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United States Environmental Protection Agency

TOXICOLOGICAL REVIEW

OF

METHANOL (NONCANCER)

(CAS No. 67-56-1)

In Support of Summary Information on the

Integrated Risk Information System (IRIS)

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ACGIH	American Conference of Governmental and Industrial Hygienists
ADH	alcohol dehydrogenase
ADH1	alcohol dehydrogenase-1
ADH3	formaldehyde dehydrogenase-3
AIC	Akaike Information Criterion
ALD	aldehyde dehydrogenase
ALDH2	mitochondrial aldehyde dehydrogenase-2
ALT	alanine aminotransferase
ANOVA	analysis of variance
AP	alkaline phosphatase
AST	aspartate aminotransferase
ATP	adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
AUC	area under the curve, representing the cumulative product of time and concentration for a substance in the blood
β-NAG	N-acetyl-beta-D-glucosaminidase
Bav	oral bioavailability
BMD	benchmark dose(s)
BMD_{1SD}	BMD for response one standard deviation from control mean
BMDL	95% lower bound confidence limit on BMD (benchmark dose)
$BMDL_{1SD}$	BMDL for response one standard deviation from control mean
BMDS	benchmark dose software
BMR	benchmark response
BSO	butathione sulfoximine
BUN	blood urea nitrogen
BW, bw	body weight
C_1 pool	one carbon pool
C_{max}	peak concentration of a substance in the blood during the exposure period
C-section	Cesarean section
CA	chromosomal aberrations
CAR	conditioned avoidance response
CASRN	Chemical Abstracts Service Registry Number
CAT	catalase
CERHR	Center for the Evaluation of Risks to Human Reproduction at the NTP
CH_3OH	methanol
CHL	Chinese hamster lung (cells)
CI	confidence interval
Cl_S	clearance rate
C_{max}	peak concentration
CNS	central nervous system
CO_2	carbon dioxide
con-A	concanavalin-A
CR	crown-rump length
CSF	Cancer slope factor
C_{ss}	steady-state concentration
CT	computed tomography
C_{VB}	concentration in venous blood
C_{VBbg}	background concentration in venous blood
C_{VBmb}	concentration in venous blood minus constant background
CYP450	cytochrome P450
d, δ, Δ	delta, difference, change
D_2	dopamine receptor
DA	dopamine
DIPE	diisopropyl ether
DMDC	dimethyl dicarbonate
DNA	deoxyribonucleic acid
DNT	developmental neurotoxicity test(ing)
DOPAC	dihydroxyphenyl acetic acid
DPC	days past conception
DTH	delayed-type hypersensitivity
EFSA	European Food Safety Authority
EKG	electrocardiogram
EO	Executive Order
EPA	U.S. Environmental Protection Agency
ERF	European Ramazzini Foundation
EtOH	ethanol
F	fractional bioavailability
F_0	parental generation
F_1	first generation
F_2	second generation
F344	Fisher 344 rat strain
FAD	folic acid deficient
FAS	folic acid sufficient
FD	formate dehydrogenase

FP	folate paired
FR	folate reduced
FRACIN	fraction inhaled
FS	folate sufficient
FSH	follicular stimulating hormone
γ-GT	gamma glutamyl transferase
g	gravity
g, kg, mg, µg	gram, kilogram, milligram, microgram
G6PD	glucose-6-phosphate dehydrogenase
GAP43	growth-associated protein (neuronal growth cone)
GD	gestation day
GFR	glomerular filtration rate
GI	gastrointestinal track
GLM	generalized linear model
GLP	good laboratory practice
GSH	glutathione
HAP	hazardous air pollutant
HCHO	formaldehyde
HCOO	formate
Hct	hematocrit
HEC	human equivalent concentration
HED	human equivalent dose
HEI	Health Effects Institute
HERO	Health and Environmental Research Online (database system)
HH	hereditary hemochromatosis
5-HIAA	5-hydroxyindolacetic acid
HMGSH	S-hydroxymethylglutathione
Hp	haptoglobin
HPA	hypothalamus-pituitary-adrenal (axis)
HPLC	high-performance liquid chromatography
HSDB	Hazardous Substances Databank
HSP70	biomarker of cellular stress
5-HT	serotonin
IL	interleukins
i.p.	intraperitoneal (injection)
IPCS	International Programme on Chemical Safety
IQ	intelligence quotient
IRIS	Integrated Risk Information System
IUR	inhalation unit risk
i.v.	intravenous (injection)
k_1	first-order urinary clearance
k_1C	first-order urinary clearance scaling constant; first order clearance of methanol from the blood to the bladder for urinary elimination
k_{ai}	first order uptake from the intestine
k_{as}	first order methanol oral absorption rate from stomach
k_{bl}	rate constant for urinary excretion from bladder
k_{iv}	respiratory/cardiac depression constant
KLH	keyhole limpet hemocyanin
KLL	alternate first order rate constant
K_m	substrate concentration at half the enzyme maximum velocity (V_{max})
K_{m2}	Michaelis-Menten rate constant for low affinity metabolic clearance of methanol
k_{si}	first order transfer between stomach and intestine
L, dL, mL	liter, deciliter, milliliter
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LH	luteinizing hormone
LLF	(maximum) log likelihood function
LMI	leukocyte migration inhibition (assay)
LOAEL	lowest-observed-adverse-effect level
M, mM, µM	molar, millimolar, micromolar
MeOH	methanol
MLE	maximum likelihood estimate
M-M	Michaelis-Menten
MN	micronuclei
MOA	mode of action
4-MP	4-methylpyrazole (fomepizole)
MRI	magnetic resonance imaging
mRNA	messenger RNA
MTBE	methyl tertiary butyl ether
MTX	methotrexate
N_2O/O_2	nitrous oxide
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenine dinucleotide
NBT	nitroblue tetrazolium (test)
NCEA	National Center for Environmental Assessment
ND	not determined
NEDO	New Energy Development Organization (of Japan)

NIEHS	National Institute of Environmental Health Sciences
NIOSH	National Institute of Occupational Safety and Health
nmol	nanomole
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NP	nonpregnant
NR	not reported
NRC	National Research Council
NS	not specified
NTP	National Toxicology Program at NIEHS
NZW	New Zealand White (rabbit strain)
OR	osmotic resistance
ORD	Office of Research and Development
OSF	oral slope factor
OU	oculus uterque (each eye)
OXA	oxazolone
P, p	probability
PB	blood:air partition coefficient
PBPK	physiologically based pharmacokinetic model
PC	partition coefficient
PEG	polyethylene glycol
PFC	plaque-forming cell
PK	pharmacokinetic
PMN	polymorphonuclear leukocytes
PND	postnatal day
POD	point of departure
ppb, ppm	parts per billion, parts per million
PR	body:blood partition coefficent
PWG	Pathology Working Group of the NTP of NIEHS
Q wave	the initial deflection of the QRS complex
Q_CC	cardiac output scaling constant
Q_P	pulmonary (alveolar) ventilation
QRS	portion of electrocardiogram corresponding to the depolarization of ventricular cardiac cells.
R^2	square of the correlation coefficient, a measure of the reliability of a linear relationship.
RBC	red blood cell
RfC	reference concentration
RfD	reference dose
RNA	ribonucleic acid
$R_{Øbg}$	zero-order endogenous production rate
ROS	reactive oxygen species
S9	microsomal fraction from liver
SAP	serum alkaline phosphatase
s.c.	subcutaneous
SCE	sister chromatid exchange
SD	Sprague-Dawley rat strain
S.D.	standard deviation
S.E.	standard error
SEM	standard error of mean
SGPT	serum glutamate pyruvate transaminase
SHE	Syrian hamster embryo
SOD	superoxide dismutase
SOP	standard operating procedure(s)
t	time
$T_{½}$, $t_{½}$	half-life
T wave	the next deflection in the electrocardiogram after the QRS complex; represents ventricular repolarization
TAME	tertiary amyl methyl ether
TAS	total antioxidant status
Tau	taurine
THF	tetrahydrofolate
TLV	threshold limit value
TNFα	tumor necrosis factor-alpha
TNP-LPS	trinitrophenyl-lipopolysaccharide
TRI	Toxic Release Inventory
U83836E	vitamin E derivative
UF(s)	uncertainty factor(s)
UF_A	UF associated with interspecies (animal to human) extrapolation
UF_D	UF associated with deficiencies in the toxicity database
UF_H	UF associated with variation in sensitivity within the human population
UF_S	UF associated with subchronic to chronic exposure
V_d	volume of distribution
V_{max}	maximum enzyme velocity
$V_{max}C$	maximum velocity of the high-affinity/low-capacity pathway
VDR	visually directed reaching test
VitC	vitamin C
VPR	ventilation perfusion ratio
v/v	volume of solute/volume of solution
VYS	visceral yolk sac

WBC	white blood cell	w/v	weight (mass of solute)/volume of solution
WOE	weight of evidence	χ^2	chi square

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EXECUTIVE SUMMARY

Introduction

Methanol is a high production volume chemical with many commercial uses. It is a basic building block for numerous chemicals. Many of its derivatives are used in the construction, housing or automotive industries. Consumer products that contain methanol include varnishes, shellacs, paints, windshield washer fluid, antifreeze, adhesives, and deicers.

Methanol can be formed in the mammalian organism as a metabolic byproduct. Endogenous background levels [naturally generated from within the body] are not the same as exogenous exposure (exposure from a source outside the body), but the combination of endogenous background levels plus exogenous exposure can lead to toxicity. Ingestion of foodstuffs, such as fruits or vegetables (Cal/EPA, 2012) and normal metabolic pathways contribute to the endogenous background levels in humans. Commercial and household uses of methanol (e.g., methanol is the major anti-freeze constituent of windshield washer fluid) can contribute to exogenous exposures. This Toxicological Review provides scientific support and rationale for a hazard and dose-response assessment of the noncancer effects associated with chronic exposure to methanol. In Section 5 (Dose Response Assessments and Characterization), the basis for a daily inhalation reference concentration (RfC) of 2×10^{1} mg/m^3 and a daily oral reference dose (RfD) of 2 mg/kg-day are described. Each represents an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime.

This health assessment focuses principally on quantifying the noncancer toxicity associated with exogenous oral or inhalation exposure to methanol that add to endogenous background levels. It does not address the potential carcinogenicity of methanol, or the health effects associated with endogenous background levels of methanol that arise from metabolic and normal dietary (e.g., fruit and juice consumption) sources. Hence, as discussed in Section 3.4.3.2 (Model Structure), responses observed in oral and inhalation studies of laboratory animals exposed to methanol are evaluated against blood concentrations of methanol in excess of endogenous background levels of methanol.

Chemical and Physical Information

Methanol is the smallest member of the family of aliphatic alcohols. Also known as methyl alcohol or wood alcohol, among other synonyms, it is a clear, colorless, very volatile, and

flammable liquid. Methanol is widely used as a solvent in many commercial and consumer products. It is freely miscible with water and other short-chain aliphatic alcohols but has little tendency to distribute into lipophilic media.

Toxicokinetics

Due to its very low oil:water partition coefficient, methanol is taken up efficiently by the lung or the intestinal tract and distributes freely in body water (blood volume, extracellular and intracellular fluid, etc.) without any tendency to accumulate in fatty tissues. Methanol can be metabolized completely to CO_2, but may also, as a regular byproduct of metabolism, enter the formic acid C_1-pool (1-carbon unit pool), and become incorporated into biomolecules. Animal studies indicate that blood methanol levels increase with the breathing rate and that metabolism becomes saturated at high exposure levels. Because of its volatility methanol can also be excreted unchanged via urine or exhaled air. As discussed in Section 3.1 (Toxicokinetics Overview), the enzymes responsible for metabolizing methanol are different in rodents and primates (Figure 3-1). Several published rat, mouse, and human PBPK models which attempt to account for these species differences are described in Section 3.4.2 (Methanol PBPK Models).

The development of methanol PBPK models was organized around a set of criteria, described in Section 3.4.1.2 (Criteria for the Development of Methanol PBPK Models), that take into account the dose routes used in key toxicity studies, the availability of pharmacokinetic information necessary for PBPK model development and the most likely toxicological mode of action (MOA). Specifically, new EPA models were developed or modified from existing models, to allow for the estimation of monkey and rat internal dose metrics. A human model was also developed to extrapolate those internal metrics to inhalation and oral exposure concentrations that would result in the same internal dose in humans (human equivalent concentrations [HECs] and human equivalent doses [HEDs]). The procedures used for the development, calibration and use of these EPA models are summarized in Section 3.4 (Physiologically Based Pharmacokinetic Models), with further details provided in Appendix B, “Development, Calibration and Application of a Methanol PBPK Model.”

Developmental malformations and anomalies in gestationally exposed fetal mice (and developmental neurotoxicity, as indicated by reduced absolute brain weight, in gestationally and lactationally exposed fetal and neonate rats) observed in inhalation studies are sensitive endpoints considered in the derivation of an RfC. However, questions remain regarding the relative involvement of parent methanol, formaldehyde, and reactive oxygen species (ROS) in the MOA for these developmental effects. Given the reactivity of formaldehyde and the lack of relevant pharmacokinetic information, PBPK models that predict levels of formaldehyde (or

subsequent metabolites of formaldehyde) in the blood would be difficult to validate.[1] However, the high reactivity of formaldehyde (see Section 3.1 [Toxicokinetics Overview]) would limit its unbound and unaltered transport as free formaldehyde from maternal to fetal blood (see discussion in Section 3.4.1.1 [MOA and Selection of a Dose Metric] and 4.7.1 [Role of Methanol and Metabolites in the Developmental Toxicity of Methanol]), and the ROS MOA requires the presence of methanol to alter embryonic catalase activity. Hence, it is likely that all of these MOAs require methanol to be present at the target site. For this reason, and because adequate pharmacokinetic information was available, PBPK models that estimate levels of parent methanol in blood were developed and validated for rats and humans. Because actual measured internal blood methanol levels suitable for use as estimates of peak concentrations (C_{max}) in mice were provided in the Rogers et al. (1993b) study, and these data were considered better than a predictive model, the mouse PBPK model was not used or discussed in detail in this toxicological review. A simple PK model for monkey methanol kinetics was also developed and used to evaluate the results of monkey developmental studies (Burbacher et al., 2004b; 2004a; 1999b; 1999a).

A pregnancy-specific PBPK model does not exist for methanol and limited data exist for the development and validation of a fetal/gestational/conceptus compartment. For this reason, and because levels of methanol in non-pregnant and pregnant adult females, and fetal blood (all measures of maternal exposure) are expected to be similar following the same oral or inhalation methanol exposure (see discussion in Section 3.4.1.2 [Criteria for the development of Methanol PBPK Models]), EPA developed and used non-pregnancy models for the appropriate species and routes of exposure for the derivation of candidate RfCs and RfDs. It is recognized that these models may not accurately represent neonate blood levels following the gestation, lactation and inhalation exposure regimen used in one of the key rat studies (NEDO, 1987), but they are considered appropriate for use in deriving HEC values from this study assuming the ratio of maternal to offspring blood methanol would be similar in rats and humans (see discussion in Sections 5.1.3.2.2 [Animal-to-Human Extrapolation UF_A]).

The rat and human methanol PBPK models fit multiple data sets for inhalation, oral, and i.v. exposures, from multiple research groups using consistent parameters that are representative of each species but are not varied within species or by dose or source of data. Also, a simple PK model calibrated to non-pregnant (NP) monkey data, which were shown to be essentially indistinguishable from pregnant monkey PK data, was used to estimate blood methanol area under the curve (AUC) values (internal doses) in that species. In the case of the mouse, a PK

[1] The PBPK models developed by EPA estimate total amount of methanol cleared by metabolic processes, but this has limited value as a metric of formaldehyde or formate dose since it ignores metabolic processes that may differ between species and between the mother and the fetus/neonate.

model developed from in vivo blood methanol levels in (Rogers et al., 1993b) resulted in more reliable estimates compared to the PBPK model and was used for derivation of effect levels in this species. Section 5 (Dose Response Assessments and Characterization) describes how the human PBPK model was used in the derivation of candidate RfCs and RfDs.

Hazard Identification

In humans, acute central nervous system (CNS) toxicity can result from relatively low ingested doses (as low as 3-20 mL of methanol), which can metabolize to formic acid and lead to metabolic acidosis. The resulting acidosis can potentially cause lasting nervous system effects such as blindness, Parkinson-like symptoms, and cognitive impairment. These effects can be observed in humans when blood methanol levels exceed 200 mg/L.

CNS effects have not been observed in rodents following acute exposures to methanol, and NEDO (1987) reported that methanol blood levels around 5,000 mg/L were necessary to cause clinical signs and CNS changes in cynomolgus monkeys. The species differences in toxicity from acute exposures appear to be the result of a limited ability of humans to metabolize formic acid.

Occupational studies and case reports offer valuable information on the effects of methanol following acute human exposures, but the relatively small amount of data for subchronic, chronic, or in utero human exposures are inconclusive. However, a number of reproductive, developmental, subchronic, and chronic toxicity studies have been conducted in mice, rats, and monkeys.

Data regarding effects from oral exposure in experimental animals exist, but they are more limited than data from the inhalation route of exposure (see Sections 4.2 [Acute, Subchronic, and Chronic Studies in Animals – Oral and Inhalation], 4.3 [Reproductive and Developmental Studies – Oral and Inhalation], and 4.4 [Neurotoxicity]). Two oral studies in rats (Soffritti et al., 2002; TRL, 1986), one oral study in mice (Apaja, 1980) and several inhalation studies in monkeys, rats and mice (NEDO, 1987, 1985a, b) of 90-days duration or longer have been reported. Some noncancer effects of methanol exposure were noted in these studies, principally in the liver and brain tissues, but they occurred at relatively high doses.

A number of studies have used the inhalation route of exposure to assess the potential of reproductive or developmental toxicity of methanol in mice, rats, and monkeys (see Section 4.3.2 [Inhalation Reproductive and Developmental Studies]). These studies indicate that fetal and neonate toxicity occurs at lower doses than maternal toxicity. At exposure concentrations of 5,000 ppm or above, methanol has been shown to cause an increase in litters with resorptions (Bolon et al., 1993), and severe malformations (exencephaly and cleft palate) in mice, the most

sensitive gestational days being GD6 and GD7 (i.e., early organogenesis) (Rogers and Mole, 1997; Rogers et al., 1993a; Rogers et al., 1993b). Increased occurrences of ossification disturbances and skeletal anomalies were observed at exposure concentrations of 2,000 ppm in mice (Rogers et al., 1993b) and at 10,000 ppm in rats (Nelson et al., 1985). NEDO (1987) conducted a series of developmental and reproductive studies, including a two generation and a follow up one generation reproductive toxicity study in rats, which used exposure times of 20 hours/day or more at concentrations between 100 and 5,000 ppm. Details were not reported (e.g., means, variances, sample sizes, pup-to-litter correlations) that would allow for an analysis of the findings from this study. However, a follow-up one-generation study conducted by NEDO (1987) contained enough information to confirm and quantify the primary endpoint identified, pup brain weight changes. This developmental neurotoxicity study is discussed in Section 4.4.2 (Inhalation Neurotoxicity Studies). Section 4.4.2 also describes another key developmental neurotoxicity study conducted in pregnant cynomolgus monkeys exposed to 200-1,800 ppm methanol for 2.5 hours/day throughout pre-mating, mating, and gestation (Burbacher et al., 2004b; 2004a; 1999b; 1999a). Potential compound-related effects noted were a shortening of the gestation period by less than 5%, and developmental neurotoxicity (particularly delayed sensorimotor development) in the monkeys.

As discussed in Section 4.6.1.2 (Key Studies, Inhalation), due largely to the lack of clear dose-response information, the data from the monkey developmental study are not conclusive, and there was insufficient evidence to determine if the primate fetus is more sensitive, or less sensitive, than rodents to the developmental or reproductive effects of methanol. Taken together, however, the NEDO (1987) rat study and the Burbacher et al. (2004b; 2004a; 1999b; 1999a) monkey study suggest that prenatal exposure to methanol can result in adverse effects on developmental neurology pathology and function, which can be exacerbated by continued postnatal exposure. Among an array of findings indicating developmental neurotoxicity and developmental malformations and anomalies that have been observed in rodents, a decrease in the brain weights of gestationally and lactationally exposed neonatal rats (NEDO, 1987) and an increase in the incidence of cervical ribs of gestationally exposed fetal mice (Rogers et al., 1993b) are considered the most robust endpoints for the purposes of RfD and RfC derivation. See Section 4.6 (Synthesis of Major Noncancer Effects) for a more extensive summary of the dose-related effects that have been observed following subchronic or chronic exposure.

Sections 4.7 (Noncancer MOA Information) and 5.3.5 (Choice of Species/Sex), provide a discussion of the uncertainty regarding human relevance of the mouse and rat developmental studies due to differences in the way humans and rodents metabolize methanol. Adult humans metabolize methanol principally via alcohol dehydrogenase (ADH1) and rodents via catalase and ADH1. Recent studies in mice have demonstrated that high catalase activity can reduce, and low

catalase activity can enhance, methanol's embryotoxic effects. However, the MOA for these effects, and the role of catalase, have not been determined. Further, while catalase does not appear to be involved in adult human methanol metabolism, less is known about the metabolism of methanol in human infants (see Section 3.3 [Human Variability in Methanol Metabolism]). Thus, the effects observed in rodents are considered relevant for the assessment of human health.

Dose-Response Assessment and Characterization

As discussed above and in Section 5.1.1 (Choice of Principal Study and Critical Effect[s]), reproductive and developmental effects are considered the most sensitive and quantifiable effects reported in studies of methanol. Because the oral reproductive and developmental studies employed single and comparatively high doses (i.e., oral versus inhalation), the developmental effects observed in the inhalation studies were used to derive the RfC and, using a route-to-route extrapolation, the RfD.

Clearly defined toxic endpoints at moderate exposure levels have been observed in inhalation studies of reproductive and developmental toxicity (see Section 5.1.1.2 [Selection of Critical Effect[s]). Three endpoints from inhalation developmental toxicity studies were critically evaluated for derivation of the RfC: (1) increased occurrences of ossification disturbances and skeletal abnormalities (i.e., formation of cervical ribs) in CD-1 mice exposed to methanol during organogenesis (Rogers et al., 1993b); (2) reduced brain weights in rats exposed to methanol from early gestation through 8 weeks of postnatal life (NEDO, 1987); and (3) deficits in sensorimotor development in the offspring of monkeys exposed to methanol throughout gestation (Burbacher et al., 2004b; 2004a; 1999b; 1999a).

Rogers et al. (1993b) exposed CD-1 mice to air concentrations of 0; 1,000; 2,000; and 5,000 ppm methanol for 7 hours/day on GD7 to GD17. A benchmark dose lower confidence limit (BMDL) of 43 mg/L was estimated for the internal peak blood methanol (C_{max}) associated with 5% extra risk for the formation of cervical ribs (see Section 5.1.2.3 [BMD Approach Applied to Cervical Rib Data in Mice] and Appendix D [RfC Derivation Options]). This $BMDL_{05}$ was then divided by 100 to account for uncertainties associated with human variability (UF_H), the animal-to-human extrapolation (UF_A) and the database (UF_D), and to reduce it to a level that is within the range of blood levels for which the human PBPK model was calibrated (see discussion in Section 5.1.3.2 [Application of UFs]). The PBPK model was then used to convert this adjusted internal $BMDL_{05}$ of 0.43 mg/L to a human equivalent candidate RfC of 20.9 mg/m^3 (see Section 5.1.3 [RfC Derivation – Including Application of Uncertainty Factors]) and a candidate RfD of 1.9 mg/kg-day (see Section 5.2.2 [RfD Derivation – Including Application of Uncertainty Factors]).

NEDO (1987) exposed fetal Sprague-Dawley rats and their dams to air concentrations of 0, 500, 1,000 and 2,000 ppm methanol from the first day of gestation (GD1) until 8 weeks of age, and brain weights were determined at 3, 6, and 8 weeks of age. A BMDL of 858 mg-hr/L was estimated for the area under the curve (AUC) internal blood methanol dose, associated with a brain weight reduction at 6 weeks equal to one standard deviation (SD) from the control mean (see Section 5.1.2.2 [BMD Approach Applied to Brain Weight Data in Rats], and Appendix D [RfC Derivation Options[). This $BMDL_{1SD}$ was then divided by 100 to account for uncertainties associated with human variability (UF_H), the animal-to-human extrapolation (UF_A) and the database (UF_D), and to reduce it to a level that is within the range of blood levels for which the human PBPK model was calibrated (see discussion in Section 5.1.3.2 [Application of UFs]). The PBPK model was then used to convert this adjusted internal $BMDL_{1SD}$ of 8.58 mg-hr/L to a human equivalent candidate RfC of 17.4 mg/m^3 (see Section 5.1.3 [RfC Derivation – Including Application of Uncertainty Factors]) and a candidate RfD of 4.0 mg/kg-day (see Section 5.2.2 [RfD Derivation – Including Application of Uncertainty Factors]).

Burbacher et al. (2004b; 2004a; 1999b; 1999a) exposed *M. fascicularis* monkeys to 0, 200, 600, or 1,800 ppm methanol 2.5 hours/day, 7 days/week during pre-mating/mating and throughout gestation (approximately 168 days). A $BMDL_{SD}$ of 19.6 mg/L was estimated for the blood methanol C_{max} associated with a one SD delay in sensorimotor development in the offspring as measured by a visually directed reaching (VDR) test (see Appendix D [RfC Derivation Options]). However, only the unadjusted VDR response for females exhibited a response that could be modeled and the dose-response was marginally significant, with only the high dose exhibiting a response significantly different from controls. Although, the metabolism of methanol in monkeys is comparable to humans (Section 3.1 [Toxicokinetics Overview]) and a delay in VDR is a potentially relevant CNS effect (Section 4.4.2 [Inhalation Neurotoxicity Studies]), EPA concluded that the use of this data for RfC/D derivation was not preferable, given the availability of more reliable dose-response data from the Rogers et al. (1993b) and NEDO (1987) rodent studies.

In summary, after the evaluation of different species, different endpoints, different protocols and different data sources, the Rogers et al. (1993b) mouse, NEDO (1987) rat, and Burbacher et al. (2004b; 2004a; 1999b; 1999a) monkey studies exhibited developmental effects at similar doses, providing consistent results. As described in Sections 5.1.1.2 (Selection of Critical Effects) and 5.2.1.1 (Expansion of the Oral Database by Route-to-Route Extrapolation), because the Rogers et al. (1993b) and NEDO (1987) studies identified relevant effects in relevant species that could be adequately quantified in a dose-response analysis, they are considered the most appropriate studies for use in the RfC and RfD derivation. The candidate RfC of 2×10^1 mg/m^3 based on decreased brain weight observed in the NEDO (1987) rat developmental study

(see Table 5-4 [Summary of POD values for critical endpoints, application of UFs and conversion to candidate RfCs using PBPK modeling]) was selected as the RfC for methanol. The candidate RfD of 2 mg/kg-day based on the formation of extra cervical ribs observed in the Rogers et al. (1993b) mouse developmental study (see Table 5-5 [Summary of POD values for critical endpoints, application of UFs and conversion to candidate RfDs using PBPK modeling]) was selected as the RfD for methanol. As described in Sections 5.1.3 (RfC Derivation – Including Application of Uncertainty Factors) and 5.2.2 (RfD Derivation – Including Application of Uncertainty Factors), the UFs employed for both the RfC and RfD derivations include a UF_H of 10 for intraspecies variability, a UF_A of 3 to address pharmacodynamic uncertainty and a UF_D of 3 for database uncertainty.

Relationship of the RfC and RfD to Endogenous Methanol Blood Levels

The approach taken by EPA in deriving the RfC and the RfD assumes that endogenous blood levels of methanol in a human population with normal background variation do not elicit adverse health effects. There is currently little evidence, epidemiological or otherwise, to challenge this assumption. Given this assumption and lack of evidence to the contrary, if the 2 mg/kg-day RfD or 2×10^1 mg/m^3 RfC were so low that the resulting (predicted) change in methanol blood levels was only a small fraction of the normal variation in background levels (e.g., 1% of one standard deviation), one could argue that this would be indistinguishable from natural variation and toxicologically irrelevant. Therefore, a comparison of the expected increase in methanol levels in blood resulting from exposure to methanol at the level of the RfC or RfD to the variation in endogenous (i.e., background) levels of methanol observed in humans is provided in Section 5.3.6 to determine if this might be the case. The increase in blood methanol levels (above background) estimated to result from exogenous exposure at the RfC alone (0.41 mg/L), at the RfD alone (0.44 mg/L), or at the RfC + RfD combined (0.86 mg/L) were compared with background methanol blood levels in humans, represented as a mean plus standard deviation of 1.5 ± 0.7 mg/L (see Section 5.3.6). From this analysis EPA concludes that the estimated increase in blood levels of methanol from exogenous exposures at the level of the RfD or the RfC (or from the RfC + RfD) are distinguishable from natural background variation, but the overall derivation of the RfD and RfC ensures that these increases will not significantly increase adverse health outcomes.

1. INTRODUCTION

This document presents background information and justification for the Integrated Risk Information System (IRIS) Summary of the hazard and dose-response assessment of methanol. IRIS Summaries may include oral reference dose (RfD) and inhalation reference concentration (RfC) values for chronic and other exposure durations, and a carcinogenicity assessment.

The RfD and RfC, if derived, provide quantitative information for use in risk assessments for noncancer health effects known or assumed to be produced through a nonlinear (presumed threshold) mode of action (MOA). The RfD (expressed in units of milligrams per kilogram per day [mg/kg-day]) is defined as an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. The inhalation RfC (expressed in units of milligrams per cubic meter [mg/m^3]) is analogous to the oral RfD but provides a continuous inhalation exposure estimate. The inhalation RfC considers toxic effects for both the respiratory system (portal-of-entry) and for effects peripheral to the respiratory system (extrarespiratory or systemic effects). Reference values are generally derived for chronic exposures (up to a lifetime), but may also be derived for acute (≤ 24 hours), short-term (>24 hours up to 30 days), and subchronic (>30 days up to 10% of lifetime) exposure durations, all of which are derived based on an assumption of continuous exposure throughout the duration specified. Unless specified otherwise, the RfD and RfC are derived for chronic exposure duration.

Development of these hazard identification and dose-response assessments for the noncancer effects of methanol has followed the general guidelines for risk assessment as set forth by the National Research Council (NRC) (1983). EPA Guidelines and Risk Assessment Forum Technical Panel Reports that may have been used in the development of this assessment include the following: *Guidelines for the Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 1986), *Recommendations for and Documentation of Biological Values for Use in Risk Assessment* (U.S. EPA, 1988), *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991), *Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies* (U.S. EPA, 1994a), *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b), *Use of the Benchmark Dose Approach in Health Risk Assessment (U.S. EPA, 1995), Guidelines for Reproductive Toxicity Risk Assessment* (U.S. EPA, 1996), *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998a), *Science Policy Council Handbook: Risk Characterization* (U.S. EPA, 2000b), *Supplementary Guidance for Conducting Health Risk Assessment of Chemical*

Mixtures (U.S. EPA, 2000c), A Review of the Reference Dose and Reference Concentration Processes (U.S. EPA, 2002), *Science Policy Council Handbook: Peer Review* (U.S. EPA, 2006c), and *A Framework for Assessing Health Risks of Environmental Exposures to Children* (U.S. EPA, 2006b), B*enchmark Dose Technical Guidance Document (U.S. EPA, 2012a)*.

Primary, peer-reviewed literature identified through January 2013 was included where that literature was determined to be relevant to the assessment. The relevant literature included publications on methanol that were identified through Toxicology Literature Online (TOXLINE), PubMed, the Toxic Substance Control Act Test Submission Database (TSCATS), the Registry of Toxic Effects of Chemical Substances (RTECS), the Chemical Carcinogenesis Research Information System (CCRIS), the Developmental and Reproductive Toxicology/Environmental Teratology Information Center (DART/ETIC), the Hazardous Substances Data Bank (HSDB), the Genetic Toxicology Data Bank (GENE-TOX), Chemical abstracts, and Current Contents. Other peer-reviewed information, including health assessments developed by other organizations, review articles, and independent analyses of the health effects data were retrieved and included in the assessment where appropriate. Studies that had not been peer-reviewed and were potentially critical to the conclusions of the assessment were separately and independently peer-reviewed. Any pertinent scientific information submitted by the public to the IRIS Submission Desk or by reviewers during internal and external peer reviews was also considered in the development of this document. It should be noted that references added to the Toxicological Review after the external peer review in response to peer reviewer‘s comments have not changed the overall qualitative and quantitative conclusions.

An initial keyword search was based on the Chemical Abstracts Service Registry Number (CASRN) and several common names for methanol. The subsequent search strategy focused on the toxicology and toxicokinetics of methanol, particularly as they pertain to target tissues, effects at low doses, different developmental stages, sensitive subpopulations, and background levels from endogenous and exogenous sources. A more targeted search was completed for the construction and parameterization of a methanol physiologically-based pharmacokinetic (PBPK) model. The focus of this targeted search included existing PBPK models for primary alcohols and pharmacokinetic information for major metabolites and related enzymes. Both the general and targeted searches identified a multitude of studies that used methanol for laboratory procedures. Exclusion terms such as ‘extract of methanol’ were used in order to cull such irrelevant studies. The literature keyword searches are narrowed down further by manual review. Selection of studies for inclusion in the Toxicological Review was based on consideration of the extent to which the study was informative and relevant to the assessment and general study quality considerations. In general, the relevance of health effect studies was evaluated as outlined in EPA guidance [*A Review of the Reference Dose and Reference Concentration Processes* (U.S.

EPA, 2002) and *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhaled Dosimetry* (U.S. EPA, 1994b)]. All animal studies of methanol involving repeated oral, inhalation, or dermal exposure that were considered to be of acceptable quality, whether yielding positive, negative, or null results, were considered in assessing the evidence for health effects associated with chronic exposure to methanol. In addition, animal toxicity studies involving short-term duration and other routes of exposure were evaluated to inform conclusions about health hazards. The references considered and cited in this document, including bibliographic information and abstracts, can be found on the Health and Environmental Research Online (HERO) website.[2]

On December 23, 2011, The Consolidated Appropriations Act, 2012, was signed into law[3]. The report language included direction to EPA for the IRIS Program related to recommendations provided by the National Research Council (NRC) in their review of EPA's draft IRIS assessment of formaldehyde. The NRC's recommendations, provided in Chapter 7 of their review report, offered suggestions to EPA for improving the development of IRIS assessments. The report language included the following:

"The Agency shall incorporate, as appropriate, based on chemical-specific datasets and biological effects, the recommendations of Chapter 7 of the National Research Council's Review of the Environmental Protection Agency's Draft IRIS Assessment of Formaldehyde into the IRIS process …. For draft assessments released in fiscal year 2012, the Agency shall include documentation describing how the Chapter 7 recommendations of the National Academy of Sciences (NAS) have been implemented or addressed, including an explanation for why certain recommendations were not incorporated."

Consistent with the direction provided by Congress, documentation of how the recommendations from Chapter 7 of the NRC report have been implemented in this assessment is provided in Appendix E. This documentation also includes an explanation for why certain recommendations were not incorporated.

For other general information about this draft assessment or other questions relating to IRIS, the reader is referred to EPA's IRIS Hotline at (202) 566-1676 (phone), (202) 566-1749 (fax), or hotline.iris@epa.gov.

[2]HERO is a database of scientific studies and other references used to develop EPA's risk assessments aimed at understanding the health and environmental effects of pollutants and chemicals. It is developed and managed in EPA's Office of Research and Development (ORD) by the National Center for Environmental Assessment (NCEA). The database includes more than 750,000 scientific articles from the peer-reviewed literature. New studies are added continuously to HERO.

[3]Pub. L. No. 112-74, Consolidated Appropriations Act, 2012.

2. CHEMICAL AND PHYSICAL INFORMATION

Methanol is also known as methyl alcohol, wood alcohol; Carbinol; Methylol; colonial spirit; columbian spirit; methyl hydroxide; monohydroxymethane; pyroxylic spirit; wood naphtha; and wood spirit. Some relevant physical and chemical properties are listed in Table 2-1 below (HSDB, 2009; IPCS, 1997).

Table 2-1 Relevant physical and chemical properties of methanol

CASRN:	67-56-1
Empirical formula:	CH_3OH
Molecular weight:	32.04
Vapor pressure:	160 mmHg at 30 ^oC
Vapor Density:	1.11
Specific gravity:	0.7866 g/mL (25 ^oC)
Boiling point:	64.7 ^oC
Melting point:	-98 ^oC
Water solubility:	Miscible
Log octanol-water partition coefficient:	-0.82 to -0.68
Conversion factor (in air):	1 ppm = 1.31 mg/m^3; 1 mg/m^3 = 0.763 ppm

Methanol is a clear, colorless liquid that has an alcoholic odor (IPCS, 1997). Endogenous levels of methanol are present in the human body as a result of both metabolism[4] and dietary sources such as fruit, fruit juices, vegetables and alcoholic beverages,[5] and can be measured in exhaled breath and body fluids (Turner et al., 2006; CERHR, 2004; IPCS, 1997). Dietary exposure to methanol also occurs through the intake of some food additives. The artificial sweetener aspartame and the beverage yeast inhibitor dimethyl dicarbonate (DMDC) release methanol as they are metabolized (Stegink et al., 1989). In general, aspartame exposure does not contribute significantly to the background body burden of methanol (Butchko et al., 2002). Oral,

[4] Methanol is generated metabolically through enzymatic pathways such as the methyltransferase system (Fisher et al., 2000).

[5] Fruits and vegetables contain methanol (Cal/EPA, 2012). Further, ripe fruits and vegetables contain natural pectin, which is degraded to methanol in the body by bacteria present in the colon (Siragusa et al., 1988). Increased levels of methanol in blood and exhaled breath have also been observed after the consumption of ethanol (Fisher et al., 2000).

dermal, or inhalation exposure to methanol in the environment, consumer products, or workplace also occur.

