanol bolus on Φ was observed (i.e., during the last 4 hours of exposure) compared to Φ in animals exposed to 5000 ppm methanol alone. Fractional absorp-

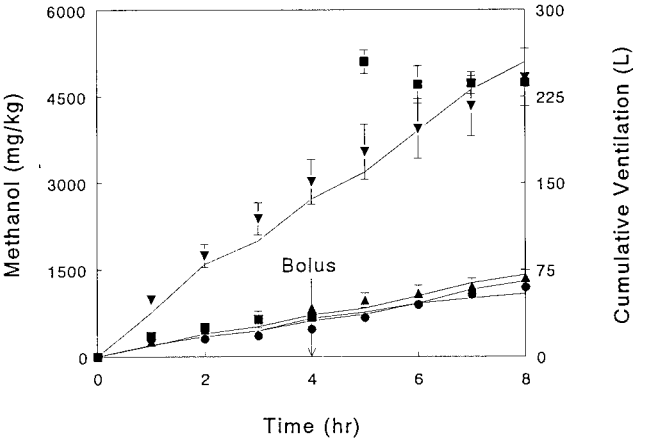

Figure 28. Influence of a 2-g/kg intravenous bolus dose of methanol (administered during inhalation exposure to 5,000 ppm methanol) superimposed on methanol blood concentration (squares), cumulative ventilation (inverted triangles), mass absorbed through time, t ($X_{A,t}$) (triangles), and mass eliminated through time, t ($X_{E,t}$) (circles). Arrow indicates time of methanol administration; symbols and bars represent means ± SD for n = 4 animals.

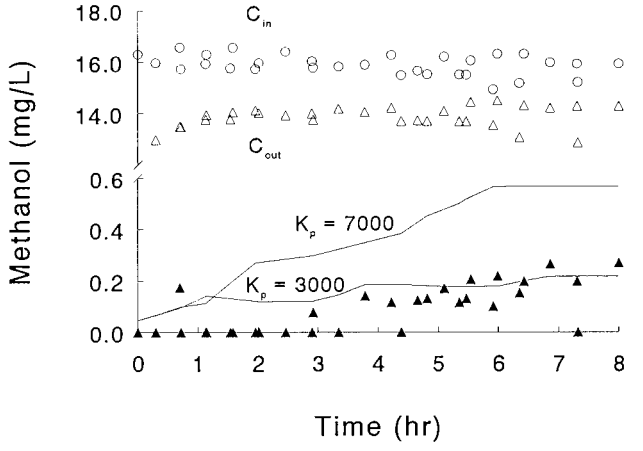

Figure 27. Methanol concentrations in a representative rat exposed via an indwelling tracheal cannula. The difference between C_{in} (circles) and C_{out} (open triangles) demonstrates methanol extracted from the airstream by the animal; methanol in tracheal air (solid triangles) was more than two orders of magnitude lower than C_{out}. Lines represent predicted tracheal air concentrations when methanol was derived entirely from the systemic circulation, and a blood-to-air partition coefficient (K_p) of 7000 or a water-to-air partition coefficient of 3000 was assumed.

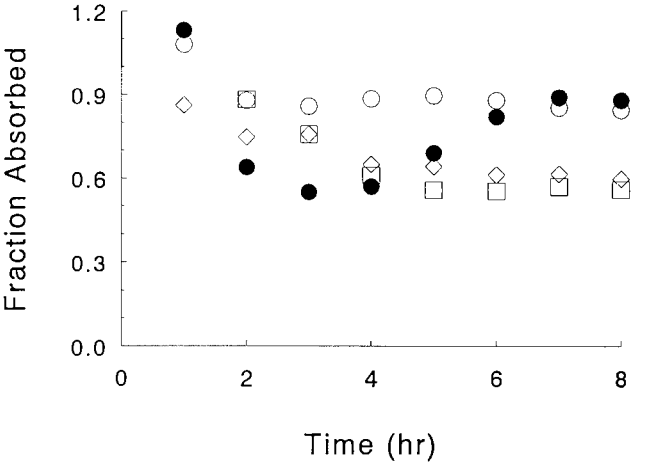

Figure 29. Time course of fractional methanol absorption during inhalation exposure of a nonpregnant rat to various concentrations of methanol (open circle = 5000 ppm; open square = 15,000 ppm; open diamond = 20,000 ppm) and a 2-g/kg intravenous methanol bolus dose superimposed on a 5000-ppm inhalation exposure (closed circles). Symbols represent mean data based on cumulative estimates of fractional absorption (see Figure 24).

tion of methanol at each hour during exposure, as opposed to the cumulative estimates of absorption, is displayed in Figure 30. The ventilation rate during the last 4 hours of exposure was lower in animals receiving the bolus dose of methanol, consistent with the higher circulating methanol concentrations in these animals and the hypothesis that methanol depresses respiration in a concentration-dependent manner (Figure 31). The degree of methanol absorption increased after 4 hours (Figure 30), again consistent with a methanol-induced decrease in ventilation (leading to more efficient absorption) as opposed to inhibition of absorption by systemic methanol. In animals exposed to 5000 ppm methanol and a superimposed intravenous bolus dose, absorption was higher than in animals with similar circulating methanol concentrations but exposed to higher atmospheric concentrations of the alcohol (15,000 to 20,000 ppm). Each of these observations suggests that the decrease in methanol absorption with increasing exposure concentration is due to the atmospheric concentration per se (i.e., the concentration in air at the site of absorption) as opposed to concentrations of methanol in blood or methanol-induced changes in respiratory function.

Despite the fact that the respiratory effects of methanol do not appear responsible for the decrease in fractional absorption with increased methanol exposure concentration, the data collected to this point in the project suggest that respiratory function is an important determinant of the total mass of methanol absorbed during an exposure period. To examine further the relationship between respiratory

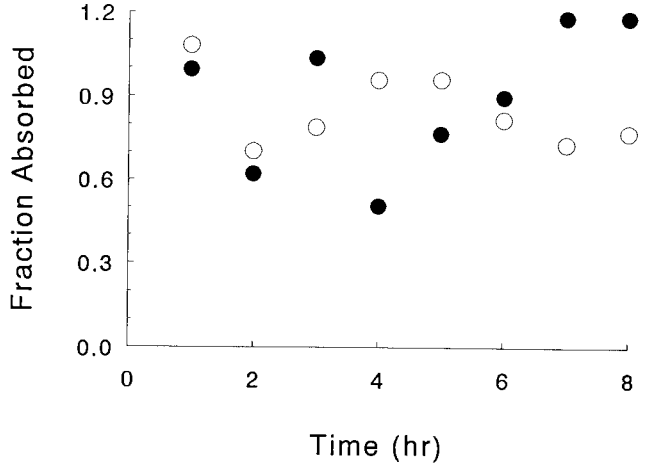

Figure 30. Time course of fractional methanol absorption during rat inhalation exposure to 5000 ppm methanol (open circles) and a 2-g/kg intravenous bolus dose of methanol superimposed at 4 hours on the 5000-ppm inhalation exposure (closed circles). Symbols represent mean data based on interval calculation of fractional absorption.

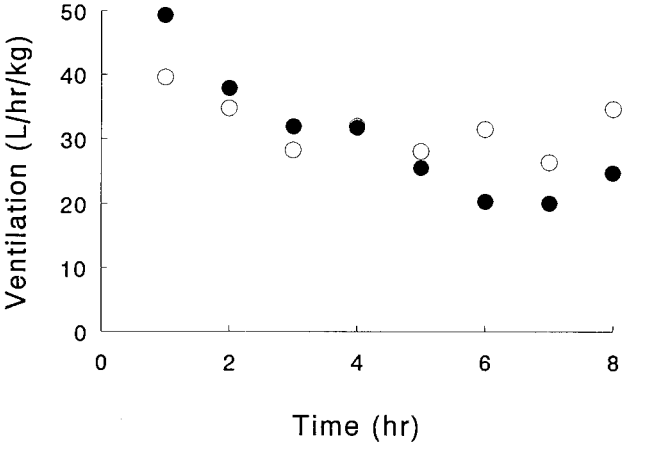

Figure 31. Time course of ventilation rate during rat inhalation exposure to 5000 ppm methanol (open circles) and 5000 ppm methanol with a 2-g/kg bolus methanol administration at 4 hours (closed circles). Symbols represent mean data.

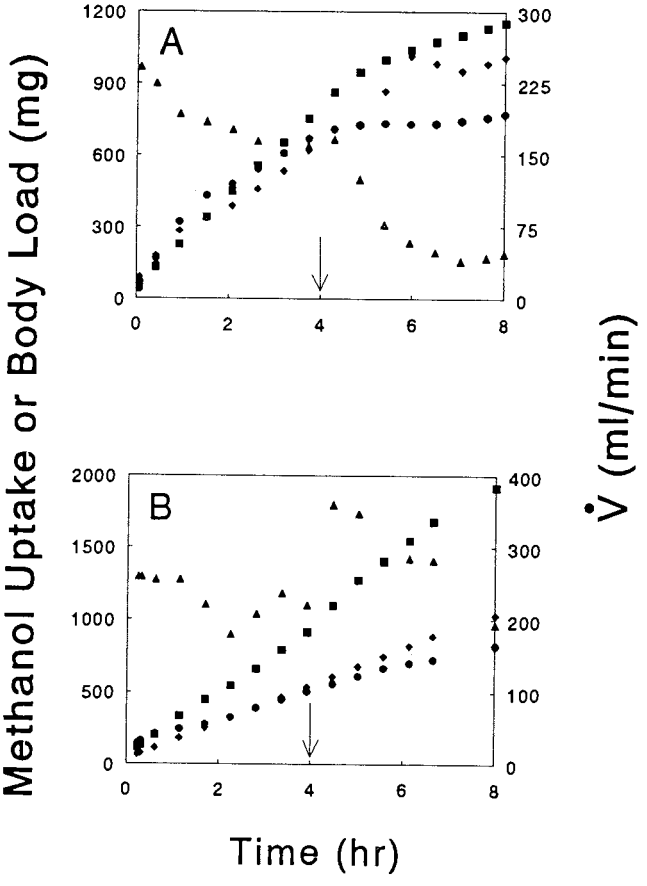

Figure 32. Influence of pentobarbital (A) or carbon dioxide (B) administration on ventilation and methanol uptake in representative rats exposed to 15,000 ppm methanol. Coadministration commenced at 4 hours (arrow). Triangles indicate ventilation rate determined over timed intervals; squares indicate the calculated $X_{A,t}$ assuming $\Phi = 1.0$; diamonds indicate calculated $X_{E,t}$; circles indicate calculated $X_{B,t}$.

function and absorbed mass, we perturbed the ventilation rate in rats by administering either pentobarbital (to depress respiration) or carbon dioxide (to stimulate respiration). Figure 32 displays the normalized methanol uptake ($X_{A,t}$) for two groups of rats exposed to 15,000 ppm methanol. In one group ventilation was decreased after 4 hours of exposure with an intravenous dose of pentobarbital; in the second group, ventilation was increased after 4 hours of exposure by administration of carbon dioxide via the influent air. Total methanol uptake increased during coexposure to carbon dioxide, and decreased after administration of pentobarbital, relative to the normal-ventilation group. This result was expected due to the changes in ventilation. The ventilation rate in the untreated rats remained relatively constant (averaged 31.7 L/hr/kg) throughout the exposure period, and was similar to the rate during the first 4 hours of the experiment in animals that subsequently received either pentobarbital or carbon dioxide (34.5 and 31.8 L/hr/kg, respectively). In the latter 4 hours of the experiment, however, the ventilation rate in rats that received pentobarbital decreased to 42% of control (13.5 L/hr/kg); in animals that received carbon dioxide, the ventilation rate increased by 34% (to 42.6 L/hr/kg). Despite the changes in respiration rate, there was little difference in the fractional absorption of methanol between the control animals and animals exposed to carbon dioxide during the last 4 hours of methanol exposure (data not shown). In contrast, the rats treated with pentobarbital showed an increase in the fraction of methanol absorbed, which corresponded to the decrease in ventilation rate (Figure 33).