Methanol is a high production volume chemical with many commercial uses and it is a basic building block for hundreds of chemical products. Many of its derivatives are used in the construction, housing or automotive industries. Consumer products that contain methanol include varnishes, shellacs, paints, windshield washer fluid, antifreeze, adhesives, de-icers, and Sterno heaters. In 2009, the Methanol Institute (2009b) estimated a global production capacity for methanol of about 35 million metric tons per year (close to 12 billion gallons), a production capacity in the United States (U.S.) of nearly 3.7 million metric tons (1.3 billion gallons), and a total U.S. demand for methanol of over 8 million metric tons. Methanol is among the highest production volume chemicals reported in the U.S. EPA's Toxic Release Inventory (TRI).[6] It is among the top chemicals on the 2008 TRI lists of chemicals with the largest total on-site and off-site recycling (6th), energy recovery (2nd) and treatment (1st) (U.S. EPA, 2009b). TRI also reports that approximately 135,000,000 pounds of methanol was released or disposed of in the United States in 2008, making methanol among the top five chemicals on the list entitled "TRI On-site and Off-site Reported Disposed of or Otherwise Released in pounds for facilities in All Industries for Hazardous Air Pollutant Chemicals U.S. 2008" (U.S. EPA, 2009d).

While production has switched to other regions of the world, demand for methanol is growing steadily in almost all end uses. A large reason for the increase in demand is its use in the production of biodiesel, a low-sulfur, high-lubricity fuel source. Global demand for biodiesel is forecast to increase by 32% per year, rising from 30 million gallons in 2004, to 150 million gallons by 2008, and to 350 million gallons by 2013 (Methanol Institute, 2009a). Power generation and fuel cells could also be large end users of methanol in the near future (Methanol Institute, 2009b).

[6] The information in TRI does not indicate whether (or to what degree) the public has been exposed to toxic chemicals. Therefore, no conclusions on the potential risks can be made based solely on this information (including any ranking information). For more detailed information on this subject refer to The Toxics Release Inventory (TRI) and Factors to Consider When Using TRI Data (U.S. EPA, 2009c).

3. TOXICOKINETICS

3.1. Overview

As has been noted, methanol occurs naturally in the human body as a product of metabolism and through intake of fruits, vegetables, and alcoholic beverages (Cal/EPA, 2012; Turner et al., 2006; CERHR, 2004; IPCS, 1997). Table 3-1 summarizes background blood methanol levels in healthy humans which were found to range from 0.25-5.2 mg/L. Formate, a metabolite of methanol, also occurs naturally in the human body (IPCS, 1997). Table 3-1 outlines background levels of formate in human blood. In most cases, methanol and formate blood levels were measured in healthy adults following restriction of methanol-producing foods from the diet.[7]

The absorption, excretion, and metabolism of methanol are well known and have been consistently summarized in reviews such as CERHR (2004), IPCS (1997), U.S. EPA (1996), Kavet and Nauss (1990), HEI (1987), and Tephly and McMartin (1984). Therefore, the major portion of this toxicokinetics overview is based upon those reviews.

Studies conducted in humans and animals demonstrate rapid absorption of methanol by inhalation, oral, and dermal routes of exposure. Table 3-2 outlines increases in human blood methanol levels following various exposure scenarios. Blood levels of methanol following various exposure conditions have also been measured in monkeys, mice, and rats, and are summarized in Tables 3-3, 3-4, and 3-5, respectively. Once absorbed, methanol pharmacokinetic (PK) data and physiologically based pharmacokinetic (PBPK) model predictions indicate rapid distribution to all organs and tissues according to water content, as an aqueous-soluble alcohol. Tissue:blood concentration ratios for methanol are predicted to be similar through different exposure routes, though the kinetics will vary depending on exposure route and timing (e.g., bolus oral exposure versus longer-term inhalation). Because smaller species generally have faster respiration rates relative to body weight than larger species, they are predicted to have a higher rate of increase of methanol concentrations in the body when exposed to the same concentration in air.

[7] Background levels among people who are on normal/non-restricted diets may be higher than those on restricted diets.

Table 3-1 Background blood methanol and formate levels in human studies

Description of human subjects	Methanol (mg/L) mean ± S.D.[a] (Range)	Formate (mg/L) mean ± S.D. (Range)	Reference
12 adults who drank no alcohol for 24 hr	1.7 ± 0.9 (0.4-4.7)	No data	Batterman and Franzblau (1997)
12 adults who drank no alcohol for 24 hr	1.8 ± 0.7 (No range data)	No data	Batterman et al. (1998)
12 males on restricted diet (no methanol-containing or methanol-producing foods) for 12 hr	0.570 ± 0.305 (0.25-1.4)	3.8 ± 1.1 (2.2-6.6)	Cook et al. (1991)
4 adult males who fasted for 8 hr, drank no alcohol for 24 hr, and took in no fruits, vegetables, or juices for 18 hr	1.75 ± 0.65 (1.2-2.6)	No data	Davoli et al. (1986)
8 adults who had no fruit, alcohol or drugs for 48 hr	No mean data (0.3-2.4)	No data	Ernstgard et al. (2005)
3 males who ate a breakfast with no aspartame-containing cereals and no juice	1.82 ± 1.21 (0.57-3.57)	9.08 ± 1.26 (7.31-10.57)	Lee et al. (1992)
5 males who ate a breakfast with no aspartame-containing cereals and no juice (second experiment)	1.93 ± 0.93 (0.54-3.15)	8.78 ± 1.82 (5.36-10.83)	Lee et al. (1992)
22 adults on restricted diet (no methanol-containing or methanol-producing foods) for 24 hr	1.8 ± 2.6 (No range data)	11.2 ± 9.1 (No range data)	Osterloh et al. (1996); Chuwers et al. (1995)
35 adults who drank no alcohol for 1 week, fasted 4 hours	0.64 ± 0.45 (No range data)	No data	Sarkola and Eriksson (2001)
12 adults fasted 5 hours	1.1 (0.4-2.2)	No data	Schmutte et al. (1988)
30 fasted adults	<4 (No range data)	19.1 (No range data)	Stegink et al. (1981)
24 fasted infants	<3.5 (No range data)	No data	Stegink et al. (1983)
30 adults. No dietary restrictions. Blood levels were estimated from concentrations in breath.	1.25 ± 0.29[b] (0.45-1.7)	No data	Turner et al.(2006)
18 males, fasted 3 hr, no other dietary restrictions	2.62 ± 1.33 (0.7-5.2)	No data	Woo et al. (2005)

[a]EPA used a random-effects model to estimate an overall mean and SD of 1.5 ± 0.7 mg/L from this data (see Section 5.3.6).
[b]Arithmetic mean and standard deviation calculated from mean values listed in Table 1 of Turner et al. (2006).

Table 3-2 Human blood methanol and formate levels following methanol exposure.

Human subjects; type of sample collected [a,b]	Exposure route	Exposure duration or method	Methanol exposure concentration	Blood methanol mean (mg/L)	Blood formate mean (mg/L)	Reference
5 adults 7 adults Fasted 5 hours	Oral	1 dose in water	7 mg/kg bw 12.5 mg/kg bw	9.04	No data	Schmutte et al. (1988)
Males; post exposure samples	Inhalation	75 min	0 ppm 191 ppm	0.570 1.881	3.8 3.6	Cook et al. (1991)
Males and females with exercise; post exposure samples	Inhalation	2 hr	0 ppm 100 ppm 200 ppm	0.64 3.72 7.82	No data	Ernsgard et al. (2005)
Males and females; post exposure samples	Inhalation	4 hr	0 ppm 200 ppm	1.8 6.5	11.2 14.3	Osterloh et al. (1996)
Males without exercise; post exposure samples	Inhalation	6 hr	0 ppm 200 ppm	1.82 6.97	9.08 8.70	Lee et al. (1992)
Males with exercise; post exposure samples	Inhalation	6 hr	0 ppm 200 ppm	1.93 8.13	8.78 9.52	
Females; post exposure samples	Inhalation	8 hr	0 ppm 800 ppm	1.8 30.7	No data	Batterman et al. (1998)

[a]Unless otherwise specified, it is assumed that whole blood was used for measurements.
[b]Information about dietary restrictions is included in Table 3-1.

Table 3-3 Monkey blood methanol and formate levels following methanol exposure

Strain-sex	Exposure route	Exposure duration	Methanol exposure concentration	Blood methanol mean (mg/L)	Blood formate mean or range (mg/L)	Reference
Monkey; cynomolgus; female; mean blood methanol and range of plasma formate at 30 min post daily exposure during premating, mating, and pregnancy	Inhalation	2.5 hr/day, 7days/wk during premating, mating, and gestation (348 days)	0 ppm	2.4	8.7	Burbacher et al. (2004b; 1999b)
			200 ppm	5	8.7	
			600 ppm	11	8.7	
			1,800 ppm	35	10	
Monkey; cynomolgus; female; Lung only inhalation of anesthetized monkeys post exposure [a]	Inhalation	2 hr	10 ppm	0.021	0.0032	Dorman et al. (1994)
			45 ppm	0.096	0.012	
			200 ppm	0.67	0.11	
			900 ppm	3.4	0.13	
			900 ppm - FD	6.8	0.44	
Monkey; Rhesus male; post exposure blood level	Inhalation	6 hr	200 ppm	3.9	5.4-13.2 at all doses	Horton et al. (1992)
			1,200 ppm	37.6		
			2,000 ppm	64.4		

FD=folate deficient

[a]Methanol and formate blood levels obtained from radiolabeled methanol and do not include background levels of methanol or formate.

Table 3-4 Mouse blood methanol and formate levels following methanol exposure

Species/strain/sex	Exposure route	Exposure duration	Methanol exposure concentration	Blood methanol mean (mg/L)	Blood formate mean (mg/L)	Reference
Mouse;CD-1;female; peak concentration (C_{max})	Injection (i.v.)	GD18	100 mg/kg bw 500 mg/kg bw 2,500 mg/kg bw	252 869 3,521	No data	Ward et al. (1997)
Mouse;CD-1;female; peak concentration (C_{max})	Oral	GD18	2,500 mg/kg bw	3,205	No data	Ward et al. (1997)
Mouse;CD-1;female; post exposure plasma methanol and peak formate level	Inhalation	6 hr on GD8	10,000 ppm	2,080	28.5	Dorman et al. (1995)
			10,000 ppm (+ 4-MP)	2,400	23	
			15,000 ppm	7,140	34.5	
Mouse;CD-1;female; post exposure blood methanol level	Inhalation	8 hr	2,500 ppm	1,883	No data	Pollack and Brouwer (1996); Perkins et al. (1995a)
			5,000 ppm	3,580		
			10,000 ppm	6,028		
			15,000 ppm	11,165		
Mouse;CD-1;female; mean post exposure plasma methanol level	Inhalation	7 hr/day on GD6–GD15	0	1.6	No data	Rogers et al. (1993b)
			1,000 ppm	97		
			2,000 ppm	537		
			5,000 ppm	1,650		
			7,500 ppm	3,178		
			10,000 ppm	4,204		
			15,000 ppm	7,330		
Mouse;CD-1;female; plasma level 1 hr post dosing	Oral-Gavage	GD6–GD15	4,000 mg/kg bw	3,856	No data	
Mouse;CD-1;female; peak plasma level	Oral-Gavage	GD8	1,500 mg/kg bw	1,610	35	Dorman et al. (1995)
			1,500 mg/kg bw (+ 4-MP)	1,450	43	

4-MP=4-methylpyrazole (fomepizole)

Table 3-5 Rat blood methanol and formate levels following methanol exposure

Species; strain/sex: type of sample collected	Exposure route	Exposure duration	Methanol exposure concentration	Blood methanol mean or range (mg/L)	Blood formate mean or range (mg/L)	Reference
Rat; Sprague-Dawley; female; post exposure blood methanol level on 3 days	Inhalation	7 hr/day for 19 days	5,000 ppm 10,000 ppm 20,000 ppm	1,000-2,170 1,840-2,240 5,250-8,650	No data	Nelson et al. (1985)
Rat; Sprague-Dawley; female; post exposure blood methanol level	Inhalation	8 hr	1,000 ppm 5,000 ppm 10,000 ppm 15,000 ppm 20,000 ppm	83 1,047 1,656 2,667 3,916	No data	Pollack and Brouwer (1996) ; Perkins et al. (1995a)
Rat; LongEvans; female; post exposure plasma level on GD7-GD12	Inhalation	7 hr/day on GD7-GD19	0 ppm 15,000 ppm	1.8-2.7 3,169-3,826	No data	Stanton et al. (1995)
Rat; LongEvans; female; 1 hr post exposure blood level	Inhalation	6 hr/day on GD6-PND21	4,500 ppm	555	No data	Weiss et al. (1996)
Rat; Long-Evans; male and female; 1 hr post exposure blood level in pups	Inhalation	6 hr/day on PND1-PND21	4,500 ppm	1,260	No data	
Rat/Fischer-344 male; post exposure blood level	Inhalation	6 hr	200 ppm 1,200 ppm 2,000 ppm	3.1 26.6 79.7	5.4-13.2 at all doses	Horton et al. (1992)
Rat; Long-Evans; male; post- exposure serum level	Inhalation	6 hr	200 ppm 5,000 ppm 10,000 ppm	7.4 680-873 1,468	No data	Cooper et al. (1992)
Rat/Fischer-344 male; 25 min post exposure blood level for 4-wk animals; ~250 min post exposure for 104-wk animals	Inhalation	19.5 hr/day for 4/104 wk	0 ppm 10 ppm 100 ppm 1,000 ppm	4.01 / 3.78 1.56 / 3.32 3.84 / 3.32 53.59 /12.08	No data	NEDO (1985b)
Rat/Fischer-344 female; 25 min post exposure blood level for 4-wk animals; ~250 min post exposure for 104-wk animals	Inhalation	19 hr/day for 4/104 wk	0 ppm 10 ppm 100 ppm 1,000 ppm	13.39 / 3.60 6.73 / 3.70 4.34 / 4.32 88.33 / 8.50	No data	NEDO (1985b)

Species; strain/sex: type of sample collected	Exposure route	Exposure duration	Methanol exposure concentration	Blood methanol mean or range (mg/L)	Blood formate mean or range (mg/L)	Reference
Rat; Long-Evans; male; peak blood formate level	Inhalation	6 hr	0 ppm FS	No data	8.3	Lee et al. (1994)
			0 ppm FR		10.1	
			1,200 ppm-FS		8.3	
			1,200 ppm-FR		46.0	
			2,000 ppm-FS		8.3	
			2,000 ppm-FR		83.0	
Rat; Sprague-Dawley; female;peak concentration (C_{max})	Injection (i.v.)	GD14	100 mg/kg bw	123.7	No data	Ward et al. (1997)
			500 mg/kg bw	612.9		
Rat;Sprague-Dawley; female;peak concentration (C_{max})	Injection (i.v.)	GD20	100 mg/kg bw	149.0	No data	Ward et al. (1997)
			500 mg/kg bw	663.6		
Rat; Long-Evans; male; peak blood methanol and formate	Oral-gavage	Single dose	2,000 mg/kg bw FS	No data	9.2	Lee et al. (1994)
			2,000 mg/kg bw FR		538	
			3,000 mg/kg bw-FS		9.2	
			3,000 mg/kg bw FR		718	
			3,500 mg/kg bw-FS	4,800	9.2	
			3,500 mg/kg bw-FP	4,800	38.2	
			3,500 mg/kg bw-FR	4,800	860	

FS = Folate sufficient; FR = Folate reduced; FP = Folate paired

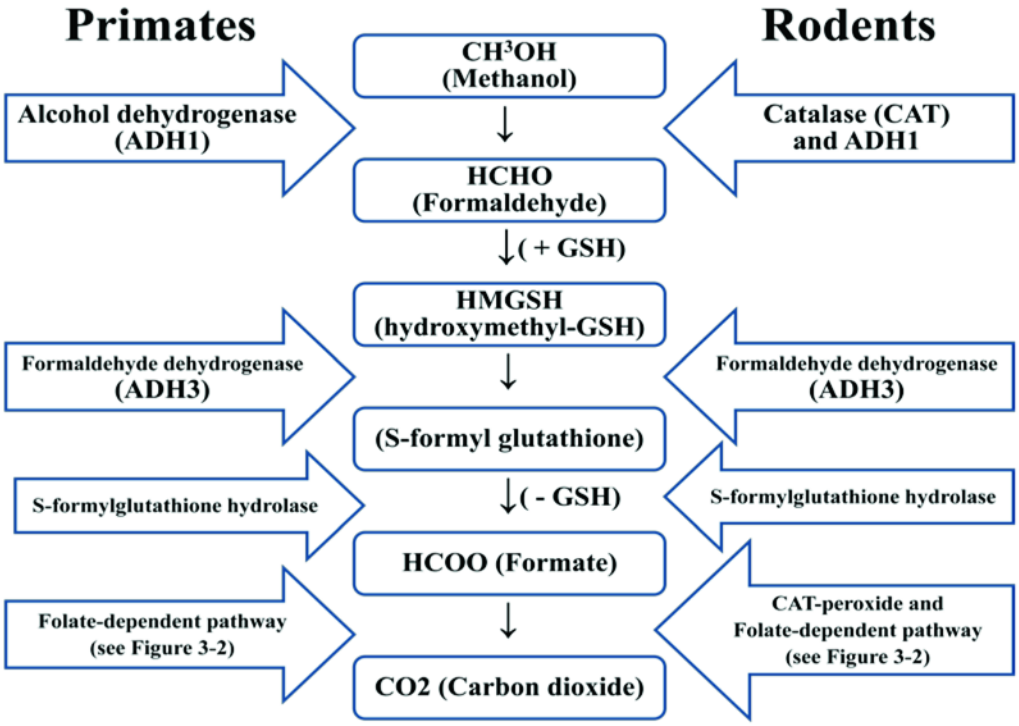
At doses that do not saturate metabolic pathways, a small percentage of methanol is excreted directly in urine. Because of the high blood:air partition coefficient for methanol and rapid metabolism in all species studied, the bulk of clearance occurs by metabolism, though exhalation and urinary clearance become more significant when doses or exposures are sufficiently high to saturate metabolism (subsequently in this document, "clearance" refers to elimination by all routes, including metabolism, as indicated by the decline in methanol blood concentrations). Metabolic saturation and the corresponding clearance shift have not been observed in humans and nonhuman primates because doses used were limited to the linear range, but the enzymes involved in primate metabolism are also saturable.

The primary route of methanol elimination in mammals is through a series of oxidation reactions that form formaldehyde, formate, and carbon dioxide (Figure 3-1). As noted in

Figure 3-1, methanol is converted to formaldehyde by alcohol dehydrogenase-1 (ADH1) in primates and by catalase (CAT) and ADH1 in rodents. Although the first step of metabolism occurs through different pathways in rodents and nonhuman primates, Kavet and Nauss (1990) report that the reaction proceeds at similar rates (V_{max} = 30 and 48 mg/hr/kg in rats and nonhuman primates, respectively). In addition to enzymatic metabolism, methanol can react with hydroxyl radicals to spontaneously yield formaldehyde (Harris et al., 2003). Mannering et al. (1969) also reported a similar rate of methanol metabolism in rats and monkeys, with 10 and 14% of a 1 g/kg dose oxidized in 4 hours, respectively; the rate of oxidation by mice was about twice as fast, 25% in 4 hours. In an HEI study by Pollack and Brouwer (1996), the metabolism of methanol was 2 times as fast in mice versus rats, with a V_{max} for elimination of 117 and 60.7 mg/hr/kg, respectively. Despite the faster elimination rate of methanol in mice versus rats, mice consistently exhibited higher blood methanol levels than rats when inhaling equivalent methanol concentrations (See Tables 3-4 and 3-5). Possible explanations for the higher methanol accumulation in mice include faster respiration (inhalation rate/body weight) and increased fraction of absorption by the mouse (Perkins et al., 1995a). Sweeting et al. (2010) examined methanol dosimetry in CD-1 mice, New Zealand white (NZW) rabbits, and cynomolgus monkeys, and found that peak plasma concentrations are not significantly different, but clearance in rabbits is approximately half that of mice following a single 0.5 or 2 g/kg i.p. (intraperitoneal) injection. This suggests that rabbit clearance is similar to that in rats and monkeys, since Mannering et al. (1969) found that rat and monkey clearance rates are also about half that in mice. Sweeting et al. (2010) did not report the clearance rates from monkeys, but the 6-hour AUC in monkeys was similar to that in rabbits. Because smaller species generally have faster breathing rates than larger species, humans would be expected to absorb methanol via inhalation more slowly than rats or mice inhaling equivalent concentrations. If humans eliminate methanol at a comparable rate to rats and mice, then humans would also be expected to accumulate less methanol than those smaller species. However, if humans eliminate methanol more slowly than rats and mice, such that the ratio of absorption to elimination stays the same, then humans would be expected to accumulate methanol to the same internal concentration but to take longer to reach that concentration.

In all species, formaldehyde is rapidly converted to formate, with the half-life for formaldehyde being ~1 minute. Formaldehyde is oxidized to formate by two metabolic pathways (Teng et al., 2001). The first pathway (not shown in Figure 3-1) involves conversion of free formaldehyde to formate by the so-called low-affinity pathway (affinity = $1/K_m$ = 0.002/μM) mitochondrial aldehyde dehydrogenase-2 (ALDH2). The second pathway (Figure 3-1) involves a two-enzyme system that converts glutathione-conjugated formaldehyde (*S*-hydroxymethylglutathione [HMGSH]) to the intermediate *S*-formylglutathione, which is

subsequently metabolized to formate and glutathione (GSH) by *S*-formylglutathione hydrolase.[8] The first enzyme in this pathway, formaldehyde dehydrogenase-3 (ADH3), is rate limiting, and the affinity of HMGSH for ADH3 (affinity = $1/K_m$ = 0.15/μM) is about a 100-fold higher than that of free formaldehyde for ALDH2. In addition to the requirement of GSH for ADH3 activity, oxidation by ADH3 is (NAD^+ [nicotinamide adenine dinucleotide])-dependent. Under normal physiological conditions NAD^+ levels are about two orders of magnitude higher than NADH, and intracellular GSH levels (mM range) are often high enough to rapidly scavenge formaldehyde (Svensson et al., 1999; Meister and Anderson, 1983); thus, the oxidation of HMGSH is favorable. In addition, genetic ablation of ADH3 results in increased formaldehyde toxicity (Deltour et al., 1999). These data indicate that ADH3 is likely to be the predominant enzyme responsible for formaldehyde oxidation at physiologically relevant concentrations, whereas ALDHs likely contribute to formaldehyde elimination at higher concentrations (Dicker and Cedebaum, 1986).

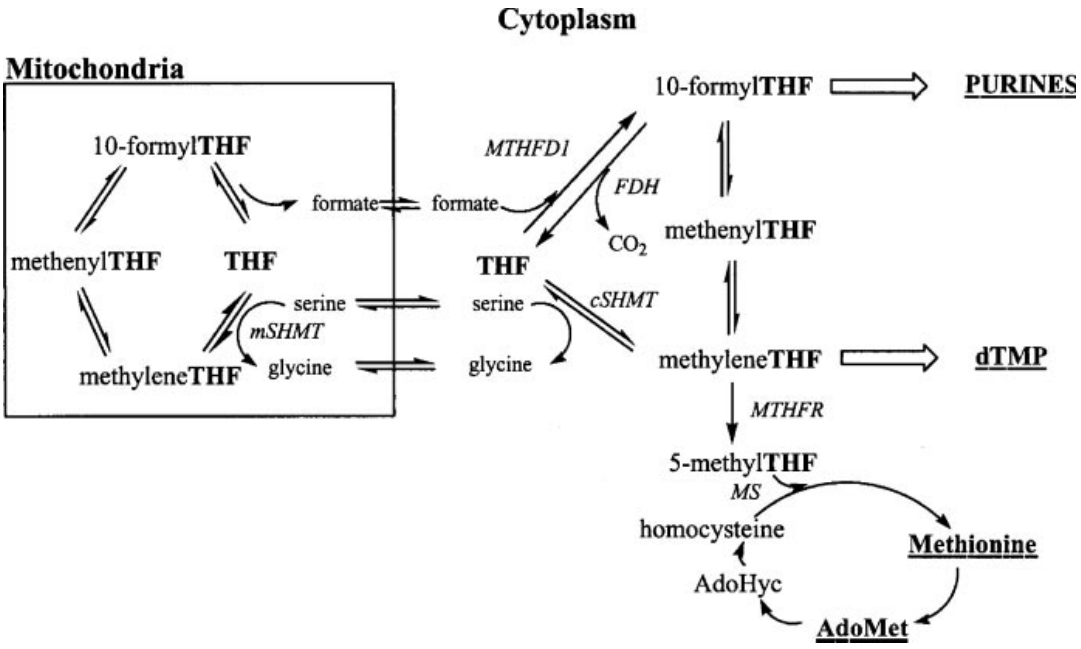


Source: IPCS (1997).

Figure 3-1 Methanol metabolism and key metabolic enzymes in primates and rodents.

[8] Other enzymatic pathways for the oxidation of formaldehyde have been identified in other organisms, but this is the pathway that is recognized as being present in humans (Caspi et al., 2006: http://metacyc.org).

Rodents convert formate to carbon dioxide (CO_2) through a folate-dependent enzyme system and a CAT-peroxide system (Dikalova et al., 2001). Formate can undergo adenosine triphosphate- (ATP-) dependent addition to tetrahydrofolate (THF), which can carry either one or two one-carbon groups. Formate can conjugate with THF to form N^{10}-formyl-THF and its isomer N^5-formyl-THF, both of which can be converted to N^5, N^{10}-methenyl-THF and subsequently to other derivatives that are ultimately incorporated into DNA and proteins via biosynthetic pathways (Figure 3-2). There is also evidence that formate generates CO_2^- radicals, and can be metabolized to CO_2 via CAT and via the oxidation of N^{10}-formyl-THF (Dikalova et al., 2001).



Source: Montserrat et al. (2006).

Figure 3-2 Folate-dependent formate metabolism. Tetrahydrofolate (THF)-mediated one carbon metabolism is required for the synthesis of purines, thymidylate, and methionine.

Unlike rodents, formate metabolism in primates occurs solely through a folate-dependent pathway. Black et al. (1985) reported that hepatic THF levels in monkeys are 60% of that in rats, and that primates are far less efficient in clearing formate than are rats and dogs. Studies of human subjects involving [^{14}C]formate suggest that ~80% is exhaled as $^{14}CO_2$, 2-7% is excreted in the urine, and ~10% undergoes metabolic incorporation (Hanzlik et al., 2005, and references therein). Sweeting et al. (2010) have reported that formic acid accumulation in primates within 6 hours of a 2 g/kg i.p. exposure to methanol was 5-fold and 43-fold higher than in rabbits and mice, respectively. Mice deficient in formyl-THF dehydrogenase exhibit no change in LD_{50} (via

intraperitoneal [i.p.]) for methanol or in oxidation of high doses of formate. Thus it has been suggested that rodents efficiently clear formate via high capacity folate-dependent pathways, peroxidation by CAT, and by an unknown third pathway; conversely, primates do not appear to exhibit such capacity and are more sensitive to metabolic acidosis following methanol poisoning (Cook et al., 2001).

Blood methanol and formate levels measured in humans under various exposure scenarios are reported in Table 3-2. As noted in Table 3-2, 75-minute to 6-hour exposures of healthy humans to 200 ppm methanol vapors, the American Council of Governmental Industrial Hygienists (ACGIH) threshold limit value (TLV) for occupational exposure (ACGIH, 2000), results in increased levels of blood methanol but not formate. A limited number of monitoring studies indicate that levels of methanol in outdoor air are orders of magnitude lower than the TLV (IPCS, 1997). Table 3-3 indicates that exposure of monkeys to 600 ppm methanol vapors for 2.5 hours increased blood methanol but not blood formate levels. Normal dietary exposure to aspartame, which releases 10% methanol during metabolism, is unlikely to significantly increase blood methanol or formate levels (Butchko et al., 2002). Exposure to high concentrations of aspartame is unlikely to increase blood formate levels; no increase in blood formate levels were observed in adults ingesting "abusive doses" (100-200 mg/kg) of aspartame (Stegink et al., 1981). Kerns et al. (2002) studied the kinetics of formate in 11 methanol-poisoned patients (mean initial methanol level of 57.2 mmol/L or 1.83 g/L) and determined an elimination half-life of 3.4 hours for formate. Kavet and Nauss (1990) estimated that a methanol dose of 11 mM or 210 mg/kg is needed to saturate folate-dependent metabolic pathways in humans. There are no data on blood methanol and formate levels following methanol exposure of humans with reduced ADH activity or marginal folate tissue levels, a possible concern regarding sensitive populations. As discussed in greater detail in Section 3.2, a limited study in folate-deficient monkeys demonstrated no increase in blood formate levels following exposure to 900 ppm methanol vapors for 2 hours. In conclusion, limited available data suggest that typical occupational, environmental, and dietary exposures are likely to increase baseline blood methanol but not formate levels in most humans.

3.2. Key Studies

Toxicokinetic and metabolism studies (Burbacher et al., 2004a; Burbacher et al., 1999a; Medinsky et al., 1997; Pollack and Brouwer, 1996; Dorman et al., 1994) provide key information on interspecies differences, methanol metabolism during gestation, metabolism in the nonhuman primate, and the impact of folate deficiency on the accumulation of formate.

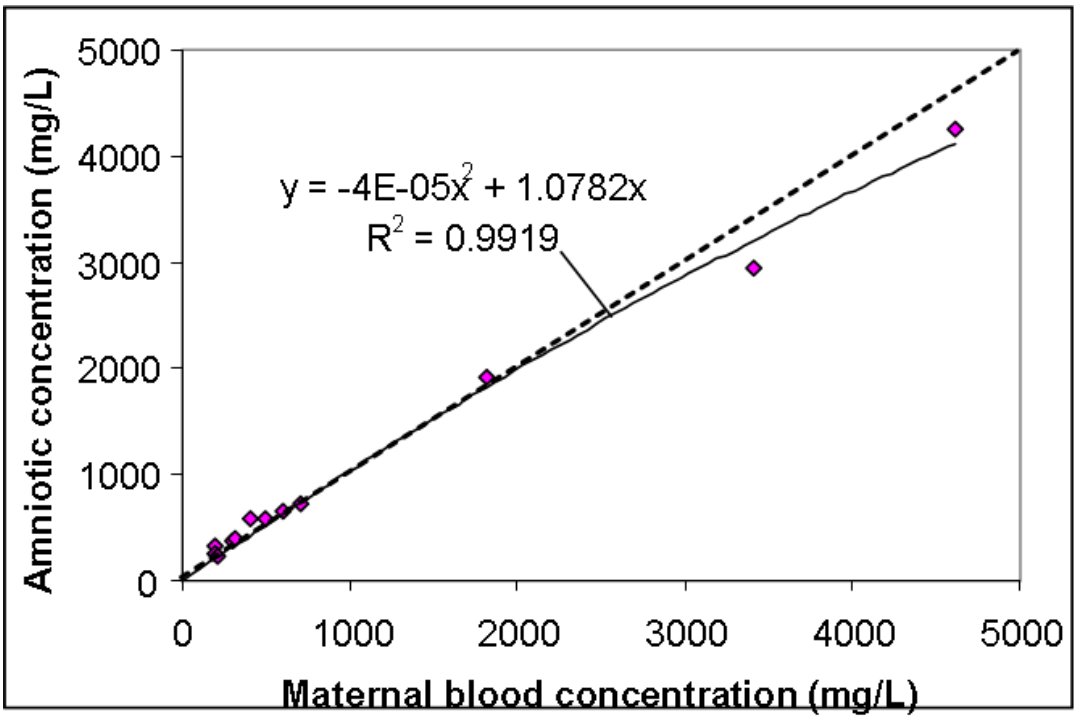
As part of an effort to develop a physiologically based toxicokinetic model for methanol distribution in pregnancy, Pollack and Brouwer (1996) conducted a large study that compared toxicokinetic differences in pregnant and nonpregnant (NP) rats and mice. Methanol disposition[9] was studied in Sprague-Dawley rats and CD-1 mice that were exposed to 100-2,500 mg/kg of body weight pesticide-grade methanol in saline by i.v. or oral routes. Exposures were conducted in NP rats and mice, pregnant rats on gestation days (GD) GD7, GD14, and GD20, and pregnant mice on GD9 and GD18. Disposition was also studied in pregnant rats and mice exposed to 1,000-20,000 ppm methanol vapors for 8 hours. Three to five animals were examined at each dose and exposure condition.

- Based on the fit of various kinetic models to methanol measurements taken from all routes of exposure, the authors concluded that high exposure conditions resulted in nonlinear disposition of methanol in mice and rats.[10] Both linear and nonlinear pathways were observed with the relative contribution of each pathway dependent on concentration. At oral doses of 100-500 mg/kg of body weight, methanol was metabolized to formaldehyde and then formic acid through the saturable nonlinear pathway. A parallel, linear route characteristic of passive-diffusion accounted for an increased fraction of total elimination at higher concentrations. Nearly 90% of methanol elimination occurred through the linear route at the highest oral dose of 2,500 mg/kg of body weight.
- Oral exposure resulted in rapid and essentially complete absorption of methanol. No significant change in blood area under the curve (AUC) methanol was seen between NP and GD7, GD14 and GD20 rats exposed to single oral gavage doses of 100 and 2,500 mg/kg, nor between NP and GD9 and GD18 mice at 2,500 mg/kg. The data as a whole suggested that the distribution of orally and i.v. administered methanol was similar in rats versus mice and in pregnant rodents versus NP rodents with the following exceptions:
- There was a statistically significant increase in the ratio of apparent volume of distribution (V_d) to fractional bioavailability (F) by ~20% (while F decreased but not significantly), between NP and GD20 rats exposed to 100 mg/kg orally. However, this trend was not seen in rats or mice exposed to 2,500 mg/kg, and the result in rats at 100 mg/kg could well be a statistical artifact since both V_d and F were being estimated from the same data, making the model effectively over-parameterized.

[9] Methanol concentrations in whole blood and urine were determined by gas chromatography with flame ionization detection (Pollack and Kawagoe, 1991).
[10] A model incorporating parallel linear and nonlinear routes of methanol clearance was required to fit the data from the highest exposure groups.

- There were statistically significant decreases in the fraction of methanol absorbed by the fast process (resulting in a slower rise to peak blood concentrations, though the peak is unchanged) and in the V_{max} for metabolic elimination between NP and GD18 mice. No such differences were observed between NP and GD9 mice.
- The authors estimated a twofold higher V_{max} for methanol elimination in mice versus rats following oral administration of 2,500 mg/kg methanol, suggesting that similar oral doses would result in lower methanol concentrations in the mouse versus rat.
- Methanol penetration from maternal blood to the fetal compartment was examined in GD20 rats by microdialysis.[11] A plot of the amniotic concentration versus maternal blood concentration (calculated from digitization of Figure 17 of Pollack and Brouwer (1996) report) is shown in Figure 3-3. The ratio is slightly less than 1:1 (dashed line in plot) and appears to be reduced with increasing methanol concentrations, possibly due to decreased blood flow to the fetal compartment. Nevertheless, this is a very minor departure from linearity, consistent with a substrate such as methanol that penetrates cellular membranes readily and distributes throughout total body water.

[11] Microdialysis was conducted by exposing the uterus (midline incision), selecting a single fetus in the middle of the uterine horn and inserting a microdialysis probe through a small puncture in the uterine wall proximal to the head of the fetus.



Source: Pollack and Brouwer (1996).

Note: Data extracted from Figure 17 by digitization, and amniotic concentration obtains as ("Fetal Amniotic Fluid/Maternal Blood Methanol") × ("Maternal Methanol").

Figure 3-3 Plot of fetal (amniotic) versus maternal methanol concentrations in GD20 rats.

Inhalation exposure resulted in less absorption in both rats and mice as concentrations of methanol vapors increased, which was hypothesized to be due to decreased breathing rate and decreased absorption efficiency from the upper respiratory tract.[12] Based on blood methanol concentrations measured following 8-hour inhalation exposures to concentrations ranging from 1,000–20,000 ppm, the study authors (Pollack and Brouwer, 1996) concluded that, across this range, methanol accumulation in the mouse occurred at a two- to threefold greater rate compared to the rat. They speculated that faster respiration rate and more complete absorption in the nasal cavity of mice may explain the higher methanol accumulation and greater sensitivity to certain developmental toxicity endpoints (see Section 4.3.2).

The Pollack and Brouwer (1996) study was useful for comparing effects in pregnant and NP rodents exposed to high doses, but the implication of these results for humans exposed to ambient levels of methanol is not clear (CERHR, 2004).

[12] Exposed mice spent some exposure time in an active state, characterized by a higher ventilation rate, and the remaining time in an inactive state, with lower (~½ of active) ventilation. The inactive ventilation rate was unchanged by methanol exposure, but the active ventilation showed a statistically significant methanol-concentration-related decline. There was also some decline in the fraction of time spent in the active state, but this was not statistically significant.