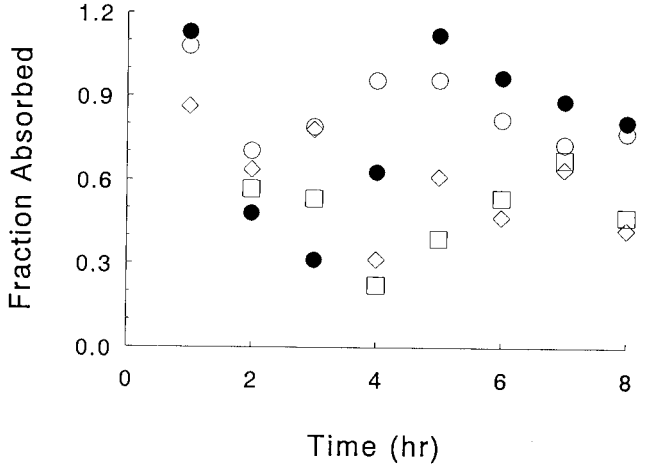

Figure 33. Fractional absorption of methanol in rats during exposure to methanol alone (open symbols; circles = 5000 ppm, squares = 15,000 ppm, diamonds = 20,000 ppm) or 5000 ppm methanol with pentobarbital coadministration at 4 hours (solid circles). Symbols represent mean data for $n = 4$ rats.

The data generated in the inhalation experiments described above served as the basis for the physiologically based pharmacokinetic model for methanol disposition during inhalation exposure. Figure 34 displays the results of model simulations of the mass of methanol in blood vs. time during an 8-hour exposure. For the three highest exposure groups, the description of the data by the model was extremely good; some divergence between the observed and predicted data occurred under the 1000- and 5000-ppm exposure conditions. The good degree of correspondence between the experimental data and model predictions indicates that the model developed is able to describe methanol disposition during inhalation exposure to methanol at concentrations up to 20,000 ppm for exposures as long as 8 hours.

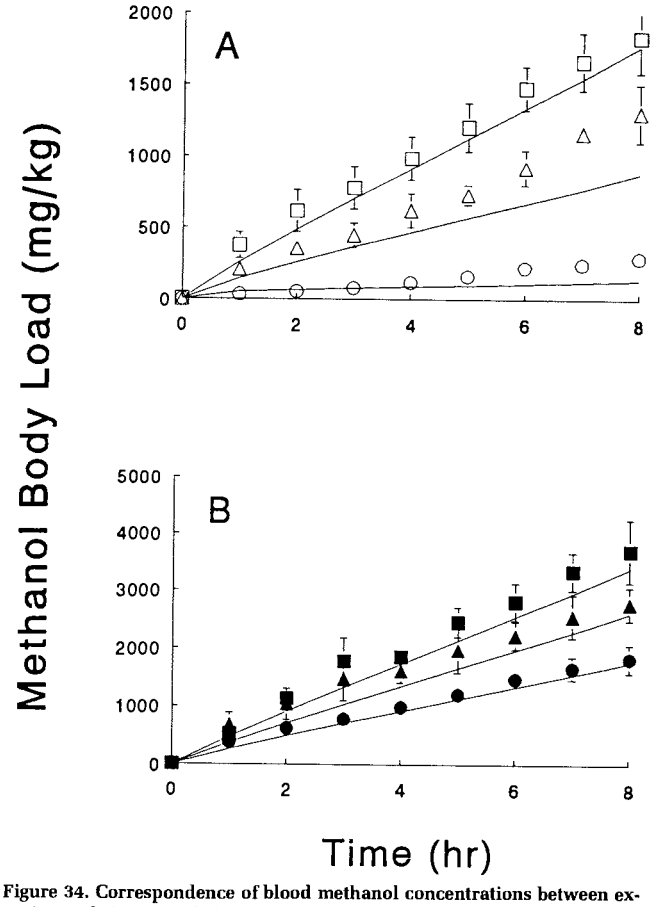

Figure 34. Correspondence of blood methanol concentrations between experimental data (symbols and bars represent means ± SD) and model predictions (lines) for nonpregnant rats exposed by inhalation to 1000 ppm (open circles), 5000 ppm (open triangles), 10,000 ppm (open squares in panel A, solid circles in panel B), 15,000 ppm (solid triangles), or 20,000 ppm (solid squares).

PHASE VI. DISPOSITION OF METHANOL DURING INHALATION EXPOSURE IN MICE

We exposed mice individually to methanol to determine respiratory function in the smaller species. For practical reasons (primarily due to the fact that several animals were killed at each time point to determine the methanol concentration-time profile), we exposed groups of mice simultaneously to methanol to obtain toxicokinetic data. The group-exposed mice and the individually exposed mice exhibited different behavior in the inhalation chamber. The group-exposed mice (similar to the individually exposed rats) evidenced a generally uniform behavior: they were moderately active for approximately 1.5 hours after introduction into the chamber, then slept (or displayed limited motor activity) for the remainder of the exposure. In contrast, the individually exposed mice displayed idiosyncratic behavior. The percentage of time that each mouse spent in active behavior is presented in Table 13, along with the weight-normalized $\dot{V}$ associated with both active and nonactive periods of behavior. The interanimal variability in the percentage of time spent in an active state was large. For example, in the 2500-ppm exposure group, the estimated time spent in active behavior varied from 18% to 62% throughout the 8-hour exposure period. There was no significant difference in mean $\dot{V}$ in the nonactive state between exposure groups. For the active state, there was more uniformity in $\dot{V}$ within the experimental groups, which led to a statistical difference among the groups ($p = 0.0387$). Thus, the respiratory response to methanol appears to be similar in mice and rats; $\dot{V}$ decreases as methanol exposure concentrations increase, secondary to increased systemic methanol and, most likely, methanol-induced respiratory depression.

The 8-hour ventilation values for individually exposed mice are summarized in Table 14. There was no statistical difference between the exposure groups due to the large intragroup variability (presumably a result of the idiosyncratic behavior displayed during the course of the experiment). To decrease the influence of interanimal variability, the data were pooled into low-exposure (0 and 2500 ppm; 835 L), intermediate-exposure (5000 ppm; 696 L) and high-exposure (10,000 and 15,000 ppm; 613 L) conditions. When the data were pooled in this fashion, a statistical decrease in Σ_{vent} as exposure concentration increases was demonstrated (Figure 35). We used this decline in ventilation as methanol concentration increased in the final kinetic model for the mouse and, as described below, allowed an approximation of the ventilation in the group-exposed mice.

Table 13. Ventilation of Mice During And Versus Passive Behavior

Exposure (ppm)	Animal	% of Time Active	$\dot{V}$(mL/min/27.5 g) Active	$\dot{V}$(mL/min/27.5 g) Passive
0	1	44	79.7	34.0
	2	0		31.1
2,500	1	62	78.1	37.3
	2	50	75.3	30.6
	3	30	76.9	35.6
	4	18	74.8	33.0
5,000	1	27	58.0	27.7
	2	45	64.7	29.8
	3	40	74.2	27.9
	4	10	51.1	30.3
10,000	1	20	55.0	33.1
	2	22	66.4	29.1
	3	33	44.2	20.3
15,000	1	40	68.2	32.7
	2	11	60.8	33.3
	3	44	48.1	23.3
Pooled average		31	65.0	30.6

Table 14. Eight-Hour Ventilation for Mice Exposed to Methanol by Inhalation[a]

	Exposure (ppm)				
Animal	0	2,500	5,000	10,000	15,000
1	967	1046	657	660	728
2	614	897	797	646	621
3	NA[b]	806	784	470	554
4	NA	682	549	NA	NA
Mean	790	858	696	592	634
SD		153	117	106	88

[a] Values are expressed in L/kg.

[b] NA = not applicable to a particular exposure concentration.

The observed methanol blood concentrations in the group-exposed mice were compared to the concentrations predicted with the kinetic model, which incorporated the ventilatory parameters obtained in individually exposed mice and the systemic disposition of methanol as determined after intravenous and oral administration of methanol (Figure 36). Both methanol cumulation during exposure (Figure 36A) and methanol washout after exposure (Figure 36B) are displayed. These model predictions incorporated the ventilation parameters in individually exposed mice, adjusted for the lower degree of physical activity in group-exposed animals according to the relationship presented in Figure 35. For both phases of the disposition profile, we noted good correspondence between model predictions and the observed data. Moreover, model predictions indicated that the fraction of inhaled methanol absorbed into the systemic circulation was approximately 0.85, and did not vary with exposure condition (Table 15). In the description of the experimental data, the results suggest that a fractional absorption of 0.85, coupled with a decreasing Σ_{vent} as inlet methanol exposure concentrations increase, yields an accurate model when combined with the systemic kinetic parameters.

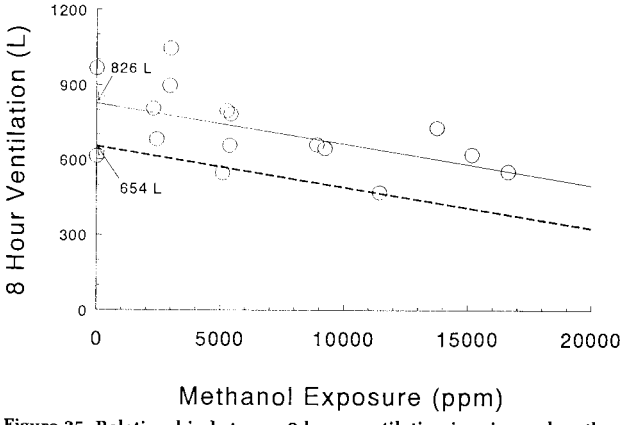

Figure 35. Relationship between 8-hour ventilation in mice and methanol inhalation exposure concentration. Symbols indicate data from individually exposed mice. Solid line represents least-squares regression of the data; dotted line indicates the predicted relationship for lower-activity mice exposed to methanol in groups (see text for details).