Sweeting et al. (2011; 2010) studied methanol and formic acid pharmacokinetics in male C57BL/6 mice, male C3H mice, male CD-1 mice, male NZW rabbits and male cynomolgous monkeys (*Macaca fascicularis*) following a 0.5 or 2 g/kg i.p. exposure to methanol. Blood samples were taken over the entire methanol elimination period for rabbits (48 hours) and CD-1 mice (12 hours for 0.5 g/kg exposure; 24 hours for 2 g/kg exposure), over a 12-hour exposure window for the C57BL/6 and C3H mice and a 6-hour post exposure window for monkeys. Following the 2 g/kg dose, methanol blood levels exhibited saturated elimination kinetics in all three species, and peak methanol concentrations were similar (68, 87 and 79 ± 10 mmol/L in C57BL/6, C3H and CD-1 mice, respectively; 114 ± 7 mmol/L in rabbits and 94 ± 14 mmol/L in monkeys), though the peak concentrations in C57BL/6 ($p < 0.01$) and CD-1 ($p < 0.05$) mice were significantly lower than rabbits. Methanol clearance rates were 2.5-fold higher in CD-1 mice than in rabbits after the 2 g/kg exposure, and 2-fold higher after the 0.5 g/kg exposure. When measured over the entire elimination period, plasma methanol AUCs in the rabbits were 175 ± 27 after a 0.5 g/kg dose and 1,893 ± 345 mmol-hr/L after a 2 g/kg dose. Comparable plasma methanol AUCs in CD-1 mice were more than 2-fold lower (71 ± 9 after a 0.5 g/kg dose, and 697 ± 50 mmol-hr/L after a 2 g/kg dose). At 12-hours (after a 2 g/kg dose), the plasma methanol AUC values for C57BL/6, C3H and CD-1 mice were 465 ± 14, 550 ± 30 and 640 ± 33 mmol-hr/L, respectively, and rabbits had an AUC value of 969 ± 77 mmol-hr/L. The elimination period for plasma formic acid AUCs in the rabbits were 3.02 ± 1.3 mmol-hr/L after a 0.5 g/kg dose, and 10.6 ± 1.4 mmol-hr/L after a 2 g/kg dose. In comparison, plasma formic acid AUCs in CD-1 mice were nearly 6-fold lower at 0.5 g/kg (71 ± 9 mmol-hr/L) and more than 3-fold lower at 2 g/kg (697 ± 50 mmol-hr/L). Twelve hours after a 2 g/kg (i.p.) dose, the plasma formic acid AUC values for C57BL/6, C3H, and CD-1 mice were 2.1 ± 0.3, 1.6 ± 0.2, and 1.9 ± 0.2 mmol-hr/L, respectively, and rabbits had a formic acid AUC value of 3.0 ± 0.3 mmol-hr/L. All of the 12-hour formic acid AUCs for the mice were significantly lower ($p < 0.05$) than the rabbit, but none of the mouse strains differed from each other ($p < 0.05$). Formic acid accumulation at 6-hours post-exposure in monkeys (7.75 ± 3.5 mmol-hr/L) was 5-fold and 43-fold higher than in rabbits (1.5 ± 0.2 mmol-hr/L) and CD-1 mice (0.15 ± 0.04 mmol-hr/L), respectively.

Burbacher, et al. (2004b; 1999b) examined toxicokinetics in *Macaca fascicularis* monkeys prior to and during pregnancy. As part of the report (*Reproductive and Offspring Developmental Effects Following Maternal Inhalation Exposure to Methanol in Nonhuman Primates* [which includes the commentary of the Institute's Health Review Committee]), the HEI review committee (Burbacher et al., 1999b) noted that this was a quality study using a relevant species. The study objectives were to assess the effects of repeated methanol exposure on disposition kinetics, determine whether repeated methanol exposures result in formate accumulation, and examine the effects of pregnancy on methanol disposition and metabolism.

Reproductive, developmental and neurological toxicity associated with this study were also examined and are discussed in Sections 4.3.2 and 4.4.2. In a 2-cohort design, 48 adult females (6 animals/dose/group/cohort) were exposed to 0, 200, 600, or 1,800 ppm methanol vapors (99.9% purity) for 2.5 hours/day, 7 days/week for 4 months prior to breeding and during the entire breeding and gestation periods. Six-hour methanol clearance studies were conducted prior to and during pregnancy. Burbacher, et al. (2004b; 1999b) reported that:

At no point during pregnancy was there a significant change in endogenous methanol blood levels, which ranged from 2.2-2.4 mg/L throughout (Table 3-6).

- PK studies were performed initially (Study 1), after 90 days of pre-exposure and prior to mating (Study 2), between GD66 and GD72 (Study 3), and again between GD126 and GD132 (Study 4). These studies were analyzed using classical PK (one-compartment) models.
- Disproportionate mean, dose-normalized, and net blood methanol dose-time profiles in the 600 and 1,800 ppm groups suggested saturation of the metabolism-dependent pathway. Data from the 600 ppm group fit a linear model, while data from the 1,800 ppm group fit a Michaelis-Menten model.
- Methanol elimination rates modestly increased between Study 1 and Study 2 (90 days prior to mating). This change was attributed to enzyme induction from the subchronic exposure.
- Blood methanol levels were measured every 2 weeks throughout pregnancy, and while there was measurement-to-measurement variation, there was no significant change or trend over the course of pregnancy (Table 3-6). An upward trend in elimination half-life appeared to correspond with a downward trend in blood methanol clearance between Studies 2, 3, and 4. However, the changes were not statistically significant and the time-courses for blood methanol concentration (elimination phase) appeared fairly similar.
- Significant differences between baseline and plasma formate levels ($p = 0.005$), and between prebreeding and pregnancy ($p = 0.0001$) were observed but were not dose dependent (Table 3-7).

- Significant differences in serum folate levels between baseline and prepregnancy ($p = 0.02$), and between prepregnancy and pregnancy ($p = 0.007$) were not dose dependent (Table 3-8).

Table 3-6 Plasma methanol concentrations in monkeys

	Mean[a] plasma methanol level (mg/L) during each exposure period			
Exposure Group	**Baseline**	**Pre-breeding**	**Breeding**	**Pregnancy[b]**
Control (n=11)	2.3 ± 0.1	2.3± 0.1	2.3 ± 0.1	2.7 ± 0.1
200 ppm (n=12)	2.2 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	5.5 ± 0.2
600 ppm (n=11)	2.4 ± 0.1	10.5 ± 0.3	10.9 ± 0.2	11.0 ± 0.2
1,800 ppm (n=12)	2.4 ± 0.1	35.6 ± 1.0	35.7 ± 0.9	35.5 ± 0.9

[a]Values are presented as means ± SE in mg/L.
[b]$n = 9$ for control, 200 ppm, and 600 ppm pregnancy groups; $n = 10$ for 1,800 ppm pregnancy group.

Source: Burbacher, et al. (1999b).

Table 3-7 Plasma formate concentrations in monkeys

	Mean[a] plasma formate level (mg/L) during each exposure period			
Exposure Group	**Baseline**	**Pre-breeding**	**Breeding**	**Pregnancy[b]**
Control (n=11)	8.3 ± 9.2	7.8 ± 0.5	10.1 ± 0.9	8.3 ± 1.4
200 ppm (n=12)	7.4 ± 0.9	8.3 ± 0.5	9.7 ± 0.5	7.8 ± 0.5
600 ppm (n=11)	6.9 ± 0.5	7.8 ± 0.5	9.2 ± 0.5	8.7 ± 1.4
1,800 ppm (n=12)	6.4 ± 0.9	8.7 ± 0.5	11 ± 0.5	10± 1.4

[a]Values are presented as means ± SE in mg/L; transformed from mM, for consistency.
[b]$n = 9$ for control, 200 ppm, and 600 ppm pregnancy groups; $n = 10$ for 1,800 ppm pregnancy group.

Source: Burbacher, et al. (1999b).

Table 3-8 Serum folate concentrations in monkeys

Exposure Group	Mean[a] serum folate level (µg/L) during each exposure period				
	Baseline	Day 70 Pre-pregnancy[a]	Day 98 Pre-pregnancy[a]	Day 55 Pregnancy[a]	Day 113 Pregnancy[b,c]
Control (n=11)	14.4 ± 1.0	14.0 ± 1.2	13.4 ± 1.2	16.0 ± 1.1	15.6 ± 1.1
200 ppm (n=12)	11.9± 1.3	13.2 ± 1.6	12.9 ± 1.3	15.5 ± 1.5	13.4 ± 1.3
600 ppm (n=11)	12.5± 1.4	15.4 ± 1.2	13.4 ± 1.0	14.8 ± 1.1	16.4 ± 1.0
1,800 ppm (n=12)	12.6 ± 0.7	14.8 ± 1.2	15.3 ± 1.1	15.9 ± 1.2	15.7 ± 1.0

[a]Values are presented as means ± SE in µg/L.

[b]Number of days exposed to methanol

[c]*n* = 9 for control, 200 ppm, and 600 ppm pregnancy groups; *n* = 10 for 1,800 ppm pregnancy group.

Source: Burbacher, et al. (1999b).

A series of studies by Medinsky et al. (1997) and Dorman et al. (1994) examined metabolism and pharmacokinetics of [^{14}C]methanol and [^{14}C]formate in normal and folate-deficient cynomolgus, *M. fascicularis* monkeys that were exposed to [^{14}C]methanol through an endotracheal tube while anesthetized. In the first stage of the study, 4 normal 12-year-old cynomolgus monkeys were each exposed to 10, 45, 200, and 900 ppm [^{14}C]methanol vapors (>98% purity) for 2 hours. Each exposure was separated by at least 2 months. After the first stage of the study was completed, monkeys were given a folate-deficient diet supplemented with 1% succinylsulfathiazole (an antibacterial sulfonamide used to inhibit folic acid biosynthesis from intestinal bacteria) for 6–8 weeks in order to obtain folate concentrations of <3 ng/mL serum and <120 ng/mL erythrocytes. Folate deficiency did not alter hematocrit level, red blood cell count, mean corpuscular volume, or mean corpuscular hemoglobin level. The folate-deficient monkeys were exposed to 900 ppm [^{14}C]methanol for 2 hours. The results of the Medinsky et al. (1997) and Dorman et al. (1994) studies showed:

- Dose-dependent changes in toxicokinetics and metabolism did not occur as indicated by a linear relationship between inhaled [^{14}C]methanol concentration and end-of-exposure blood [^{14}C]methanol level, [^{14}C]methanol AUC and total amounts of exhaled [^{14}C]methanol and [^{14}C]carbon dioxide.
- Methanol concentration had no effect on elimination half-life (<1 hour) and percent urinary [^{14}C]methanol excretion (<0.01%) at all doses.
- Following exposure to 900 ppm methanol, urinary excretion or exhalation of [^{14}C]methanol did not differ significantly between monkeys in the folate sufficient and deficient state. There was no significant [^{14}C]formate accumulation at any dose.

- Peak blood [^{14}C]formate levels were significantly higher in folate-deficient monkeys, but did not exceed endogenous blood levels reported by the authors to be between 0.1 and 0.2 mmol/L (4.6-9.2 mg/L).

An HEI review committee (Medinsky et al., 1997) noted that absolute values in this study cannot be extrapolated to humans because the use of an endotracheal tube in anesthetized animals results in an exposure scenario that is not relevant to humans. However, the data in this study suggest that a single exposure to methanol (10- 900 ppm for 2 hours) is unlikely to result in a hazardous elevation in formate levels, even in individuals with moderate folate deficiency.

3.3. Human Variability in Methanol Metabolism

The ability to metabolize methanol may vary among individuals as a result of genetic, age, and environmental factors. Reviews by Agarwal (2001), Burnell et al.(1989), Bosron and Li (1986), and Pietruszko (1980), discuss genetic polymorphisms for ADH. Class I ADH, the primary ADH in human liver, is a hetero- or homodimer composed of randomly associated polypeptide units encoded by three separate gene loci (ADH1A, ADH1B, and ADH1C). Polymorphisms have been found to occur at the ADH1B (ADH1B*2, ADH1B*3) and ADH1C (ADH1C*2) gene loci; however, no human allelic polymorphism has been found in ADH1A. The ADH1B*2 phenotype is estimated to occur in ~15% of Caucasians of European descent, 85% of Asians, and <5% of African Americans. Fifteen percent of African Americans have the ADH1B*3 phenotype, while it is found in <5% of Caucasian Europeans and Asians. To date, there are two reports of polymorphisms in ADH3 (Cichoz-Lach et al., 2007; Hedberg et al., 2001), yet the functional consequence(s) for these polymorphisms remains unclear.

Although racial and ethnical differences in the frequency of the occurrence of ADH alleles in different populations have been reported, ADH enzyme kinetics (V_{max} and K_m) have not been reported for methanol. There is an abundance of information pertaining to the kinetic characteristics of the ADH dimers to metabolize ethanol in vitro; however, the functional and biological significance is not well understood due to the lack of data documenting metabolism and disposition of methanol or ethanol in individuals of known genotype. While potentially significant, the contribution of ethnic and genetic polymorphisms of ADH to the interindividual variability in methanol disposition and metabolism cannot be reliably quantified at this time.

Because children generally have higher baseline breathing rates and are more active, they may receive higher methanol doses than adults exposed to equivalent concentrations of any air pollutant (CERHR, 2004). There is evidence that children under 5 years of age have reduced ADH activity. A study by Pikkarainen and Raiha (1967) measured liver ADH activity using

ethanol as a substrate and found that 2-month-old fetal livers have ~3-4% of adult ADH liver activity. ADH activity in 4 to 5 month old fetuses is ~10% of adult activity, and an infant's activity is ~20% of adult activity. ADH continues to increase in children with age and reaches a level that is within adult ranges at 5 years of age. Adults were found to have great variation in ADH activity (1,625 to 6,530/g liver wet weight or 2,030 to 5,430 mU/100 mg soluble protein). Smith et al. (1971) also compared liver ADH activity in 56 fetuses (9 to 22 weeks gestation), 37 infants (premature to <1 year old), and 129 adults (>20 years old) using ethanol as a substrate. ADH activity was 30% of adult activity in fetuses and 50% of adult activity in infants. There is evidence that some human infants are able to efficiently eliminate methanol at high exposure levels, however, possibly via CAT (Tran et al., 2007).

ADH3 exhibits little or no activity toward small alcohols, thus the previous discussion is not relevant to the ontogeny of formaldehyde elimination (clearance). While such data on ADH3 activity does not exist, ADH3 mRNA is abundantly expressed in the mouse fetus (Ang et al., 1996) and is detectible in human fetal tissues (third trimester), neonates and children (Hines and Mccarver, 2002; Estonius et al., 1996).

As noted earlier in this section, folate-dependent reactions are important in the metabolism of formate. Individuals who are commonly folate deficient include those who are pregnant or lactating, have gastrointestinal (GI) disorders, have nutritionally inadequate diets, are alcoholics, smoke, have psychiatric disorders, have pernicious anemia, or are taking folic acid antagonist medications such as some antiepileptic drugs (CERHR, 2004; IPCS, 1997). Groups which are known to have increased incidence of folate deficiencies include Hispanic and African American women, low-income elderly, and mentally ill elderly (CERHR, 2004).
A polymorphism in methylene tetrahydrofolate reductase reduces folate activity and is found in 21% of Hispanics in California and 12% of Caucasians in the United States. Genetic variations in folic acid metabolic enzymes and folate receptor activity are theoretical causes of folate deficiencies.

3.4. Physiologically Based Pharmacokinetic Models

In accordance with the needs of this human health assessment, particularly the derivation of human health effect benchmarks from studies of the developmental effects of methanol inhalation exposure in mice (Rogers et al., 1993b), monkeys (Burbacher et al., 2004a; Burbacher et al., 1999a) and rats (NEDO, 1987) models were evaluated for their ability to estimate mouse, monkey and rat internal dose metrics. A human model was developed to extrapolate those internal metrics to inhalation and oral exposure concentrations that would result in the same

internal dose in humans (HECs and HEDs). The procedures used for the development, calibration and use of these models are summarized in this section, with further details provided in Appendix B, "Development, Calibration and Application of a Methanol PBPK Model."

3.4.1. Model Requirements for EPA Purposes

3.4.1.1. MOA and Selection of a Dose Metric

Dose metrics closely associated with one or more key events that lead to the selected critical effect are preferred for dose-response analyses compared to metrics not clearly correlated. For instance, internal (e.g., blood, target tissue) measures of dose are preferred over external measures of dose (e.g., atmospheric or drinking water concentrations), especially when, as with methanol, blood methanol concentrations increase disproportionally with dose (Rogers et al., 1993b). This is likely due to the saturable metabolism of methanol. In addition, respiratory and GI absorption may vary between and within species. Mode of action (MOA) considerations can also influence whether to model peak concentrations (C_{max}) or a time-dependent metric such as area under the curve (AUC), and whether to model the parent compound with or without its metabolites for selection of the most adequate dose metric.

As discussed in Section 4.3, developmental effects following methanol exposures have been noted in both rats and mice (Rogers et al., 1993a; Rogers et al., 1993b; NEDO, 1987; Nelson et al., 1985), but are not as evident or clear in primate exposure studies (Burbacher et al., 2004a; Clary, 2003; Andrews et al., 1987). The report of the New Energy Development Organization (NEDO, 1987) of Japan, which investigated developmental effects of methanol in rats, indicated that there is a potential that developing rat brain weight is reduced following maternal and neonatal exposures. These exposures included both in utero and postnatal exposures. The methanol PBPK models developed for this assessment do not explicitly describe these exposure routes. Mathematical modeling efforts have focused on the estimation of human equivalent external exposures that would lead to an increase in maternal blood levels of methanol or its metabolites presumed to be associated with developmental effects as reported in rats (NEDO, 1987), mice (Rogers et al., 1993b) and monkeys (Burbacher et al., 2004a; Burbacher et al., 1999a). PBPK models were developed for all species, but because measured internal blood methanol levels suitable for use as estimates of peak concentrations (C_{max}) are provided by Rogers et al. (1993b), a mouse PBPK model is not used or discussed in this toxicological review. However, limited discussion of the mouse models is included, as they are useful in evaluating model structure.

In a recent review of the reproductive and developmental toxicity of methanol, a panel of experts concluded that methanol, not formate, is likely to be the proximate teratogen and determined that blood methanol level is a useful biomarker of exposure (CERHR, 2004; Dorman et al., 1995). The CERHR Expert Panel based their assessment of potential methanol toxicity on an assessment of circulating blood levels (CERHR, 2004). While recent in vitro evidence indicates that formaldehyde is more embryotoxic than methanol and formate (Harris et al., 2004; 2003), the high reactivity of formaldehyde would limit its unbound and unaltered transport as free formaldehyde from maternal to fetal blood (Thrasher and Kilburn, 2001), and the capacity for the metabolism of methanol to formaldehyde is likely lower in the fetus and neonate versus adults (see discussion in Section 3.3). Thus, even if formaldehyde is ultimately identified as the proximate teratogen, methanol would likely play a prominent role, at least in terms of transport to the target tissue.

Given the reactivity of formaldehyde, models that predict levels of formaldehyde in the blood are difficult to validate. However, production of formaldehyde or formate following exposure to methanol can be estimated by summing the total amount of methanol cleared by metabolic processes.[13] This metric of formaldehyde or formate dose has limited value since it ignores important processes that may differ between species, such as elimination (all routes) of these two metabolites, but it can be roughly equated to the total amount of metabolites produced and may be the more relevant dose metric if formaldehyde is found to be the proximate toxic moiety. Thus, both blood methanol and total metabolism metrics are considered to be important components of the PBPK models. Dose metric selection and MOA issues are discussed further in Section 4.7.

3.4.1.2. Criteria for the Development of Methanol PBPK Models

The development of methanol PBPK models that would meet the needs of this assessment was organized around a set of criteria that reflect: (1) the MOA(s) being considered for methanol; (2) absorption, distribution, metabolism, and elimination characteristics; (3) dose routes necessary for interpreting toxicity studies or estimating HECs; and (4) general parameters needed for the development of predictive PK models.

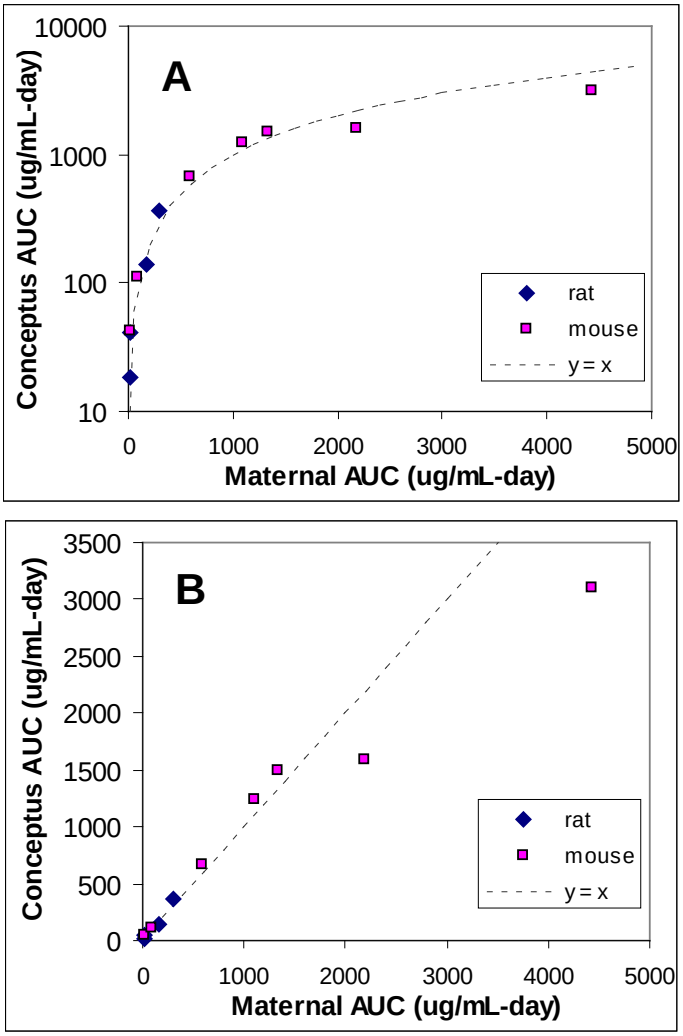
The criteria with a brief justification are provided below:

- (1) Must simulate blood methanol concentrations and total methanol metabolism. Blood methanol is the recommended dose metric for developmental effects, but total metabolism may be a useful metric.

[13] This assumption is more likely to be appropriate for formaldehyde than formate as formaldehyde is a direct metabolite of methanol.

- (2) Must be capable of simulating experimental blood methanol and total metabolism for the inhalation route of exposure in rats (a) and humans (b), and the oral route in humans (c). These routes are important for determining dose metrics in the most sensitive test species under the conditions of the toxicity study and in the relevant exposure routes in humans.
- (3) The model code should easily allow designation of respiration rates during inhalation exposures. A standard variable in inhalation route assessments is ventilation rate. Blood methanol concentrations will depend strongly on ventilation rate, which varies significantly between species.
- (4) Must address the potential for saturable metabolism of methanol. Saturable metabolism has the potential to bring nonlinearities into the exposure: tissue dose relationship.
- (5) Model complexity should be consistent with modeling needs and limitations of the available data. Model should adequately describe the biological mechanisms that determine the internal dose metrics (blood methanol and total metabolism) to assure that it can be reliably used to predict those metrics in exposure conditions and scenarios where data are lacking. Compartments or processes should not be added that cannot be adequately characterized by the available data.

Although existing rat models are useful for the evaluation of the dose metrics associated with methanol's developmental effects and the relevant toxicity studies, including gestational exposures, no pregnancy-specific PBPK model exists for methanol, and limited data exists for the development and validation of a fetal/gestational/conceptus compartment. However, EPA determined that nonpregnancy models for the appropriate species and routes of exposure could prove to be valuable because, as discussed in Section 3.2, levels of methanol in NP, pregnant and fetal blood are expected to be similar following the same oral or inhalation exposure. Pollack and Brouwer (1996) determined that methanol distribution in rats and mice following repeated oral and i.v. exposures up to day 20 of gestation is "virtually unaffected by pregnancy, with the possible exception of the immediate perinatal period." Ward et al. (1997) report a "nonlinear" relationship between the maternal blood and conceptus, but the nonlinear perception given by Figure 8 in their paper is the result of the data being plotted on a log-y/linear-x scale. Replotting the data from their Table 5 (AUC) shows the results to be linear, especially in the low-dose region which is of the greatest concern (Figure 3-4).



Source: Ward et al. (1997).

Note: Plotted (A) on a log-linear scale, as in Figure 8 of Ward et al. (1997), and (B) on a linear-linear scale. In both panels the line y = x is plotted (dashed line) for comparison.

Figure 3-4 Conceptus versus maternal blood AUC values for rats and mice.

The critical window for methanol induction of cervical rib malformations in CD-1 mice has been identified as occurring between GD6 and GD7 (Rogers and Mole, 1997; Rogers et al., 1993a), a developmental period roughly equivalent to week 3 of human development (Chernoff and Rogers, 2004). Methanol blood kinetics measured during and after inhalation exposure in NP and pregnant mice on GD6-GD10 and GD6-GD15 (Perkins et al., 1996a; Dorman et al., 1995; Perkins et al., 1995a; Rogers et al., 1993b) are also similar. Further, the available data indicate that the maternal blood:fetal partition coefficient is approximately 1 at dose levels most relevant to this assessment (Ward et al., 1997; Horton et al., 1992). Further supporting data exist for ethanol, which is quite similar to methanol in its partitioning and transport properties. In rats

(Zorzano and Herrera, 1989; Guerri and Sanchis, 1985), sheep (Brien et al., 1985; Cumming et al., 1984), and guinea pigs (Clarke et al., 1986), fetal and maternal blood concentrations of ethanol are virtually superimposable; maternal to fetal blood ratios are very close to 1, including during late gestation. Also, fetal brain concentrations in guinea pigs (Clarke et al., 1986) were very similar to the maternal concentrations. Consequently, fetal methanol concentrations are expected to be roughly equivalent to that in the mother's blood. Thus, pharmacokinetics and blood dose metrics for NP rats and humans are expected to provide reasonable approximations of pregnancy levels and fetal exposure, particularly during early gestation, that improve upon default estimations from external exposure concentrations.

In addition to the absolute maternal-fetal concentration similarity noted above, it is common practice to use blood concentrations as an appropriate metric for risk extrapolation via PBPK modeling for effects in various tissues, based on the reasonable expectation that any tissue:blood differences will be similar in both the test species and humans. For example, even if the brain:blood ratio was around 1.2 in the mouse or rat, because tissue:blood ratios depend on tissue composition which is expected to be quite similar in rats and humans, the brain:blood levels in humans is also expected to be close to 1.2. Therefore, the potential error that might occur by using blood instead of brain concentration in evaluating the dose-response in rats will be cancelled out by using blood instead of brain concentration in the human. Measured fetal blood levels are virtually identical to maternal levels for methanol (and ethanol) thus indicating that the rate of metabolism in the fetus is not sufficient to significantly reduce the fetal concentration of methanol versus maternal. Use of a PBPK model to predict maternal levels will give a better estimate of fetal exposure than use of the applied dose or exposure, because there are animal-human differences in adult PK of methanol for which the model accounts, based on PK data from humans as well as rodents.

3.4.2. Methanol PBPK Models

As has been discussed, methanol is well absorbed by both inhalation and oral routes and is readily metabolized to formaldehyde, which is rapidly converted to formate in both rodents and humans. As was discussed in Section 3.1, the enzymes responsible for metabolizing methanol are different in adult rodents and humans. Several rat, mouse and human PBPK models that attempt to account for these species differences have been published (Fisher et al., 2000; Ward et al., 1997; Perkins et al., 1995a; Horton et al., 1992). Two methanol PK models (Bouchard et al., 2001; Ward et al., 1997) were identified as potentially appropriate for use in animal-to-human extrapolation of methanol metabolic rates and blood concentrations. An additional methanol PBPK model by Fisher et al. (2000) was considered principally because it

had an important feature – pulmonary compartmentalization (see below for details) – worth adopting in the final model.

3.4.2.1. Ward et al. (1997)

The PBPK model of Ward et al. (1997) describes inhalation, oral and i.v. routes of exposure and is parameterized for both NP and pregnant mice and rats (Table 3-9). The model has not been parameterized for humans.

Respiratory uptake of methanol is described as a constant infusion into arterial blood at a rate equal to the minute ventilation times the inhaled concentration and includes a parameter for respiratory bioavailability, which for methanol is <100%. This simple approach is nonstandard for volatile compounds but is expected to be appropriate for a compound like methanol, for which there is little clearance from the blood via exhalation. Oral absorption is described as a biphasic process, dependent on a rapid and a slow first-order rate constant.

Methanol elimination in the Ward et al. (1997) model is primarily via saturable hepatic metabolism. The parameters describing this metabolism come from the literature, primarily previous work by Ward and Pollack (1996) and Pollack et al. (1993). A first-order elimination of methanol from the kidney compartment includes a lumped metabolic term that accounts for both renal and pulmonary excretion.

The model adequately fits the experimental blood kinetics of methanol in rats and mice and is therefore suitable for simulating blood dosimetry in the relevant test species and routes of exposure (oral and i.v.). The Ward et al. (1997) model meets criteria 1, 2a, 2c, 3, 4, and 5 outlined in Section 3.4.1.2. The most significant limitation is the absence of parameters for the oral and inhalation routes in the human. A modified version of this model that includes human parameters and a standard PBPK lung compartment might be suitable for the purposes of this assessment.

3.4.2.2. Bouchard et al. (2001)

The Bouchard et al. (2001) model is not actually a PBPK model but is an elaborate classical PK model, since the transfer rates are not determined from blood flows, ventilation, partition coefficients, and the like. The Bouchard et al. (2001) model uses a single compartment for methanol: a central compartment represented by a volume of distribution where the concentration is assumed to equal that in blood. The model was developed for inhalation and i.v. kinetics only. Methanol is primarily eliminated via saturable metabolism. The model adequately simulates blood kinetics in NP rats and humans following inhalation exposure and in NP rats

following i.v. exposure; there is no description for oral absorption. Because methanol distributes with total body water (Ward et al., 1997; Horton et al., 1992), this simple model structure is sufficient for predicting blood concentrations of methanol following inhalation and i.v. dosing.

The Bouchard et al. (2001) model has the advantage of simplicity, reflecting the minimum number of compartments necessary for representing blood methanol pharmacokinetics. Because volume of distribution can be easily and directly estimated for water-soluble compounds like methanol or fit directly to experimental kinetics data, concern over the scalability of this parameter is absent. The model has been parameterized for a required human exposure route, inhalation (Table 3-9). The model meets criteria 1, 2b, 3, 4, and 5 described in Section 3.4.1.2. However, the Bouchard model has a specific and significant limitation. The model has not been parameterized for the oral route in humans. As such, the model cannot be used to conduct the necessary interspecies extrapolation.

Table 3-9 Routes of exposure optimized in models – optimized against blood concentration data

	Ward et al.			Bouchard et al.		
Route	**Mouse**	**Rat**	**Human**	**Mouse**	**Rat**	**Human**
Injection (i.v.)	P/NP	P/NP	--	--	NP	--
Inhalation	P/NP	--	--	--	NP	NP
Oral	P/NP	NP	--	--	--	--

P = Pregnant NP = Nonpregnant

Source: Bouchard et al. (2001); Ward et al. (1997).

3.4.3. Selected Modeling Approach

As discussed earlier regarding model criteria, fetal methanol concentrations can reasonably be assumed to equal maternal blood concentration. Thus, methanol pharmacokinetics and blood dose metrics for NP laboratory animals and humans are expected to improve upon default extrapolations from external exposures as estimates of fetal exposure during early gestation. The same level of confidence cannot be placed on the whole-body rate of metabolism, in particular as a surrogate for formaldehyde dose. Because of formaldehyde's reactivity and the limited fetal metabolic (ADH) activity (see Sections 3.3 and 4.9.1), fetal formaldehyde concentration increases (from methanol) will probably not equal maternal increases in formaldehyde concentration. But since there is no model that explicitly describes formaldehyde concentration in the adult, let alone the fetus, the metabolism metric is the closest one can come

to predicting fetal formaldehyde dose. This metric is expected to be a better predictor of formaldehyde dose than applied methanol dose or even methanol blood levels, which do not account for species differences in conversion of methanol to formaldehyde.

Most of the published rodent kinetic models for methanol describe the metabolism of methanol to formaldehyde as a saturable process but differ in the description of metabolism to and excretion of formate (Bouchard et al., 2001; Fisher et al., 2000; Ward et al., 1997). The model of Ward et al. (1997) used one saturable and one first-order pathway to describe methanol elimination in mice. The saturable pathway described in Ward et al. (1997) can specifically be ascribed to metabolic formation of formaldehyde in the liver, while the renal first-order elimination described in that paper represents nonspecific clearance of methanol (e.g., metabolism, excretion, or exhalation), since it was not fit to route-specific elimination data. However, Pollack and Brouwer (1996) obtained a rate constant for the urinary elimination rate from rat urine excretion data, so it can be made specific to that route by use of that parameter. The model of Ward et al. (1997) does not describe kinetics of formaldehyde subsequent to its formation and does not include any description of formate.

Bouchard et al. (2001) employed a metabolic pathway for conversion of methanol to formaldehyde and a second pathway described as urinary elimination of methanol in rats and humans. They then explicitly describe two pathways of formaldehyde transformation, one to formate and the other to "other, unobserved formaldehyde byproducts." Finally, formate removal is described by two pathways, one to urinary elimination, and one via metabolism to CO_2 (which is exhaled). All of these metabolic and elimination steps are described as first-order processes, but the explicit descriptions of formaldehyde and formate kinetics significantly distinguish the model of Bouchard et al. (2001) from that of Ward et al. (1997), which only describes methanol.

There are two other important distinctions between the Ward et al. (1997) and Bouchard et al. (2001) models. The former is currently capable of simulating blood data for all exposure routes in mice but not humans, while the latter is capable of simulating human inhalation route blood pharmacokinetics but not those in mice. The Ward et al. (1997) model has more compartments than is necessary to adequately represent methanol disposition but has been fit to PK data in pregnant and NP mice for all routes of exposure (i.v., oral, and inhalation). The Ward et al. (1997) model has also been fit to i.v. and oral route PK data in rats. Based primarily on the extensive amount of fitting that has already been demonstrated for this model, it was determined that a modified Ward et al. (1997) model, with the addition of a lung compartment as described by Fisher et al. (2000), should be used for the purposes of this assessment. The ability of the Ward et al. (1997) mouse PBPK model to describe dosimetry in that species supports the biological basis for this model structure; and hence, the expectation that it can be used to predict dosimetry in humans. However, as mentioned previously, the mouse parameterized PBPK model

is not used in this assessment. See Appendix B for a more complete discussion of the selected modeling approach and modeling considerations.

3.4.3.1. Available PK Data

Although limited human data are available, several studies exist that contain PK and metabolic data in mice, rats, and nonhuman primates for model parameterization (Table 3-10).

3.4.3.2. Model Structure

As described in detail in Appendix B, a model was developed which includes compartments for alveolar air/blood methanol exchange, liver, fat, bladder (human simulations) and the rest of the body (Figure 3-5). This model is a revision of the model reported by Ward et al. (1997), reflecting significant simplifications (removal of compartments for placenta, embryo/fetus, and extra-embryonic fluid) and two elaborations (addition of a second GI lumen compartment to the existing stomach lumen compartment and addition of a bladder compartment), while maintaining the ability to describe methanol blood kinetics in rats and humans. A fat compartment was included because it is the only tissue with a tissue:blood partitioning coefficient appreciably different than 1, and the liver is included because it is the primary site of metabolism. A bladder compartment was also added for use in simulating human urinary excretion to capture the difference in kinetics between changes in blood methanol concentration and urinary methanol concentration. The model code describes inhalation, oral, and i.v. dose routes, and data exist from studies (Table 3-10) that were used to fit parameters and evaluate model predictions for all three of those routes. In humans, inhalation exposure data an i.v. study and a single short-duration oral PK study were available for model calibration and validation.

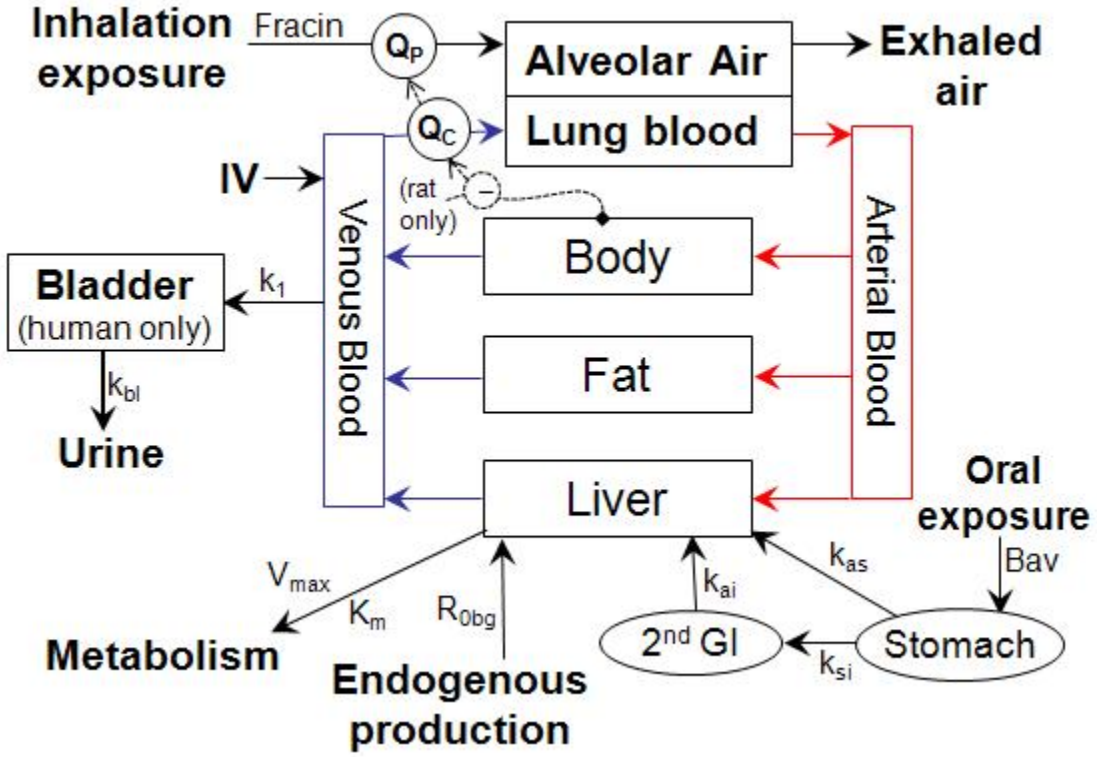
Table 3-10 Key methanol kinetic studies for model validation

Reference	i.v. dose (mg/kg)	Inhalation (ppm)	Oral/ dermal/ i.p.	Species	Samples	Digitized figures[a]
Batterman & Franzblau (1997)			Dermal	Human Male/female	Blood	Figure 1
Batterman et al. (1998)		800 (8 hr)		Human Male/female	Blood, urine, exhaled	
Ernstgàrd et al. (2005)		100 (2hr) 200 (2hr)		Human Male/female	Blood, exhaled	
Haffner et al. (1992)	10			Human males	Blood	Figure 1
Osterloh et al. (1996); Chuwers et al. (1995); D'Alessandro et al. (1994)		200 (4 hr)		Human Male/female	Blood, urine	Figure 1in Osterloh et al. (1996)
Schmutte et al. (1988)			Oral 1.1 mg/L	Human	Blood	Figure 1
Sedivec et al. (1981)		78-231 (8 hr)		Human Male	Urine, blood	Figures 2, 3, 6, 7, 8
Burbacher, et al. (2004b); Burbacher, et al. (2004a)		0-1,800 (2.5 hr, 4 mo)		Monkeys Cynomolgus Pregnant, NP	Blood	
Medinsky et al. (1997); Dorman et al., (1994)		10-900 (2 hr)		Monkeys Cynomolgus Folate deficient	Blood, urine, exhaled	
Horton et al. (1992)	100 (rats only)	50-2,000 (6 hr)		Monkey Rhesus, and Rat Fischer-344	Blood, urine, exhaled	Figure 7
Perkins et al. (1996a, 1995a, b)		1,000-20,000 (8 hr)		Mouse and Rat	Blood, urine	
Pollack and Brouwer (1996) Pollack et al. (1993)	100-2,500	1,000-20,000 (8 hr)	Oral: 100-2,500 mg/kg	Rat: Sprague-Dawley, & Mouse; CD-1 Pregnant, NP	Blood	
Ward et al. (1997) Ward and Pollack (1996)	100, 500 (Rat)		Oral: 2,500 mg/kg	Mouse CD-1, GD18; Rat Sprague-Dawley, GD14 & GD20	Blood, conceptus	
Rogers and Mole (1997) Rogers et al. (1993b)		1,000-15,000 (7 hr, 10 days)		Mouse CD-1 Pregnant	Blood	

[a]Data obtained from the reported figure, from the corresponding reference.

The approach to model calibration and specific data sets used for Sprague-Dawley (SD) rats and humans are described in detail in Appendix B. The metabolism of methanol was described using Michaelis-Menten kinetics. Simulated metabolic elimination of methanol is not

linked in the PBPK model to production of formaldehyde or formate; it is simply another route of methanol elimination. Metabolism of formaldehyde (to formate) is not explicitly simulated by the model, and this model tracks neither formate nor formaldehyde. Since the metabolic conversion of formaldehyde to formate is rapid (<1 minute) in all species (Kavet and Nauss, 1990), the rate of methanol metabolism may approximate a formate production rate, though this has not been verified.



Note: Parameters: Fracin (FRACIN), fraction of exposure concentration reaching gas exchange region in lungs; Bav, oral bioavailability; k_{as}, first-order oral absorption rate from stomach; k_{ai}, first-order uptake from 2nd GI compartment; k_{si}, first-order transfer between stomach and 2nd GI; V_{max} and K_m Michaelis-Menten rate constants for metabolism in liver; k_1, first-order rate constant for urinary elimination; k_{bl}, rate constant for urinary excretion from bladder. For the rat only, high levels of methanol in the body compartment lead to respiratory and cardiac depression, indicated by the dashed line. Rat data were consistent with Bav = 100%, but humans with Bav = 83%.

Figure 3-5 Schematic of the PBPK model used to describe the inhalation, oral, and i.v. route pharmacokinetics of methanol.

The primary purpose of this assessment is for the determination of noncancer risk associated with exogenous oral or inhalation exposure to methanol that add to endogenous background levels. However, because background methanol levels can impact model parameter estimation and internal dose predictions, the PBPK models developed for this assessment incorporate a zero-order liver infusion term for methanol designed to approximate reported

background levels. The PBPK model estimate of background levels was then subtracted for benchmark dose (BMD) modeling. For example, when the metric is blood AUC, BMD analysis used the PBPK-predicted difference, AUC (exposed rats) – AUC (control rats), as the dose metric. In short the level of effect was correlated with the internal dose above background in the test animal (POD_{AUC}). The human PBPK model was then used to estimate the human equivalent oral dose (POD_{HED}) or inhalation concentration (POD_{HEC}) associated with this internal dose. To do this, the human PBPK model used an average endogenous level of 1.5 mg/L (except when study specific data were available during model calibration) and the POD_{HED} or POD_{HEC} was selected such that the predicted increase in human blood levels over this background matched the POD_{AUC}.

3.4.3.3. Model Parameters

The EPA methanol model uses a consistent set of physiological parameters obtained predominantly from the open literature (Appendix B, Table B-1); the Ward et al. (1997) model employed a number of data-set specific parameters.[14] Parameters for blood flow, ventilation, and metabolic capacity were scaled as a function of body weight raised to the 0.75 power, according to the methods of Ramsey and Andersen (1984).The process by which the rat and human inhalation and oral models were calibrated and analyzed for parameter sensitivity is discussed in Appendix B, "Development, Calibration and Application of a Methanol PBPK Model." An evaluation of the importance of selected parameters on the model estimates of blood methanol was performed using the subroutines within acslX v2.3 (Aegis Technologies, Huntsville, Alabama).

3.4.4. Monkey PK Data and Analysis

In order to estimate internal doses (blood C_{max} and AUC values) for the monkey health-effects study of Burbacher, et al. (1999a) and further elucidate the potential differences in methanol pharmacokinetics between NP and pregnant individuals (2nd and 3rd trimester), a focused reanalysis of the data of Burbacher, et al. (1999b) was performed. The monkeys in this study were exposed for 2.5 hours/day, with the methanol concentration raised to approximately the target concentration for the first 2 hours of each exposure and the last 30 minutes providing a chamber "wash-out" period, when the exposure chamber concentration was allowed to drop to 0. Blood samples were taken and analyzed for methanol concentration at 30 minutes, 1, 2, 3, 4, and

[14] Some data sets provided in the Ward et al. (1997) model code were corrected to be consistent with figures in the published literature describing the experimental data.

6 hours after removal from the chamber (or 1, 1.5, 2.5, 3.5, 4.5, and 6.5 hours after the end of active exposure). These data were analyzed to compare the PK in NP versus pregnant animals, and fitted with a simple PK model to estimate 24-hour blood AUC values for each exposure level. Details of this analysis are provided in Appendix B. The chamber concentrations for "pregnancy" exposures recorded by Burbacher, et al. (1999b: Table 2) and average body weights for each exposure group at the 2nd trimester time point were used along with the model described in Appendix B to calculate C_{max} above background and 24-hour blood methanol AUC above background (Table B-6) for the dose-response analysis of data from the Burbacher et al. (1999b; 1999a) developmental study in monkeys described in Appendix D.

3.4.5. Summary and Conclusions

Rat and human versions of a methanol PBPK model have been developed and calibrated to data available in the open literature. The model simplifies the structure used by Ward et al.(1997), while adding specific refinements such as a standard lung compartment employed by Fisher et al. (2000) and a two-compartment GI tract.

Although the developmental endpoints of concern are effects which occur following in utero and (to a lesser extent) lactational exposure, no pregnancy-specific PBPK model exists for methanol and limited data exists for the development and validation of a fetal/gestational/conceptus compartment. The fact that the unique physiology of pregnancy and the fetus/conceptus are not represented in a methanol model would be important if methanol pharmacokinetics differed significantly during pregnancy or if the observed partitioning of methanol into the fetus/conceptus versus the mother showed a concentration ratio significantly greater than or less than 1. Methanol pharmacokinetics during GD6–GD10 in the mouse are not different from NP mice (Pollack and Brouwer, 1996), and the maternal blood:fetus/conceptus partition coefficient is reported to be near 1 (Ward et al., 1997; Horton et al., 1992). Maternal blood kinetics in monkeys differs little from those in NP animals (see Section 3.2 for details). Further, in both mice and monkeys, to the extent that late-pregnancy blood levels differ from NP for a given exposure, they are higher; i.e., the difference between model predictions and actual concentrations is in the same direction. These data support the assumption that the ratio of actual target-tissue methanol concentration to (predicted) NP maternal blood concentrations will be about the same across species, and hence, that using NP maternal blood levels in place of fetal concentrations will not lead to a systematic error when extrapolating risks.

The critical gestational window for the reduced brain weight effect observed in the NEDO (1987) rat study is broader than for the mouse cervical rib effect. In addition, NEDO (1987) rats were exposed not only to methanol gestationally but also lactationally and via

inhalation after parturition. The findings in the mice and rats up to GD20 (similar blood methanol kinetics between NP and pregnant animals and a maternal blood:fetal partition coefficient close to 1) are assumed to be applicable to the rat later in pregnancy. However, the additional routes of exposure presented to the pups in this study present uncertainties (see additional discussion in Sections 5.1.2.2 and 5.1.3.2.2) and suggest that average blood levels in pups might be greater than those of the dam.

Methanol is transported directly from the maternal circulation to fetal circulation via the placenta, but transfer via lactation involves distribution to the breast tissue, then milk, then uptake from the pup's GI tract. Therefore blood or target-tissue levels in the breast-feeding infant or rat pup are likely to differ more from maternal levels than do fetal levels. In addition, the health-effects data indicate that most of the effects of concern are due to fetal exposure, with a relatively small influence due to postnatal exposures. Therefore, it would be extremely difficult to distinguish the contribution of postnatal exposure from prenatal exposure to a given effect in a way that would allow the risk to be estimated from estimates of both exposure levels, even if one had a lactation/child PBPK model that allowed for prediction of blood (or target-tissue) levels in the offspring. Finally, one would still expect the target-tissue concentrations in the offspring to be closely related to maternal blood levels (which depend on ambient exposure and determine the amount delivered through breast milk), with the relationship between maternal levels and those in the offspring being similar across species. Further, as discussed in Section 5.1.3.2.2, it is likely that the difference in blood levels between rat pups and dams would be similar to the difference between mothers and human offspring. Therefore, it is assumed that the potential differences between pup and dam blood methanol levels do not have a significant impact on this assessment and the estimation of HECs.

Therefore, the development of a lactation/child PBPK model is not necessary, given the minimal change that is likely to result in risk extrapolations, and use of NP maternal blood levels as a measure of risk in the offspring is considered preferable over use of default extrapolation methods. In particular, the existing human data allow for predictions of maternal blood levels, which depend strongly on the rate of maternal methanol clearance. Since bottle-fed infants do not receive methanol from their mothers, they are expected to have lower or, at most, similar overall exposures for a given ambient concentration than the breast-fed infant, so that use of maternal blood levels for risk estimation should also be adequately protective for that group.

The final rat and human methanol PBPK models fit multiple data sets for inhalation, oral, and i.v. (rat only) exposures, using consistent parameters that are representative of each species but are not varied within species or by dose or source of data. Also, a simple PK model calibrated to early gestation monkey data, which were shown to be essentially indistinguishable from NP and late-gestation pregnant monkey PK data, was used to estimate blood methanol peak

concentrations (internal doses) in that species. The models are used to estimate chronic human exposure concentrations from internal dose metrics for use in the RfC and RfD derivations discussed in Section 5.

4. HAZARD IDENTIFICATION

4.1. Studies in Humans – Case Reports, Occupational and Controlled Studies

4.1.1. Case Reports

An extensive library of case reports has documented the consequences of acute accidental/intentional methanol poisoning. Nearly all have involved ingestion, but a few have involved percutaneous and/or inhalation exposure.

As many of the case reports demonstrate, the association of Parkinson-like symptoms with methanol poisoning is related to the observation that lesions in the putamen are a common feature both in Parkinson's disease and methanol overexposure. These lesions are commonly identified using Computed Tomography (CT) or by Magnetic Resonance Imaging (MRI). Other areas of the brain (e.g., the cerebrum, cerebellum, and corpus callosum) also have been shown to be adversely affected by methanol overexposure. The associated effects are further discussed in Appendix C, Human Case Studies.

Various therapeutic procedures [e.g., infusion of ADH1 inhibitors ethanol or fomepizole (4-methylpyrazole)], sodium bicarbonate or folic acid administration, and hemodialysis) have been used in many of these methanol overexposures, and the reader is referred to the specific case reports for details in this regard (see Appendix C). The reader also is referred to Kraut and Kurtz (2008) and Barceloux et al. (2002) for a more in-depth discussion of the treatments in relation to clinical features of methanol toxicity.

Most cases of accidental/intentional methanol poisoning reveal a common set of symptoms, many of which are likely to be presented upon hospital admission. These include:

- blurred vision and bilateral or unilateral blindness
- convulsions, tremors, and coma
- nausea, headache, and dizziness
- abdominal pain
- diminished motor skills
- acidosis

- dyspnea
- behavioral and/or emotional deficits
- speech impediments

Acute symptoms generally are nausea, dizziness, and headache. In the case reports cited in Appendix C, the onset of symptom sets as well as their severity varies depending upon how much methanol was ingested, whether or not and when appropriate treatment was administered, and individual variability. A longer time between exposure and treatment, with few exceptions, results in more severe outcomes (e.g., convulsions, coma, blindness, and death). The diminution of some acute and/or delayed symptoms may reflect concomitant ingestion of ethanol or how quickly therapeutic measures (one of which includes ethanol infusion) were administered in the hospital setting.

Those individuals who are in a metabolic acidotic state (e.g., pH <7.0) are typically the individuals who manifest the more severe symptoms. Many case reports stress that, unlike blood pH levels <7.0, blood levels of methanol are not particularly good predictors of health outcome. According to a publication of the American Academy of Clinical Toxicology (Barceloux et al., 2002), "the degree of acidosis at presentation most consistently correlates with severity and outcome."

Table 4-1 Mortality rate for subjects exposed to methanol-tainted whisky in relation to their level of acidosis

Subjects[a]	Number	Percent deaths
All patients	323	6.2
Acidotic (CO_2 <20 mEq)	115	19
Acidotic (CO_2 <10 mEq)	30	50

[a]These data do not include those who died outside the hospital or who were moribund on arrival.

Source: Bennett et al. (1953).

As the case reports (Appendix C) demonstrate, those individuals who present with more severe symptoms (e.g., coma, seizures, and severe acidosis) generally exhibit higher mortality (even after treatment) than those without such symptoms. In survivors of poisoning, persistence or permanence of vision decrements and particularly blindness often have been observed. Because of the strong correlation between outcomes of methanol poisoning with severity of acidosis (e.g., Table 4-1), formate is usually assumed to be the proximal cause of the acute effects of methanol. Most of the symptoms of methanol poisoning (listed in the individual

studies in Appendix C) are common to the several other types of metabolic acidosis (Berkow and Fletcher, 1992). However, several of the CNS effects of methanol poisoning are not seen in other cases of acidosis, including irreversible blindness and Parkinsonian effects. It has been postulated that formaldehyde may be the toxic moiety for the symptoms of methanol poisoning that are seemingly distinct from acidotic symptoms (Hayasaka et al., 2001). Since formaldehyde has a very short half life, it is unlikely to be distributed from the liver to the brain or eye fast enough to cause CNS or ocular damage. However, methanol is distributed to multiple organ systems and there is evidence that it can be metabolized to formaldehye *in situ* by other organ systems, including several studies that have found ADH activity in non-liver cells including several sites in or around the brain (Jelski et al., 2006; Motavkin et al., 1988; Bühler et al., 1983) and a rat study that reports dose-dependent increases of formaldehyde DNA adducts derived from exogenous methanol exposure in multiple tissues such as liver, lung, spleen, thymus, bone marrow, kidney, and WBC (exogenous adduct levels were less than 10% of endogenous adduct levels for most organ systems; ocular tissue was not examined) of rats (Lu et al., 2012).

Correlation of symptomatology with blood levels of methanol has been shown to vary appreciably between individuals. Blood methanol levels in the case reports involving ingestion ranged from values of 300 to over 10,000 mg/L. The lowest value (200 mg/L) reported (Adanir et al., 2005) involved a case of percutaneous absorption (with perhaps associated inhalation exposure) that led to vision and CNS deficits after hospital discharge. In one case report (Rubinstein et al., 1995) involving ingestion, coma and subsequent death were associated with an initial blood methanol level of 360 mg/L.

Upon MRI and CT scans, the more seriously affected individuals typically have focal necrosis in both brain white matter and more commonly, in the putamen. Bilateral hemorrhagic and nonhemorrhagic necrosis of the putamen is considered by many radiologists as the most well-known sequelae of methanol overexposure.

4.1.2. Occupational Studies

Occupational health studies have been carried out to investigate the potential effects of chronic exposure to lower levels of methanol than those seen in acute poisoning cases such as those described in Appendix C. For example, Frederick et al. (1984) conducted a health hazard evaluation on behalf of the National Institute for Occupational Safety and Health (NIOSH) to determine if vapor from duplicating fluid (which contains 99% methanol) used in mimeograph duplicating machines caused adverse health effects in exposed persons. A group of 84 teacher's aides were selected for study, 66 of whom responded with a completed medical questionnaire. A group of 297 teachers (who were not exposed to methanol vapors to the same extent as the

teacher's aides) completed questionnaires as a control group. A 15-minute breathing zone sample was taken from 21 duplicators, 15 of which were greater than the NIOSH-recommended short term ceiling concentration of 800 ppm (1,048 mg/m^3). The highest breathing zone concentrations were in the vicinity of duplicators for which no exhaust ventilation had been provided (3,080 ppm [4,036 mg/m^3] was the highest value recorded). Upon comparison of the self-described symptoms of the 66 teacher's aides with those of 66 age-matched teachers chosen from the 297 who responded, the number of symptoms potentially related to methanol were significantly higher in the teacher's aides. These included blurred vision (22.7 versus 1.5%), headache (34.8 versus 18.1%), dizziness (30.3 versus 1.5%), and nausea (18 versus 6%). By contrast, symptoms that are not usually associated with methanol exposure (painful urination, diarrhea, poor appetite, and jaundice) were similar in incidence among the groups.

To further investigate these disparities, NIOSH physicians (not involved in the study) defined a hypothetical case of methanol toxicity by any of the following four symptom aggregations: (1) visual changes; (2) one acute symptom (headache, dizziness, numbness, giddiness, nausea or vomiting) combined with one chronic symptom (unusual fatigue, muscle weakness, trouble sleeping, irritability, or poor memory); (3) two acute symptoms; or (4) three chronic symptoms. By these criteria, 45% of the teacher's aides were classified as being adversely affected by methanol exposure compared to 24% of teachers ($p < 0.025$). Those teacher's aides and teachers who spent a greater amount of time using the duplicators were affected at a higher rate than those who used the machines for a lower percentage of their work day.

Tanner (1992) reviewed the occupational and environmental causes of Parkinsonism, spotlighting the potential etiological significance of manganese, carbon monoxide, repeated head trauma (such as suffered by boxers), and exposure to solvents. Among the latter, Tanner (1992) discussed the effects of methanol and n-hexane on the nervous system. Acute methanol intoxication resulted in inebriation, followed within hours by GI pain, delirium, and coma. Tanner (1992) pinpointed the formation of formic acid, with consequent inhibition of cytochrome oxidase, impaired mitochondrial function, and decreased ATP formation as relevant biochemical and physiological changes for methanol exposure. Nervous system injury usually includes blindness, Parkinson-like symptoms, dystonia, and cognitive impairment, with injury to putaminal neurons most likely underlying the neurological responses.

Kawai et al. (1991) carried out a biomarker study in which 33 occupationally exposed workers in a factory making methanol fuel were exposed to concentrations of methanol of up to 3,577 ppm (4,687 mg/m^3), as measured by personal samplers of breathing zone air. Breathing zone exposure samples were correlated with the concentrations of methanol in urine at the end of the shift in 38 exposed individuals and 30 controls (r = 0.82). Eleven of 22 individuals who

experienced high exposure to methanol (geometric mean of 459 ppm [601 mg/m^3]) complained of dimmed vision during work while 32% of this group of workers experienced nasal irritation. These incidences were statistically significant compared to those of persons who worked in low-exposure conditions (geometric mean of 31 ppm [41 mg/m^3]). One 38-year-old female worker who had worked at the factory for only 4 months reported that her visual acuity had undergone a gradual impairment. She also displayed a delayed light reflex.

Lorente et al. (2000) carried out a case control study of 100 mothers whose babies had been born with cleft palates. Since all of the mothers had worked during the first trimester, Lorente et al. (2000) examined the occupational information for each subject in comparison to 751 mothers whose babies were healthy. Industrial hygienists analyzed the work histories of all subjects to determine what, if any, chemicals the affected mothers may have been exposed to during pregnancy. Multivariate analysis was used to calculate odds ratios, with adjustments made for center of recruitment, maternal age, urbanization, socioeconomic status, and country of origin. Occupations with positive outcomes for cleft palate in the progeny were hairdressing (OR = 5.1, with a 95% confidence interval [CI] of 1.0-26) and housekeeping (OR = 2.8, with a 95% CI of 1.1-7.2). Odds ratios for cleft palate only and cleft lip with or without cleft palate were calculated for 96 chemicals. There seemed to be no consistent pattern of association for any chemical or group of chemicals with these impairments, and possible exposure to methanol was negative for both outcomes.

4.1.3. Controlled Human Studies

Two controlled studies have evaluated humans for neurobehavioral function following exposure to ~200 ppm (262 mg/m^3) methanol vapors in a controlled setting. The occupational TLV established by the American Conference of Governmental Industrial Hygienists (2000) is 200 ppm (262 mg/m^3). In a pilot study by Cook et al. (1991), 12 healthy young men (22-32 years of age) served as their own controls and were tested for neurobehavioral function following a random acute exposure to air or 191 ppm (250 mg/m^3) methanol vapors for 75 minutes. The majority of results in a battery of neurobehavioral endpoints were negative. However, statistical significance was obtained for results in the P-200 and N1-P2 component of event-related potentials (brain wave patterns following light flashes and sounds), the Sternberg memory task, and subjective evaluations of concentration and fatigue. As noted by the Cook et al.(1991), effects were mild and within normal ranges. Cook et al. (1991) acknowledged limitations in their study design, such as small sample size, exposure to only one concentration for a single duration time, and difficulties in masking the methanol odor from experimental personnel and study subjects.

In a randomized double-blind study, neurobehavioral testing was conducted on 15 men and 11 women (healthy, aged 26-51 years) following exposure to 200 ppm (262 mg/m^3) methanol or water vapors for 4 hours (Chuwers et al., 1995); subjects served as their own controls in this study. Exposure resulted in elevated blood and urine methanol levels (up to peak levels of 6.5 mg/L and 0.9 mg/L, respectively) but not formate concentrations. The majority of study results were negative. No significant findings were noted for visual, neurophysiological, or neurobehavioral tests except for slight effects ($p < 0.05$) on P-300 amplitude (brain waves following exposure to sensory stimuli) and Symbol Digit testing (ability to process information and psychomotor skills). Neurobehavioral performance was minimally affected by methanol exposure at this level. Limitations noted by Chuwers et al. (1995) are that studies of alcohol's affect on P-300 amplitude suggest that this endpoint may be biased by unknown factors and some experimenters and subjects correctly guessed if methanol was used.

Although the slight changes in P-200 and P-300 amplitude noted in both the Chuwers et al. (1995) and Cook et al. (1991) studies may be an indication of moderate alterations in cognitive function, the results of these studies are generally consistent and suggest that the exposure concentrations employed were below the threshold for substantial neurological effects. This is consistent with the data from acute poisoning events which have pointed to a serum methanol threshold of 200 mg/L for the instigation of acidosis, visual impairment, and CNS deficits.

Mann et al. (2002) studied the effects of methanol exposure on human respiratory epithelium as manifested by local irritation, ciliary function, and immunological factors. Twelve healthy men (average age 26.8 years) were exposed to 20 and 200 ppm (26.2 and 262 mg/m^3, respectively) methanol for 4 hours at each concentration; exposures were separated by 1-week intervals. The 20 ppm (26.2 mg/m^3) concentration was considered to be the control exposure since previous studies had demonstrated that subjects can detect methanol concentrations of 20 ppm (26.2 mg/m^3) and greater. Following each single exposure, subclinical inflammation was assessed by measuring concentrations of interleukins (IL-8, IL-1β, and IL-6) and prostaglandin E2 in nasal secretions. Mucociliary clearance was evaluated by conducting a saccharin transport time test and measuring ciliary beat frequency. Interleukin and prostaglandin data were evaluated by a 1-tailed Wilcoxon test, and ciliary function data were assessed by a 2-tailed Wilcoxon test. Exposure to 200 (262 mg/m^3) versus 20 ppm (26.2 mg/m^3) methanol resulted in a statistically-significant increase in IL-1β (median of 21.4 versus 8.3 pg/mL) and IL-8 (median of 424 versus 356 pg/mL). There were no significant effects on IL-6 and prostaglandin E2 concentration, ciliary function, or on the self-reported incidence of subjective symptoms of irritation. The authors concluded that exposure to 200 ppm (262 mg/m^3) methanol resulted in a subclinical inflammatory response.

In summary, adult human subjects acutely exposed to 200 ppm (262 mg/m^3) methanol have experienced slight neurological (Chuwers et al., 1995) and immunological effects (increased subclinical biomarkers for inflammation) with no self-reported symptoms of irritation (Mann et al., 2002). These exposure levels were associated with peak methanol blood levels of 6.5 mg/L (Chuwers et al., 1995), which is more than 4-fold higher than mean background methanol blood levels reported for adult human subjects on methanol-restrictive diets (Table 3-1). Nasal irritation effects have been reported by adult workers exposed to 459 ppm (601 mg/m^3) methanol (Kawai et al., 1991). Frank effects such as blurred vision, bilateral or unilateral blindness, coma, convulsions/tremors, nausea, headache, abdominal pain, diminished motor skills, acidosis, and dyspnea begin to occur as blood levels approach 200 mg methanol/L, while 800 mg/L appears to be the threshold for lethality. Data for subchronic, chronic or in utero human exposures are very limited and inconclusive.

4.2. Acute, Subchronic and Chronic Studies in Animals – Oral and Inhalation

A number of studies in animals have investigated the acute, subchronic, and chronic toxicity of methanol. Most are via the inhalation route. Presented below are summaries of the noncancer effects reported in these bioassays. Carcinogenic effects are not described or discussed in this assessment.

4.2.1. Oral Studies

4.2.1.1. Acute Toxicity

Although there are few studies that have examined the short-term toxic effects of methanol via the oral route, a number of median lethal dose (LD_{50}) values have been published for the compound. As listed in Lewis (1992), these include 5,628 mg/kg in rats, 7,300 mg/kg in mice, and 7,000 mg/kg in monkeys.

4.2.1.2. Subchronic Toxicity

An oral repeat dose study was conducted by the U.S. EPA (TRL, 1986) in rats. Sprague-Dawley rats (30/sex/dose) at no less than 30 days of age were gavaged with 0, 100, 500, or 2,500 mg/kg-day of methanol. Six weeks after dosing, 10 rats/sex/dose group were subjected to interim sacrifice, while the remaining rats continued on the dosing regimen until the final

sacrifice (90 days). This study generated data on weekly body weights and food consumption, clinical signs of toxicity, ophthalmologic evaluations, mortality, blood and urine chemistry (from a comprehensive set of hematology, serum chemistry, and urinalysis tests), and gross and microscopic evaluations for all test animals. Complete histopathologic examinations of over 30 organ tissues were done on the control and high-dose rats. Histopathologic examinations of livers, hearts, and kidneys and all gross lesions seen at necropsy were done on low-dose and mid-dose rats. There were no differences between dosed animals and controls in body weight gain, food consumption, or upon gross or microscopic evaluations. Elevated levels ($p \leq 0.05$ in males) of serum alanine transaminase (ALT)[15] and serum alkaline phosphatase (SAP), and increased (but not statistically significant) liver weights in both male and female rats suggest possible treatment-related effects in rats bolus dosed with 2,500 mg methanol/kg-day despite the absence of supportive histopathologic lesions in the liver. Brain weights of high-dose group (2,500 mg/kg-day) males and females were significantly less than those of the control group at terminal sacrifice. The only histopathology noted was a higher incidence of colloid in the hypophyseal cleft of the pituitary gland in the high-dose versus control group males (13/20 versus 0/20) and females (9/20 versus 3/20). Based on these findings, 500 mg/kg-day of methanol is considered an NOAEL from this rat study.

4.2.1.3. Chronic Noncancer Toxicity

A report by Soffritti et al. (2002) summarized a European Ramazzini Foundation (ERF) chronic duration experimental study of methanol[16] in which the compound was provided to 100 Sprague-Dawley rats/sex/group ad libitum in drinking water at concentrations of 0, 500, 5,000, and 20,000 ppm (v/v). The animals were 8 weeks old at the beginning of the study. In general, ERF does not randomly assign animals to treatment groups, but assigns all animals from a given litter to the same treatment group (Bucher, 2002). All rats were exposed for up to 104 weeks, and then maintained until they died naturally. Rats were housed in groups of 5 in Makrolon cages (41 × 25 × 15 cm) in a room that was maintained at 23 ± 2°C and 50–60% relative humidity. The in-life portion of the experiment ended at 153 weeks with the death of the last animal. Mean daily drinking water, food consumption, and body weights were monitored weekly for the first 13 weeks, every 2 weeks thereafter for 104 weeks, then every 8 weeks until the end of the experiment. Clinical signs were monitored 3 times/day, and the occurrence of

[15] Also known as serum glutamate pyruvate transaminase (SGPT)
[16] Soffritti et al. (2002) report that methanol was obtained from J.T. Baker, Deventer, Holland, purity grade 99.8%.

gross changes was evaluated every 2 weeks. All rats were necropsied at death then underwent histopathologic examination of organs and tissues.[17]

Soffritti et al. (2002) reported no substantial dose-related differences in survival, but no data were provided. Using individual animal data available from the ERF website,[18] Cruzan (2009) reports that male rats treated with methanol generally survived better than controls, with 50% survival occurring at day 629, 686, 639 and 701 in the 0, 500, 5,000, and 20, 000 mg/L groups, respectively. There were no significant differences in survival between female control and treatment groups, with 50% survival occurring at day 717, 691, 678 and 708 in the 0, 500, 5,000, and 20,000 mg/L groups, respectively. Body weight and water and food consumption were monitored in the study, but the data were not documented in the published report.

Soffritti et al. (2002) reported that water consumption in high-dose females was reduced compared to controls between 8 and 56 weeks and that the mean body weight in high-dose males tended to be higher than that of control males. Overall, there was no pattern of compound-related clinical signs of toxicity, and the available data did not provide any indication that the control group was not concurrent with the treated group (Cruzan, 2009). Soffritti et al. (2002) further reported that there were no compound-related signs of gross pathology or histopathologic lesions indicative of noncancer toxicological effects in response to methanol.

Apaja (1980) performed dermal and drinking water chronic bioassays in which male and female Eppley Swiss Webster mice (25/sex/dose group; 8 weeks old at study initiation) were exposed 6 days per week until natural death to various concentrations of malonaldehyde and methanol. The stated purpose of the study was to determine the carcinogenicity of malonaldehyde, a product of oxidative lipid deterioration in rancid beef and other food products in advanced stages of degradation. However, due to its instability, malonaldehyde was obtained from the more stable malonaldehyde bis (dimethylacetal), which was hydrolyzed to malonaldehyde and methanol in dilute aqueous solutions in the presence of a strong mineral acid. In the drinking water portion of this study, mice were exposed to 3 different concentrations of the malonaldehyde/methanol solution and three different control solutions of methanol alone, 0.222%, 0.444% and 0.889% methanol in drinking water (222, 444 and 889 ppm, assuming a density of 1 g/mL), corresponding to the stoichiometric amount of methanol liberated by hydrolysis of the acetal in the three test solutions. The methanol was described as Mallinckrodt

[17] Histopathology was performed on the following organs and tissues: skin and subcutaneous tissue, brain, pituitary gland, Zymbal glands, parotid glands, submaxillary glands, Harderian glands, cranium (with oral and nasal cavities and external and internal ear ducts) (5 sections of head), tongue, thyroid and parathyroid, pharynx, larynx, thymus and mediastinal lymph nodes, trachea, lung and mainstem bronchi, heart, diaphragm, liver, spleen, pancreas, kidneys, adrenal glands, esophagus, stomach (fore and glandular), intestine (four levels), urinary bladder, prostate, gonads, interscapular fat pad, subcutaneous and mesenteric lymph nodes, and any other organs or tissues with pathologic lesions.

[18] http://www.ramazzini.it/fondazione/foundation.asp.

analytical grade. No unexposed control groups were included in these studies. However, the author provided pathology data from historical records of untreated Swiss mice of the Eppley colony used in two separate chronic studies, one involving 100 untreated males and 100 untreated females (Toth et al., 1977) and the other involving 100 untreated females histopathological analyzed by Apaja (Apaja, 1980).

Mice in the Apaja (1980) study were housed five/plastic cage and fed Wayne Lab-Blox pelleted diet. Water was available ad libitum throughout life. Liquid consumption per animal was measured at 3 times/week. The methanol dose in the dermal study (females only) was 21.3 mg (532 mg/kg-day using an average weight of 0.04 kg as approximated from Figure 4 of the study), three times/week. The methanol doses in the drinking water study were reported as 22.6, 40.8 and 84.5 mg/day (560, 1,000 and 2,100 mg/kg-day using an average weight of 0.04 kg as approximated from Figures 14-16 of the study) for females, and 24.6, 43.5 and 82.7 mg/day (550, 970, and 1,800 mg/kg-day using an average weight of 0.045 kg as approximated from Figures 14-16 of the study) for males, 6 days/week. The animals were checked daily and body weights were monitored weekly. The in-life portion of the experiment ended at 120 weeks with the death of the last animal. Like the Soffritti et al. (2002) study, test animals were sacrificed and necropsied when moribund.[19]

The authors reported that survival of the methanol exposed females of the drinking water study was lower than untreated historical controls ($p < 0.05$), but no significant differences in survival was noted for males. An increase in liver parenchymal cell necrosis was reported in the male and female high-dose groups, with the incidence in females (8%) being significant ($p < 0.01$) relative to untreated historical controls. Incidence of acute pancreatitis was higher in high-dose males ($p < 0.001$), but did not appear to be dose-related in females, increasing at the mid- ($p < 0.0001$) and low-doses ($p < 0.01$) when compared to historical controls but not appearing at all in the high-dose females. Significant increases relative to untreated historical controls were noted in amyloidosis of the spleen, nephropathy and pneumonia, but the increases did not appear to be dose related.

[19] The following tisues were fixed in 10% formalin (pH 7.5), embedded in paraffin, sectioned, stained routinely with hematoxylineosin (special stains used as needed) and histologically evaluated: skin, lungs, liver spleen, pancreas, kidneys, adrenal glands, esophagus, stomach, small and large intestines, rectum, urinary bladder, uterus and ovaries or testes, prostate glands and tumors or other gross pathological lesions.

4.2.2. Inhalation Studies

4.2.2.1. Acute Toxicity

Lewis (1992) reported a 4-hour median lethal concentration (LC_{50}) for methanol in rats of 64,000 ppm (83,867 mg/m^3).

Japan's NEDO sponsored a series of toxicological tests on monkeys (*M. fascicularis*), rats, and mice, using inhalation exposure.[20] These are unpublished studies; accordingly, they were externally peer reviewed by EPA in 2009.[21] A short-term exposure study evaluated monkeys (sex unspecified) exposed to 3,000 ppm (3,931 mg/m^3), 21 hours/day for 20 days (1 animal), 5,000 ppm (6,552 mg/m^3) for 5 days (1 animal), 5,000 ppm (6,552 mg/m^3) for 14 days (2 animals), and 7,000 and 10,000 ppm (9,173 and 13,104 mg/m^3, respectively) for up to 6 days (1 animal at each exposure level) (NEDO, 1987). Most of the experimental findings were discussed descriptively in the report, without specifying the extent of change for any of the effects in comparison to seven concurrent controls. However, the available data indicate that clinical signs of toxicity were apparent in animals exposed to 5,000 ppm (all exposure durations) or higher concentrations of methanol. These included reduced movement, crouching, weak knees, involuntary movements of hands, dyspnea, and vomiting. In the discussion section of the summary report, the authors stated that there was a sharp increase in the blood levels of methanol and formic acid in monkey exposed to >3,000 ppm (3,931 mg/m^3) methanol. They reported that methanol and formic acid concentrations in the blood of monkeys exposed to 3,000 ppm or less were 80 mg/L and 30 mg/L, respectively.[22] In contrast, monkeys exposed to 5,000 ppm or higher concentrations of methanol had blood methanol and formic acid concentrations of 5,250 mg/L and 1,210 mg/L, respectively. Monkeys exposed to 7,000 ppm and 10,000 ppm became critically ill and had to be sacrificed prematurely. Food intake was said to be little affected at 3,000 ppm, but those exposed to 5,000 ppm or more showed a marked reduction. Clinically, the monkeys exposed to 5,000 ppm or more exhibited reduced movement, weak knees, and involuntary movement of upper extremities, eventually losing consciousness and dying.

[20] In their bioassays, NEDO (NEDO, 1987) used inbred rats of the F344 or Sprague-Dawley strain, inbred mice of the B6C3F1 strain and wild-caught *M. fascicularis* monkeys imported from Indonesia. The possibility of disease among wild-caught animals is a concern, but NEDO (NEDO, 1987) state that the monkeys were initially quarantined for 9 weeks and measures were taken throughout the studies against the transmission of pathogens for infectious diseases. The authors indicated that "no infectious disease was observed in monkeys" and that "subjects were healthy throughout the experiment."

[21] An external peer review (ERG, 2009) was conducted by EPA in 2009 to evaluate the accuracy of experimental procedures, results, and interpretation and discussion of the findings presented in these study reports.

[22] Note that Burbacher, et al. (1999b) and Burbacher, et al. (2004b) measured blood levels of methanol and formic acid in control monkeys of 2.4 mg/L and 8.7 mg/L, respectively (see Table 3-3).