Comparison of the systemic methanol concentrations achieved at the end of a given exposure regimen between species is informative because we developed a kinetic model capable of describing methanol disposition during inhalation exposure in both the rat and the mouse. Such a comparison is particularly useful because of the differences in systemic disposition of methanol observed after intravenous and oral administration of the alcohol. Figure 37 compares experimentally determined blood methanol concentrations between the two species. Table 16 displays predicted concentrations under environmental exposure conditions (i.e., when the mass of methanol extracted from the ambient air is insignificant with respect to the total mass of methanol available for inhalation). Despite the more rapid elimination of methanol from the systemic circulation of the mouse, due predominantly to a higher V_{max} for methanol elimination in the smaller species, blood methanol concentrations at the end of an 8-hour exposure were approximately twofold higher in mice than in rats. This

Table 15. Fractional Absorption of Inhaled Methanol in Mice

	Exposure (ppm)			
Subject	2,500	5,000	10,000	15,000
1	1.15	0.86	0.76	0.97
2	0.76	0.89	0.76	0.94
3	0.78	0.72	0.97	0.61
4	0.75	0.94	NA[a]	NA
Mean	0.86	0.85	0.83	0.84
SD	0.19	0.09	0.12	0.20

[a] Values are expressed in L/kg.

interspecies difference was observed for both the experimentally determined and model-predicted methanol concentrations, and is due to a slightly more efficient absorption of methanol and higher ventilation rate in mice as compared to rats.

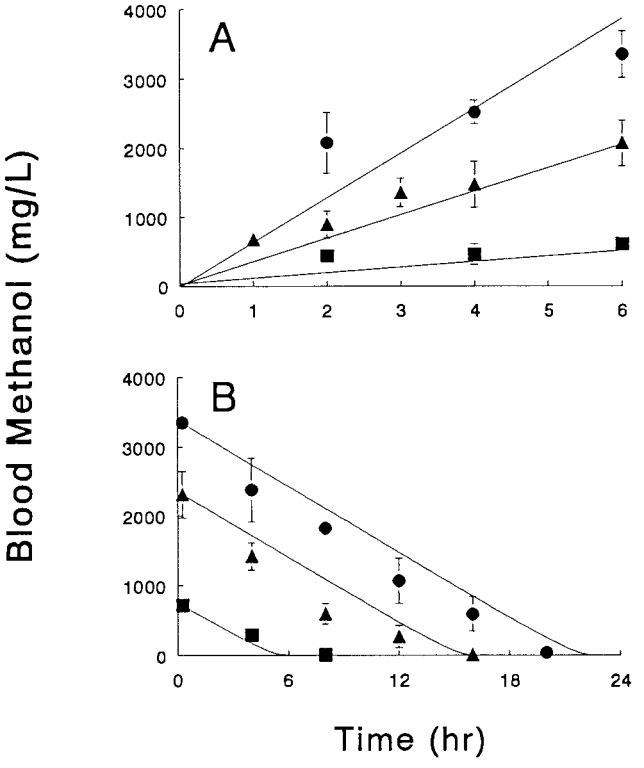

Figure 36. Correspondence between experimental data and model predictions (lines) for methanol disposition in mice during (A) and after (B) inhalation exposure at 2500 ppm (squares), 5000 ppm (triangles), or 10,000 ppm (circles). Symbols and bars represent means ± SD for n = 3 to 4 mice per time point; lines represent model predictions.

DISCUSSION

PHASE I. SYSTEMIC METHANOL DISPOSITION IN NONPREGNANT RATS

The disposition of methanol in the rat was markedly nonlinear, as anticipated for a short-chain aliphatic alcohol. The blood methanol concentration-time data were consistent with a significant capacity-limited elimination pathway, which presumably represents biotransformation to formaldehyde. It was not possible, however, to describe the disposition of methanol over the dosage range of 100 mg/kg to 2500 mg/kg with a single set of kinetic parameters. After administration of 100 mg/kg or 500 mg/kg intravenously, we described the concentration-time data adequately with

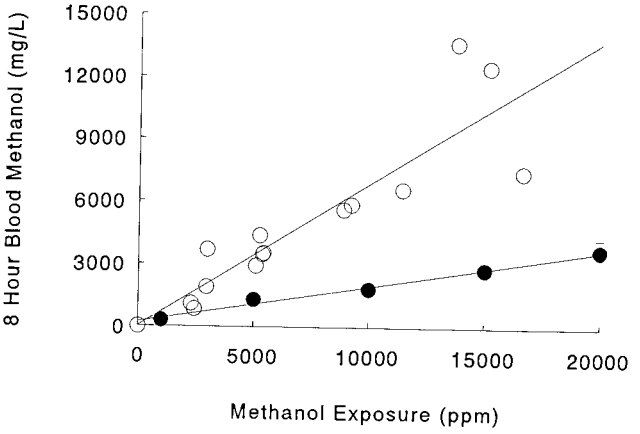

Figure 37. Relationship between 8-hour blood methanol concentrations and inhalation exposure to methanol in rats (solid circles and bars indicates; means ± SD for *n* = 4 rats) and mice (open circles show individual data). Lines indicate the result of linear least-squares regression.

Table 16. Predicted[a] and Observed Blood Methanol Concentrations After an Eight-Hour Exposure by Inhalation

	Rat		Mouse	
Methanol (ppm)	Observed	Predicted	Observed	Predicted
1,000	83 ± 15	93.5	ND[b]	268
2,500	ND	422	1,883 ± 278	1,729
5,000	1,047 ± 298	1,018	3,580 ± 599	4,188
10,000	1,656 ± 330	2,130	6,028 ± 506	8,303
15,000	2,667 ± 372	3,158	11,165 ± 1,329	11,273
20,000	3,916 ± 907	4,132	ND	ND

[a] Predicted concentrations were based on environmental exposures (see text for details); observed concentrations are reported as means ± SD. All values are expressed in µg/mL.

[b] ND = Not determined.

a classic two-compartment Michaelis-Menten elimination model. In contrast, it was necessary to incorporate a parallel linear clearance pathway to simulate methanol disposition in rats that received the 2500-mg/kg dose.

When we modeled the contribution of the linear pathway to total elimination (Figure 4), at blood methanol concentrations produced by the two lower doses the linear process was the lesser contributor to total methanol elimination. This is consistent with an observed increase in the AIC value when Equation 2 was fit to these data as compared to Equation 1. In contrast, at the high blood concentrations produced by the 2500-mg/kg dose, the linear pathway accounted for nearly 90% of methanol elimination. The results of this simulation suggest that the presence of the linear elimination pathway at lower methanol concentrations is masked by the predominant nonlinear pathway. This conclusion is similar to that reported for the disposition of [^{14}C]-methanol in male Fischer 344/N rats (Horton et al. 1992). After a 100-mg/kg intravenous bolus dose, 3.4% of the parent compound was recovered in urine and expired air (presumably representing linear methanol elimination). Based on a time-averaged methanol blood concentration of approximately 20 μg/mL in these rats, the current simulation predicts a 4% contribution of the linear pathway to overall methanol elimination.

A similar kinetic pattern was reported previously for ethanol disposition in the rabbit (Fujimiya et al. 1989) in which parallel Michaelis-Menten and first-order elimination were observed after high (3000 mg/kg) intravenous doses. A comparison of the kinetic parameters governing ethanol disposition in the rabbit with those that govern methanol disposition in the rat is informative. The V_{max} for ethanol (410 μg/mL/hr) in the rabbit is similar to the methanol V_{max} in rats (an average of 106 μg/mL/hr across the three intravenous dose groups in nonpregnant animals), correcting for differences in body weight and the molecular weights of the two alcohols. Values for K_m (30 μg/mL for ethanol in the rabbit vs. 39 μg/mL for methanol in the rat [again averaged across the three intravenous dose groups in nonpregnant animals]), V_c (approximately 300 to 500 mL/kg for both alcohols in both species), and the rate constant associated with the linear elimination pathway (0.06 hr^{-1} for ethanol in the rabbit; 0.0689 hr^{-1} for methanol in the rat) also were similar. Furthermore, the contribution of the linear elimination pathway to total alcohol disposition was quantitatively similar between the two studies. The linear pathway accounted for 50% of total ethanol elimination at a concentration of approximately 2200 μg/mL (47.8 mM) while first-order elimination of methanol accounted for 50% of total elimination at 1320 μg/mL (41.2 mM) methanol (Figure 4). Thus, similar kinetic processes appear to govern the disposition of both methanol and ethanol, and only minor kinetic differences between these short-chain alcohols are observed between the rat and rabbit.

The kinetic pattern observed in the present study, as well as that reported by Fujimiya and colleagues (1989), is apparently a general one that is not restricted to a single species or substrate. A similar pattern was observed for ethanol disposition in rats (Wendell and Thurman 1979), baboons (Salaspuro and Lieber 1978), and humans (Rangno et al. 1981). Thus, parallel linear and saturable elimination pathways for short-chain alcohols appear to exist in mammals. While the saturable elimination of short-chain alcohols is mediated by enzymatic catabolism involving both alcohol dehydrogenase (ADH) and catalase systems (Goldberg and Rydberg 1969; Bradford et al. 1993), the process underlying the first-order elimination of methanol is unknown. It is likely, however, that this parallel pathway represents the sum of excretion by at least two routes (exhaled breath and urine). Both processes are controlled by passive diffusion of the alcohol and are therefore first-order. The estimated sum of pulmonary and renal clearances of ethanol in humans (approximately 10 mL/hr/kg) (Holford 1987) is similar to the linear clearance of methanol in the rat as calculated by the product of λ and V_c in nonpregnant rats that received the 2500-mg/kg intravenous dose (i.e., 16.9 ± 4.6 mL/hr/kg). In the present study, renal clearance accounted for approximately one-half of the total linear elimination of the alcohol. In view of the rate of pulmonary blood flow in the rat (~170 mL/min/kg), a transpulmonary methanol extraction ratio of only 0.001 would be sufficient to account for the remaining linear clearance. This low degree of extraction is consistent with the low range of pulmonary methanol extraction reported herein (0.0324 ± 0.0232).

The apparent change in methanol disposition from essentially a Michaelis-Menten system to linear and nonlinear elimination routes that operate in parallel occurs at what is considered unrealistically high blood concentrations in humans. However, such a change in the kinetic profile has important consequences on the ability to perform high-dose to low-dose toxicologic extrapolations. Steady-state blood methanol concentrations produced by varying the daily methanol exposure can be predicted (Figure 38). The solid line depicts the simulated relationship based upon Equation 2, and incorporates the values of the kinetic parameters determined in the present study (2500-mg/kg dose). The dashed line shows the relationship predicted if blood methanol data were generated at a methanol

dose rate of 3 g/kg/day, analyzed according to Equation 1, and back-extrapolated to lower doses based upon the calculated value of V_{max} and K_m. In the latter case, assumption of a single saturable elimination pathway resulted in an overestimate of V_{max} (3.03 g/day/kg based upon Equation 1 vs. 0.876 g/day/kg based upon Equation 2). It is usually assumed that, when nonlinear kinetics prevail, risk estimates based on high-dose data will overestimate risks associated with low-dose exposure due to a disproportionate increase in systemic concentrations with increasing dose. In the presence of parallel linear and nonlinear elimination pathways, predictions based upon high-dose data may actually underestimate the systemic concentration of a parent chemical after low-dose exposure if a simple Michaelis-Menten model is used, as opposed to a kinetic model that incorporates both elimination pathways. This kinetic behavior underscores the importance of delineating the complete disposition profile of a potential toxicant for purposes of risk assessment. However, comprehensive risk assessment must include consideration of derived metabolites (e.g., formate) when those metabolites contribute to the toxic effects of the parent. Investigations continue to determine the potential role of derived formate in methanol-induced embryotoxicity in rodents.

PHASE II. INFLUENCE OF PREGNANCY ON METHANOL DISPOSITION IN THE RAT

Changes in the disposition of xenobiotics in pregnant mammals were observed for numerous compounds elimi-

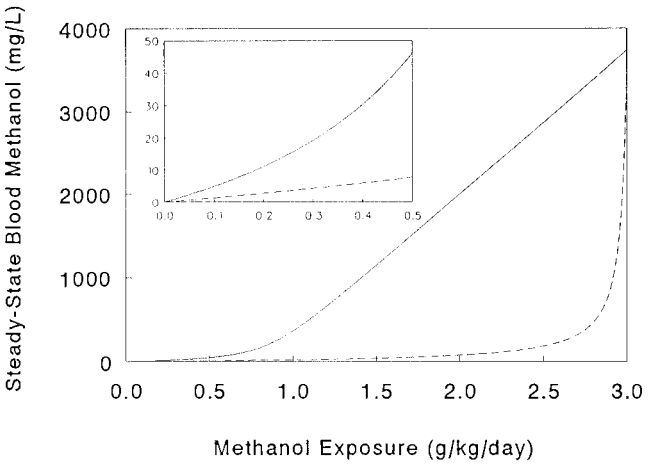

Figure 38. Predicted relationship between steady-state blood methanol concentrations and daily methanol exposure in nonpregnant rats based on a simple Michaelis-Menten analysis of the data obtained after a 2500-mg/kg intravenous dose (broken line) and the parallel linear and nonlinear model (solid line). The comparison indicates the necessity for complete characterization of the kinetic system for accurate high-dose to low-dose extrapolation. The insert enlarges the lower levels of methanol.

nated primarily by biotransformation, including the cytochrome P-450 model substrate antipyrine (Brouwer and Vore 1985), caffeine (Dorrbecker et al. 1988a), and a variety of therapeutic agents (Cummings 1983). Some of the dispositional changes observed are explained on the basis of pregnancy-induced alterations in organ blood flow, particularly to the liver and kidney, the primary organs of xenobiotic elimination (Buelke-Sam et al. 1982). We anticipated, therefore, that methanol disposition in the pregnant rat may be different from that in nonpregnant animals, and that systemic methanol disposition may change as gestation progresses. Such changes alter the relationship between the exposure dose and methanol concentrations in the maternal circulation, and potentially perturb the relationship between the magnitude of exposure to the dam and methanol exposure to the developing conceptus.

The pregnant state, whether considered a distinct condition that does not change during the course of gestation or a time-dependent change in physiology, did not influence the systemic disposition of methanol significantly. After intravenous administration, the parameters associated with methanol toxicokinetics did not vary consistently between pregnant and nonpregnant rats or across gestational stage (Tables 3 and 4). Although a general tendency was observed for V_{max} to decline with increasing gestational stage, with an associated increase in AUC, this trend was not statistically significant for either parameter; the magnitude of the observed changes in mean V_{max} between groups, if reflective of actual changes in methanol disposition, would result in only minor perturbations in methanol cumulation during prolonged exposure. Similarly, we observed no significant changes in methanol disposition during pregnancy after administration of the alcohol by gavage (Tables 5 and 6). As was the case with methanol disposition after intravenous administration, the changes observed in methanol toxicokinetics after an oral dose are not anticipated to result in significant alterations in methanol cumulation during prolonged exposure.

In summary, the results of this experiment suggest that pregnancy exerts only a minor influence on the systemic disposition of methanol. Thus, development of a comprehensive physiologically based pharmacokinetic model for methanol disposition need not consider pregnancy-related changes in toxicokinetics within the maternal component of the maternal-fetal model. Total systemic exposure to the alcohol, as expressed by the AUC, may increase slightly in pregnancy at low methanol doses; this is consistent with a progressive decrease in the V_{max} for methanol elimination during gestation. However, these observations do not preclude a gestational dependency in the kinetics of methanol flux between the maternal circulation and the fetus and

fetal environment. Indeed, the observed changes in distributional rate constants suggest that the rate of methanol distribution throughout the maternal system decreases as gestation progresses.

PHASE III. SYSTEMIC DISPOSITION OF METHNAOL IN THE MOUSE

The experiments that constitute this portion of the project were designed to characterize methanol disposition in the female CD-1 mouse, and to compare methanol disposition in the nonpregnant mouse to that in the nonpregnant rat. These experiments indicate that the maximal rate of methanol elimination after a 2500-mg/kg dose of methanol was approximately two-fold higher in the mouse than in the rat. This interspecies difference in V_{max} was responsible for the more rapid decline in mouse blood methanol concentrations as compared to that observed in the rat. These results confirm earlier work by Mannering and coworkers (1969), which demonstrates that the rate of oxidation of a single dose of ^{14}C-methanol is more than twofold higher in mice than in rats. The apparently low K_m for both species (< 50 mg/L) indicates that methanol elimination proceeds as an essentially zero-order process throughout the majority of the elimination profile at the doses administered in these experiments.

These observations may have significant implications for chronic methanol toxicity or teratogenicity studies in rodents. Similar daily exposure conditions in both rats and mice would result in higher blood methanol concentrations in rats (even after a single dose as shown in the present study). In rats exposed chronically to high daily oral doses, methanol would be expected to accumulate significantly over the duration of the study; mice, however, would eliminate essentially the entire methanol dose within 24 hours, which would result in limited accumulation. For example, Figure 39 displays a simulated multiple-dose concentration-time profile based upon the toxicokinetic parameters reported in Table 7, which assumes that a single dose of methanol (2500 mg/kg) per day was administered intravenously for 7 consecutive days. The differences in predicted methanol concentrations between species highlight the importance of considering the toxicokinetics of a compound when species differences in toxicity are examined, especially when extrapolating between species for risk assessment.

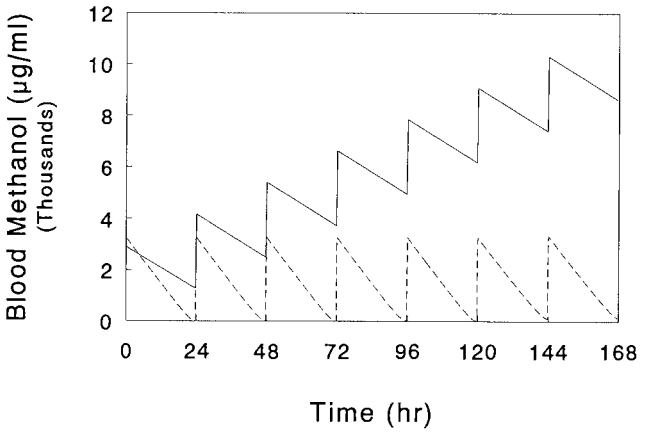

Figure 39. Simulated methanol cumulation profiles during daily intravenous administration of 2500 mg/kg methanol in rats (solid lines) and mice (broken lines) based on the parameters presented in Table 7 (for oral administration). The higher degree of accumulation in rats is due to the lower V_{max} for methanol elimination.

When we considered the absorption characteristics of methanol from the gastrointestinal tract, we anticipated rapid and complete absorption within a short time after exposure, as shown in earlier experiments in this project and work in the laboratory of other investigators (Leaf and Zatman 1952; Stegink et al. 1981). Mice absorbed a significant fraction of the methanol dose very rapidly, with the remainder absorbed for up to 10 hours after gavage. This phenomenon was manifested as a period from 2 to 10 hours after exposure during which the blood methanol concentration declined much less rapidly than expected based upon methanol disposition after an intravenous dose (Figure 10), suggestive of the continued absorption of methanol into the systemic circulation. Slow methanol absorption also was observed by Lee and coworkers (1994) after an oral dose of 3500 mg/kg to male Long-Evans rats. In that study, blood methanol concentrations increased for at least 6 hours after gavage, although a complete toxicokinetic profile for methanol was not developed. Physiologically, such a process could be explained by several factors. Preliminary experiments in this laboratory indicate that methanol distributes much more readily into water than into lipids. Thus, the absorption of methanol from the gastrointestinal tract is dependent largely upon water absorption, which occurs at different rates in different segments of the intestine (Kutchai 1988). An additional consideration is that the animals used in this experiment were not deprived of food or water prior to gavage. An oral dose of methanol could distribute into aqueous intestinal contents, and be released slowly as the contents moved down the intestine. Further clarification of this effect may be obtained by comparing the absorption characteristics of methanol in fasted animals to those in animals allowed food and water ad libitum.

There was a large degree of variability in the data obtained in the first 2 hours after oral administration of methanol to mice (Figure 9). Initially, this variability was hypothesized to be an artifact of the decapitation method used to obtain the samples. However, while there was variability between different time points across this period, the variability within a given time point typically was small, which indicates that interanimal differences were not an artifact of the sample collection technique. When blood samples were obtained by decapitation, the blood methanol concentration 1 hour after exposure was consistently lower than in samples at both earlier and later time points. While the higher concentrations prior to 1 hour could be due to methanol contamination from the gastrointestinal tract to trunk blood immediately after decapitation, it is unlikely that the consistently higher concentrations at later times were an artifact of the decapitation procedure. The studies performed on jugular vein–cannulated mice to obtain blood samples after an oral exposure did not demonstrate this effect, but the limited number of samples do not prove or disprove whether the variability is entirely an artifact of decapitation. Several possible explanations exist for this apparent variability in the methanol disposition profile. As discussed previously, only a portion of the methanol dose is absorbed rapidly from the gastrointestinal tract. While some of the variability, especially at the early time points, is a result of nonuniform methanol absorption, the slow absorption process does not supply sufficient methanol to account for all of the variability, such as that between 1 and 1.5 hours. The generation of hydrogen peroxide in the liver is likely to be the rate-limiting step in the biotransformation of methanol by the catalase system (Tephly and McMartin 1984). The hormonally controlled degradation of endogenous substrates such as fats and purines by oxidase enzymes is the primary means for the generation of hydrogen peroxide (Röe 1982; Voet and Voet 1990). Because methanol was shown to alter normal hormone levels in rodents (Cooper et al. 1992), and stress is associated with the various experimental procedures typically employed to characterize the disposition of xenobiotics (for example, substrate administration by gavage), hormonal variations after methanol exposure is one possible explanation for the variability of blood methanol concentrations observed in the first 2 hours after exposure.