There were no significant changes in growth, with the exception of animals exposed to the highest concentration, where body weight was reduced by 13%. There were few compound-related changes in hematological or clinical chemistry effects, although animals exposed to 7,000 and 10,000 ppm showed an increase in white blood cells. A marked change in blood pH values at the 7,000 ppm and 10,000 ppm levels (values not reported) was attributed to acidosis due to accumulation of formic acid. The authors reported that no clinical or histopathological effects of the visual system were apparent, but that exposure to 3,000 ppm (3,931 mg/m^3) or more caused dose-dependent fatty degeneration of the liver, and exposure to 5,000 ppm (6,552 mg/m^3) or more caused vacuolar degeneration of the kidneys, centered on the proximal uriniferous tubules. A range of histopathologic changes to the CNS was apparently related to treatment. Severity of the effects was increased with exposure concentration. Lesions included characteristic degeneration of the bilateral putamen, caudate nucleus, and claustrum, with associated edema in the cerebral white matter. CNS effects reported in this and the NEDO chronic monkey inhalation study are discussed in greater detail in sSection 4.4.2 "Inhalation Neurotoxicity Studies."

The NEDO (1987) studies in nonhuman primates, including the chronic study discussed below, have multiple deficiencies that make them difficult to interpret. The reports lack a full description of the materials and methods and raw data from the experiments. The data gaps (e.g., materials and methods, statistical methods, data) are profound and the group sizes are too small to support rigorous statistical analysis. At best, they provide a descriptive, rather than quantitative, evaluation of the inhalation toxicity of methanol (ERG, 2009).

4.2.2.2. Subchronic Toxicity

A number of experimental studies have examined the effects of subchronic exposure to methanol via inhalation. For example, Sayers et al. (1944) employed a protocol in which 2 male dogs were repeatedly exposed (8 times daily for 3 minutes/exposure) to 10,000 ppm (13,104 mg/m^3) methanol for 100 days. One of the dogs was observed for a further 5 days before sacrifice; the other dog was observed for 41 days postexposure. There were no clinical signs of toxicity, and both gained weight during the study period. Blood samples were drawn on a regular basis to monitor hematological parameters, but few if any compound-related changes were observed. Ophthalmoscopic examination showed no incipient anomalies at any point during the study period. Median blood concentrations of methanol were 65 mg/L (range 0–280 mg/L) for one dog, and 140 mg/L (70–320 mg/L) for the other.

White et al. (1983) exposed 4 male Sprague-Dawley rats/group, 6 hours/day, 5 days/week to 0, 200, 2,000, or 10,000 ppm (0, 262, 2,621, and 13,104 mg/m^3) methanol for periods of 1, 2, 4, and 6 weeks. Additional groups of 6-week-exposure animals were granted a 6-week

postexposure recovery period prior to sacrifice. The lungs were excised intact and lavaged 6 times with known volumes of physiological saline. The lavage supernatant was then assayed for lactate dehydrogenase (LDH) and *N*-acetyl-*β*-*D*-glucosaminidase (*β*-NAG) activities. Other parameters monitored in relation to methanol exposure included absolute and relative lung weights, lung DNA content, protein, acid RNase and acid protease, pulmonary surfactant, number of free cells in lavage/unit lung weight, surface protein, LDH, and *β*-NAG. As discussed by the authors, none of the monitored parameters showed significant changes in response to methanol exposure.

Andrews et al. (1987) carried out a study of methanol inhalation in five Sprague-Dawley rats/sex/group and three *M. fascicularis* monkeys/sex/group, 6 hours/day, 5 days/week, to 0, 500, 2,000, or, 5,000 ppm (0, 660, 2,620, and 6,552 mg/m^3) methanol for 4 weeks. Clinical signs were monitored twice daily, and all animals were given a physical examination once a week. Body weights were monitored weekly, and animals received an ophthalmoscopic examination before the start of the experiment and at term. Animals were sacrificed at term by exsanguination following i.v. barbiturate administration. A gross necropsy was performed, weights of the major organs were recorded, and tissues and organs taken for histopathologic examination. As described by the authors, all animals survived to term with no clinical signs of toxicity among the monkeys and only a few signs of irritation to the eyes and nose among the rats. In the latter case, instances of mucoid nasal discharges appeared to be dose related. There were no differences in body weight gain among the groups of either rats or monkeys, and overall, absolute and relative organ weights were similar to controls. The only exception to this was a decrease in the absolute adrenal weight of female high-concentration monkeys and an increase in the relative spleen weight of mid-concentration female rats. These changes were not considered by the authors to have biological significance. For both rats and monkeys, there were no compound-related changes in gross pathology, histopathology, or ophthalmoscopy. These data suggest a NOAEL of 5,000 ppm (6,600 mg/m^3) for Sprague-Dawley rats and monkeys under the conditions of the experiment.

Two studies by Poon et al. (1995; 1994) examined the effects of methanol on Sprague-Dawley rats, when inhaled for 4 weeks. The effects of methanol were evaluated in comparison to those of toluene and toluene/methanol mixtures (Poon et al., 1994), and to gasoline and gasoline/methanol mixtures (Poon et al., 1995). In the first case (Poon et al., 1994), 10 Sprague-Dawley rats/sex/group were exposed via inhalation, 6 hours/day, 5 days/week to 0, 300, or 3,000 ppm (0, 393, 3,930 mg/m^3) methanol for 4 weeks. Clinical signs were monitored daily, and food consumption and body weight gain were monitored weekly. Blood was taken at term for hematological and clinical chemistry determinations. Weights of the major organs were recorded at necropsy, and histopathologic examinations were carried out. A 10,000 ×*g* liver supernatant

was prepared from each animal to measure aniline hydroxylase, aminoantipyrine N-demethylase, and ethoxyresorufin-O-deethylase activities. For the most part, the responses to methanol alone in this experiment were unremarkable. All animals survived to term, and there were no clinical signs of toxicity among the groups. Body weight gain and food consumption did not differ from controls, and there were no compound-related effects in hematological or clinical chemistry parameters or in hepatic mixed function oxidase activities. However, the authors described a reduction in the size of thyroid follicles that was more obvious in female than male rats. The authors considered this effect to possibly have been compound related, although the incidence of this feature for the 0, 300, and 3,000 ppm-receiving females was 0/6, 2/6, and 2/6, respectively.

The second experimental report by Poon et al. (1995) involved the exposure of 15 Sprague-Dawley rats/sex/group, 6 hours/day, 5 days/week for 4 weeks to 0 or 2,500 ppm (0 and 3,276 mg/m^3) to methanol as part of a study on the toxicological interactions of methanol and gasoline. Many of the toxicological parameters examined were the same as those described in Poon et al. (1994) study. However, in this study urinalysis featured the determination of ascorbic and hippuric acids. Additionally, at term, the lungs and tracheae were excised and aspirated with buffer to yield bronchoalveolar lavage fluid that was analyzed for ascorbic acid, protein, and the activities of gamma-glutamyl transferase (γ-GT), AP and LDH. Few if any of the monitored parameters showed any differences between controls and those animals exposed to methanol alone. However, two male rats had collapsed right eyes, and there was a reduction in relative spleen weight in females exposed to methanol. Histopathologic changes in methanol-receiving animals included mild panlobular vacuolation of the liver in females and some mild changes to the upper respiratory tract, including mucous cell metaplasia. The incidence of the latter effect, though higher, was not significantly different than controls in rats exposed to 2,500 ppm (3,267 mg/m^3) methanol. However, there were also signs of an increased severity of the effect in the presence of the solvent. No histopathologic changes were seen in the lungs or lower respiratory tract of rats exposed to methanol alone.

4.2.2.3. Chronic Noncancer Toxicity

Information on the chronic noncancer toxicity of inhalation exposure to methanol has come from NEDO (1987) which includes the results of experiments on (1) monkeys exposed for up to 3 years, (2) rats and mice exposed for 12 months, (3) mice exposed for 18 months, and (4) rats exposed for 2 years. These are unpublished studies; accordingly, they were externally

peer reviewed by EPA in 2009.[23] Neurotoxic effects reported in the monkey studies are discussed in more detail in Section 4.4.2.

In the monkeys, 8 animals (sex unspecified) were exposed to 10, 100, or 1,000 ppm (13, 131, and 1,310 mg/m^3) methanol, 21 hours/day, for 7 months (2 animals), 19 months, (3 animals), or 29 months (3 animals). There was no indication in the NEDO (1987) report that this study employed a concurrent control group. One of the 3 animals receiving 100 ppm methanol and scheduled for sacrifice at 29 months was terminated at 26 months. Clinical signs were monitored twice daily, body weight changes and food consumption were monitored weekly, and all animals were given a general examination under anesthetic once a month. Blood was collected for hematological and clinical chemistry tests at term, and all animals were subject to a histopathologic examination of the major organs and tissues.

While there were no clinical signs of toxicity in the low-concentration animals, there was some evidence of nasal exudate in monkeys in the mid-concentration group. High-concentration (1,000 ppm) animals also displayed this response and were observed to scratch themselves over their whole body and crouch for long periods. Food and water intake, body temperature, and body weight changes were the same among the groups. NEDO (1987) reported that there was no abnormality in the retina of any monkey. When animals were examined with an electrocardiogram, there were no abnormalities in the control or 10 ppm groups. However, in the 100 ppm group, one monkey showed a negative change in the T wave. All 3 monkeys exposed to 1,000 ppm (1,310 mg/m^3) displayed this feature, as well as a positive change in the Q wave. This effect was described as a slight myocardial disorder and suggests that 10 ppm (13.1 mg/m^3) is a NOAEL for chronic myocardial effects of methanol and mild respiratory irritation. There were no compound-related effects on hematological parameters. However, 1 monkey in the 100 ppm (131 mg/m^3) group had greater than normal amounts of total protein, neutral lipids, total and free cholesterol, and glucose, and displayed greater activities of ALT and aspartate transaminase (AST). The authors expressed doubts that these effects were related to methanol exposure and speculated that the animal suffered from liver disease.[24]

Histopathologically, no degeneration of the optical nerve, cerebral cortex, muscles, lungs, trachea, tongue, alimentary canal, stomach, small intestine, large intestine, thyroid gland, pancreas, spleen, heart, aorta, urinary bladder, ovary or uterus were reported (neuropathological findings are discussed in Section 4.4.2). Most of the internal organs showed no compound-related histopathologic lesions. However, there were signs of incipient fibrosis and round cell infiltration of the liver in monkeys exposed to 1,000 ppm (1,310 mg/m^3) for 29 months. NEDO

[23] An external peer review (ERG, 2009) was conducted by EPA in 2009 to evaluate the accuracy of experimental procedures, results, and interpretation and discussion of the findings presented in these study reports.
[24] Ordinarily, the potential for liver disease in test animals would be remote, but may be a possibility in this case given that these monkeys were captured in the wild.

(1987) indicated that this fibrosis occurred in 2/3 monkeys of the 1,000 ppm group to a "strictly limited extent." They also qualitatively reported a dose-dependent increase in "fat granules" in liver cells "centered mainly around the central veins" at all doses, but did not provide any response data. The authors state that 1,000 ppm (1,310 mg/m^3) represents a chronic lowest-observed-adverse-effect level (LOAEL) for hepatic effects of inhaled methanol, suggesting that the no effect level would be 100 ppm (131 mg/m^3). However, this is a tenuous determination given the lack of information on the pathological progression and significance of the appearance of liver cell fat granules at exposures below 1,000 ppm and the lack detail (e.g., time of sacrifice) for the control group.

Dose-dependent changes were observed in the kidney; NEDO (1987) described the appearance of Sudan-positive granules in the renal tubular epithelium at 100 ppm (131 mg/m^3) and 1,000 (1,310 mg/m^3) and hyalinization of the glomerulus and penetration of round cells into the renal tubule stroma of monkeys exposed to methanol at 1,000 (1,310 mg/m^3). The former effect was more marked at the higher concentration and was thought by the authors to be compound-related. This would indicate a no effect level at 10 ppm (13.1 mg/m^3) for the chronic renal effects of methanol. The authors observed atrophy of the tracheal epithelium in four monkeys. However, the incidence of these effects was unrelated to dose and therefore, could not be unequivocally ascribed to an effect of the solvent. No other histopathologic abnormalities were related to the effects of methanol. Confidence in these determinations is considerably weakened by limited study details (e.g., materials and methods, statistical methods, data), small group sizes and uncertainty over whether a concurrent control group was used in the chronic study.[25] In general, external peer reviewers of the NEDO (1987) monkey studies stated that the deficiencies in these reports were broad and significant, precluding the use of these studies for quantitative dose-response assessment (ERG, 2009). Although the limited information available from the NEDO (1987) summary report suggests that 100 ppm (131 mg/m^3) may be an effect level for myocardial effects, renal effects and neurotoxicity (see Section 4.4.2) following continuous, chronic exposure to methanol, NOAEL and LOAEL values are not derived for any of the NEDO (1987) monkey studies.

NEDO also performed 12-months inhalation studies in rats and mice (NEDO, 1987), an 18-month inhalation study in mice (NEDO, 1985) and a 24-month inhalation study in rats (NEDO, 1985b). External peer reviewers generally indicated that these rodent studies used good experimental designs, group sizes, endpoints and quality assurance procedures that were consistent with the OECD guidelines in place at the time. However, the reports available for the chronic studies (NEDO, 1985a, b) were far more detailed than the summary reports available for

[25] All control group responses were reported in a single table in the section of the NEDO (1987) report that describes the acute monkey study, with no indication as to when the control group was sacrificed.

the 12-month studies (NEDO, 1987), which suffered from many of the same reporting issues identified for the NEDO monkey studies, including a lack a full description of the materials and methods and raw data from the experiments. For all of the NEDO (1987) mouse, rat and monkey studies, parameters should have been assessed by one way analysis of variance (ANOVA), rather than the t-test comparisons with controls that were apparently performed (ERG, 2009).

NEDO (1987) describes a 12-month inhalation study in which 20 F344 rats/sex/group were exposed to 0, 10, 100, or 1,000 ppm (0, 13.1, 131, and 1,310 mg/m^3) methanol, approximately 20 hours/day, for a year. Clinical signs of toxicity were monitored daily; body weights and food consumption were recorded weekly for the first 13 weeks, then monthly. Blood samples were drawn at term to measure hematological and clinical chemistry parameters. Weights of the major organs were monitored at term, and a histopathologic examination was carried out on all major organs and tissues. Survival was high among the groups; one high-concentration female died on day 337 and one low-concentration male died on day 340. As described by the authors, a number of procedural anomalies arose during this study. For example, male controls in two cages lost weight because of an interruption to the water supply. Another problem was that the brand of feed was changed during the study. Fluctuations in some clinical chemistry and hematological parameters were recorded. The authors considered the fluctuations to be minor and within the normal range. Likewise, a number of histopathologic changes were observed, which, in every case, were considered to be unrelated to exposure level or due to aging.

A companion experiment featured the exposure of 30 B6C3F1 mice/sex/group for 1 year to the same concentrations as the F344 rats (NEDO, 1987). Broadly speaking, the same suite of toxicological parameters was monitored as described above, with the addition of urinalysis. 10 mice/sex/group were sacrificed at 6 months to provide interim data on the parameters under investigation. A slight atrophy in the external lacrimal gland was observed in both sexes and was significant in the 1,000 ppm male group compared with controls. An apparently dose-related increase in moderate fatty degeneration of hepatocytes was observed in males (1/20, 4/20, 6/20 and 8/20 in the 0, 10, 100, and 1,000 ppm dose groups, respectively) which was significantly increased over controls at the 1,000 ppm dose. However a high (10/20) incidence of moderate to severe fatty degeneration was observed in untreated animals maintained outside of the chamber. In addition, there was a clear correlation between fatty degeneration and body weight (a change which was not associated with treatment at 12 months); heavier animals tended to have more severe cases of fatty degeneration. Thus, methanol's role in fatty liver degeneration in mice is questionable, especially given the failure to confirm the finding in the 18-month study described below (ERG, 2009). The possibility of renal deficits due to methanol exposure was suggested by the appearance of protein in the urine. However, this effect was also seen in controls and did not

display a dose-response effect. Therefore, it is unlikely to be a consequence of exposure to methanol. NEDO (1987) reported other histopathologic and biochemical (e.g., urinalysis and hematology) findings that do not appear to be related to treatment, including a number of what were considered to be spontaneous tumors in both control and exposure groups.

NEDO (1987, 1985)[26] exposed 52 male and 53 female B6C3F1 mice/group for 18 months at the same concentrations of methanol (0, 10, 100 and 1,000 ppm) and with a similar experimental protocol to that described in the 12-month studies.[27] Animals were sacrificed at the end of the 18-month exposure period. NEDO (1985) reported that "there was no microbiological contamination that may have influenced the result of the study" and that the study included an assessment of general conditions, body weight change, food consumption rate, laboratory tests (urinalysis, hematological, and plasma biochemistry) and pathological tests (pathological autopsy,[28] organ weight check and histopathology[29]). As stated in the summary report (NEDO, 1987), a few animals showed clinical signs of toxicity, but the incidence of these responses was not related to dose. Likewise, there were no compound-related changes in body weight increase, food consumption,[30] urinalysis, hematology, or clinical chemistry parameters. High-concentration males had lower testis weights compared to control males. Significant differences were detected for both absolute and relative testis weights. One animal in the high-dose group had severely atrophied testis weights, approximately 25% of that of the others in the dose group. Exclusion of this animal in the analysis still resulted in a significant difference in absolute testis weight compared to controls but resulted in no difference in relative testis weight. High-concentration females had higher absolute kidney and spleen weights compared to controls, but there was no significant difference in these organ weights relative to body weight. At necropsy, there were signs of swelling in spleen, preputial glands, and uterus in some animals. Some animals developed nodes in the liver and lung although, according to the authors, none of these changes were treatment-related. NEDO (1985) reported that all nonneoplastic changes were "nonspecific and naturally occurring changes that are often experienced by 18-month old

[26] This study is described in a summary report (NEDO, 1987) and a more detailed, eight volume translation of the original chronic mouse study report (NEDO, 1985). The translation was submitted to EPA by the Methanol Institute and has been certified by NEDO as accurate and complete (Hashimoto, 2008). An external peer review (ERG, 2009) was conducted by EPA in 2009 to evaluate the accuracy of experimental procedures, results, and interpretation and discussion of the findings presented in these study reports.

[27] The authors reported that "[t]he levels of methanol turned out to be ~4 ppm in low level exposure group (10 ppm) for ~11 weeks from week 43 of exposure due to the analyzer malfunction" and that "the average duration of methanol exposure was 19.1 hours/day for both male and female mice."

[28] Autopsy was performed on all cases to look for gross lesions in each organ.

[29] Complete histopathological examinations were performed for the control group and high-dose (1,000 ppm) groups. Only histopathological examinations of the liver were performed on the low- and medium-level exposure groups because no chemical-related changes were found in the high-level exposure group and because liver changes were noted in the 12-month mouse study (NEDO, 1987).

[30] NEDO (NEDO, 1985) reports sporadic reductions in food consumption of the 1,000 ppm group, but no associated weight loss or abnormal test results.

B6C3F1 mice" and that fatty degeneration of liver that was suspected to occur dose-dependently in the 12-month NEDO (1987) study was not observed in this study.

Another study reported in NEDO (1987, 1985b)[31] was a 24-month bioassay in which 52 F344 rats/sex/group were kept in whole body inhalation chambers containing 0, 10, 100, or 1,000 ppm (0, 13.1, 131, and 1,310 mg/m^3) methanol vapor. Animals were maintained in the exposure chambers for approximately 19.5 hours/day for a total of 733-736 days (males) and 740-743 days (females). Animals were monitored once a day for clinical signs of toxicity, body weights were recorded once a week, and food consumption was measured weekly from a 24-animal subset from each group. Urinalysis was carried out on the day prior to sacrifice for each animal, the samples being monitored for pH, protein, glucose, ketones, bilirubin, occult blood, and urobilinogen. Routine clinical chemistry and hematological measurements were carried out and all animals were subject to necropsy at term, with a comprehensive histopathological examination of tissues and organs.[32]

There was some fluctuation in survival rates among the groups in the rat study, though apparently unrelated to exposure concentration.[33] In all groups, at least 60% of the animals survived to term. A number of toxicological responses were described by the authors, including atrophy of the testis, cataract formation, exophthalmia, small eye ball, alopecia, and paralysis of the hind leg. However, according to the authors, the incidence of these effects were unrelated to dose and more likely represented effects of aging. NEDO (1985b) reported a mild, nonsignificant (4%) body weight suppression among 1,000 ppm females between 51 and 72 weeks, but that body weight gain was largely similar among the groups for the duration of the experiment. Food consumption was significantly lower than controls in high-concentration male rats during the day 210–365 time interval, but no corresponding weight loss was observed. Among hematological parameters, mid- and high-concentration females had a significantly ($p > 0.05$) higher differential leukocyte count than controls, but dose dependency was not observed. Serum total cholesterol, triglyceride, free fatty acid, and phospholipid concentrations were significantly ($p > 0.05$) lower in high-concentration females compared to controls. Likewise, serum sodium concentrations were significantly ($p > 0.05$) lower in mid- and high-concentration males compared to controls.

[31] This study is described in a summary report (NEDO, 1987) and a more detailed, 10-volume translation of the original chronic rat study report (NEDO, 1985b). The translation was submitted to EPA by the Methanol Institute and has been certified by NEDO as accurate and complete (Hashimoto, 2008). An external peer review (ERG, 2009) was conducted by EPA in 2009 to evaluate the accuracy of experimental procedures, results, and interpretation and discussion of the findings presented in these study reports.

[32] Complete histopathological examinations were performed on the cases killed on schedule (week 104) among the control and high-exposure groups, and the cases that were found dead/ killed in extremis of all the groups. Because effects were observed in male and female kidneys, male lungs as well as female adrenal glands of the high-level exposure group, these organs were histopathologically examined in the low- and mid-exposure groups.

[33]Survival at the time of exposure termination (24 months) was 69%, 65%, 81%, and 65% for males and 60%, 63%, 60% and 67% for females of the control, low-, mid- and high-exposure groups, respectively.

High-concentration females had significantly lower ($p > 0.05$) serum concentrations of inorganic phosphorus but significantly ($p > 0.05$) higher concentrations of potassium compared to controls. Glucose levels were elevated in the urine of high-concentration male rats relative to controls, and female rats had lower pH values and higher bilirubin levels in mid- and high-concentration groups relative to controls. In general, NEDO (1987, 1985b) reported that these variations in urinary, hematology, and clinical chemistry parameters were not related to chemical exposure.

NEDO (1987) reported that there was little change in absolute or relative weights of the major organs or tissues. When the animals were examined grossly at necropsy, there were some signs of swelling in the pituitary and thyroid, but these effects were judged to be unrelated to treatment. The most predominant effect was the dose-dependent formation of nodes in the lung of males (2/52, 4/52, 5/52, and 10/52 [$p < 0.01$] for control, low-, mid-, and high-concentration groups, respectively). Histopathologic examination pointed to a possible association of these nodes with the appearance of pulmonary adenoma (1/52, 5/52, 2/52, and 6/52 for control, low-, mid- and high-concentration groups, respectively) and a single pulmonary adenocarcinoma in the high-dose group (1/52).

4.3. Reproductive and Developmental Studies – Oral and Inhalation

Many studies have been conducted to investigate the reproductive and developmental toxicity of methanol. The purpose of these studies was principally to determine if methanol has a similar toxicology profile to another widely studied teratogen, ethanol.

4.3.1. Oral Reproductive and Developmental Studies

Three studies were identified that investigated the reproductive and developmental effects of methanol in rodents via the oral route (Fu et al., 1996; Sakanashi et al., 1996; Rogers et al., 1993b). Two of these studies also investigated the influence of folic acid-deficient (FAD) diets on the effects of methanol exposures (Fu et al., 1996; Sakanashi et al., 1996).

Rogers et al. (1993b) conducted a developmental toxicity study in which methanol in water was administered to pregnant female CD-1 mice via gavage on GD6–GD15. Eight test animals received 4 g/kg-day methanol given in 2 daily doses of 2 g/kg; 4 controls received distilled water. By analogy to the protocol of an inhalation study of methanol that was described in the same report, it is assumed that dams were sacrificed on GD17, at which point implantation sites, live and dead fetuses, resorptions/litter, and the incidences of external and skeletal anomalies and malformations were determined. In the brief summary of the findings provided by

the authors, it appears that cleft palate (43.5% per litter versus 0% in controls) and exencephaly (29% per litter versus 0% in controls) were the prominent external defects following maternal methanol exposure by gavage. Likewise, an increase in totally resorbed litters and a decrease in the number of live fetuses per litter were evident. However, it is possible that these effects may have been caused or exacerbated by the high bolus dosing regimen employed. It is also possible that effects were not observed due to the limited study size. The small number of animals in the control group relative to the test group limits the power of this study to detect treatment-related responses.

Sakanashi et al. (1996) tested the influence of dietary folic acid intake on various reproductive and developmental effects observed in CD-1 mice exposed to methanol. Starting 5 weeks prior to breeding and continuing for the remainder of the study, female CD-1 mice were fed folic acid free diets supplemented with 400 (low), 600 (marginal) or 1,200 (sufficient) nmol folic acid/kg. After 5 weeks on their respective diets, females were bred with CD-1 male mice. On GD6–GD15, pregnant mice in each of the diet groups were given twice-daily gavage doses of 2.0 or 2.5 g/kg-day methanol (total dosage of 4.0 or 5.0 g/kg-day). On GD18, mice were weighed and killed, and the liver, kidneys and gravid uteri removed and weighed. Maternal liver and plasma folate levels were measured, and implantation sites, live and dead fetuses, and resorptions were counted. Fetuses were weighed individually and examined for cleft palate and exencephaly. One third of the fetuses in each litter were examined for skeletal morphology. They observed an approximate 50% reduction in liver and plasma folate levels in the mice fed low versus sufficient folic acid diets in both the methanol exposed and unexposed groups. Similar to Rogers et al. (1993b), Sakanashi et al. (1996) observed that an oral dose of 4-5 g/kg-day methanol during GD6-GD15 resulted in an increase in cleft palate in mice fed sufficient folic acid diets, as well as an increase in resorptions and a decrease in live fetuses per litter. They did not observe an increase in exencephaly in the FAS group at these doses, and the authors suggest that this may be due to diet and the source of CD-1 mice differing between the two studies.

In the case of the animals fed the folate deficient diet, there was a 50% reduction in maternal liver folate concentration and a threefold increase in the percentage of litters affected by cleft palate (86.2% versus 34.5% in mice fed sufficient folic acid) and a 10-fold increase in the percentage of litters affected by exencephaly (34.5% versus 3.4% in mice fed sufficient folic acid) at the 5 g/kg methanol dose. Sakanashi et al. (1996) speculate that the increased methanol effect from the FAD diet could have been due to an increase in tissue formate levels (not measured) or to a critical reduction in conceptus folate concentration following the methanol exposure. Plasma and liver folate levels at GD18 within each dietary group were not significantly different between exposed versus unexposed mice. However, these measurements were taken 3 days after methanol exposure. Dorman et al. (1995) observed a transient decrease in

maternal red blood cells (RBCs) and conceptus folate levels within 2 hours following inhalation exposure to 15,000 ppm methanol on GD8. Thus, it is possible that short-term reductions in available folate during GD6-GD15 may have affected fetal development.

Fu et al. (1996) also tested the influence of dietary folic acid intake on reproductive and developmental effects observed in CD-1 mice exposed to methanol. This study was performed by the same laboratory and used a similar study design and dosing regimen as Sakanashi et al. (1996), but exposed the pregnant mice to only the higher 2.5 g/kg-day methanol (total dosage of 5.0 g/kg-day) on GD6-GD10. Like Sakanashi et al. (1996), Fu et al. (1996) measured maternal liver and plasma folate levels on GD18 and observed similar, significant reductions in these levels for the FAD versus FAS mice. However, Fu et al. (1996) also measured fetal liver folate levels at GD18. This measurement does not address the question of whether methanol exposure caused short-term reductions in fetal liver folate because it was taken 8 days after the GD6-GD10 exposure period. However, it did provide evidence regarding the extent to which a maternal FAD diet can impact fetal liver folate levels in this species and strain. Significantly, the maternal FAD diet had a greater impact on fetal liver folate than maternal liver folate levels. Relative to the FAS groups, fetal liver folate levels in the FAD groups were reduced 2.7-fold for mice not exposed to methanol (1.86 ± 0.15 nmol/g in the FAD group versus 5.04 ± 0.22 nmol/g in the FAS group) and 3.5-fold for mice exposed to methanol (1.69 ± 0.12 nmol/g in the FAD group versus 5.89 ± 0.39 nmol/g in the FAS group). Maternal folate levels in the FAD groups were only reduced twofold both for mice not exposed (4.65 ± 0.37 versus 9.54 ± 0.50 nmol/g) and exposed (4.55 ± 0.19 versus 9.26 ± 0.42 nmol/g). Another key finding of the Fu et al. (1996) study is that methanol exposure during GD6-GD10 appeared to have similar fetotoxic effects, including cleft palate, exencephaly, resorptions, and decrease in live fetuses, as the same level of methanol exposure administered during GD6-GD15 (Sakanashi et al., 1996; Rogers et al., 1993b). This is consistent with the hypothesis made by Rogers et al. (1993b) that the critical period for methanol-induced cleft palate and exencephaly in CD-1 mice is within GD6-GD10. As in the studies of Sakanashi et al. (1996) and Rogers et al. (1993b), Fu et al. (1996) reported a higher incidence of cleft palate than exencephaly.

4.3.2. Inhalation Reproductive and Developmental Studies

Nelson et al. (1985) exposed 15 pregnant Sprague-Dawley rats/group to 0, 5,000, 10,000, or 20,000 ppm (0, 6,552, 13,104, and 26,209 mg/m^3) methanol (99.1% purity) for 7 hours/day. Exposures were conducted on GD1–GD19 in the two lower concentration groups and GD7-GD15 in the highest concentration group, apparently on separate days. Two groups of 15 control rats were exposed to air only. Day 1 blood methanol levels measured 5 minutes after

the termination of exposure in NP rats that had received the same concentrations of methanol as those animals in the main part of the experiment were 1.00 ± 0.21, 2.24 ± 0.20, and 8.65 ± 0.40 mg/mL for those exposed to 5,000, 10,000 and 20,000 ppm methanol, respectively. Evidence of maternal toxicity included a slightly unsteady gait in the 20,000 ppm group during the first few days of exposure. Maternal bodyweight gain and food intake were unaffected by methanol. Dams were sacrificed on GD20, and 13-30 litters/group were evaluated. No effect was observed on the number of corpora lutea or implantations or the percentage of dead or resorbed fetuses. Statistical evaluations included analysis of variance (ANOVA) for body weight effect, Kruskal-Wallis test for endpoints such as litter size and viability and Fisher's exact test for malformations. Fetal body weight was significantly reduced at concentrations of 10,000 and 20,000 ppm by 7% and 12–16%, respectively, compared to controls. An increased number of litters with skeletal and visceral malformations were observed at ≥10,000 ppm, with statistical significance obtained at 20,000 ppm. Numbers of litters with visceral malformations were 0/15, 5/15, and 10/15 and with skeletal malformations were 0/15, 2/15, and 14/15 at 0, 10,000, and 20,000 ppm, respectively. Visceral malformations included exencephaly and encephaloceles. The most frequently observed skeletal malformations were rudimentary and extra cervical ribs. The developmental and maternal NOAELs for this study were identified as 5,000 ppm (6,552 mg/m^3) and 10,000 ppm (13,104 mg/m^3), respectively.

NEDO (1987) sponsored a teratology study in Sprague-Dawley rats that included an evaluation of postnatal effects in addition to standard prenatal endpoints. Thirty-six pregnant females/group were exposed to 0, 200, 1,000, or 5,000 ppm (0, 262, 1,310, and 6,552 mg/m^3) methanol vapors (reagent grade) on GD7–GD17 for 22.7 hours/day. Statistical significance of results was evaluated by t-test, Mann-Whitney U test, Fisher's exact test, and/or Armitage's χ^2 test.

Contrary to the Nelson et al. (1985) report of a 10,000 ppm NOAEL for this rat strain, in the prenatal portion of the NEDO (1987) study, reduced body weight gain and food and water intake during the first 7 days of exposure were reported for dams in the 5,000 ppm group. However, it was not specified if these results were statistically significant. One dam in the 5,000 ppm group died on GD19, and one dam was sacrificed on GD18 in moribund condition. On GD20, 19-24 dams/group were sacrificed to evaluate the incidence of reproductive deficits and such developmental parameters as fetal viability, weight, sex, and the occurrence of malformations. The reported reproductive and fetal effects are summarized in Table 4-2. The authors suggest that adverse effects (an increase in late-term resorptions, decreased live fetuses, reduced fetal weight, and increased frequency of litters with fetal malformations, variations, and delayed ossifications) were limited to the 5,000 ppm group. However, dose-response analyses indicate statistically significant linear trends for more than one reproductive/fetal effect in the

F_1 rats, including number of pre-implantation resorptions ($p < 0.01$), pre-implantation resorption rate ($p < 0.01$) and bifurcated vertebral center ($p < 0.01$) (ERG, 2009).

Postnatal effects of methanol inhalation were evaluated in the remaining 12 dams/group that were permitted to deliver and nurse their litters. Again, the authors suggest that effects were limited to the 5,000 ppm group, including a 1-day prolongation of the gestation period and reduced post-implantation survival, number of live pups/litter, and survival on PND4 (Table 4-3). However, dose-response analyses indicate statistically significant linear trends for post-implantation embryo survival rate ($p < 0.01$) and number of surviving pups on postnatal day 4 ($p < 0.03$) (ERG, 2009). When the delay in parturition was considered, methanol treatment had no effect on attainment of developmental milestones such as eyelid opening, auricle development, incisor eruption, testes descent, or vaginal opening. There were no adverse body weight effects in offspring from methanol treated groups. The weights of some organs (brain, thyroid, thymus, and testes) were reduced in 8-week-old offspring exposed to 5,000 ppm methanol during prenatal development.

Table 4-2 Reproductive and developmental toxicity in pregnant Sprague-Dawley rats exposed to methanol via inhalation during gestation

	Exposure concentration (ppm)			
Effect	**0**	**200**	**1,000**	**5,000**
Reproductive effects				
Number of pregnant females examined	19	24	22	21
Number of corpora lutea	17.0 ± 2.6	17.2 ± 2.7	16.4 ± 1.9	16.5 ± 2.4
Number of implantations	15.7 ± 1.6	15.0 ± 3.0	15.5 ± 1.2	14.5 ± 3.3
No. of pre-implantation resorptions	0.79 ± 0.85	0.71 ± 1.23	0.95 ± 0.65	1.67 ± 2.03
Early resorption	0.68 ± 0.75	0.71 ± 1.23	0.91 ± 0.61	0.67 ± 0.97
Late resorption	0.11 ± 0.32	0.0 ± 0.0	0.05 ± 0.21	1.00 ± 1.79
Number of live fetuses	14.95 ± 1.61	14.25 ± 3.54	14.55 ± 1.1	12.86 ± 4.04[a]
Sex ratio (M/F)	144/140	177/165	164/156	134/136
Fetal weight (male)	3.70 ± 0.24	3.88 ± 0.23	3.82 ± 0.29	3.02 ± 0.27[c]
Fetal weight (female)	3.51 ± 0.19	3.60 ± 0.25	3.60 ± 0.30	2.83 ± 0.26[c]
Total resorption rate (%)	11.2 ± 9.0	15.6 ± 21.3	10.6 ± 8.4	23.3 ± 22.7[a]
Pre-implantation resorption rate (%)[d]	6.6 ± 8.2	11.8 ± 18.7	4.9 ± 7.9	12.7 ± 16.5
Pre-implantation resorption rate (%)[e]	4.9 ± 5.2	5.4 ± 12.1	6.1 ± 4.0	14.5 ± 23.3
Early resorption rate (%)	4.3 ± 4.7	5.4 ± 12.1	5.8 ± 3.9	4.2 ± 6.1
Late resorption rate (%)	0.6 ± 1.9	0.0 ± 0.0	0.3 ± 1.3	10.4 ± 23.4[a]
Soft tissue malformations				
Number of fetuses examined	136	165	154	131
Abnormality at base of right subclavian	0.7 ± 2.87 (1)	0	0	0
Excessive left subclavian	0	0	0	3.5 ± 9.08 (3)
Ventricular septal defect	0	0.6 ± 2.92 (1)	0	47.6 ± 36.51 (16)[b]
Residual thymus	2.9 ± 5.91 (4)	2.4 ± 5.44 (4)	2.6 ± 5.73 (4)	53.3 ± 28.6 (20)[b]
Serpengious urinary tract	43.0 ± 24.64 (18)	35.2 ± 31.62 (19)	41.8 ± 38.45 (15)	22.1 ± 22.91 (13)
Skeletal abnormalities				
Number of fetuses examined	148	177	165	138
Atresia of foramen costotransversarium	23.5 ± 5.47 (3)	7.7 ± 1.3 (8)	3.5 ± 8.88 (4)	45.2 ± 25.18 (20)[b]
Patency of foramen costotransversium	0	0	0.6 ± 2.67 (1)	13.7 ± 20.58 (7)
Cleft sternum	0	0	0	5.6 ± 14.14 (3)
Split sternum	0	0	0	7.0 ± 14.01 (5)
Bifurcated vertebral center	0.8 ± 3.28 (1)	1.6 ± 5.61 (2)	3.0 ± 8.16 (3)	14.5 ± 16.69 (11)[b]

Effect	Exposure concentration (ppm)			
	0	200	1,000	5,000
Cervical rib	0	0	0	65.2 ± 25.95 (19)[b]
Excessive sublingual neuropore	0	0	0	49.9 ± 27.31 (19)
Curved scapula	0	0	0	0.7 ± 3.19 (1)
Waved rib	0	0	0	6.1 ± 11.84 (5)
Abnormal formation of lumbar vertebrae	0	0	0	0.7 ± 3.19 (1)

[a] $p < 0.05$
[b] $p < 0.01$
[c] $p < 0.001$, as calculated by the authors.
[d]Pre-implantation resorption/corpora lutea x 100 (%)
[e](Early + late resorption) / implantation x 100 (%)
Values are means ± S.D. Values in parentheses are the numbers of litters.

Source: NEDO (1987).

Table 4-3 Reproductive parameters in Sprague-Dawley dams exposed to methanol during pregnancy, and then allowed to deliver their pups

Parameter	Exposure concentration (ppm)			
	0	200	1,000	5,000
Number of dams	12	12	12	12
Duration of gestation (days)	21.9 ± 0.3	21.9 ± 0.3	21.9 ± 0.3	22.6 ± 0.5[c]
Number of implantations	15.8 ± 1.6	14.8 ± 1.2	15.3 ± 1.3	14.6 ± 1.1[a]
Number of pups	15.2 ± 1.6	14.4 ± 1.3	14.5 ± 1.4	13.1 ± 2.2[a]
Number of live pups	15.2 ± 1.6	14.1 ± 1.4	14.3 ± 1.4	12.6 ± 2.5[b]
Number of live pups on PND4	15.0 ± 1.7 (2)	13.8 ± 1.5 (3)	14.2 ± 1.6 (1)	10.3 ± 2.8 (9)[c]
Sex ratio (M/F)	88/94	87/85	103/70[a]	75/81
Postimplantation embryo survival rate	96.3 ± 4.2	94.9 ± 5.1	93.6 ± 6.1	86.2 ±16.2[a]

[a] $p < 0.05$
[b] $p < 0.01$
[c] $p < 0.001$
p values presented as calculated by the authors.
Values are means ± S.D. Values in parentheses are the numbers of litters.

Source: NEDO (1987).

NEDO (1987) contains an account of a two-generation reproductive study that evaluated the effects of pre- and postnatal methanol (reagent grade) exposure (20 hours/day) on reproductive and other organ systems of Sprague-Dawley rats. The F_0 generation (30 males and

30 females per exposure group)[34] was exposed to 0, 10, 100, and 1,000 ppm (0, 13.1, 131, and 1,310 mg/m^3) from 8 weeks old to the end of mating (males) or to the end of lactation period (females). The F_1 generation was exposed to the same concentrations from birth to the end of mating (males) or to weaning of F_2 pups 21 days after delivery (females). Males and females of the F_2 generation were exposed from birth to 21 days old (one animal/sex/litter was exposed to 8 weeks of age). NEDO (1987) noted reduced brain, pituitary, and thymus weights, and early testicular descent in the offspring of F_0 and F_1 rats exposed to 1,000 ppm methanol. The early testicular descent is believed to be an indication of earlier fetal development as indicated by the observation that it was correlated with increased pup body weight. However, no histopathologic effects of methanol were observed. As discussed in the report, NEDO (1987) sought to confirm the possible compound-related effect of methanol on the brain by carrying out an additional study in which Sprague-Dawley rats were exposed to 0, 500, 1,000, and 2,000 ppm (0, 655, 1,310, and 2,620 mg/m^3) methanol from the first day of gestation through the F_1 generation (see Section 4.4.2).

Rogers et al. (1993b) evaluated development toxicity in pregnant female CD-1 mice exposed to air or 1,000, 2,000, 5,000, 7,500, 10,000, or 15,000 ppm (0, 1,310, 2,620, 6,552, 9,894, 13,104, and 19,656 mg/m^3) methanol vapors (≥ 99.9% purity) in a chamber for 7 hours/day on GD6-GD15 in a 3-block design experiment. The numbers of mice exposed at each dose were 114, 40, 80, 79, 30, 30, and 44, respectively. During chamber exposures to air or methanol, the mice had access to water but not food. In order to determine the effects of the chamber exposure conditions, an additional 88 control mice were not handled and remained in their cages; 30 control mice were not handled but were food deprived for 7 hours/day on GD6-GD15. Effects in dams and litters were statistically analyzed using the General Linear Models procedure and multiple *t*-test of least squares means for continuous variables and the Fisher's exact test for dichotomous variables. An analysis of plasma methanol levels in 3 pregnant mice/block/treatment group on GD6, GD10, and GD15 revealed a dose-related increase in plasma methanol concentration that did not seem to reach saturation levels, and methanol plasma levels were not affected by gestation stage or number of previous exposure days. Across all 3 days, the mean plasma methanol concentrations in pregnant mice were approximately 97, 537, 1,650, 3,178, 4,204, and 7,330 µg/mL in the 1,000, 2,000, 5,000, 7,500, 10,000, and 15,000 ppm exposure groups, respectively.

The dams exposed to air or methanol in chambers gained significantly less weight than control dams that remained in cages and were not handled. There were no methanol-related reductions in maternal body weight gain or overt signs of toxicity. Dams were sacrificed on

[34] A second control group of 30 animals/sex was maintained in a separate room to "confirm that environmental conditions inside the chambers were not unacceptable to the animals."

GD17 for a comparison of developmental toxicity in methanol-treated groups versus the chamber air-exposed control group. Fetuses in all exposure groups were weighed, assessed for viability, and examined for external malformations. Fetuses in the control, 1,000, 2,000, 5,000, and 15,000 ppm groups were also examined for skeletal and visceral defects. Incidence of developmental effects is listed in Table 4-4. A statistically significant increase in cervical ribs/litter was observed at concentrations of 2,000, 5,000, and 15,000 ppm. At doses of ≥5,000 ppm the incidences of cleft palates/litter and exencephaly/litter were increased with statistical significance achieved at all concentrations with the exception of exencephaly which increased but not significantly at 7,500 ppm.[35] A significant reduction in live pups/litter was noted at ≥ 7,500 ppm, with a significant increase in fully resorbed litters occurring at ≥ 10,000 ppm. Fetal weight was significantly reduced at ≥ 10,000 ppm. Rogers et al. (1993b) identified a developmental NOAEL and LOAEL of 1,000 ppm and 2,000 ppm, respectively. They also provide BMD maximum likelihood estimates (referred to by the authors as MLE) and estimates of the lower 95% confidence limit on the BMD (BMDL; referred to by the authors as benchmark dose [BMD]) for 5% and 1% added risk, by applying a log-logistic dose-response model to the mean percent/litter data for cleft palate, exencephaly and resorption. The BMD_{05} and $BMDL_{05}$ values for added risk estimated by Rogers et al. (1993b) are listed in Table 4-5. From this analysis, the most sensitive indicator of developmental toxicity was an increase in the proportion of fetuses per litter with cervical rib anomalies. The most sensitive BMDL and BMD from this effect for 5% added risk were 305 ppm (400 mg/m^3) and 824 ppm (1,080 mg/m^3), respectively.[36]

[35] Due to the serious nature of this response and the relative lack of a response in controls, all incidence of exencephaly reported in this study at 5,000 ppm or higher are considered biologically significant.

[36] The BMD analysis of the data described in Section 5 was performed similarly using, among others, a similar nested logistic model. However, the Rogers et al. (1993b) analysis was performed using added risk and external exposure concentrations, whereas the analyses in Section 5 used extra risk and internal dose metrics that were then converted to human equivalent exposure concentrations.

Table 4-4 Embryonic and Developmental effects in CD-1 mice after methanol inhalation

	Exposure concentration (ppm)						
Effects	**0**	**1,000**	**2,000**	**5,000**	**7,500**	**10,000**	**15,000**
Endpoint							
No. live pups/litter	9.9	9.5	12.0	9.2	8.6[b]	7.3[c]	2.2[c]
No. fully resorbed litters	0	0	0	0	3	5[a]	14[c]
Fetus weight (g)	1.20	1.19	1.15	1.15	1.17	1.04[c]	0.70[c]
Cleft palate/ litter (%)	0.21	0.65	0.17	8.8[b]	46.6[c]	52.7[c]	48.3[c]
Exencephaly/litter (%)	0	0	0.88	6.9[a]	6.8	27.4[c]	43.3[c]
Anomalies							
Cervical ribs/litter (%)	28	33.6	49.6[b]	74.4[c]	ND	ND	60.0[a]
Sternebral defects/litter (%)	6.4	7.9	3.5	20.2[c]	ND	ND	100[c]
Xiphoid defects/litter (%)	6.4	3.8	4.1	10.9	ND	ND	73.3[c]
Vertebral arch defects/litter (%)	0.3	ND	ND	1.5	ND	ND	33.3[c]
Extra lumbar ribs/litter (%)	8.7	2.5	9.6	15.6	ND	ND	40.0[c]
Ossifications (values are means of litter means)							
Sternal	5.96	5.99	5.94	5.81	ND	ND	5.07[c]
Caudal	5.93	6.26	5.71[a]	5.42	ND	ND	3.20[a]
Metacarpal	7.96	7.92	7.96	7.93	ND	ND	7.60[b]
Proximal phalanges	7.02	7.04	7.04	6.12	ND	ND	3.33[c]
Metatarsals	9.87	9.90	9.87	9.82	ND	ND	8.13[c]
Proximal phalanges	7.18	7.69	6.91	5.47	ND	ND	0[c]
Distal phalanges	9.64	9.59	9.57	8.46[b]	ND	ND	4.27[c]
Supraoccipital score+	1.40	1.65	1.57	1.48	ND	ND	3.20[c]

[a]$p < 0.05$
[b]$p < 0.01$
[c]$p < 0.001$
p values as calculated by the authors.
ND = Not determined. [+] = on a scale of 1–4, where 1 is fully ossified and 4 is unossified.

Source: Rogers et al. (1993b).

Table 4-5 Benchmark doses at two added risk levels

Endpoint	BMD_{05} (ppm)	$BMDL_{05}$ (ppm)	BMD_{01} (ppm)	$BMDL_{01}$ (ppm)
Cleft Palate (CP)	4,314	3,398	2,717	1,798
Exencephaly (EX)	5,169	3,760	2,122	784
CP and EX	3,713	3,142	2,381	1,816
Resorptions (RES)	5,650	4,865	3,749	2,949
CP, EX, and RES	3,667	3,078	2,484	1,915
Cervical ribs	824	305	302	58

Source: Rogers et al. (1993b).

Bolon et al. (1993) performed an inhalation exposure developmental study in CD-1 mice under conditions similar to Rogers et al. (1993b). To determine the determine the developmental phase specificity of methanol induced fetal effects, they evaluated developmental toxicity in CD-1 mice (n = 20-27/group) following inhalation exposure (6 hr/day) to 5,000, 10,000, or 15,000 ppm methanol either throughout organogenesis (GD 6–15), during the period of neural tube development and closure (GD 7–9), or during a time of potential neural tube reopening (GD9-GD11). To better define the critical gestational window of susceptibility, mice (n = 8-15/group) were exposed to 15,000 ppm on GD 7, GD8 or GD9 or for 2 days on GD7-GD8 or GD8-GD9. The results of the dose-response portion of the study are shown in Table 4-6 and the results of the "window of susceptibility" portion of the study are shown in Table 4-7.

Table 4-6 Developmental Phase-Specific Embryotoxicity and Teratogenicity in CD-1 mice after methanol inhalation

Gestational Days of Exposure	**GD7 to GD9**				**GD9 to GD11**
Methanol Concentration	**0**	**5,000 ppm**	**10,000 ppm**	**15,000 ppm**	**15,000 ppm**
No. of pregnant dams	22	27	20	20	17
No. implants/litter[a]	12.5 ± 0.4	11.6 ± 0.5	12.8 ± 0.4	13.4 ± 0.4	12.8 ± 0.4
Embryotoxicity					
% Resorptions/Litter[b]	2.7	10.5	16.6	46.2[c]	6.9
% Litters with ≥ 1 Resorption[b]	27.3	55.6[c]	75.0[c]	90.0[c]	41.2
No. (%) of live fetuses/litter[a]	12.0 ± 0.4 (98)	10.8 ± 0.5 (99)	11.2 ± 0.6 (100)	7.9 ± 1.1 (91)	10.5 ± 0.9 (87)
Fetal body wt (GD 17)[a], in grams(g)	0.92 ± 0.05	0.96 ± 0.01	0.91 ± 0.01	0.82 ± 0.02	0.83 ± 0.01
Maternal body wt (GD 17)[a], in grams (g)	51.2 ± 0.9	49.7 ± 0.8	51.1 ± 1.1	45.9 ± 1.8	51.1 ± 1.1
Developmental Toxicity	**Percentage of affected litters (percentage affected fetuses)**				
No. of litters examined	22	27	20	17	17
Neural tube defects	0	0	30 (3.6)	65[c] (14.7)	0
Cleft palate	9 (0.7)	4 (0.3)	50[c] (14.6)	88[c] (50.4)	53 (20.1)
Renal pelvic dilatation					
Cavitation	41 (4.3)	100[c] (49.4)	90[c] (31.2)	75[c] (44.9)	100 (36.9)
Hydronephrosis	0	7 (0.9)	45[c] (13.9)	53[c] (11.3)	18 (5.9)
Ocular defects	0	0	10[c] (1.3)	53[c] (17.2)	0
Limb anomalies	0	0	5 (0.5)	0	41 (24.7)
Tail anomalies	0	0	40[c] (8.8)	65[c] (15.1)	71 (12.4)

[a]Values represent mean ± standard error.

[b]Embryos from 3/20 litters completely resorbed at 15,000 ppm.

[c]Denotes lowest dose that was significantly different from control by Shirley's test, $p < 0.05$

Source: Bolon et al. (1993).

Table 4-7 Developmental phase-specific embryotoxicity in CD-1 mice induced by methanol inhalation (15,000 ppm) during neurulation

	Gestational Days of Exposure						
	Control (GD7-GD9)[b]	**GD7**	**GD8**	**GD9**	**GD7-GD8**	**GD8-GD9**	**GD7-GD9[b]**
No. of pregnant dams	22	15	13	8	14	11	20
No. of implants/litter[a]	12.5 ± 0.4	11.3 ± 0.9	12.9 ± 0.6	13.2 ± 0.8	12.9 ± 0.5	12.7 ± 1.1	13.4 ± 0.4
% Resorptions/litter	2.7	38.6 [c]	4.2	2.3	41.9 [c]	10.7	46.2[c]
% Litters with ≥ 1 Resorption[b]	27.3	86.7 [c]	30.8	25.0	100 [c]	45.5	90.0 [c]
No. (%) of live fetuses/litter[a]	12.0 ± 0.04 (98.3)	7.7 ± 1.2[c] (92.3)	12.2 ± 0.6 (98.9)	12.9 ± 0.8 (99.1)	8.4 ± 1.0 (95.5)	11.7 ± 1.3 (98.7)	7.9 ± 1.1 (91.0)
Fetal body wt (GD17)[a], (grams[g])	0.92 ± 0.05	0.99 ± 0.03	0.93 ± 0.02	0.99 ± 0.02	0.81 ± 0.02	0.90 ± 0.03	0.82± 0.02[c]
Maternal body wt (GD 17)[a], (grams [g])							
Dam with uterus	51.2 ± 0.9	45.3 ± 2.0	54.0 ± 1.3	54.3 ± 2.5	46.1 ± 1.8	52.9 ± 2.5	45.9 ± 1.8
Dam minus uterus	36.9 ± 2.1	34.8 ± 0.9	35.8 ± 0.4	34.3 ± 1.4	33.5 ± 0.8	35.1 ± 1.0	Not Done
Neural tube defects[d]	0	8 (1.4)	15 (2.2)	0	67 [c] (15.6)	27 (1.9)	65 [c] (14.7)

[a]Values represent mean ± standard error

[b]Values from Table 4-6

[c]Significantly different from controls by Dunn's test, $a_c = 0.05$

[d]Percentage affected litters (Percentage affected fetuses)

Source: Bolon et al. (1993).

Bolon et al. (1993) reported that transient neurologic signs and reduced body weights were observed in up to 20% of dams exposed to 15,000 ppm. Embryotoxicity (increased resorptions, reduced fetal weights, and/or fetal malformations) was apparent at 10,000 and 15,000 ppm, while 3-day exposures at 5,000 ppm yielded only an increase in the percentage of litters with one or more resorptions. Developmental toxicity included neural and ocular defects, cleft palate, hydronephrosis, deformed tails, and limb (paw and digit) anomalies at 10,000 ppm (GD 7-9). The only endpoint increased at 5,000 ppm was renal pelvic dilatation (cavitation). Neural tube defects and ocular lesions occurred after methanol inhalation between GD7-GD9, while limb anomalies were induced only during GD9-GD11; cleft palate and hydronephrosis were observed after exposure during either period. Table 5 (of the Bolon et al. study) shows that neural tube effects are most likely to develop from exposure on GD8 and resorptions are most likely to occur from exposure on GD7. These findings indicate that the spectrum of teratogenic effects depended upon both the timing (i.e., stage of embryonic development) and the number of methanol exposures.

Bolon et al. (1994) observed a spectrum of cephalic neural tube defects in near-term (gestation day 17 [GD17]) mouse fetuses following maternal inhalation of methanol at a high concentration (15,000 ppm) for 6 hr/day during neurulation (GD7-GD9). Their results suggest that (1) exposure to a high concentration of methanol injures multiple stem cell populations in the neurulating mouse embryo and (2) significant neural pathology may remain in older conceptuses even in the absence of gross lesions.

Rogers and Mole (1997) investigated the critical period of sensitivity to the developmental toxicity of inhaled methanol in the CD-1 mouse by exposing 12-17 pregnant females to 0 or 10,000 ppm (0 and 13,104 mg/m^3), 7 hours/day on 2 consecutive days during GD6–GD13, or to a single exposure to the same methanol concentration during GD5-GD9. Another group of mice received a single 7-hour exposure to methanol at 10,000 ppm. The latter animals were sacrificed at various time intervals up to 28 hours after exposure. Blood samples were taken from these animals to measure the concentration of methanol in the serum. Serum methanol concentrations peaked at ~4 mg/mL 8 hours after the onset of exposure. Methanol concentrations in serum had declined to pre-exposure levels after 24 hours. All mice in the main body of the experiment were sacrificed on GD17, and their uteri removed. The live, dead, and resorbed fetuses were counted, and all live fetuses were weighed, examined externally for cleft palate, and then preserved. Skeletal abnormalities were determined after the carcasses had been cleaned and eviscerated. Cleft palate, exencephaly, and skeletal defects were observed in the fetuses of exposed dams. For example, cleft palate was observed following 2-day exposures to methanol on GD6-GD7 through GD11-GD12. These effects also were apparent in mice receiving a single exposure to methanol on GD5-GD9. This effect peaked when the dams were exposed on GD7. Exencephaly showed a similar pattern of development in response to methanol exposure. However, the data indicated that cleft palate and exencephaly might be competing malformations, since only one fetus displayed both features. Skeletal malformations included exoccipital anomalies, atlas and axis defects, the appearance of an extra rudimentary rib on cervical vertebra No.7, and supernumerary lumbar ribs. In each case, the maximum time point for the induction of these defects appeared to be when the dams were exposed to methanol on or near GD7. When dams were exposed to methanol on GD5, there was also an increased incidence of fetuses with 25 presacral vertebrae (26 is normal). However, an increased incidence of fetuses with 27 presacral vertebrae was evident when dams were exposed on GD7. These results indicate that gastrulation and early organogenesis is a period of increased embryonic sensitivity to methanol.

Burbacher, et al. (1999b; 1999a) carried out toxicokinetic and reproductive/developmental studies of methanol in *M. fascicularis* monkeys that were published by the Health Effects Institute (HEI) in a two-part monograph. Some of the data were

subsequently published in the open scientific literature (Burbacher et al., 2004b; Burbacher et al., 2004a). The experimental protocol featured exposure to 2 cohorts of 12 monkeys/group to low exposure levels (relative to the previously discussed rodent studies) of 0, 200, 600, or 1,800 ppm (0, 262, 786, and 2,359 mg/m^3) methanol vapors (99.9% purity), 2.5 hours/day, 7 days/week, during a premating period and mating period (~180 days combined) and throughout the entire gestation period (~168 days). The monkeys were 5.5–13 years old. The study included an evaluation of maternal reproductive performance and tests to assess infant postnatal growth and newborn health, reflexes, behavior, and development of visual, sensorimotor, cognitive, and social behavioral function (see Section 4.4.2 for a review of the developmental neurotoxicity findings from this study). Blood methanol levels, clearance, and the appearance of formate were also examined and are discussed in Section 3.2.

With regard to reproductive parameters, there was a statistically significant decrease (p = 0.03) in length of pregnancy in all treatment groups, as shown in Table 4-8. Cesarean section (C-section) deliveries performed in the methanol exposure groups did not impact this finding (decreased length of pregnancy was observed in vaginally delivered animals). C-section deliveries were performed in response to "signs of difficulty" in the pregnancy, but it is not clear whether this is an indication of either reproductive dysfunction or fetal risk due to methanol exposure. Maternal menstrual cycles, conception rate, and live birth index were all unaffected by exposure. There were also no signs of an effect on maternal weight gain or clinical toxicity among the dams.

While pregnancy duration was virtually the same in all exposure groups, there were some indications of increased pregnancy duress only in methanol-exposed monkeys. C-sections were done in 2 monkeys from the 200 ppm group and 2 from the 600 ppm group due to vaginal bleeding, presumed, but not verified, to be from placental detachment.[37] A monkey in the 1,800 ppm group also received a C-section after experiencing nonproductive labor for 3 nights. In addition, signs of prematurity were observed in 1 infant from the 1,800 ppm group that was born after a 150-day gestation period. The authors speculated that the shortened gestation length could be due to a direct effect of methanol on the fetal hypothalamus-pituitary-adrenal (HPA) axis or an indirect effect of methanol on the maternal uterine environment. Other fetal parameters such as crown-rump length and head circumference were unchanged among the groups. Infant growth and tooth eruption were unaffected by prenatal methanol exposure.

[37] Burbacher, , et al. (2004a) and Burbacher, et al. (2004b) note, however, that in studies of pregnancy complication in alcohol- exposed human subjects, an increased incidence of uterine bleeding and abrutio placenta has been reported.

Table 4-8 Reproductive parameters in monkeys exposed via inhalation to methanol during prebreeding, breeding, and pregnancy

Exposure (ppm)	Conception rate	Weight gain (kg)	Pregnancy duration (days)[a]	Live born delivery rate
0	9/11	1.67 ± 0.07	168 ± 2	8/9
200	9/12	1.27 ± 0.14	160 ± 2[b]	9/9
600	9/11	1.78 ± 0.25	162 ± 2[b]	8/9
1,800	10/12	1.54 ± 0.20	162 ± 2[b]	9/10

[a]Live-born offspring only;
[b] $p < 0.05$, as calculated by the authors.
Values are means ± SE.

Source: Burbacher, et al. (2004a).

In later life, 2 females out of the total of 9 offspring in the 1,800 ppm group experienced a wasting syndrome at 12 and 17 months of age. Food intake was normal and no cause of the syndrome could be determined in tests for viruses, hematology, blood chemistry, and liver, kidney, thyroid, and pancreas function. Necropsies revealed gastroenteritis and severe malnourishment. No infectious agent or other pathogenic factor could be identified. Thus, it appears that a highly significant toxicological effect on postnatal growth can be attributed to prenatal methanol exposure at 1,800 ppm (2,300 mg/m^3).

In summary, the Burbacher, et al. (1999b; 1999a) studies suggest that methanol exposure can cause reproductive effects, manifested as a shortened mean gestational period, pregnancy complications that precipitated delivery via a C-section, and developmental neurobehavioral effects which may or may not be related to the shortened gestational period (see Section 4.4.2). The low exposure of 200 ppm may signify a LOAEL for reproductive effects. However, the decrease in gestational length was marginally significant. Also, this effect did not appear to be dose related, the greatest gestational period decrease having occurred at the lowest (200 ppm) exposure level. Thus, a clear NOAEL or LOAEL cannot be determined from this study.

In a study of the testicular effects of methanol, Cameron et al. (1984) exposed 5 male Sprague-Dawley rats/group to methanol vapor, 8 hours/day, 5 days/week for 1, 2, 4, and 6 weeks at 0, 200, 2,000, or 10,000 ppm (0, 262, 2,620, and 13,104 mg/m^3). The authors examined the possible effects of methanol on testicular function by measuring blood levels of testosterone, luteinizing hormone (LH), and follicular stimulating hormone (FSH) using radioimmunoassay. When the authors tabulated their results as a percentage of the control value for each duration series, the most significant changes were in blood testosterone levels of animals exposed to 200 ppm methanol, the lowest concentration evaluated. At this exposure level, animals exposed for 6 weeks had testosterone levels that were 32% of those seen in controls; however, higher

concentrations of methanol were associated with testosterone levels that were closer to those of controls. The lack of a clear dose-response is not necessarily an indication that the effect is not related to methanol. The higher concentrations of methanol could be causing other effects (e.g., liver toxicity) which can influence the results. Male rats exposed to 10,000 ppm methanol for 6 weeks displayed blood levels of LH that were about 3 times higher (mean ± S.D.) than those exposed to air (311 ± 107% versus 100 ± 23%). In discussing their results, the authors placed greater emphasis on the observation that an exposure level equal to the ACGIH TLV (200 ppm) had caused a significant depression in testosterone formation in male rats.

A follow-up study report by the same research group (Cameron et al., 1985) described the exposure of 5 male Sprague-Dawley rats/group, 6 hours/day for either 1 day or 1 week, to methanol, ethanol, n-propanol, or n-butanol at their respective TLVs. Groups of animals were sacrificed immediately after exposure or after an 18-hour recovery period, and the levels of testosterone, LH, and corticosterone measured in serum. As shown in Table 4-9, the data were consistent with the ability of these aliphatic alcohols to cause a transient reduction in the formation of testosterone. Except in the case of n-butanol, rapid recovery from these deficits can be inferred from the 18-hour postexposure data.

Table 4-9 Mean serum levels of testosterone, luteinizing hormone, and corticosterone (± S.D.) in male Sprague-Dawley rats after inhalation of methanol, ethanol, n-propanol or n-butanol at threshold limit values

Condition	TLV (ppm)	Single-day exposure		One-week exposure	
		End of exposure	18 hr postexposure	End of exposure	18 hr postexposure
Testosterone (as a percentage of control)					
Control	--	100 ± 17	100 ± 20	100 ± 26	100 ± 17
Methanol	200	41 ± 16[a]	98 ± 18	81 ± 22	82 ± 27
Ethanol	1,000	64 ± 12[a]	86 ± 16	88 ± 14	101 ± 13
n-Propanol	200	58 ± 15[a]	81 ± 13	106 ± 28	89 ± 17
n-Butanol	50	37 ± 8[a]	52 ± 22[a]	73 ± 34	83 ± 18
Luteinizing hormone					
Control	--	100 ± 30	100 ± 35	100 ± 28	100 ± 36
Methanol	200	86 ± 32	110 ± 40	78 ± 13	70 ± 14
Ethanol	1,000	110 ± 22	119 ± 54	62 ± 26	81 ± 17
n-Propanol	200	117 ± 59	119 ± 83	68 ± 22	96 ± 28
n-Butanol	50	124 ± 37	115 ± 28	78 ± 26	98 ± 23
Corticosterone					
Control	--	100 ± 20	ND	100 ± 21	ND
Methanol	200	115 ± 18	ND	74 ± 26	ND
Ethanol	1,000	111 ± 32	ND	60 ± 25	ND
n-Propanol	200	112 ± 21	ND	79 ± 14	ND
n-Butanol	50	143 ± 11[a]	ND	85 ± 26	ND

[a]$p < 0.05$, as calculated by the authors.
ND = No data.

Source: Cameron et al. (1985).

In a series of studies that are relevant to the reproductive toxicity of methanol in males, Lee et al. (1991) exposed 8-week-old male Sprague-Dawley rats (9-10/group) to 0 or 200 ppm (0 and 262 mg/m^3) methanol, 8 hours/day, 5 days/week, for 1, 2, 4, or 6 weeks to measure the possible treatment effects on testosterone production. Study results were evaluated by one factor ANOVA followed by Student's *t*-test. In the treated rats, there was no effect on serum testosterone levels, gross structure of reproductive organs, or weight of testes and seminal vesicles. Lee et al. (1991) also studied the in vitro effect of methanol on testosterone production from isolated testes, but saw no effect on testosterone formation either with or without the addition of human chorionic gonadotropin hormone.

In a third experiment from the same report, Lee et al. (1991) examined testicular histopathology to determine if methanol exposure produced lesions indicative of changing testosterone levels; the effects of age and folate status were also assessed. This is relevant to the potential toxicity of methanol because folate is the coenzyme of tetrahydrofolate synthetase, an enzyme that is rate limiting in the removal of formate. Folate deficiency would be expected to cause potentially toxic levels of formate to be retained. The same authors examined the relevance of folate levels, and by implication, the overall status of formate formation and elimination in mediating the testicular functions of Long-Evans rats. Groups of 4-week-old male Long-Evans rats were given diets containing either adequate or reduced folate levels plus 1% succinylsulfathiazole, an antibiotic that, among other activities, [38] would tend to reduce the folate body burden. At least 9 rats/dietary group/dose were exposed to 0, 50, 200, or 800 ppm (0, 66, 262, and 1,048 mg/m^3) methanol vapors starting at 7 months of age while 8-12 rats/dietary group/dose were exposed to 0 or 800 ppm methanol vapors at 15 months of age. The methanol exposures were conducted continuously for 20 hours/day for 13 weeks. Without providing details, the study authors reported that visual toxicity and acidosis developed in rats fed the low folate diet and exposed to methanol. No methanol-related testicular lesions or changes in testes or body weight occurred in rats that were fed either the folate sufficient or deficient diets and were 10 months old at the end of treatment. Likewise, no methanol-lesions were observed in 18-month-old rats that were fed diets with adequate folate. However, the incidence but not severity of age-related testicular lesions was increased in the 18-month-old rats fed folate-deficient diets. Subcapsular vacuoles in germinal epithelium were noted in 3/12 control rats and 8/13 rats in the 800 ppm group. One rat in the 800 ppm group had atrophied seminiferous tubules and another had Leydig cell hyperplasia. These effects, as well as the transient decrease in testosterone levels observed by Cameron et al. (1985; 1984), could be the result of chemically-related strain on the rat system as it attempts to maintain hormone homeostasis.

Dorman et al. (1995) conducted a series of in vitro and in vivo studies of developmental toxicity in ICR BR (CD-1) mice associated with methanol and formate exposure. The studies used HPLC grade methanol and appropriate controls. PK and developmental toxicity parameters were measured in mice exposed to a 6-hour methanol inhalation (10,000 or 15,000 ppm), methanol gavage (1.5 g/kg) or sodium formate (750 mg/kg by gavage) on GD8. In the in vivo inhalation study, 12-14 dams/group were exposed to 10,000 ppm methanol for 6 hours on GD8,[39] with and without the administration of fomepizole to inhibit the metabolism of methanol by ADH1. Dams were sacrificed on GD10, and folate levels in maternal RBC and conceptus

[38] Succinylsulfathiazone antibiotic may have a direct impact on the effects being measured, the extent of which was not addressed by the authors of this study.

[39] Dorman et al. (1995) state that GD8 was chosen because it encompasses the period of murine neurulation and the time of greatest vulnerability to methanol-induced neural tube defects.

(decidual swelling) were measured, as well as fetal neural tube patency (an early indicator of methanol-induced dysmorphogenic response). The effects observed included a transient decrease in maternal RBC and conceptus folate levels within 2 hours following exposure and a significant ($p < 0.05$) increase in the incidence of fetuses with open neural tubes (9.65% in treated versus 0 in control). These responses were not observed following sodium formate administration, despite peak formate levels in plasma and decidual swellings being similar to those observed following the 6-hour methanol inhalation of 15,000 ppm. This suggests that these methanol-induced effects are not related to the accumulation of formate. As this study provides information relevant to the identification of the proximate teratogen associated with developmental toxicity in rodents, it is discussed more extensively in Section 4.7.1.

4.3.3. Other Reproductive and Developmental Studies

Additional information relevant to the possible effects of methanol on reproductive and developmental parameters has been provided by experimental studies that have exposed experimental animals to methanol during pregnancy via i.p. injections (Sweeting et al., 2011; Degitz et al., 2004b; Rogers et al., 2004). Relevant to the developmental impacts of the chemical, a number of studies also have examined the effects of methanol when included in whole-embryo culture (Miller and Wells, 2011; Hansen et al., 2005; Harris et al., 2003; Andrews et al., 1998; Andrews et al., 1995; Andrews et al., 1993).

Pregnant female C57BL/6J mice received two i.p. injections of methanol on GD7 (Rogers et al., 2004). The injections were given 4 hours apart to provide a total dosage of 0, 3.4, and 4.9 g/kg. Animals were sacrificed on GD17 and the litters were examined for live, dead, and resorbed fetuses. Rogers et al. (2004) monitored fetal weight and examined the fetuses for external abnormalities and skeletal malformations. Methanol-related deficits in maternal and litter parameters observed by Rogers et al. (2004) are summarized in Table 4-10.

Table 4-10 Maternal and litter parameters when pregnant female C57BL/6J mice were injected i.p. with methanol

	Methanol dose (g/kg)		
Parameter	**0**	**3.4**	**4.9**
No. pregnant at term	43	13	24
Wt gain GD7–GD8 (g)	0.33 ± 0.10	0.37 ± 0.15	-0.24 ± 0.14[a]
Wt gain GD7–GD10 (g)	1.63 ± 0.18	2.20 ± 0.20	1.50 ± 0.20
Live fetuses/litter	7.5 ± 0.30	6.3 ± 0.5[a]	3.7 ± 0.4[a]
Resorbed fetuses/litter	0.4 ± 0.1	1.3 ± 0.4[a]	4.4 ± 0.4[a]
Dead fetuses/litter	0.1 ± 0.1	0	0.1 ± 0.1
Fetal weight (g)	0.83 ± 0.02	0.82 ± 0.03	0.70 ± 0.02[a]

[a]$p < 0.05$, as calculated by the authors.
Values are means ± SEM.

Source: Rogers et al. (2004).

Rogers et al. (2004) used a number of sophisticated imaging techniques, such as confocal laser scanning and fluorescence microscopy, to examine the morphology of fetuses excised at GD7, GD8, and GD9. They identified a number of external craniofacial abnormalities, the incidence of which was, in all cases, significantly increased in the high-dose group compared to controls. For some responses, such as microanophthalmia and malformed maxilla, the incidence was also significantly increased in animals receiving the lower dose. Fifteen compound-related skeletal malformations were tabulated in the report. In most cases, a dose-response effect was evident, resulting in statistically significant incidences in affected fetuses and litters, when compared to controls. Apparent effects of methanol on the embryonic forebrain included a narrowing of the anterior neural plate, missing optical vesicles, and holoprosencephaly (failure of the embryonic forebrain to divide). The authors noted that there was no sign of incipient cleft palate or exencephaly, as had been observed in CD-1 mice exposed to methanol via the oral and inhalation routes (Rogers et al., 1993b).

In order to collect additional information on cell proliferation and histological changes in methanol-treated fetuses, Degitz et al. (2004b) used an identical experimental protocol to that of Rogers et al. (2004) by administering 0, 3.4, or 4.9 g methanol/kg in distilled water i.p. (split doses, 4 hours apart) to C57BL/6J mice on GD7. Embryos were collected at various times on GD8 and GD10. Embryos from dams exposed to 4.9 g/kg and examined on GD8 exhibited reductions in the anterior mesenchyme, the mesenchyme subjacent to the mesencephalon and the base of the prosencephalon (embryonic forebrain), and in the forebrain epithelium. The optic pits were often lacking; where present their epithelium was thin and there were fewer neural crest cells in the mid- and hindbrain regions. At GD9, there was extensive cell death in areas

populated by the neural crest, including the forming cranial ganglia. Dose-related abnormalities in the development of the cranial nerves and ganglia were seen on GD7. In accordance with an arbitrary dichotomous scale devised by the authors, scores for ganglia V, VIII, and IX were significantly (not otherwise specified) reduced at all dose levels, and ganglia VII and X were reduced only at the highest dose. At the highest dose (4.9 g/kg), the brain and face were poorly developed and the brachial arches were reduced in size or virtually absent. Flow cytometry of the head regions of the embryos from the highest dose at GD8 did not show an effect on the proportion of cells in S-phase.

Cell growth and development were compared in C57BL/6J and CD-1 mouse embryos cultured in methanol (Degitz et al., 2004a). GD8 embryos, with 5-7 somites, were cultured in 0, 1, 2, 3, 4, or 6 mg methanol/mL for 24 hours and evaluated for morphological development. Cell death was increased in both strains in a developmental stage- and region-specific manner at 4 and 6 mg/mL after 8 hours of exposure. The proportions of cranial region cells in S-phase were significantly ($p < 0.05$) decreased at 6 mg/mL following 8- and 18-hour exposures to methanol. After 24 hours of exposure, C57BL/6J embryos had significantly ($p < 0.05$) decreased total protein at 4 and 6 mg/kg. Significant ($p < 0.05$) developmental effects were seen at 3, 4, and 6 mg/kg, with eye dysmorphology being the most sensitive endpoint. CD-1 embryos had significantly decreased total protein at 3, 4, and 6 mg/kg, but developmental effects were seen only at 6 mg/kg. It was concluded that the C57BL/6J embryos were more severely affected by methanol in culture than the CD-1 embryos.