Pregnancy altered methanol disposition in female mice in a manner similar to rats. Absorption (a decrease in the near-term rapid methanol absorption process), distribution (a decrease in apparent midgestation distributional volume), and elimination (a trend, albeit not statistically significant, toward decreased V_{max} across gestational stages) processes all appeared to be affected somewhat by pregnancy. Decreased V_{max} for elimination in gestation was confirmed by in vitro metabolism studies in both the mouse and the rat (Table 9). However, as was the case in rats, blood methanol concentrations were similar between nonpregnant and near-term pregnant mice during most of the disposition profile after a single oral dose of methanol (Figure 12); differences between pregnant and nonpregnant animals became apparent during the latter stages (12 to 24 hours in mice, 40 to 50 hours in rats) of the profile in both rodent species.

With regard to the teratogenic potential of methanol, some question still remains as to whether methanol itself is the proximal toxicant or if formate, the ultimate biotransformation product of methanol, causes the embryotoxic effects. In this study, mice eliminated methanol much more rapidly than rats. The majority of this elimination is mediated by biotransformation to formaldehyde and then to formate (Kavet and Nauss 1990). Thus, overall formation of formate should proceed more rapidly in mice than rats. However, Johlin and coworkers (1987) demonstrated that the maximal rate of formate oxidation also is more rapid in mice (300 mg/kg/hr vs. 78 mg/kg/hr in rats). Based upon these observations, it seems unlikely that the mouse experiences a greater body burden of formate than the rat. Only limited information is available about the disposition of methanol or formate in the developing conceptus of either species. Given the large species differences in the elimination of methanol from the nonpregnant rodent, differences in maternal-fetal disposition of methanol and formate may play a role in the differential embryotoxic sensitivity of rats and mice to methanol; this is an ongoing research topic in this laboratory.

One scenario in which formate may be the proximal toxicant despite limited accumulation in the maternal circulation is if methanol is metabolized to a significant extent in the conceptus, which results in relatively high concentrations of formate in the target tissue. Near-term rat and mouse fetal liver metabolized methanol with a V_{max} of only approximately 5% to 6% of that in the corresponding adult liver. Although it was not possible to test experimentally, it is unlikely that liver at earlier gestational stages would metabolize methanol at a rate faster than that in the near-term fetus. Thus, it is probable that production of formate in the conceptus is insufficient to generate significant concentrations of the metabolite (in comparison to the amount that reaches the conceptus from the maternal circulation), although further experimentation is required to confirm this hypothesis. It is interesting to note that the species difference in methanol metabolism in adult liver (V_{max} in the mouse nearly two-fold higher than in the rat) also is

present in the near-term fetus to a certain extent (V_{max} in mouse fetal liver approximately 50% higher than in rat fetal liver). The toxicologic implications of these differences, and their potential importance in extrapolation across species for risk assessment, remains unconfirmed.

In summary, the experiments that constitute this portion of the project demonstrate that methanol disposition is similar between rats and mice, although mice eliminate methanol nearly twice as rapidly as rats. This information will be useful in risk assessment based on extrapolation from rodent studies, and should be considered in the design of chronic exposure experiments with methanol are planned. Further studies are needed to clarify the implications of these data on the differences in species' sensitivity to teratogenic effects associated with methanol. Finally, we characterized the absorption of methanol from the gastrointestinal tract in mice as biphasic, with both a fast and a slow component.

PHASE IV. METHANOL DELIVERY TO THE FETAL ENVIRONMENT

Data regarding xenobiotic translocation between the maternal circulation and the developing conceptus or its immediate environment (or both) are relatively limited. Such translocation is primarily assumed to be governed by passive diffusion (Levy 1981; Krauer and Krauer 1983; Brazier et al. 1984). For example, caffeine and its metabolite paraxanthine readily penetrate the developing rabbit conceptus, and distribute homogeneously throughout the fetus and fetal environment (Dorrbecker et al. 1988b). Thus, the concentration gradient typically drives the net transfer of substrates from maternal blood to fetus, which is influenced by the ability of the substrate to permeate cellular membranes (Sibley et al. 1983). While carrie-mediated translocation was documented for some substrates (Leichtweiss et al. 1987), such saturable transfer should not occur for small molecules such as methanol that rapidly penetrate the lipid domain of cellular membranes. Fetal tissue also mediates biotransformation of some compounds, although available data are somewhat limited in scope (Juchau et al. 1983).

In the experiment reported herein, the rate of methanol permeation from the maternal circulation to amniotic fluid, presumably representative of fetal exposure, decreased with increased maternal blood methanol concentrations. In general, such an inverse relationship between permeation rate (or extravascular-to-blood partitioning, as demonstrated in the present experiment) and blood concentration suggests either active transport into the site of interest or a decrease in blood flow to the site of interest. As discussed above, it is unlikely that methanol permeation into the fetal environment is a carrier-mediated event. However, in view of the cardiovascular effects (mediated via central nervous system depression) of short-chain alcohols, it is possible that high circulating methanol concentrations decrease blood flow to the fetal environment, and thereby limit methanol delivery to this particular site. If such a change in blood flow occurs, and is significant, it is possible that the resulting decrease in oxygen delivery to the conceptus could have deleterious effects on organ differentiation and development. The potential hemodynamic influences of methanol exposure in pregnancy require further evaluation.

PHASE V. METHANOL DISPOSITION DURING INHALATION EXPOSURE IN RATS

Experiments performed in this phase of the project helped to develop the appropriate techniques to characterize methanol disposition during inhalation exposure, and to apply those techniques to generating the data required to develop a physiologically based pharmacokinetic model for methanol uptake and disposition. An advantage of the method developed is the ability to measure respiratory parameters in individual animals throughout the exposure period. Thus, data from the literature in other experimental settings is not required. It is important to note respiratory function can be treated as a dynamic rather than static process, which allows exposure conditions to influence ventilation rate in the kinetic model (as in the laboratory).

Clearly the large degree of interanimal variability in ventilation observed in this study, as well as the large differences between various published values of ventilation (see references in Table 11), indicate that care is required in selecting ventilation rates for use in inhalation toxicokinetic models. In detailed kinetic analyses, any error in the estimated value of V results in a proportional error in the administered dose. The errors associated with estimation of the inhaled dose from fixed values of respiratory function are undoubtedly important contributors to the failure of physiologically based toxicokinetic models to predict accurately the disposition of many vapor-phase xenobiotics. The present method allows measurement of V during the experiment; coupled with quantitation of C_{in} and C_{out} in the well-stirred chamber, the mass absorbed in any given experimental interval may be estimated precisely. Significant vapor-phase toxicant adsorption to the fur complicates kinetic analysis. Based on the lack of methanol adsorption by dead animals documented in preliminary experiments, we assumed nonspecific methanol loss onto the fur of animals to be negligible.

The apparatus we constructed in this experiment allowed us to collect blood samples throughout the exposure period. Toxicokinetic models of blood methanol concentrations, together with concentrations of methanol in chamber

air, can therefore be based on parameters generated from direct administration of the alcohol into the systemic circulation. In the present experiment, we multiplied blood methanol concentrations by the apparent volume of distribution, estimated in the previously described experiments in which we administered methanol intravenously, to obtain the total methanol body load at any point in time during inhalation exposure. Through summation of the mass of methanol eliminated over each experimental interval (again based on parameters derived from data after intravenous methanol administration), the cumulative mass of methanol eliminated from the animal could be estimated at each point in time. Total methanol uptake through a given time point is then the sum of these two mathematical functions.

Compared with the standard nose-only exposure method, the present approach has the additional advantage of using ambulatory animals. Thus, perturbations in ventilation due to restraint per se are not an issue. Such perturbations limit the applicability of physiologically based models between experimental settings or, potentially, between animal species. The nose-only method provides an accurate measurement of ventilation, and hence inhalation kinetics, but predictions made from the results of those data would be based on the much higher ventilation rates in restrained compared to unrestrained or lightly restrained rodents. Caution is therefore required to extrapolate the relationship between blood methanol concentrations and inhalation exposure in restrained animals to the unrestrained condition in which toxicity measurements typically would be obtained.

It should be noted that the breathing frequency reported in rats exposed via the nose-only method is approximately twice that observed in the present approach, while V_T is approximately the same (Table 11 and references contained therein). The estimation of V_T in the present method is subject to error due to variations in three key parameters: calibration of the volume displaced during respiration to the deflection of the recorder pen; the temperature and humidity of the chamber; and both body core and end-of-nose temperatures. However, the good correspondence between the observed V_T and published estimates of this parameter suggests that any bias associated with the present approach is minimal. Because V_T is approximately the same, breathing frequency accounts for the wide discrepancy in ventilation rates between the nose-only method and the present approach, with breathing frequency in the nose-only experiments approximately double that in the present study. In addition, measurements taken in the first few minutes after insertion of an animal into the exposure chamber indicated that both $\dot{V}$ and breathing frequency are consistent with estimates from the nose-only exposure protocol. Heightened arousal upon entrance into the new environment stimulated respiration, albeit transiently, to levels observed in restrained animals.

An additional advantage of the present approach is the generation of two semi-independent estimates of total systemic exposure to methanol. Either $X_{E,t}$ or $X_{B,t}$ can be used to express total systemic exposure through time t, which is a critical parameter to estimate absorption efficiency during inhalation exposure. Moreover, since there was generally a good correspondence between exposure estimates based on methanol extraction from the airstream and those based on determination of blood concentration, future studies could eliminate blood sampling without an associated loss of analytic power.

The relatively large amount of methanol recovered in urine required that we quantitate the urine in the chamber for this highly water-soluble compound. Methanol concentrations in urine at the end of the experiment were approximately 7000-fold higher than C_{in}; 7000 is approximately the Henry's law constant for methanol and water (Perry 1973). Thus, the methanol concentration in urine provides no information about urinary excretion in the exposed animal, but allows correction for the nonspecific loss of methanol from chamber air into excreted urine.