Sweeting et al. (2011) performed a series of experiments in NZW rabbits, C57BL/6J mice and C3H mice to compare plasma pharmacokinetics of methanol and formic acid and embryotoxicity. For the teratology portion of the study, pregnant female mice and rabbits were given two i.p. doses of 2 g methanol/kg body weight on GD7 or GD8, for a total daily dose of 4 g methanol/kg body weight, or two i.p. doses of a saline vehicle control. Methanol exposure did not significantly impact fetal body weights for any of the species and strains tested. No statistically significant effects were reported on rabbit growth parameters and mortality. A 4.4-fold increase in tail abnormalities per litter, including shortening and absence, was reported in rabbit fetuses. However, due to the variability of this endpoint among litters, this difference was not statistically significant. Non-significant increases were reported in exposed rabbit litters for several other effects that were not observed in controls, including two fetuses with open posterior neuropores, one with an abdominal wall defect (prune belly), and three with frontal nasal hyperplasia. In C3H mice, methanol in utero exposure caused a 2-fold increase in fetal resorptions, but this increase was not statistically significant over saline treated controls ($p < 0.01$). In C57BL/6, methanol caused a 66% incidence of fetal ophthalmic abnormalities ($p < 0.001$) compared to a non-significant 3% incidence in C3H mice. Ophthalmic anomalies

were not observed in saline-exposed controls of either strain. Methanol also caused a 17% increase in fetal cleft palates in C57BL/6 mice ($p < 0.05$) compared to 0% in saline controls, and 0% in C3H mice treated with either methanol or saline. No increase in cephalic NTDs, an endpoint commonly observed in CD-1 mice, was observed in C57BL/6 or C3H mice. The different teratological results across these mouse strains could not be explained by differences in methanol or formic acid disposition (the pharmacokinetic results of this study are described in Section 3.2). The authors hypothesize that these differences in embryotoxicity could be due to strain differences in ADH activity and the amount of catalase available for ROS detoxification, or differences in other pathways that involve ROS formation. Sweeting et al. (2011) suggest that their findings indicate that rabbits are resistant to the teratogenic effects of methanol. However, because the critical gestational window for developmental effects could be different for rabbits versus mice, this claim needs to be verified over several gestational days, as has been done for mice. Postpartum lethality was nearly 2-fold higher in the methanol exposed (11%) versus control (5%) rabbit fetuses, and stillbirths were also increased (4% versus 0%). Though these increased incidences were not statistically significant, they may prove to be biologically significant given that postpartum lethality ("wasting syndrome") and a shortened gestational period were possible adverse outcomes observed in methanol exposed monkeys (see discussion of Burbacher, et al., (2004a; 1999a) in Section 4.3.2).

Table 4-11 displays the results of three studies of whole rodent embryos exposed to methanol (Miller and Wells, 2011; Hansen et al., 2005; Andrews et al., 1993). These data suggest that mouse embryos are more sensitive than rat embryos to the developmental effects of methanol. The Miller and Wells (2011) results also demonstrate that developmental effects from methanol exposure are increased in acatalasemic (aCat) mouse embryos over their wild type controls (C3HWT) and decreased in mouse embryos expressing human catalase (hCat) over their wild type controls (C57WT). These results suggest that embryonic catalase activity may be a determinant for teratological risk in mice following methanol-exposure.

Table 4-11 Developmental studies of rodent embryos exposed to methanol

Species/Strain/GD	Embryo Culture Dose & Duration	Effect	Reference
Mouse/CD-1/GD8	0, 2, 4, 6, or 8 mg/mL for 24 hrs	Decrease in developmental score and crown-rump length at 4 mg/mL and above. Embryo lethality at 8 mg/mL.	Andrews et al. (1993)
Rat/Sprague-Dawley/GD9	0, 2, 4, 8, 12 or 16 mg/mL for 24 hrs	Decrease in somite number, head length, and developmental score at 8 mg/mL and above. Embryo lethality at 12 mg/mL.	Andrews et al. (1993)
Mouse/CD-1/GD8	4 - 12 mg/mL for 24 hrs	Reduced VYS DNA and rotation at 4 mg/mL; reduced embryo DNA and protein, neural tube closure and viability at 8 mg/L; reduced VYS protein at 10 mg/L	Hansen et al. (2005)
Rat/Sprague-Dawley/GD10	8 - 20 mg/mL for 24 hrs	Reduced embryo protein and rotation at 8 mg/mL; reduced VYS DNA and protein, embryo DNA, and neural tube closure at 8 mg/L; reduced viability at 16 mg/L	Hansen et al. (2005)
Mouse/wild-type control (C57WT)/GD9	0 or 4 mg/L for 24 hrs	Decreased somites developed and turning, and increased heart rate at 4 mg/L relative to 0 mg/L. Decreased neuropore closure at 4 mg/L relative to 0 mg/L and hCat	Miller and Wells (2011)
Mouse/C57BL/6 with human catalase (hCat)/GD9	0 or 4 mg/L for 24 hrs	Increased crown rump length and heart rate relative to 0 mg/L. Increased somites at 4 mg/L relative to C57WT	Miller and Wells (2011)
Mouse/wild-type control C3HeB/FeJ (C3HWT)/GD9	0 or 4 mg/L for 24 hrs	Decreased somites developed at 4 mg/L relative to 0 mg/L.	Miller and Wells (2011)
Mouse/C3Ga.Cg-Catb/J acatalasemic (aCat)/GD9	0 or 4 mg/L for 24 hrs	Decreased somites developed at 4 mg/L relative to 0 mg/L. Reduced anterior neuropore closure and head length at 4 mg/L relative to 0 mg/L and C3HWT. Lower yolk sac diameters at 4 mg/L relative to C3HWT.	Miller and Wells (2011)

GD = Gestation Day; WT = Wild Type; VYS = visceral yolk sac

In contrast to the in vitro and in vivo findings of Dorman et al. (1995), Andrews et al. (1995) demonstrated that formate can induce similar developmental lesions in whole rat and mouse conceptuses. Using a similar experimental system as Andrews et al. (1993) to examine the developmental toxicity of formate and formic acid in comparison to methanol, Andrews et al. (1995) report that the formates are embryotoxic at doses that are four times lower than equimolar doses of methanol. Among the anomalies observed were open anterior and posterior neuropores, plus rotational defects, tail anomalies, enlarged pericardium, and delayed heart development. Andrews et al. (1998) showed that exposure to combinations of methanol and formate was less embryotoxic than would be expected based on simple toxicity additivity, suggesting that the

embryotoxicity observed following low-level exposure to methanol is mechanistically different from that observed following exposure to formate.

The whole embryo study by Hansen et al. (2005) also determined the comparative toxicity of methanol and its metabolites, formaldehyde and sodium formate, in GD8 mouse (CD-1) and GD10 rat (Sprague-Dawley) conceptuses. Whole embryos were incubated for 24 hours in media containing methanol (mouse: 4-12 mg/mL; rat: 8-20 mg/mL), formaldehyde (mouse: 1-6 µg/mL; rat: 1-8 µg/mL) and sodium formate (mouse: 0.5-4 mg/mL; rat: 0.5-8 mg/mL). In other experiments, the chemicals were injected directly into the amniotic space. The embryos were examined morphologically to determine growth and developmental parameters such as viability, flexure and rotation, crown-rump length, and neuropore closure. For each response, Table 4-12 provides a comparison of the concentrations or amounts of methanol, formaldehyde, and formate that resulted in statistically significant changes in developmental abnormalities compared to controls. For a first approximation, these concentrations or amounts may be taken as threshold-dose ranges for the specific responses under the operative experimental conditions. The data show consistently lower threshold values for the effects of formaldehyde compared to those of formate and methanol. The mouse embryos were more sensitive towards methanol toxicity than rat embryos, consistent with in vivo findings, whereas the difference in sensitivity disappeared when formaldehyde was administered. Hansen et al. (2005) hypothesized that, while the MOA for the initiation of the organogenic defects is unknown, the relatively low threshold levels of formaldehyde for most measured effects suggest formaldehyde involvement in the embryotoxic effects of methanol. Consistent with this hypothesis, formate, a subsequent metabolite of methanol and putative toxicant for the acute effects of methanol poisoning (acidosis, neurological deficits), did not appear to reproduce the methanol-induced teratogenicity in these whole embryo culture experiments.

Table 4-12 Reported thresholds concentrations (and author-estimated ranges) for the onset of embryotoxic effects when rat and mouse conceptuses were incubated in vitro with methanol, formaldehyde, and formate

	Mouse			Rat		
Parameter	**Methanol**	**Formaldehyde**	**Formate**	**Methanol**	**Formaldehyde**	**Formate**
In vitro incubation (mg/mL)						
Viability (%)	8.0	0.004	NS	16.0	0.006	2.0
Normal rotation (%)	4.0	0.003	0.5	8.0	0.003	4.0
CR [a] length	No change	No change	No change	No change	No change	No change
Neural tube closure (%)	8.0	0.001	2.0	12.0	No change	No change
Reduced embryo protein	8.0	0.003	4.0	8.0	0.004	2.0
Reduced VYS [b] protein	10.0	0.004	4.0	12.0	0.004	NR
Reduced embryo DNA	8.0	0.003	No change	12.0	0.003	NR
Reduced VYS DNA	4.0	0.001	0.5	12.0	0.003	NR
Microinjection (author-estimated dose ranges in µg)						
Viability (%)	46-89	0.003-0.5	1.01-1.5	46-89	1.01-1.5	1.51-4.0
Normal rotation (%)	1-45	0.003-0.5	0.03-0.5	46-89	1.01-1.5	0.51-1.0
CR [a] length	No change	No change	No change	No change	No change	No change
Neural tube closure (%)	1-45	0.003-0.5	1.01-1.5	No change	No change	1.01-1.5
Reduced embryo protein	1-45	0.501-1.0	No change	No change	1.51-2.0	0.51-1.0
Reduced VYS [b] protein	135-178	1.01-1.5	No change	No change	No change	1.01-1.5
Reduced embryo DNA	46-89	0.501-1.0	No change	No change	No change	0.51-1.0
Reduced VYS [b] DNA	1-45	0.003-0.5	0.03-0.5	No change	No change	0.51-1.0

[a]CR = crown-rump length,
[b]VYS = visceral yolk sac.
NR = not reported

Source: Hansen et al. (2005); Harris et al. (2004) (adapted).

Harris et al. (2003) provided biochemical evidence consistent with the concept that formaldehyde might be the ultimate embryotoxicant of methanol by measuring the activities of enzymes that are involved in methanol metabolism in mouse (CD-1) and rat (Sprague-Dawley) whole embryos at different stages of development. Specific activities of the enzymes ADH1, ADH3, and CAT, were determined in rat and mouse conceptuses during the organogenesis period of 8-25 somites. Activities were measured in heads, hearts, trunks, and VYS from early- and late-stage mouse and rat embryos. While CAT activities were similar between rat and mouse embryos, mouse ADH1 activities in the VYS were significantly lower throughout organogenesis

when compared to the rat VYS or embryos of either species. ADH1 activities of heads, hearts, and trunks from mouse embryos were significantly lower than those from rats at the 7-12 somite stage. However, these interspecies differences were not evident in embryos of 20-22 somites. ADH3 activities were lower in mouse versus rat VYS, irrespective of the stage of development. However, while ADH3 activities in mouse embryos were markedly lower than those of rats in the early stages of development, the levels of activity were similar to at the 14-16 somite stage and beyond. A lower capacity to transform formaldehyde to formate might explain the increased susceptibility of mouse versus rat embryos to the toxic effects of methanol. The hypothesis that formaldehyde is the ultimate embryotoxicant of methanol is supported by the demonstration of diminished ADH3 activity in mouse versus rat embryos and by the demonstration by Hansen et al. (2005) that formaldehyde has a far greater embryotoxicity than either formate or methanol itself.

4.4. Neurotoxicity

A substantial body of information exists on the toxicological consequences to humans who consume or are exposed to large amounts of methanol. As discussed in Section 4.1, neurological consequences of acute methanol intoxication in humans include Parkinson-like responses, visual impairment, confusion, headache, and numerous subjective symptoms. The occurrence of these symptoms has been shown to be associated with necrosis of the putamen when neuroimaging techniques have been applied (Salzman, 2006). Such profound changes have been linked to tissue acidosis that arises when methanol is metabolized to formaldehyde and formic acid through the actions of ADH1 and ADH3. However, the well-documented impact of the substantial amounts of formate that are formed when humans and animals are exposed to large amounts of methanol may obscure the potentially harmful effects that may arise when humans and animals are exposed to smaller amounts. Human acute exposure studies (Chuwers et al., 1995; Cook et al., 1991) (See Section 4.1.3) at TLV levels of 200 ppm would indicate that some measures of neurological function (e.g., sensory evoked potentials, memory testing and psychomotor testing) were impaired in the absence of measurable formate production.

4.4.1. Oral Neurotoxicity Studies

As discussed in Section 4.2.1.2, an oral subchronic (90 days, beginning at roughly 30 days of age) gavage study noted reduced brain weight in high-dose group (2,500 mg/kg-day) male and female SD rats (30/sex/dose) (TRL, 1986). They also reported a higher incidence of

colloid in the hypophyseal cleft of the pituitary gland in the high-dose versus control group males (13/20 versus 0/20) and females (9/20 versus 3/20). Based on these findings, a 500 mg/kg-day NOAEL was identified for this study

Two rodent studies investigated the neurological effects of developmental methanol exposure via the oral route (Aziz et al., 2002; Infurna and Weiss, 1986). One of these studies also investigated the influence of FAD diets on the effects of methanol exposures (Aziz et al., 2002). In the first, Infurna and Weiss (1986) exposed 10 pregnant female Long-Evans rats/dose to 2% methanol (purity not specified) in drinking water on either GD15-GD17 or GD17-GD19. Daily methanol intake was calculated at 2,500 mg/kg-day by the study authors. Dams were allowed to litter and nurse their pups. Data were analyzed by ANOVA with the litter as the statistical unit. Results of the study were equivalent for both exposure periods. Treatment had no effect on gestational length or maternal bodyweight. Methanol had no effect on maternal behavior as assessed by the time it took dams to retrieve pups after they were returned to the cage following weighing. Litter size, pup birth weight, pup postnatal weight gain, postnatal mortality, and day of eye opening did not differ from controls in the methanol treated groups. Two neurobehavioral tests were conducted in offspring. Suckling ability was tested in 3-5 pups/treatment group on PND1. An increase in the mean latency for nipple attachment was observed in pups from the methanol treatment group, but the percentage of pups that successfully attached to nipples did not differ significantly between treatment groups. Homing behavior, the ability to detect home nesting material within a cage containing one square of shavings from the pup's home cage and four squares of clean shavings, was evaluated in 8 pups/group on PND10. Pups from both of the methanol exposure groups took about twice as long to locate the home material and took less direct paths than the control pups. Group-specific values differed significantly from controls. This study suggests that developmental toxicity can occur at this drinking water dose without readily apparent signs of maternal toxicity.

Aziz et al. (2002) investigated the role of developmental deficiency in folic acid and methanol-induced developmental neurotoxicity in PND45 rat pups. Wistar albino female rats (80/group) were fed FAD[40] and FAS diets separately. Following 14-16 weeks on the diets, liver folate levels were estimated and females exhibiting a significantly low folic acid level were mated. Throughout their lactation period, dams of both the FAD and the FAS group were given 0, 1, 2, or 4% v/v methanol via drinking water, equivalent to approximately 480, 960 and 1,920 mg/kg-day.[41] Pups were exposed to methanol via lactation from PND1-PND21. Litter size was culled to 8 with equal male/female ratios maintained as much as possible. Liver folate levels

[40] Along with the FAD diet, 1% succinylsulphathiazole was also given to inhibit folic acid biosynthesis from intestinal bacteria.

[41] Assuming that Wistar rat drinking water consumption is 60 mL/kg-day (Rogers et al., 2002), 1% methanol in drinking water would be equivalent to 1% × 0.8 g/mL × 60 mL/kg-day = 0.48 g/kg-day = 480 mg/kg-day.

were determined at PND21 and neurobehavioral parameters (motor performance using the spontaneous locomotor activity test and cognitive performance using the conditioned avoidance response [CAR] test), and neurochemical parameters (dopaminergic and cholinergic receptor binding and dopamine levels) were measured at PND45. The expression of growth-associated protein (GAP 43), a neuro-specific protein in the hippocampus that is primarily localized in growth cone membranes and is expressed during developmental regenerative neurite outgrowth, was examined using immunohistochemistry and Western blot analysis.

A loss in body weight gain was observed at PND7, PND14, and PND21 in animals exposed to 2% (11, 15 and 19% weight gain reduction) and 4% (17, 24 and 29% weight gain reduction) methanol in the FAD group and only at 4% (9, 14 and 17% weight gain reduction) methanol in the FAS group. No significant differences in food and water intake were observed among the different treatment groups. Liver folate levels in the FAD group were decreased by 63% in rats prior to mating and 67% in pups on PND21.

Based on reports of Parkinson-like symptoms in survivors of severe methanol poisoning (see Section 4.1), Aziz et al. (2002) hypothesized that methanol may cause a depletion in dopamine levels and degeneration of the dopaminergic nigrostriatal pathway.[42] Consistent with this hypothesis, they found dopamine levels were significantly decreased (32% and 51%) in the striatum of rats in the FAD group treated with 2% and 4% methanol, respectively. In the FAS group, a significant decrease (32%) was observed in the 4% methanol-exposed group.

Methanol treatment at 2% and 4% was associated with significant increases in activity, in the form of distance traveled in a spontaneous locomotor activity test, in the FAS group (13% and 39%, respectively) and more notably, in the FAD group (33% and 66%, respectively) when compared to their respective controls. Aziz et al. (2002) suggest that these alterations in locomotor activity may be caused by a significant alteration in dopamine receptors and disruption in neurotransmitter availability. Dopamine receptor (D_2) binding in the hippocampus of the FAD group was significantly increased (34%) at 1% methanol, but was significantly decreased at 2% and 4% methanol exposure by 20% and 42%, respectively. In the FAS group, D_2 binding was significantly increased by 22% and 54% in the 2% and 4% methanol-exposed groups.

At PND45, the CAR in FAD rats exposed to 2% and 4% methanol was significantly decreased by 48% and 52%, respectively, relative to nonexposed controls. In the FAS group, the CAR was only significantly decreased in the 4% methanol-exposed animals and only by 22% as compared to their respective controls. Aziz et al. (2002) suggest that the impairment in CAR of

[42] The nigrostriatal pathway is one of four major dopamine pathways in the brain that are particularly involved in the production of movement. Loss of dopamine neurons in the substantia nigra is one of the pathological features of Parkinson's disease (Kim et al., 2003).

the methanol-exposed FAD pups may be due to alterations in the number of cholinergic (muscarinic) receptor proteins in the hippocampal region of the brain. Muscarinic receptor binding was significantly increased in the 2% (20%) and 4% (42%) methanol-exposed group in FAD animals, while FAS group animals had a significant increase in cholinergic binding only in the 4% methanol exposed group (21%). High concentrations of methanol may saturate the body's ability to remove toxic metabolites, including formaldehyde and formate, and this may be exacerbated in FAD pups having a low store of folate.

Immunohistochemistry showed an increase in the expression of GAP-43 protein in the dentate granular and pyramidal cells of the hippocampus in 2% and 4% methanol-exposed animals in the FAD group. The FAS group showed increased expression only in the 4% methanol-exposed group. The Western blot analysis also confirmed a higher expression of GAP-43 in the 2% and 4% methanol-exposed FAD group rats. Aziz et al. (2002) suggested that up-regulation of GAP-43 in the hippocampal region may be associated with axonal growth or protection of the nervous system from methanol toxicity.

The Aziz et al. (2002) study provides evidence that hepatic tetrahydrofolate is an important contributing factor in methanol-induced developmental neurotoxicity in rodents. The immature blood-brain barrier and inefficient drug-metabolizing enzyme system make the developing brain a particularly sensitive target organ to the effects of methanol exposure.

4.4.2. Inhalation Neurotoxicity Studies

A review by Carson et al. (1981) has summarized a number of older reports of studies on the toxicological consequences of methanol exposure. In one example relevant to the potential for neurotoxicity from repeat, low level exposure to methanol, the review cites a research report of Chen-Tsi (1959) who exposed 10 albino rats/group (sex and strain unstated) to 1.77 and 50 mg/m^3 (1.44 and 40.7 ppm) methanol vapor, 12 hours/day, for 3 months. Deformation of dendrites, especially the dendrites of pyramidal cells, in the cerebral cortex was included in the description of histopathological changes observed in adult animals following exposure to 50 mg/m^3 (40.7 ppm) methanol vapor. One out of ten animals exposed to the lower methanol concentration also displayed this feature.

Information on the neurotoxicity of methanol inhalation exposure in adult cynomolgus monkeys (*M. fascicularis*) has come from NEDO (1987) which describes the results of a number of inhalation experiments that have already been discussed in Section 4.2.2. The monkey studies that will be discussed here with respect to their neurotoxicity implications include an acute study, a chronic study, and a repeated exposure experiment (of variable duration depending upon exposure level), followed by recovery period (1-6 months), and an experiment looking at chronic

formaldehyde exposure (1 or 5 ppm), a metabolite of methanol. This last experiment was only a pilot study and included only one monkey per exposure condition.

As noted in Section 4.2.2.1, histopathologic changes to the CNS reported in monkeys following acute exposure to methanol included characteristic degeneration of the bilateral putamen, caudate nucleus, and claustrum, with associated edema in the cerebral white matter (NEDO, 1987). These lesions increased in severity with increasing exposure. Necrosis of the basal ganglia was noted following exposure to 5,000 ppm for 5 days (1 animal) and 14 days (1 animal). The authors reported that at 3,000 ppm the monkeys experienced little more than minimal fibrosis of "responsive stellate cells" of the thalamus, hypothalamus and basal ganglion. This effect was also observed following chronic exposure and is discussed more extensively below.

In the chronic experiment, 8 monkeys were included per exposure level (control, 10, 100, 1,000 ppm or 13, 131, and 1,310 mg/m^3, respectively, for 21 hours/day); however, animals were serially sacrificed at 3 time points: 7 months, 19 months, or >26 months. This design reduced the number of monkeys at each exposure level to 2 subjects at 7 months and 3 subjects at the subsequent time points (see Section 4.2.2). One of the 3 animals receiving 100 ppm methanol and scheduled for sacrifice at 29 months was terminated at 26 months.

Histopathologically, no overt degeneration of the retina, optical nerve, cerebral cortex, or other potential target organs (liver and kidney) was reported in the chronic experiment. Regarding the peripheral nervous system, 1/3 monkeys exposed to 100 ppm (131 mg/m^3) and 2/3 exposed to 1,000 ppm (1,310 mg/m^3) for 29 months showed slight but clear changes in the peroneal nerves. The most pervasive effect noted across the exposure concentrations and durations was "fibrosis of responsive stellate cells," characterized as "neurological disease" in the NEDO (1987) summary report. These "stellate cells" are likely to be astrocytes, star-shaped glial cells in the brain that are among the most numerous cells in all regions of the CNS. As was noted in an independent peer review of this study (ERG, 2009), the degree of fibrosis of responsive stellate cells is an appropriate CNS endpoint of consideration given that stellate astroglia are believed to play a key role in the pathogenesis of CNS disorders and an essential role in response to tissue injury and inflammation by hypertrophy, proliferation, production of growth factors and cytokines, and involvement in extracellular matrix deposition characteristic of fibrosis (De Keyser et al., 2008). A peer reviewer also recommended that, because there did not appear to be an effect of duration on the incidence of this neurological endpoint, the results can be pooled across durations to obtain a clearer view of dose-response results (ERG, 2009). As reported in "appended Table 3" of the NEDO (1987) report, the incidence of stellate cell fibrosis at 10 ppm (13.1 mg/m^3), 100 ppm (131 mg/m^3) or 1,000 ppm (1,310 mg/m^3) for exposure durations of 7 months or longer were: [3/8, 7/8 and 7/8 within the cerebral white matter]; [0/8,

3/8 and 3/8 inside the nucleus of the thalamus]; [3/8, 6/8 and 4/8 in the hypothalamus]; [4/8, 7/8 and 7/8 in the mesencephalon central gray matter]; [2/8, 7/8 and 7/8 in the pons tegmentum]; and [0/8, 5/8 and 4/9 in the medulla oblongata tegmentum]. All monkeys that had degeneration of the inside nucleus of the thalamus also had degeneration of the cerebral white matter.

According to NEDO (1987), the stellate cell response was transient and "not characteristic of degeneration." However, the authors also noted that the stellate cell response was "nearly absent in normal monkeys in the control group" and that "in the groups exposed to a large quantity of methanol or for a long time their presence tended to become permanent, so a relation to the long term over which the methanol was inhaled is suspected." There is a question concerning whether an appropriate, concurrent control was used as all control group responses are reported in a single table in the section of the NEDO (1987) report that describes the acute monkey study, with no indication as to when the control group was sacrificed. However, responses in the mid- and high- dose groups appeared to be increased over responses in the low-dose groups.

In the recovery experiment, monkeys were exposed for 7 months to 1,000 ppm (3 animals), for 20 days to 2,000 ppm (3 animals), for 20 days to 3,000 ppm (4 animals), for 5-14 days to 5,000 ppm (5 animals) or for 6 days to 7,000 ppm (2 animals) methanol, followed by recovery periods of various durations. Monkeys exposed to 3,000 ppm for 20 days followed by a 6-month recovery period experienced relatively severe fibrosis of responsive stellate cells and elucidation of the medullary sheath. However, resolution of some of the glial responses was noted in the longer duration at lower exposure levels, with no effects observed on the cerebral white matter in monkeys exposed for 7 months to 1,000 ppm methanol followed by a 6-month recovery period. In general, the results from the recovery experiment corroborated results observed in the chronic experiment. NEDO (1987) interpreted the lack of glial effects after a 6-month recovery as an indication of a transient effect. However, glial responses to neural damage do not necessarily persist following resolution of neurodegeneration (Aschner and Kimelberg, 1996). In addition, the reported data do not fully support that changes in cerebral white matter were transient (ERG, 2009). Two of three monkeys exposed to 2,000 ppm exhibited stellate cell changes in at least one lobe after 1 and 11 months recovery. Also, the only monkey exposed 7 months with a 1 month recovery period exhibited such changes at autopsy. While the monkeys exposed to 1,000 ppm for 7 months with a 5 month 20 day recovery period were devoid of stellate cell changes, the small sample size (n=2) does not allow for the stellate cell effect to be characterized as transient.

The limited information available from the NEDO (1987) summary report suggests that 100 ppm (131 mg/m^3) may be an effect level following continuous, chronic exposure to methanol. However, as noted in Section 4.2.2.1, the NEDO (1987) studies in nonhuman

primates, have multiple reporting deficiencies and data gaps that make them difficult to interpret. In addition, confidence in the dose-response data from this study is weakened by the apparent lack of a concurrent control group and the small number of animals at each exposure level for each serial sacrifice (2-3 monkeys/time point/exposure level). In general, peer reviewers of this study stated that it provides descriptive, rather than quantitative, support for the evaluation of the inhalation toxicity of methanol (ERG, 2009).

Weiss et al. (1996) exposed 4 cohorts of pregnant Long-Evans rats (10-12 dams/ treatment group/cohort) to 0 or 4,500 ppm (0 and 5,897 mg/m^3) methanol vapor (high-performance liquid chromatography [HPLC] grade), 6 hours/day, from GD6 to PND21. Pups were exposed together with the dams during the postnatal period. Average blood methanol levels in pups on PND7 and PND14 were about twice the level observed in dams. However, methanol exposure had no effect on maternal gestational weight gain, litter size, or postnatal pup weight gain up to PND18[43]. Neurobehavioral tests were conducted in neonatal and adult offspring; the data generated from those tests were evaluated by repeated measures ANOVA. Three neurobehavioral tests conducted in 13-26 neonates/group included a suckling test, conditioned olfactory aversion test, and motor activity test. In contrast to earlier test results reported by Infurna and Weiss (1986), methanol exposure had no effect on suckling and olfactory aversion tests conducted on PND5 and PND10, respectively. Results of motor activity tests in the methanol group were inconsistent, with decreased activity on PND18 and increased activity on PND25. Tests that measured motor function, operant behavior, and cognitive function were conducted in 8-13 adult offspring/group. Some small performance differences were observed between control and treated adult rats in the fixed wheel running test only when findings were evaluated separately by sex and cohort. The test requires the adult rats to run in a wheel and rotate it a certain amount of times in order to receive a food reward. A stochastic spatial discrimination test examined the rats' ability to learn patterns of sequential responses. Methanol exposure had no effect on their ability to learn the first pattern of sequential responses, but methanol-treated rats did not perform as well on the reversal test. The result indicated possible subtle cognitive deficits as a result of methanol exposure. A morphological examination of offspring brains conducted on PND1 and PND21 indicated that methanol exposure had no effect on neuronal migration, numbers of apoptotic cells in the cortex or germinal zones, or myelination. However, neural cell adhesion molecule (NCAM) 140 and NCAM 180 gene expression in treated rats was reduced on PND4 but not 15 months after the last exposure.

[43] The fact that this level of exposure caused effects in the Sprague-Dawley rats of the NEDO (1987) study but did not cause a readily apparent maternal effect in Long-Evans rats of this study could be due to diffences in strain susceptibility.

NCAMs are glycoproteins required for neuron migration, axonal outgrowth, and establishing mature neuronal function patterns.

Stanton et al. (1995) exposed 6-7 pregnant female Long-Evans rats/group to 0 or 15,000 ppm (0 and 19,656 mg/m^3) methanol vapors (≥ 99.9% purity) for 7 hours/day on GD7-GD19. Mean serum methanol levels at the end of the 1st, 4th, 8th, and 12th days of exposure were 3,836, 3,764, 3,563, and 3,169 µg/mL, respectively. As calculated by authors, dams received an estimated methanol dose of 6,100 mg/kg-day. A lower body weight on the first 2 days of exposure was the only maternal effect; there was no increase in postimplantation loss. Dams were allowed to deliver and nurse litters. Parameters evaluated in pups included mortality, growth, pubertal development, and neurobehavioral function. Examinations of pups revealed that two pups from the same methanol-exposed litter were missing one eye; aberrant visually evoked potentials were observed in those pups. A modest but significant reduction in body weight gain on PND1, PND21, and PND35 was noted in pups from the methanol group. For example, by PND35, male pups of dams exposed to methanol had a mean body weight of 129 grams versus 139 grams in controls ($p < 0.01$). However, postnatal mortality was unaffected by exposure to methanol. The study authors did not consider a 1.7-day delay in vaginal opening in the methanol group to be an adverse effect. Preputial separation was not affected by prenatal methanol exposure. Neurobehavioral status was evaluated using 8 different tests on specific days up to PND160. Tests included motor activity on PND13-PND21, PND30, and PND60, olfactory learning and retention on PND18 and PND25, behavioral thermoregulation on PND20-21, T-maze delayed alternation learning on PND23-PND24, acoustic startle reflex on PND24, reflex modification audiometry on PND61-PND63, passive avoidance on PND73, and visual evoked potentials on PND160. A single pup/sex/litter was examined in most tests, and some animals were subjected to multiple tests. The statistical significance of neurobehavioral testing was assessed by one-way ANOVA, using the litter as the statistical unit. Results of the neurobehavioral testing indicated that methanol exposure had no effect on the sensory, motor, or cognitive function of offspring under the conditions of the experiment. However, given the comparatively small number of animals tested for each response, it is uncertain whether the statistical design had sufficient power to detect small compound-related changes.

NEDO (1987) sponsored a teratology study that included an evaluation of postnatal effects in addition to standard prenatal endpoints in Sprague-Dawley rats. Thirty-six pregnant females/group were exposed to 0, 200, 1,000, or 5,000 ppm (0, 262, 1,310, and 6,552 mg/m^3) methanol vapors (reagent grade) on GD7-GD17 for 22.7 hours/day. Statistical significance of results was evaluated by t-test, Mann-Whitney U test, Fisher's exact test, and/or Armitage's χ^2 test.

Postnatal effects of methanol inhalation were evaluated in the remaining 12 dams/group that were permitted to deliver and nurse their litters. Effects were only observed in the 5,000 ppm. There were no adverse effects on offspring body weight from methanol exposure. However, the weights of some organs (brain, thyroid, thymus, and testes) were reduced in 8-week-old offspring following prenatal-only exposure to 5,000 ppm methanol. An unspecified number of offspring were subjected to neurobehavioral testing or necropsy, but results were incompletely reported.

As described in Section 4.3.2, NEDO (1987) performed a two-generation reproductive study that evaluated the effects of pre- and postnatal methanol exposure (20 hours/day) on reproductive and other organ systems of Sprague-Dawley rats and in particular the brain. They reported reduced brain, pituitary, and thymus weights, in the offspring of F_0 and F_1 rats exposed to 1,000 ppm methanol. In particular, they noted a reduction in absolute brain weights in F1 pups at 8 weeks (male and female), 16 weeks (males) and 24 weeks (females) and in F2 pups at 8 weeks (male and female). Details were not reported (e.g., means, variances, sample sizes, pup-to-litter correlations) that would allow for further analysis of these findings.

Seeking to confirm the possible compound-related effect of methanol on the brain NEDO (1987) conducted an additional developmental study in which Sprague-Dawley rats were exposed to 0, 500, 1,000, and 2,000 ppm (0, 655, 1,310, and 2,620 mg/m^3) methanol from the first day of gestation through the F_1 generation. According to NEDO (1987 page 201), another purpose of the supplementary study was “to know from what period after birth such changes would appear.” Information important for a determination of possible litter correlations (e.g., pup litter assignments) was not reported for the supplemental experiment. However, the number of pups per dose group per “period after birth” was reported (11-14/sex/dose/postnatal period) and it is reasonable to assume that, consistent with the standard culling protocol used for both the F1 and F2 generations of the two-generation study (NEDO, 1987 pages 185 and 189), the pups for each gender, dose and exposure time combination came from a different litter (to avoid problems associated with litter correlation). Brain weights were measured in the 11-14 offspring/sex/group at 3, 6, and 8 weeks of age. As illustrated in Table 4-13, brain weights were significantly reduced in 3-week-old males and females exposed to ≥ 1,000 ppm. At 6 and 8 weeks of age, brain weights were significantly reduced in males exposed to ≥ 1,000 ppm and females exposed to 2,000 ppm. Due to the toxicological significance of this postnatal effect, the brain weight changes observed by NEDO (1987) following gestational and postnatal exposures and following gestation-only exposure (in the teratology study discussed above) are evaluated quantitatively and discussed in more detail in Section 5 of this review.

Table 4-13 Brain weights of rats exposed to methanol vapors during gestation and lactation

		Brain weight (g) (% control) at each exposure level					
Offspring age	**Sex**	**0 ppm**	**200 ppm**	**500 ppm**	**1,000 ppm**	**2,000 ppm**	**5,000 ppm**
3 wk[a]	Male	1.45 ± 0.06	--	1.46 ± 0.08 (101%)	1.39 ± 0.05[c] (96%)	1.27 ± 0.06[e] (88%)	--
3 wk[a]	Female	1.41 ± 0.06	--	1.41 ± 0.07 (100%)	1.33 ± 0.07[d] (94%)	1.26 ± 0.09[e] (89%)	--
6 wk[a]	Male	1.78 ± 0.07	--	1.74 ± 0.09 (98%)	1.69 ± 0.06[d] (95%)	1.52 ± 0.07[e] (85%)	--
6 wk[a]	Female	1.68 ± 0.08	--	1.71 ± 0.08 (102%)	1.62 ± 0.07 (96%)	1.55 ± 0.05[e] (92%)	--
8 wk[a]	Male	1.99 ± 0.06	--	1.98 ± 0.09 (99%)	1.88 ± 0.08[d] (94%)	1.74 ± 0.05[e] (87%)	--
8 wk[a]	Female	1.85 ± 0.05	--	1.83 ± 0.07 (99%)	1.80 ± 0.08 (97%)	1.67 ± 0.06[e] (90%)	--
8 wk[b]	Male	2.00 ± 0.05	2.01 ± 0.08 (100%)	--	1.99 ± 0.07 (100%)	--	1.81 ± 0.16[d] (91%)
8 wk[b]	Female	1.86 ± 0.08	1.91 ± 0.06 (103%)	--	1.90 ± 0.08 (102%)	--	1.76 ± 1.09 (95%)

[a]Exposed throughout gestation and F_1 generation.

[b]Exposed on gestational days 7-17 only.

[c]$p < 0.05$; [d]$p < 0.01$; [e]$p < 0.001$; p values as calculated by the authors.Values are means ± S.D.

Source: NEDO (1987).

Burbacher, et al. (1999b; 1999a) carried out toxicokinetic, reproductive, developmental and postnatal neurological and neurobehavioral studies of methanol in *M. fascicularis* monkeys that were published by HEI in a two-part monograph. Some of the data were subsequently published in the open scientific literature (Burbacher et al., 2004b; Burbacher et al., 2004a). The experimental protocol featured exposure to 2 cohorts of 12 monkeys/group to low-exposure levels (relative to the previously discussed rodent studies) of 0, 200, 600, or 1,800 ppm (0, 262, 786, and 2,359 mg/m^3) methanol vapors (99.9% purity), 2.5 hours/day, 7 days/week, during a premating period and mating period (–180 days combined) and throughout the entire gestation period (–168 days). The monkeys were 5.5-13 years old. The outcome study included an evaluation of maternal reproductive performance (discussed in Section 4.3.2) and tests to assess infant postnatal growth and newborn health, neurological outcomes included reflexes, behavior, and development of visual, sensorimotor, cognitive, and social behavioral function. Blood methanol levels, elimination, and the appearance of formate were also examined and are

discussed in Section 3.2. The effects observed were in the absence of appreciable increases in maternal blood formate levels.