Use of this apparatus to examine methanol disposition during inhalation exposure revealed several fundamental aspects of the behavior of this compound. As anticipated for a general central nervous system depressant, methanol decreased ventilation in a concentration-dependent manner (Figure 19). Although the data were variable, we developed a statistical relationship between the change in $\dot{V}$ and blood methanol concentrations. Because the systemic dose is linked directly to ventilation for inhaled toxicants, incorporation of probable changes in ventilation during exposure (due to cumulation of methanol in the systemic circulation) is important. Furthermore, the efficiency of methanol absorption from the airstream decreased with increasing exposure concentration (Figure 23). The dose of methanol that reached the systemic circulation from the environmental atmosphere therefore is dependent on the atmospheric concentration of methanol in two respects: increasing methanol concentrations in air result in higher systemic concentrations and decreased ventilation, and increasing methanol concentrations in air are associated with decreased absorption efficiency from the airstream. The decreased absorption efficiency is perhaps more striking in view of the fact that, at a fixed exposure concentra-

tion, absorption efficiency increased with decreased respiratory rate (Figure 26). Thus, one would anticipate that increased methanol exposure concentration should increase absorption efficiency by virtue of the associated decrease in respiration. Taken together, these two factors result in a less than proportionate increase in the systemic dose with increased methanol concentrations in the exposure chamber.

It is interesting to note that the standard physiologically based pharmacokinetic models predict approximately 70% absorption for methanol, based on essentially complete absorption due to the very high blood-to-air partition coefficient with approximately 30% loss to dead space in the respiratory tract. The average fractional absorption across all exposure groups was 71% (Table 12), which agrees with the predicted value. However, the prediction of 70% absorption is based on the assumption that methanol absorption is mediated entirely in the alveoli. As discussed below, this assumption appears erroneous for methanol. Moreover, the standard assumption yields a concentration-independent fractional absorption for methanol, again due to the high blood-to-air partition coefficient that leads to a low degree of washout into alveolar air. This prediction clearly is not borne out by the experimental data. Thus, the good correspondence between the average fractional absorption across experiments and predictions based on previously developed inhalation models appears to be merely fortuitous.

Use of inhalation data for risk assessment requires adequate knowledge about the mechanism or mechanisms of changes in absorption so that variation in any mechanism can be accounted for in extrapolating between species. We performed several experiments to elucidate the mechanisms of changes in methanol absorption from the airstream. An initial hypothesis that the nonlinear absorption was due to methanol-induced changes in respiration was not supported by the experimental data. Changes in methanol absorption associated with perturbations in respiration with pentobarbital (decreased ventilation) or carbon dioxide (increased respiration) resulted in changes in absorption opposite to those observed with decreased respiration due to increased concentrations of systemic methanol. Fractional absorption was increased marginally after pentobarbital administration, and decreased after introduction of carbon dioxide into the inhalation chamber. Furthermore, systemic methanol did not appear to control methanol absorption beyond the changes associated with altered respiratory function. We administered a large intravenous dose of methanol during inhalation exposure that altered respiratory rate, but did not perturb methanol absorption from the airstream (Figure 28).

Because we failed to demonstrate the required relationship between respiratory rate and fraction absorbed, we hypothesized that methanol is absorbed entirely in the nasal cavity. The limited aqueous volume in this space may be saturated at high atmospheric concentrations of methanol, which could result in significant washout during exhalation. To address this hypothesis, we obtained air samples from rats with indwelling tracheal cannulae. Only a small amount of methanol could be measured in tracheal air at an exposure concentration of 15,000 ppm, despite the fact that methylene chloride, a substrate known to be absorbed into the alveoli and used herein as a positive control, was clearly present in these air samples. Also, the air concentrations of methanol measured in the tracheal samples were consistent with the blood methanol concentration divided by the water-to-air partition coefficient. Thus, it is apparent that methanol was absorbed entirely in the upper respiratory tract in the rat, and that the methanol that reached the tracheal air diffused from the blood through the mucous lining of the trachea (hence the closer correspondence to the wate-to-air, as opposed to the blood-to-air, partition coefficient).

The data obtained in these experiments are consistent with a "wash-in, wash-out" explanation of methanol absorption. In this hypothesis, all of the inhaled methanol is extracted from the airstream in the upper respiratory tract. There is a time lag associated with the diffusion of the absorbed methanol through the mucus and upper respiratory tract tissue to the upper respiratory tract capillaries; essentially methanol-free air exhaled from the lungs is available for methanol uptake. When exhaled, methanol may diffuse from mucus or tissue (or both) into the airstream. The net effect of this phenomenon is that Φ is less than 1.0 despite the thermodynamically favorable partitioning of methanol from air into an aqueous matrix. This hypothesis is supported by the observation that, during very slow breathing (for example, after anesthesia with pentobarbital), the efficiency of absorption increases, consistent with the fact that the methanol in mucus and tissue has sufficient time to diffuse further away from the lumen of the respiratory tract, thereby limiting washout. Also, methanol concentrations in blood have a limited effect on absorption per se, and this appears to be mediated by ventilatory effects of the alcohol. In view of the high water- (or blood)-to-air partition coefficient, blood concentrations 3000-fold (or 7000-fold) higher than those in air are required for complete equilibrium (that is, for mass transfer by diffusion to cease). Quantitatively, for C_{in} of 5000 ppm (6.55 mg/L at complete equilibration), C_{blood} is approximately 20,000 mg/L. After the superimposed bolus dose

during methanol inhalation exposure, C_{blood} was 5000 mg/L (only 25% of the equilibrium condition). Thus, the lack of effect of systemic methanol on mass transfer from air to blood (i.e., the fractional absorption of methanol) is not surprising.

To date, work in this laboratory on the physiologically based toxicokinetic model for methanol inhalation indicates that absorption takes place entirely in the upper respiratory tract, and that knowledge about variations in absorption with dose and exposure time, as well as variations in ventilation, is required to translate ambient methanol concentrations in air into methanol concentrations in blood. Once methanol is absorbed into the blood, the kinetic parameters developed from experiments that use intravenous administration of methanol allow the kinetic behavior of inhaled methanol in the rat to be satisfactorily modeled (Figure 34). It should be noted that some degree of bias may be inherent in the final model developed in this project. Experiments performed to determine (in part) the relationship between $\dot{V}$ or Φ and methanol concentrations in blood also provide the observed concentration-time data that demonstrated the efficacy of the model in the rat. However, concerns about such bias are minimized for several reasons: (1) the majority of the parameters in the final model (Equation 15) were either known without error (Q, C_{in}) or were generated from independent experiments that did not involve inhalation exposure (V_{max}, K_m, V_{ss}); (2) multiple inhalation exposure conditions were used to estimate values of $\dot{V}$ and Φ, which were able to describe the data from all inhalation exposures; and (3) given the fact that methanol is eliminated predominantly by saturable mechanisms, the model contained the minimum number of parameters possible to incorporate the relevant processes that determine methanol disposition.

PHASE VI. DISPOSITION OF METHANOL DURING INHALATION EXPOSURE IN MICE

Application of the experimental protocol developed for the rat to experiments in female mice was straightforward. In many respects, results obtained in mice were consistent with those from the rat experiments. Respiration in mice was inhibited somewhat when we increased concentrations of methanol (Figure 35). Although the parameters that govern the relationship between methanol concentration and respiratory depression were different between the two species, knowledge of the relationship in mice allowed appropriate corrections in respiratory rate during kinetic modeling. The dose of methanol transferred from the environmental air to the mice was a function of $\dot{V}$ and Φ integrated over the exposure time. However, in mice the efficiency of absorption did not change with the methanol exposure concentration, and Φ averaged 0.85 across exposure groups. Application of this concentration-independent fractional absorption in the physiologically based pharmacokinetic model resulted in acceptable description of the methanol concentration-time data during and after inhalation exposure (Figure 36). One complicating factor that was not present in rats was that $\dot{V}$ in the individually exposed mice was dependent more strongly on behavior than on the ambient methanol concentration. In turn, animal-to-animal variability in behavior was so wide that the likely mechanistic relationship between methanol-induced central nervous system depression and decreases in $\dot{V}$ was masked. Quantitative extrapolation (Figure 35) provided some insight into the likely ventilation of group-exposed mice in chambers and provided input into a model that yielded accurate results.

The wide variation of Φ and the lack of a correlation with ventilation in mice seems to conflict with the "wash-in, wash-out" mechanistic explanation of less than complete absorption of very water-soluble vapors. The "wash-in, wash-out" postulate predicts that higher ventilation rates decrease absorption because methanol in the upper respiratory tract has less time to diffuse toward the capillaries before exhalation. This phenomenon was demonstrated in the previous experiment for methanol inhalation in the rat. An additional complexity is that, despite the fact that the mice ventilated two- to threefold more (on a per kilogram basis) than the rat, the mice absorbed more methanol per breath ($\Phi = 0.85$) than the rat (Φ typically was 0.70 to 0.75). There are several explanations for this apparent failure of the "wash-in, wash-out" postulate in the mouse. The phenomenon may be present, but may be obscured by the idiosyncratic behavior of individual mice with respect to ventilation. Moreover, although the mice ventilated more than the rat on a per kilogram basis, the length of the nasal passages is shorter on an absolute basis in the smaller species. Hence the mouse nasal passages must warm and humidify larger amounts of air in a shorter distance than the rat. The same morphology and physiology that permits this rapid warming and humidification may result in such rapid absorption of methanol that the time interval between exhalation and inhalation may not be an important factor. In addition, there are differences in the breathing patterns of mice and rats. The ratio of the time of inspiration (T_i) to the total time of the respiratory cycle (T_t) was approximately 0.5 in the rat. Some rats at lower breathing frequencies demonstrated a definite respiratory pause at the end of expiration. The mice observed in the present experiment had an average T_i/T_t of 0.3 and lacked respiratory pause,

although some respiratory breaking was observed. The mechanistic relationship of these differences in breathing pattern to differences in absorption between the species is unclear, but demonstrates the importance of evaluating differences in absorption between species.

The chamber model is contaminated by an "air cleaning" effect of the animal in the chamber (that is, concentrations in ambient air decrease to some extent due to extraction from the airstream, whereas truly environmental exposures would not be associated with a significant loss of substrate due to extraction by the animal). However, once Φ and $\dot{V}$ are determined from experiments in the chamber, a very simple model for the blood concentration following environmental exposures can be constructed:

$$dC_{blood}/dt = -[dX_{elimin}/dt] + C_{environ} \bullet \Phi \bullet \dot{V}/V_{ss} \quad (16)$$

where the flux of methanol via elimination ($dX_{eliminated}/dt$) and V_{ss} are estimates obtained from direct systemic administration of methanol, and Φ and $\dot{V}$ are estimates from inhalation experiments as defined above.

Table 16 shows the 8-hour blood concentration in rats based on experimentally determined variations in $\dot{V}$ and Φ with C_{blood} and C_{out}; methanol concentrations in mice also are shown for comparison. Both experimentally derived and model-predicted blood methanol concentrations in mice are approximately two- to threefold higher than those in rats at the same exposure concentration. This larger degree of methanol accumulation in mice occurs despite the fact that mice eliminate methanol at a rate approximately twofold higher than rats. The comparison of the model predictions in Table 16 with the observed blood concentrations demonstrates the utility of the model; chamber-exposed animals evidenced slightly lower blood concentrations than the model predictions because the animals in the chamber breathe the chamber outlet concentration, C_{out}, which is a lower concentration than the environmental exposure concentration.

Clearly, methanol accumulation in the mouse far exceeds that in the rat during inhalation exposure. The mechanism of this species difference is the more efficient absorption of methanol in the mouse, coupled with a higher rate of delivery to the absorption site secondary to more rapid breathing in the smaller animal. There are obvious implications of this observation with respect to the teratogenic potential of methanol. Based on the data here, and if one assumes that methanol is the proximal teratogen (or that the proximal teratogen varies in proportion to the body load of methanol), the conclusion that remains is that the higher incidence of fetal abnormalities in the mouse as compared to the rat is strictly due to toxicokinetic differences between the two species. Further experimentation is required to verify this conclusion.

CONCLUSIONS

Several conclusions may be drawn from the work performed in this project:

1. Methanol disposition is nonlinear in female rats and mice, regardless of the mode of methanol administration. However, the disposition profile is not straightforward; both linear and nonlinear pathways for methanol elimination exist, and the relative contribution of the two pathways to instantaneous methanol elimination is concentration-dependent. Knowledge of the complete disposition profile, including the presence of the two parallel pathways, is crucial for comprehensive toxicokinetic modeling for this compound.
2. Methanol disposition is virtually unaffected by pregnancy, with the possible exception of the immediate perinatal period, in rodents. Minor changes in distributional volume were observed, possibly related to recompartmentalization of total body water during progression of gestation. These distributional changes may exert some effect on the methanol concentration-time profile after a single dose, but would have no influence on the relationship between steady-state methanol concentrations and exposure rate during chronic methanol exposure. A trend toward a decreasing V_{max} for methanol elimination during gestation was observed in vivo in both species, consistent with parallel in vitro metabolism experiments.
3. Disposition of methanol in the mouse was similar to that in the rat, although the mouse evidenced a twofold higher V_{max} for methanol elimination as compared to the rat. This observation suggests that, at fixed systemic doses, methanol concentrations should be lower in the mouse than in the rat.
4. Penetration of methanol from maternal blood to the fetal environment is not a simple function of maternal blood concentration. Increases in maternal blood methanol were associated with decreases in the partition coefficient between amniotic fluid and maternal blood, as well as decreases in the initial rate of permeation into the fetal compartment. The data collected to date are consistent with a methanol-induced decrease in blood flow to the fetal compartment. Further experimentation is required to characterize this process completely. Despite these limitations, it appears that, after distribution equilibrium is achieved, methanol concentrations in the conceptus are approximately equal to those in maternal blood, as anticipated for a substrate that penetrates cellular membranes readily and distributes throughout total body water.

5. As in the case of systemic disposition, the absorption of methanol is nonlinear in the rat. Decreased absorption with increased exposure concentration was observed, and is due to both a lower efficiency of absorption in the upper respiratory tract and a decreased rate of breathing. The degree of nonlinearity was lower in the mouse than in the rat because absorption efficiency did not decrease with exposure concentration.
6. Methanol accumulation in the mouse exceeded that in the rat two- to threefold during inhalation exposure. This difference persisted despite the more rapid systemic elimination of methanol in the mouse, and is due to more rapid breathing and more efficient absorption of methanol in the mouse than the rat. This particular interspecies difference is important because the mouse appears to be more sensitive to the teratogenic effects of methanol than the rat, and raises the question of which rodent species is a better model of methanol toxicokinetics in humans. Application of the current modeling approach to blood methanol concentration-time data after inhalation exposure in humans or nonhuman primates addresses this question.
7. It is likely that the differences in incidence of teratogenicity between rats and mice are, at least in part, a function of maternal toxicokinetic differences between the species. Moreover, the differences that may predispose mice to such adverse events undoubtedly are related to absorption from air rather than disposition in the systemic circulation. It is obvious, therefore, that a critical need exists to determine the efficiency of methanol absorption after inhalation exposure in humans. Without such knowledge, accurate predictions of systemic concentrations under particular exposure conditions, even given relevant (and existing) parameters for the systemic disposition of methanol in humans, are not possible.

It should be emphasized that the results obtained from the experiments described herein may not extrapolate readily to humans. The exposure conditions used were selected primarily to match previous experiments by other investigators, which attempted to determine the teratogenic potential of methanol in rats and mice. Therefore, both the inhaled concentration and the duration of exposure were well above any anticipated inhalation exposure scenarios in humans. Certainly, metabolic differences between primates and lower mammals are anticipated based on differences in prevailing enzyme systems; these differences may be exaggerated by the higher methanol concentrations used in the present rodent studies as compared to those generated in experiments with nonhuman primates or anticipated during methanol exposure in humans. Although the model that resulted from the present project can scale adequately between mice and rats, the ability of this approach to predict methanol disposition in primates, including humans, requires further prospective experimentation.

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ABBREVIATIONS

ADH	alcohol dehydrogenase
AIC	Akaike's information criterion
ANOVA	analysis of variance
AUC	area under the curve (of the blood methanol concentration-time profile)
C_{in}	inlet airstream concentration
C_{out}	effluent airstream concentration
CV	coefficient of variation
Cl_R	renal clearance
dX_L/dt	rate of nonspecific loss from exposure chamber
E	extraction ratio
f	breathing frequency
F	systemic bioavailability
HCHO	formaldehyde
k_0	zero-order infusion rate
k_{12}, k_{21}	first-order distributional rate constants
k_a	first-order absorption rate constant
k_{AF}	first-order absorption rate constant for the rapid absorption process
k_{AS}	first-order absorption rate constant for the slow absorption process
KCl	potasium chloride
K_m	Michaelis constant
K_p	partition coefficient
λ	first-order rate constant for linear elimination pathway
$MgCl_2$	magnesium chloride
NaOH	sodium hydroxide
ppm	parts per million
Q	air flow
Σ_{vent}	total ventilation over exposure period
T_i	inspiration time
T_t	duration of respiratory cycle
$\dot{V}$	minute ventilation
V_T	tidal volume
V_c	apparent distributional volume for the central compartment
V_{max}	metabolic capacity
V_{ss}	steady-state volume of distribution
Φ	fractional absorption from airstream
X_A	mass absorbed
X_B	total body load
X_E	mass extracted from the airstream
$X_{E,t}$	mass eliminated through time t
X_F	mass absorbed by the rapid absorption process
X_S	mass absorbed by the slow absorption process
X_u	mass in urine
$ZnSO_4$	zinc sulfate

INTRODUCTION

Many urban locales remain unable to meet air quality standards due to the atmospheric ozone produced when volatile organic compounds and oxides of nitrogen are emitted from motor vehicles. In an attempt to address this situation, the Clean Alternative Fuels Program was initiated in the 1980s, and the development of alternative fuels such as methanol was encouraged. The Clean Air Act Amendments of 1990 also changed existing laws related to the use of methanol as an alternative fuel, defining it in Section 219 as a "low-polluting fuel" and in Section 241 as a "clean alternative fuel." Methanol use is expected to lower the emissions of carbon monoxide and volatile organic compounds from motor vehicles; lowering the latter emissions should help to reduce the photochemical reactivity of the combustion products of methanol.

The Energy Policy Act of 1992 was a major impetus in determining how government and private industry might proceed in fulfilling the low-polluting fuel and clean fuel mandates of the 1990 Amendments to the Clean Air Act. Congress enacted the Energy Policy Act to stimulate the private sector to manufacture and provide alternative fuels for a variety of vehicles. As a result of the incentives contained in this Act, the petroleum industry has developed high-octane methanol fuels. Given the activities supported by the Energy Policy Act, methanol and other alcohol fuels are expected to contribute to the mix of fuels that will be used by the year 2000.

Although methanol as an alternative fuel could have significant remedial effects on pollution, its use raises substantial concerns about potential health risks posed by increased exposure of the general population to methanol vapors. As a result of this concern, the Health Effects Institute has funded research on the health effects of methanol exposure (including the study described in this report) to investigate the effects of exposure to methanol and to understand interspecies differences in its uptake and disposition in developing fetuses, a potentially susceptible population.

METHANOL TOXICITY

Concern about the potential toxicity of methanol originates from the well-documented and severe effects of acute methanol poisoning from both accidental and intentional ingestion (Hunter 1975; Hayreh et al. 1980; LeWitt and Martin 1988). For those individuals who do not die from such exposures, the most severe effects are blindness due to optic nerve damage (Hayreh et al. 1980) and residual motor dysfunction that results from basal ganglia damage (LeWitt and Martin 1988). The biochemical mechanisms responsible for methanol toxicity during acute exposures are well understood: excessive accumulation of formate or formic acid (methanol metabolites) leads to metabolic acidosis (reviewed by Tephly and McMartin 1984; Health Research Committee 1987; Kavet and Nauss 1990; Tephly 1991).

Most analysts predict that the levels of methanol to which humans would be exposed via vapors from motor vehicles would be much lower than those seen in acute methanol poisoning by ingestion. On the basis of limited data on emissions, projected exposure concentrations for methanol vapors from motor vehicle evaporative and exhaust emissions have been estimated to be 1 to 10 parts per million (ppm)* in typical traffic situations and as high as 200 ppm in a worst-case scenario such as a malfunctioning vehicle in an enclosed garage (Health Research Committee 1987; Gold and Moulis 1988; Kavet and Nauss 1990). Given what is known about the rate of methanol metabolism to formate or formic acid, these most toxic metabolites of methanol should not accumulate in blood even at the highest estimated levels of exposure to methanol vapors from auto emissions (Health Research Committee 1987; Kavet and Nauss 1990).

Although these projections suggest little risk of toxicity for healthy humans who inhale methanol vapors from motor vehicles, potentially susceptible populations are still of concern. In fact, some animal studies have shown that exposure to high concentrations of methanol vapors (5,000 to 20,000 ppm) can have effects on mammalian development (including exencephaly, neural tube defects, skeletal and limb abnormalities, and cleft palate) that were dependent upon the stage of fetal development when exposed, the timing and magnitude of exposure, and the species being studied (Nelson et al. 1985; Bolon et al. 1993; Rogers et al. 1993). Although differences in methanol metabolism among species (McMartin 1977) make it difficult to compare and extrapolate results from animals to humans, the data support the suggestion that the developing fetus may be particularly sensitive to inhaled methanol.

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* A list of abbreviations appears at the end of the Investigators' Report for your reference.

Even low-level methanol exposures are a concern because it is known that developmental neurotoxicity can result from prolonged exposures to low levels of some neurotoxins (Needleman and Bellinger 1994). For example, subtle neurotoxic effects occur in the offspring of women who consume ethanol at far lower levels than those necessary to cause the characteristic malformations of Fetal Alcohol Syndrome (Streissguth et al. 1986). From these observations came the concern that the developing human fetus may be a potential population at risk when pregnant women are exposed to methanol vapors. At the time the present study was funded, little information was available concerning the relationship between methanol exposure and its systemic disposition in either pregnant or nonpregnant females. Such data would be useful for interspecies extrapolation and for assessing the risks associated with exposure to low environmental levels of methanol in humans (Klaassen and Eaton 1991).

JUSTIFICATION FOR THE STUDY

Because of the concerns relating to the potential effects of low-level methanol exposure, the HEI Research Committee issued RFA 89-1 in 1989, titled "Health Effects of Methanol Exposure: Metabolism and Pharmacokinetics; Fetal and Perinatal Neurotoxicity; Reproductive Toxicity," which requested applications on these topics in order to improve the understanding of the exposure-dose-response relationship for methanol. The RFA was broadly based because, as described above, although there was a substantial literature on the acute toxicity of exposures to high levels of methanol, very little information was available on the metabolism and health effects of low-level exposures, especially in the developing fetus.

In response to this RFA, Drs. Gary Pollack and Kim Brouwer of the University of North Carolina submitted an application titled "Maternal-Fetal Pharmacokinetics of Methanol." The rationale for this study, which the HEI funded, was to construct a physiologically based pharmacokinetic model for methanol distribution in the maternal-fetal unit in rats and mice. This four-year project, which began in October 1990, had total expenditures of $323,548. The investigators submitted their final research report in November 1994, and the Health Review Committee accepted their revised report in November 1995. This study was part of a larger program that also encompassed research in nonhuman primates and focused not only on uptake and disposition, but also on effects on fetal development.

During the review process, the Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Health Review Committee's Commentary. The following is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the Investigators' Report into scientific and regulatory perspective.

OBJECTIVES AND STUDY DESIGN

The major objective of this study was to determine the distribution of methanol in rats and mice at different stages of gestation after exposure to this alcohol by different routes. In order to accomplish this goal, the investigators designed experiments to achieve four specific aims. They were to (1) characterize the systemic disposition of methanol in rats and mice, (2) determine the kinetics of methanol delivery to the fetus, (3) elucidate the kinetics of methanol absorption during inhalation exposure, and (4) construct a physiologically based model capable of predicting methanol disposition in rats and mice.

In order to achieve the first specific aim, the investigators exposed nonpregnant and pregnant rats and mice at several stages of gestation (7, 14, and 21 days in rats; 9 and 18 days in mice) to methanol (100 to 2,500 mg/kg body weight) by intravenous and oral routes of administration. They then measured methanol concentrations in the blood and urine of adults using gas chromatographic techniques. For the second specific aim, the investigators examined fetal methanol exposure as a consequence of intravenous maternal methanol exposure. They sampled amniotic fluid by microdialysis at the three different gestational stages to study the kinetics of methanol delivery to the fetus. For the third specific aim, the investigators measured the uptake of methanol vapors during whole-body exposure (1,000 to 20,000 ppm for 8 hours) of rats and mice in flow-through chambers that allowed them to estimate methanol concentrations and respiratory parameters. Finally, to address the fourth specific aim, they used the data collected in the other three specific aims to construct a pharmacokinetic model of inhaled methanol absorption and disposition.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The investigators conducted a valuable study of methanol vapor exposure that described the physiological factors governing methanol delivery to the developing fetus after maternal exposure. In addition, they successfully developed a physiologically based pharmacokinetic model of methanol disposition in pregnant mice and rats. Overall, the investigators were able to meet the goals stated in their objectives and specific aims.

STUDY DESIGN AND METHODS

The investigators used an appropriate study design and applicable methods. They generated a substantial amount of complex data. In general, the statistical approaches employed were appropriate, clearly presented, and interpreted correctly. The models for the kinetic analyses were justified and the parameters of these models were properly estimated by nonlinear methods. Akaike's Information Criterion (Yamaoka et al. 1978) also was correctly employed for selecting models. In a number of cases, multiple comparisons were performed and the investigators used suitable adjustments to control for multiple testing.

The one issue that detracts from the overall positive presentation of the statistical methods is that the investigators did not provide any justification for the ad hoc decisions they made when pooling the data, other than to state that it was done to decrease interanimal variability. Although the investigators' decisions appear to be appropriate, it is worthy of note that they were ad hoc in nature.

RESULTS AND INTERPRETATION

At the inhalation exposure concentrations used in this study, decreasing absorption of methanol in rats and mice was seen with increasing exposure concentrations. This was due to a decreased rate of breathing and a concomitant lower efficiency of absorption in the upper respiratory tract. Also, the rate of methanol accumulation in the mouse was two- to threefold greater than that in the rat. This difference persisted in spite of the two fold higher rate of methanol elimination observed in the mouse. The investigators hypothesized that this difference between rats and mice was due to more rapid breathing in the mouse and the apparently complete absorption of methanol in the nasal cavity of this species. They also believed that the greater accumulation of methanol seen in the mouse may help to explain the greater sensitivity of this species to the teratogenic effects of methanol.

The investigators found that methanol disposition in rodents was virtually unaffected by pregnancy, although some minor changes in distributional volume were observed during the progress of gestation. These latter changes were hypothesized to be related to recompartmentalization of total body water during gestation. Although methanol concentrations in the fetus were approximately the same as those in the mother, the rate of permeation into the fetus appeared to be inversely proportional to the maternal blood methanol concentration. The investigators believed that this relationship was more consistent with a decreased blood flow to the fetus induced by methanol than with a change in permeability coefficients. Overall, methanol distribution between the mother and fetus appeared to reach equilibrium, as the investigators expected it would given that methanol readily penetrates cellular membranes and completely distributes throughout total body water.

Using their results from both species of rodents, the investigators constructed a pharmacokinetic model that describes the disposition of methanol in the rat and mouse. The disposition profile can be partitioned into saturable and linear methanol elimination pathways whose relative contributions are dependent on concentration. The saturable pathway was evident at lower doses (100 and 500 mg/kg) and displayed classic Michaelis-Menten kinetics that are indicative of a rate-limiting step mediated by a carrier system. The linear pathway appeared at the highest dose (2,500 mg/kg) and displayed the first-order kinetics of elimination that are characteristic of passive-diffusion mechanisms. In fact, at the highest intravenous dose of 2,500 mg/kg, the linear pathway accounted for nearly 90% of methanol elimination. This linear elimination pathway consists of pulmonary and urinary clearance of methanol in approximately equal amounts.

The investigators chose inhalation exposure levels (1,000 to 20,000 ppm) to approximate those of previous animal studies (5,000 to 20,000 ppm) in which teratogenic effects of methanol have been demonstrated (Nelson et al. 1985; Bolon et al. 1993; Rogers et al. 1993). However, when interpreting these findings, it is important to note that the lowest inhalation exposure used in this study (1,000 ppm for 8 hours) is 2 to 3 orders of magnitude greater than the estimated typical traffic situation (1 to 10 ppm) and exceeds even the worst-case scenario of a malfunctioning vehicle in an enclosed garage (200 ppm for 15 minutes) (Health Research Committee 1987; Gold and Moulis 1988; Kavet and Nauss 1990). Therefore, the results of these experiments must be interpreted and extrapolated cautiously when applied to ambient exposure situations. The models constructed by these investigators need to be validated at lower exposure levels.

IMPLICATIONS FOR FUTURE RESEARCH

The investigators' results showing that mice accumulate methanol at a rate two- to threefold faster than rats led them to hypothesize that this could explain the greater teratogenicity of methanol in mice. If this hypothesis is correct, studies in which the association between increased methanol accumulation and a greater level of teratogenicity is further evaluated and the ultimate teratogen identified, whether it be methanol itself or one of its metabolites, could help in analyzing the toxic effects of methanol in various

species. However, the differences seen in these two closely related rodent species indicate that extrapolation to humans is likely to be difficult.

Although pregnancy itself had little effect on the disposition of methanol in pregnant rats and mice, the investigators found that the permeation rate into the fetus was inversely proportional to the concentration of methanol in maternal blood. Although they hypothesized that this was due to changes in blood flow at higher blood methanol concentrations, and not a reduction in permeability coefficients, the underlying mechanism remains unknown. However, this decreased blood flow to the fetal environment could limit the delivery of oxygen and other nutrients, thereby having deleterious effects on organ differentiation and development. Given that the investigators may have uncovered a physiological response to methanol that is present in pregnant females exposed to high levels and could also be operating at lower exposures to reduce the availability of nutrients to the fetus, further work on characterizing the mechanism of this response is warranted.

At the 2,500-mg/kg intravenous dose of methanol, the investigators were able to identify a parallel linear elimination pathway that apparently operates by passive diffusion and involves pulmonary and urinary clearance mechanisms. Although the exposures in this study were designed to parallel previous work on the teratogenicity of methanol and, therefore, are well above those expected for human inhalation exposures, both in concentration and duration, the discovery of this second pathway of methanol elimination could have important consequences in performing high- to low-dose and interspecies extrapolations. As a result, the elucidation of the mechanisms by which this pathway operates and its presence or absence in other species is potentially a critical issue in risk assessment that needs further study.

CONCLUSIONS

Drs. Pollack and Brouwer exposed rats and mice at several different stages of gestation (7, 14, and 21 days in rats; 9 and 18 days in mice) to methanol intravenously and orally (100 to 2,500 mg/kg) or by inhalation exposure (1,000 to 20,000 ppm for 8 hours). They measured concentrations of methanol in blood, urine, and amniotic fluid. The investigators found that mice accumulated methanol at a rate two- to threefold faster than rats. The latter was true in spite of a twofold higher rate of methanol elimination observed in the mouse. They also found that methanol disposition was virtually unaffected by pregnancy and that fetal methanol concentrations were approximately the same as in the mother.

Using their results, the investigators constructed a pharmacokinetic model that describes the disposition of methanol in rats and mice. The disposition profile can be partitioned into saturable and linear methanol elimination pathways; the former displays classic carrier-mediated Michaelis-Menten kinetics with a rate-limiting step, and the latter consists of passive elimination via pulmonary and urinary clearance mechanisms. At higher methanol exposures, such as the 2,500-mg/kg intravenous dose, the passive elimination pathway predominated.

The construction of this physiologically based model describing methanol disposition in pregnant mice and rats is a critical step in making the interspecies extrapolations necessary for risk assessment. However, the data sets from these two rodent species show some important differences, which make it difficult to use this rodent model to extrapolate to humans. Also, the exposures used in this study were significantly higher than those predicted for ambient exposures resulting from the use of methanol fuels (1 to 10 ppm in typical traffic situations, and as high as 200 ppm in a worst-case scenario such as a malfunctioning vehicle in an enclosed garage). As a result, the usefulness of the pharmacokinetic model developed for high-level exposures in rodents may be limited in predicting the effects of the lower ambient exposures expected for humans. Therefore, validation of this model at lower exposure levels may be necessary.

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