Neurobehavioral function was assessed in 8-9 infants/group during the first 9 months of life (Burbacher et al., 2004a; Burbacher et al., 1999a). Although results in 7/9 tests were negative, 2 effects were possibly related to methanol exposure. The Visually Directed Reaching (VDR) test is a measure of sensorimotor development and assessed the infants' ability to grasp for a brightly colored object containing an applesauce-covered nipple. Beginning at 2 weeks after birth, infants were tested 5 times/day, 4 days/week. Performance on this test, measured as age from birth at achievement of test criterion (successful object retrieval on 8/10 consecutive trials over 2 testing sessions), was reduced in all treated male infants. The times (days after birth) to achieve the criteria for the VDR test were 23.7 ± 4.8 (n = 3), 32.4 ± 4.1 (n = 5), 42.7 ± 8.0 (n = 3), and 40.5 ± 12.5 (n = 2) days for males and 34.2 ± 1.8 (n = 5), 33.0 ± 2.9 (n = 4), 27.6 ± 2.7 (n = 5), and 40.0 ± 4.0 (n = 7) days for females in the control to 1,800 ppm groups, respectively. Statistical significance was obtained in the 1,800 ppm group when males and females were evaluated together (p = 0.04) and in the 600 ppm (p = 0.007) for males only. However, there was no significant difference between responses and/or variances (indicating lack of a dose-response trend) among the dose levels for males and females combined (p = 0.244), for males only (p = 0.321) and for males only, excluding the high-dose group (p = 0.182). However, there was a significant dose-response trend for females only (p = 0.0265). The extent to which VDR delays were due to a direct effect of methanol on neurological development or a secondary effect due to the methanol-induced decrease in length of pregnancy and subsequent prematurity is not clear. Studies of reaching behavior have shown that early motor development in pre-term human infants without major developmental disorders differs from that of full-term infants (Fallang et al., 2003). Clinical studies have indicated that the quality of reaching and grasping behavior in pre-term infants is generally less than that in full-term infants (Fallang et al., 2003; Plantinga et al., 1997). For this reason, measures of human infant development generally involve adjustment of a child's "test age" if he or she had a gestational age of fewer than 38 weeks, often by subtracting weeks premature from the age measured from birth (Wilson and Cradock, 2004). When this type of adjustment is made to the Burbacher et al. (2004a; 1999a) VDR data, the dose-response trend for males only remains unacceptable (p = 0.448) and, while the dose-response trend for the females only remains adequate (p = 0.009), the variance in the data could not be modeled adequately. Thus, only the unadjusted VDR response for females only exhibited a dose-response that could be adequately modeled (see Appendix D).

At 190-210 days of age, the Fagan Test of infant intelligence was conducted. The paradigm makes use of the infant's proclivity to direct more visual attention to novel stimuli rather than familiar stimuli. The test measures the time infants spend looking at familiar versus

novel items. Deficits in the Fagan task can qualitatively predict deficits in intelligence quotient (IQ) measurements assessed in children at later ages (Fagan and Singer, 1983). Control monkey infants in the Burbacher et al. (2004a; 1999a) study spent more than 62% ± 4% (mean for both cohorts) of their time looking at novel versus familiar monkey faces, while the treated monkeys did not display a statistically significant preference for the novel faces (59% ± 2%, 54% ± 2% and 59% ± 2% in 200, 600 and 1,800 ppm groups, respectively). Unlike the VDR results discussed previously, results of this test did not appear to be gender specific and were neither statistically significant (ANOVA $p = 0.38$) nor related to exposure concentration. The findings indicated a cohort effect which appeared to reduce the statistical power of this analysis. The authors' exploratory analysis of differences in outcomes between the 2 cohorts indicated an effect of exposure in the second cohort and not the first cohort due to higher mean performance in controls of cohort 2 (70% ± 5% versus 55% ± 4% for cohort 1). In addition, this finding could reflect the inherent constraints of this endpoint. If the control group performs at the 60% level and the most impaired subjects perform at approximately the 50% chance level (worse than chance performance would not be expected), the range over which a concentration-response relationship can be expressed is limited. Because of the longer latency between assessment and birth, these results would not be confounded with the postulated methanol-induced decrease in gestation length of the exposed groups of this study. Negative results were obtained for the remaining seven tests that evaluated early reflexes, gross motor development, spatial and concept learning and memory, and social behavior. Infant growth and tooth eruption were unaffected by methanol exposure.

4.4.3. Neurotoxicity Studies Employing i.p. and in vitro Methanol Exposures

Table 4-14 describes three i.p. injection studies that attempt to determine the biochemical changes associated with the effects of repeat methanol exposures on the brain, retina, optic nerve (Rajamani et al., 2006; Gonzalez-Quevado et al., 2002) and the hypothalamus-pituitary-adrenal (HPA) axis of the neuroendocrine system (Parthasarathy et al., 2006a). The goal of the Gonzalez-Quevado et al. (2002) study was to determine whether a sustained increase in formate levels, [44] at concentrations below those known to produce toxic effects from acute exposures, can induce biochemical changes in the retina, optical nerve, or certain regions of the brain. [45] The amino acids aspartate, glutamate, asparagine, serine, histidine, glutamine, threonine, glycine, arginine,

[44] Formate levels were increased by treating test rats with methotrexate (MTX), which depletes folate stores by interfering with tetrahydrofolate (THF) regeneration (Dorman et al., 1994).
[45] A subset of exposed rats were also exposed to taurine, which plays an important role in the retina and optical nerve, to explore its possible protective effect (Gonzalez-Quevado et al., 2002).

alanine, hypotaurine, gamma-aminobutyric acid (which is also a neurotransmitter), and tyrosine were measured in blood, brain, and retinal regions.

The increased level of aspartate in the optic nerve of animals treated with MTX-methanol and Tau-MTX-methanol may indicate a relation to formate accumulation. The authors note that L-aspartate is a major excitatory amino acid in the brain and that increased levels of excitatory amino acids can trigger neuronal cell damage and death (Albin and Greenamyre, 1992). Increased levels of aspartate and glutamine in the hippocampus could provide an explanation for some of the CNS symptoms observed in methanol poisonings on the basis of their observed impact on cerebral arteries (Huang et al., 1994). The observation that these increases resulted primarily from methanol without MTX could be significant in that it indicates methanol can cause excitotoxic effects without formate mediation. The neurotransmitters serotonin (5-HT) and dopamine (DA) and their respective metabolites, 5-hydroxyindolacetic acid (5-HIAA) and dihydroxyphenylacetic acid (DOPAC), were also measured in various brain regions. The levels of these monoamines were not affected by formate accumulation, as the only increases were observed for 5-HT and 5-HIAA following methanol-only exposure. DA and DOPAC levels were not altered by the treatments in any of the areas measured. The posterior cortex did not show any changes in monoamine levels for any treatment group.

Rajamani et al. (2006) examined several oxidative stress parameters in male Wistar rats following methotrexate-induced folate deficiency. The optic nerve, retina, and brain were collected and the brain was dissected into the following regions: cerebral cortex, cerebellum, mid-brain, pons medulla, hippocampus and hypothalamus. Each region was examined for indicators of oxidative stress including increases in the free radical scavengers superoxide dismutase (SOD), CAT, glutathione peroxidase (GPx), and reduced GSH levels. The levels of protein thiols, protein carbonyls, and amount of lipid peroxidation were also measured. More recently, investigators from the same laboratory measured increased methanol blood levels and corresponding increases in these indicators of oxidative stress in discrete regions of the brain in Wistar strain albino rats exposed to 75 mg/kg/day aspartame (Iyyaswamy and Rathinasamy, 2012). Overall, the results reported in these studies suggest that folate-deficient rats exposed to methanol exhibit signs of oxidative stress (e.g., increased SOD, GPx and CAT activity and decreased levels of GSH and protein thiol) in discrete regions of the brain, retina and optic nerve.

To determine the effects of methanol on the HPA axis, Parthasarathy et al. (2006a) evaluated a combination of oxidative stress, immune and neurobehavioral parameters following methanol exposure. Oxidative stress parameters examined included SOD, CAT, GSH peroxidase, GSH, and ascorbic acid (Vitamin C). Plasma corticosterone levels were measured, and lipid peroxidation was measured in the hypothalamus and the adrenal gland. An assay for DNA fragmentation was conducted in tissue from the hypothalamus, the adrenal gland and the spleen.

Immune function tests conducted included the footpad thickness test for delayed type hypersensitivity (DTH), a leukocyte migration inhibition assay, the hemagglutination assay (measuring antibody titer), the neutrophil adherence test, phagocytosis index, and a nitroblue tetrazolium (NBT) reduction and adherence assay used to measure the killing ability of polymorphonuclear leukocytes (PMNs). The open field behavior test was used to measure general locomotor and explorative activity during methanol treatment in the 30-day treatment group, with tests conducted on days 1, 4, 8, 12, 16, 20, 24, and 28.

The results for this study shown in Table 4-14 suggest that exposure to methanol-induced oxidative stress, disturbs HPA-axis function, altering corticosterone levels and producing effects in several nonspecific and specific immune responses.

Table 4-14 Intraperitoneal injection neurotoxicity studies

Species/Strain/N	Dose & Duration	Effect Relative to Control	Reference
Rat/Sprague-Dawley/ (5-7 per group; 100-150 g)	Control: tap water (wk 1); saline s.c. (wks 2-4)	--	Gonzalez-Quevado et al. (2002)
	MeOH: tap water (wk 1); s.c. saline (wk 2); 2 g/kg-day MeOH i.p. (wks 3-4)	Increased blood formate (<2-fold); Increased aspartate, glutamine and Tau in hippocampus; Increased 5-HT and 5-HIAA in hippocampus; Increased 5-HT in retina	
	MTX: tap water (wk 1); 0.2 mg/kg-day MTX s.c. (wk 2); 0.1 mg/kg-day MTX s.c. & saline i.p. (wks 2-4)	No change in blood formate or any other measured parameter	
	MTX-MeOH: tap water (wk 1); 0.2 mg/kg-day MTX s.c. (wk 2); 0.1 mg/kg-day MTX s.c. & 2 g/kg -day MeOH i.p. (wks 3-4)	Increased blood formate (>3-fold); Increased aspartate in optic nerve; Increased aspartate and Tau in hippocampus	
	Tau: 2% Tau in DW (wks 1-4); saline s.c. (wks 2-4)	No change in blood formate; Increased blood histidine and Tau	
	Tau-MTX-MeOH: 2% Tau in DW (wks 1-4); 0.2 mg/kg-day MTX s.c. (wk 2); 0.1 mg/kg-day MTX s.c. & 2 g/kg-day MeOH i.p. (wks 3-4)	Increased blood formate (>3-fold) and Tau; Increased aspartate in optic nerve; Increased aspartate, glutamine and Tau in hippocampus	
Rat/Wistar/ 6 per group	Control: saline i.p. (day 8)	--	Rajamani et al. (2006)
	MTX: 0.2 mg/kg-day MTX (wk 1); saline i.p. (day 8)	Increased SOD, CAT, GSH peroxidase, oxidized GSH, protein carbonyls and lipid peroxidation in all brain regions; Decreased GSH and protein thiols in all brain regions; Increased HSP70 in hippocampus	
	MTX-MeOH: 0.2 mg/kg-day MTX (wk 1); 3 g/kg-day MeOH i.p. (day 8)	Increased SOD, CAT, GSH peroxidase, oxidized GSH, protein carbonyls and lipid peroxidation in all brain regions over control and MTX group; Decreased GSH and protein thiols in all brain regions over control and MTX group; Increased HSP70 in hippocampus	

Species/Strain/N	Dose & Duration	Effect Relative to Control	Reference
Rat/Wistar/ 6 per group	0 or 2.37 g/kg-day MeOH i.p. for 1, 15 or 30 days	All antioxidants increased at 1-day, but decreased at 15 and 30 days; Increased lipid peroxidation in hypothalamus and adrenal gland at 1, 15, and 30 days; Increased leukocyte migration and antibody titer at all time points; Decreased footpad thickness at 15 and 30 days; Decreased neutrophil adherence at 1 and 30 days. Decreased NBT reduction and adherence in PMNs at 30-days versus PMNs at 15-days; Decrease in ambulation from 4th day on; Decrease in rearing and grooming from 20th day on. Increase in immobilization from 8th day on; Increase fecal bolus from 24th day on; Increase in corticosterone levels at 1 and 15 days; Decrease in corticosterone levels at 30 days; Fragmentation of DNA from hypothalamus, adrenal gland, and spleen at 30 days.	Parthasarathy et al. (2006a)

wk = week; MeOH = methanol; s.c. = subcutaneous injection; i.p.= intraperitoneal injection; MTX = methotrexate; Tau = taurine; DW = drinking water ad libitum exposure

There is some experimental evidence that the presence of methanol can affect the activity of acetylcholinesterase (Tsakiris et al., 2006). Although these experiments were carried out on erythrocyte membranes in vitro, the apparent compound-related changes may have implications for possible impacts of methanol and/or its metabolites on acetylcholinesterase at other centers, such as the brain. Tsakiris et al. (2006) prepared erythrocyte ghosts from blood samples of healthy human volunteers by repeated freezing-thawing. The ghosts were incubated for 1 hour at 37^{o}C in 0, 0.07, 0.14, 0.6 or 0.8 mmol/L methanol, and the specific activities of acetylcholinesterase monitored. Respective values (in change of optical density units/minute-mg protein) were 3.11 ± 0.15, 2.90 ± 0.10, 2.41 ± 0.10 ($p < 0.05$), 2.05 ± 0.11 ($p < 0.01$), and 1.81 ± 0.09 ($p < 0.001$). More recently, Simintzi et al. (2007) carried out an in vitro experiment to investigate the effects of aspartame metabolites, including methanol, on 1) a pure preparation of acetylcholinesterase, and 2) the same activity in homogenates of frontal cortex prepared from the brains of (both sexes of) Wistar rats. The activities were measured after incubations with 0, 0.14, 0.60, or 0.8 mmoles/L (0, 4.5, 19.2, and 25.6 mg/L) methanol, and with methanol mixed with the other components of aspartame metabolism, phenylalanine and aspartic acid. After incubation at 37^{o}C for 1 hour, the activity of acetylcholinesterase was measured spectrophotometrically. As shown in Table 4-15, the activities of the acetylcholinesterase preparations were reduced dose dependently after incubation in methanol. Similar results were also obtained with the other aspartame metabolites, aspartic acid, and phenylalanine, both individually or as a mixture with methanol. While the implications of this result to the acute neurotoxicity of methanol are uncertain, the authors speculated that methanol may bring about these changes through either interactions with the lipids of rat frontal cortex or perturbation of proteinaceous components.

Table 4-15 Effect of methanol on Wistar rat acetylcholinesterase activities

Methanol concentration (mmol/L)	Acetylcholinesterase activity (ΔOD/min-mg)	
	Frontal cortex	Pure enzyme
Control	0.269 ± 0.010	1.23 ± 0.04
0.14	0.234 ± 0.007[a]	1.18 ± 0.06
0.60	0.223 ± 0.009[b]	1.05 ± 0.04[b]
0.80	0.204 ± 0.008[b]	0.98 ± 0.05[b]

[a]$p < 0.01$.
[b]$p < 0.001$.
Values are means ± S.D. for four experiments. The average value of each experiment was derived from three determinations of each enzyme activity.

Source: Simintzi et al. (2007).

4.5. Immunotoxicity

Parthasarathy et al. (2005b) provided data on the impact of methanol on neutrophil function in an experiment in which 6 male Wistar rats/group were given a single i.p. exposure of 2,370 mg/kg methanol mixed 1:1 in saline. Another group of 6 animals provided blood samples that were incubated with methanol in vitro at a methanol concentration equal to that observed in the in vivo-treated animals 30 and 60 minutes postexposure. Total and differential leukocyte counts were measured from these groups in comparison to in vivo and in vitro controls. Neutrophil adhesion was determined by comparing the neutrophil index in the untreated blood samples to those that had been passed down a nylon fiber column. The cells' phagocytic ability was evaluated by their ability to take up heat-killed *Candida albicans*. In another experiment, neutrophils were assessed for their killing potential by measuring their ability to take up then convert NBT to formazan crystals.[46] One hundred neutrophils/slides were counted for their total and relative percent formazan-positive cells.

The blood methanol concentrations 30 and 60 minutes after dosing were 2,356 ± 162 and 2,233 ± 146 mg/L, respectively. The mean of these values was taken as the target concentration for the in vitro methanol incubation. In the in vitro studies, there were no differences in total and differential leukocyte counts, suggesting that no lysis of the cells had occurred at this methanol concentration. This finding contrasts with the marked difference in total leukocytes observed as a result of methanol incubation in vivo, in which, at 60 minutes after exposure, 16,000 ± 1,516

[46] Absence of NBT reduction indicates a defect in some of the metabolic pathways involved in intracellular microbial killing.

cells/mm^3 were observed versus 23,350 ± 941 in controls ($p < 0.001$). Some differences in neutrophil function were observed in blood samples treated with methanol in vitro and in vivo. These differences are illustrated for the 60-minute postexposure samples in Table 4-16.

Table 4-16 Effect of methanol on neutrophil functions in in vitro and in vivo studies in male Wistar rats

	In vitro studies (60 minutes)		In vivo studies (60 minutes)	
Parameter	**Control**	**Methanol**	**Control**	**Methanol**
Phagocytic index (%)	89.8 ± 3.07	81.6 ± 2.2[a]	66.0 ± 4.8	84.0 ± 7.0[b]
Avidity index	4.53 ± 0.6	4.47 ± 0.7	2.4 ± 0.1	3.4 ± 0.3[a]
NBT reduction (%)	31.6 ± 4.6	48.6 ± 4.3[b]	4.6 ± 1.2	27.0 ± 4.6[b]
Adherence (%)	50.2 ± 5.1	39.8 ± 2.4[a]	49.0 ± 4.8	34.6 ± 4.0[b]

[a]$p < 0.01$.
[b]$p < 0.001$.
Values are means ± S.D. for six animals.

Source: Parthasarathy et al. (2005b).

Parthasarathy et al. (2005b) observed differences in the neutrophil functions of cells exposed to methanol in vitro versus in vivo, most notably in the phagocytic index that was reduced in vitro but significantly increased in vivo. However, functions such as adherence and NBT reduction showed consistency in the in vitro and in vivo responses. The authors noted that, by and large, the in vivo effects of methanol on neutrophil function were more marked than those in cells exposed in vitro.

Another study by Parthasarathy et al. (2005a) also exposed 6 male Wistar rats/group i.p. to methanol at approximately 1/4 the LD_{50} (2.4 g/kg). The goal was to further monitor possible methanol-induced alterations in the activity of isolated neutrophils and other immunological parameters. The exposure protocol featured daily injections of methanol for up to 30 days in the presence or absence of sheep RBCs. Blood samples were assessed for total and differential leukocytes, and isolated neutrophils were monitored for changes in phagocytic and avidity indices, NBT reduction, and adherence. In the latter test, blood samples were incubated on a nylon fiber column, then eluted from the column and rechecked for total and differential leukocytes. Phagocytosis was monitored by incubating isolated buffy coats from the blood samples with heat-killed *C. albicans*. NBT reduction capacity examined the conversion of the dye to formazan crystals within the cytoplasm. The relative percentage of formazan-positive cells in each blood specimen gave a measure of methanol's capacity to bring about cell death. As tabulated by the authors, there was a dose-dependent reduction in lymphoid organ weights (spleen, thymus, and lymph node) in rats exposed to methanol for 15 and 30 days via i.p.

injection, irrespective of the presence of sheep RBCs. Methanol also appeared to result in a reduction in the total or differential neutrophil count. These and potentially related changes to neutrophil function are shown in Table 4-17.

Table 4-17 Effect of intraperitoneally injected methanol on total and differential leukocyte counts and neutrophil function tests in male Wistar rats

	Without sheep red blood cell treatment			With sheep red blood cell treatment		
Parameter	**Control**	**15-day methanol**	**30-day methanol**	**Control**	**15-day methanol**	**30-day methanol**
Organ weights (mg)						
Spleen	1,223 ± 54	910 ± 63[a]	696 ± 83[a,b]	1,381 ± 27	1,032 ± 39[a]	839 ± 35[a,b]
Thymus	232 ± 12	171 ± 7[a]	121 ± 10[a,b]	260 ± 9	172 ± 10[a]	130 ± 24[a,b]
Lymph node	32 ± 2	24 ± 3[a]	16 ± 2[a,b]	39 ± 2	28 ± 1[a]	23 ± 1[a,b]
Leukocyte counts						
Total leukocytes	23,367 ± 946	16,592 ± 1,219[a]	13,283 ± 2,553[a,b]	18,633 ± 2,057	16,675 ± 1,908	14,067 ± 930[a,b]
% neutrophils	24 ± 8	21 ± 3	16 ± 3[a]	8 ± 3	23 ± 4[a]	15 ± 5[a,b]
% Lymphocytes	71 ± 7	76 ± 3	79 ± 5	89 ± 4	78.5 ± 4[a]	82 ± 6
Neutrophil function tests						
Phagocytic index (%)	91.0 ± 2.0	80.0 ± 4.0[a]	79.0 ± 2.0[a]	87.0 ± 4.0	68.0 ± 3.0[a]	63.0 ± 4.0[a]
Avidity index	2.6 ± 0.3	3.2± 0.5[a]	3.2 ± 0.1[a]	4.1 ± 0.1	2.6 ± 0.3[a]	2.1 ± 0.3[a]
NBT reduction (%)	6.3 ± 2.0	18.2 ± 2.0[a]	15.0 ± 1.0[a,b]	32.0 ± 3.3	22.0 ± 3.0[a]	19.0 ± 2.4[a]
Adherence (%)	49.0 ± 5.0	44.0 ± 5.0	29.5 ± 5.0[a,b]	78.0 ± 9.2	52.0 ± 9.0[a]	30.0 ± 4.3[a,b]

[a]$p < 0.05$ from respective control.
[b]$p < 0.05$ between 15-and 30-day treatment groups.
Values are means ± S.D. (n = 6).

Source: Parthasarathy et al. (2005a).

The study provided data that showed altered neutrophil functions following repeated daily exposures of rats to methanol for periods up to 30 days. This finding is indicative of a possible effect of methanol on the immunocompetence of an exposed host.

Parthasarathy et al. (2006b) reported on additional immune system indicators as part of a study to determine the effects of methanol intoxication on the HPA axis. As described in Section 4.4.3, immune function tests conducted included the footpad thickness test for DTH, a leukocyte migration inhibition assay, the hemagglutination assay (measuring antibody titer), the neutrophil adherence test, phagocytosis index, and a NBT reduction and adherence assay used to measure the killing ability of PMNs.

Leukocyte migration and antibody titer were both significantly increased over controls for all time points, while footpad thickness was significantly deceased in 15- and 30-day treated animals. Neutrophil adherence was significantly decreased after 1 and 30 days of exposure. A significant decrease in the NBT reduction and adherence was found when comparing PMNs from the 30-day treated animals with cells from the 15-day methanol-treated group.

Parthasarathy et al. (2007) reported the effects of methanol on a number of specific immune functions. As before, 6 male Wistar rats/group were treated with 2,370 mg/kg methanol in a 1:1 mixture in saline administered intraperitoneally for 15 or 30 days. Animals scheduled/designated for termination on day 15 were immunized intraperitoneally with 5×10^9 sheep RBCs on the 10th day. Animals scheduled for day 30 termination were immunized on the 25th day. Controls were animals that were not exposed to methanol but immunized with sheep RBCs as described above. Blood samples were obtained from all animals at sacrifice and lymphoid organs including the adrenals, spleen, thymus, lymph nodes, and bone marrow were removed. Cell suspensions were counted and adjusted to 1×10^8 cells/mL. Cell-mediated immune responses were assessed using a footpad thickness assay and a leukocyte migration inhibition (LMI) test, while humoral immune responses were determined by a hemagglutination assay, and by monitoring cell counts in spleen, thymus, lymph nodes, femoral bone marrow, and in splenic lymphocyte subsets. Plasma levels of corticosterone were measured along with levels of such cytokines as TNF-α, IFN-γ, IL-2, and IL-4. DNA damage in splenocytes and thymocytes was also monitored using the Comet assay.

Table 4-18 shows decreases in the animal weight/organ weight ratios for spleen, thymus, lymph nodes and adrenal gland as a result of methanol exposure. However, the splenocyte, thymocyte, lymph node, and bone marrow cell counts were time-dependently lower in methanol-treated animals.

Table 4-18 Effect of methanol exposure on animal weight/organ weight ratios and on cell counts in primary and secondary lymphoid organs of male Wistar rats

		Immunized	
Organ	**Control**	**15 days**	**30 days**
Animal weight/organ weight ratio			
Spleen	3.88 ± 0.55	2.85 ± 0.36[a]	2.58 ± 0.45[a]
Thymus	1.35 ± 0.29	0.61 ± 0.06[a]	0.63 ±0.04[a]
Lymph node	0.10 ± 0.01	0.08 ± 0.01[a]	0.06 ± 0.02[a]
Adrenal	0.14 ± 0.01	0.15 ± 0.01	0.12 ± 0.01[a, b]
Cell counts			
Splenocytes (× 10^8)	5.08 ± 0.06	3.65 ±0.07[a]	3.71 ± 0.06[a]
Thymocytes (× 10^8)	2.66 ± 0.09	1.95 ± 0.03[a]	1.86 ± 0.09[a]
Lymph node (× 10^7)	3.03 ± 0.04	2.77 ± 0.07[a]	2.20 ± 0.06[a, b]
Bone marrow (× 10^7)	4.67 ± 0.03	3.04 ± 0.09[a]	2.11 ± 0.05[a,b]

Values are means ± six animals. [a] $p < 0.05$ versus control groups. [b] $p < 0.05$ versus 15-day treated group.

Source: Parthasarathy et al. (2007).

Parthasarathy et al. (2007) also documented their results on the cell-mediated and humoral immunity induced by methanol. Leukocyte migration was significantly increased compared to control animals, an LMI of 0.82 ± 0.06 being reported in rats exposed to methanol for 30 days. This compares to an LMI of 0.73 ± 0.02 in rats exposed for 15 days and 0.41 ± 0.10 in controls. By contrast, footpad thickness and antibody titer were decreased significantly in methanol-exposed animals compared to controls (18.32 ± 1.08, 19.73 ± 1.24, and 26.24 ± 1.68% for footpad thickness; and 6.66 ± 1.21, 6.83 ± 0.40, and 10.83 ± 0.40 for antibody titer in 30-day, 15-day exposed rats, and controls, respectively).

Parthasarathy et al. (2007) also provided data in a histogram that showed a significant decrease in the absolute numbers of Pan T cells, CD4, macrophage, major histocompatibility complex (MHC) class II molecule expressing cells, and B cells of the methanol-treated group compared to controls. The numbers of CD8 cells were unaffected. Additionally, as illustrated in the report, DNA single strand breakage was increased in immunized splenocytes and thymocytes exposed to methanol versus controls. Although some fluctuations were seen in corticosterone levels, the apparently statistically significant change versus controls in 15-day exposed rats was offset by a decrease in 30-day exposed animals. Parthasarathy et al. (2007) also tabulated the impacts of methanol exposure on cytokine levels; these values are shown in Table 4-19.

Table 4-19 The effect of methanol on serum cytokine levels in male Wistar rats

		Immunized	
Cytokines (pg/mL)	**Control**	**15 days**	**30 days**
IL-2	1,810 ± 63.2	1,303.3 ± 57.1[a]	1,088.3 ± 68.8[a,b]
IL-4	44.8 ± 2.0	74.0 ± 5.1[a]	78.8 ± 4.4[a]
TNF-α	975 ± 32.7	578.3 ± 42.6[a]	585 ± 45[a]
IFN-γ	1,380 ± 55.1	961.6 ± 72.7[a]	950 ± 59.6[a]

[a] $p < 0.05$ versus control groups.
[b] $p < 0.05$ versus 15-day treated group.
Values are means ± six animals.

Source: Parthasarathy et al.(2007).

Drawing on the results of DNA single strand breakage in this experiment, the authors speculated that methanol-induced apoptosis could suppress specific immune functions such as those examined in this research report. Methanol appeared to suppress both humoral and cell-mediated immune responses in exposed Wistar rats.

4.6. Synthesis of Major Noncancer Effects

4.6.1. Summary of Key Studies in Methanol Toxicity

A substantial body of information exists on the toxicological consequences to humans who consume or are acutely exposed to large amounts of methanol. Neurological and immunological effects have been noted in adult human subjects acutely exposed to as low as 200 ppm (262 mg/m^3) methanol (Mann et al., 2002; Chuwers et al., 1995). Nasal irritation effects have been reported by adult workers exposed to 459 ppm (601 mg/m^3) methanol. Frank effects such as blurred vision and bilateral or unilateral blindness, coma, convulsions/tremors, nausea, headache, abdominal pain, diminished motor skills, acidosis, and dyspnea begin to occur as blood levels approach 200 mg methanol/L, and 800 mg/L appears to be the threshold for lethality. Data for subchronic, chronic or in utero human exposures are very limited. Determinations regarding longer term effects of methanol are based primarily on animal studies.

An end-point-by-end-point survey of the primary noncancer effects of methanol in experimental animals is given in the following paragraphs. Tabular summaries of the principal toxicological studies that have examined the noncancer effects of methanol when experimental animals were exposed to methanol via the oral or inhalation routes are provided in Tables 4-20 and 4-21. Figures 4-1 and 4-2 graphically depict the oral and inhalation exposure–response

information from these studies, illustrating the relationship between NOAELs and LOAELs that have been identified. Most studies focused on developmental and reproductive effects. A large number of the available studies were performed by routes of exposure (e.g., i.p.) that are less relevant to the assessment. The data are summarized in separate sections that address oral exposure (Section 4.6.1.1) and inhalation exposure (Section 4.6.1.2).

Table 4-20 Summary of noncancer effects reported in repeat exposure and developmental studies of methanol toxicity in experimental animals (oral)

Species, strain, number/sex	Dose/duration	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	Effect	Reference
Rat Sprague-Dawley 30/sex/group	0, 100, 500, and 2,500 mg/kg-day for 13 wk	500	2,500	Reduction of brain weights, increase in the serum activity of ALT and AP. Increased liver weights	TRL (1986)
Rat Sprague-Dawley 100/sex/group	0, 500, 5,000, or 20,000 ppm (v/v) in drinking water, for 104 wk. Doses were approx. 0, 46.6, 466, and 1,872 mg/kg-day (male) and 0, 52.9, 529, and 2,101 mg/kg-day (female)	ND	ND	No noncancer effects were reported	Soffritti et al. (2002)
Mouse Swiss	560, 1,000 and 2,100 mg/kg/day (female) and 550, 970, and 1,800 mg/kg/day (male), 6 days/wk for life	970-1000	1,800-2,100	Increased incidence of liver parenchymal cell necrosis	Apaja (1980)
Reproductive/developmental toxicity studies					
Rat Long-Evans 10 pregnant females/group	0 and 2,500 mg/kg-day on either GD15-GD17 or GD17-GD19.	NA	2,500	Neurobehavioral deficits (such as homing behavior, suckling ability	Infurna and Weiss (1986)
Mouse CD-1 8 pregnant females and 4 Controls	4 g/kg-day in 2 daily doses on GD6-GD15	NA	4,000	Increased incidence of totally resorbed litters, cleft palate and exencephaly. A decrease in the number of live fetuses/litter	Rogers et al. (1993b)

NA = Not applicable; ND = Not determined; M= male, F=female.

Table 4-21 Summary of repeat exposure and developmental studies of methanol toxicity in experimental animals (inhalation exposure)

Species, strain, number/sex	Dose/duration	NOAEL (ppm)	LOAEL (ppm)	Effect	Reference
Monkey *M. fascicularis*, 1 or 2 animals/group	0, 3,000, 5,000, 7,000, or 10,000 ppm, 21 hr/day, for up to 14 days	ND	ND [a]	Clinical signs of toxicity, CNS changes, including degeneration of the bilateral putamen, caudate nucleus, and claustrum. Edema of cerebral white matter.	NEDO (1987)
Dog (2)	10,000 ppm for 3 min, 8 times/day for 100 days	NA	NA	None	Sayers et al. (1944)
Rat Sprague-Dawley 5 males/ group	0, 200, 2000, or 10,000 ppm, 8 hr/day, 5 days/wk for up to 6 wk	NA	200	Transient reduction in plasma testosterone levels	Cameron et al. (1984)
Rat Sprague-Dawley 5 males/ group	0, or 200 ppm, 6 hr/day, for either 1 or 7 days	NA	200	Transient reduction in plasma testosterone levels	Cameron et al. (1985)
Rat Sprague-Dawley 5/sex/group	0, 500, 2,000, or 5,000 ppm, 5 days/wk for 4 wk	5,000	NA	No compound-related effects	Andrews et al. (1987)
Monkey *M. fascicularis* 3/sex/group	0, 500, 2,000, or 5,000 ppm, 5 days/wk for 4 wk	5,000	NA	No compound-related effects	
Rat Sprague-Dawley 10/sex/group	0, 300, or 3,000 ppm, 6 hr/day, 5 days/wk for 4 wk	NA	300	Reduction in size of thyroid follicles	Poon et al. (1994)
Rat Sprague-Dawley 15/sex/group	0 or 2,500 ppm, 6 hr/day, 5 days/wk for 4 wk	NA	2,500	Reduction of relative spleen weight in females, histopathologic changes to the liver, irritation of the upper respiratory tract	Poon et al. (1995)
Monkey *M. fascicularis* 2 or 3 animals/ group/time point	0, 10, 100, or 1,000 ppm, 21 hr/day for either 7, 19, or 29 mo	ND	ND [a]	Limited fibrosis of the liver; Possible myocardial and renal effects; ; Fibrosis of responsive stellate cells in the brain	NEDO (1987)
Rat F344 20/sex/group	0, 10, 100, or 1,000 ppm, 20 hr/day, for 12 mo	NA	NA	No compound-related effects	
Mouse $B6C3F_1$ 30/sex/group	0, 10, 100, or 1,000 ppm, 20 hr/day, for 12 mo	NA	NA	No clear-cut compound-related effects	

Species, strain, number/sex	Dose/duration	NOAEL (ppm)	LOAEL (ppm)	Effect	Reference
Mouse $B6C3F_1$ 52-53/sex/group	0, 10, 100, or 1,000 ppm, 20 hr/day, for 18 mo	100	1,000	Increase in kidney weight, decrease in testis and spleen weights	
Rat F344 52/sex/group	0, 10, 100, or 1,000 ppm, ~20 hr/day for 2 yr	100	1,000	Fluctuations in a number of urinalysis, hematology, and clinical chemistry parameters.	
Rat Sprague-Dawley 15/pregnant females/group	0, 5,000, 10,000, or 20,000 ppm, 7 hr/day on either GD1-GD19 or GD7-GD15.	5,000	10,000	Reduced fetal body weight, increased incidence of visceral and skeletal abnormalities, including rudimentary and extra cervical ribs	Nelson et al. (1985)
Rat Sprague-Dawley 36/pregnant females/group	0, 200, 1,000, or 5,000 ppm, 22.7 hr/day, on GD7-GD17	1,000	5,000	Late-term resorptions, reduced fetal viability, increased frequency of fetal malformations, variations and delayed ossifications.	
Rat Sprague-Dawley F_1 and F_2 generations of a two-generation study	0, 10, 100, or 1,000 ppm, 20 hr/day; F_1- birth to end of mating (M) or weaning (F); F2- birth to 8 wks	100	1,000	Reduced weight of brain, pituitary, and thymus at 8, 16 and 24 wk postnatal in F_1 and at 8 wk in F_2	NEDO (1987)
Rat Sprague-Dawley Follow-up study of brain weights in F_1 generation of 10-14/sex/group in F_1 generation	0, 500, 1,000, and 2,000 ppm; GD0 through F_1 8 wks	500	1,000	Reduced brain weight at 3 wk and 6 wk (males only). Reduced brain and cerebrum weight at 8 wk (males only)	
Mouse CD-1 30-114 pregnant females/group	0, 1,000, 2,000, 5,000, 7,500, 10,000, or 15,000 ppm, 7 hr/day on GD6-GD15.	1,000	2,000	Increased incidence of extra cervical ribs, cleft palate, exencephaly; reduced fetal weight and pup survival, Delayed ossification	Rogers et al. (1993b)
Mouse CD-1 12-17 pregnant females/group	0 and 10,000 ppm 7 hr/day, 2 consecutive days during GD6-GD13 or on one day during GD5-GD9	NA	10,000	Cleft palate, exencephaly, skeletal malformations	Rogers and Mole (1997)

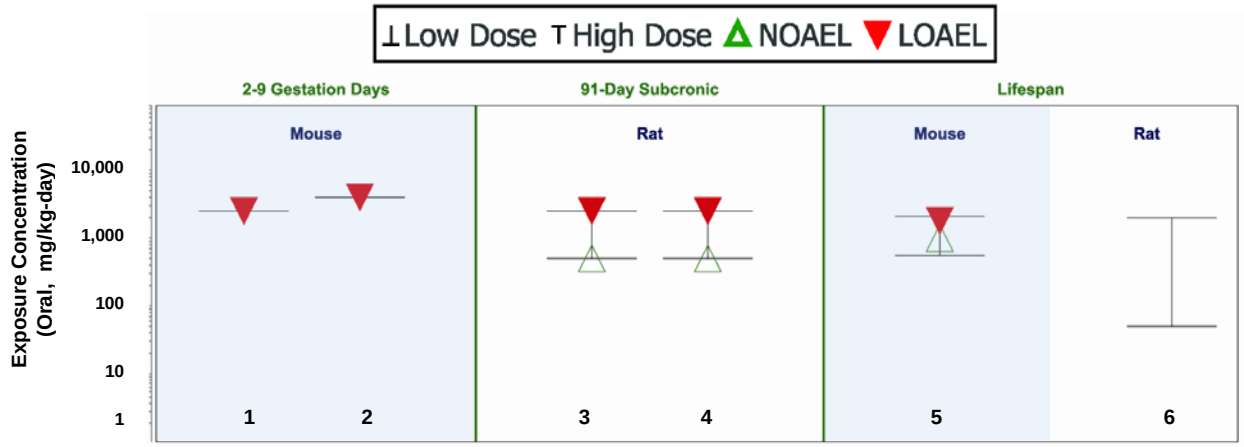
Species, strain, number/sex	Dose/duration	NOAEL (ppm)	LOAEL (ppm)	Effect	Reference
Rat Long-Evans 6-7 pregnant females/group	0 or 15,000 ppm, 7 hr/day on GD7-GD19	NA	15,000	Reduced pup weight	Stanton et al. (1995)
Rat Long-Evans 10-12 pregnant females/group	0 or 4,500 ppm from GD10 to PND21.	NA	4,500	Subtle cognitive deficits	Weiss et al. (1996)
Monkey *M. fascicularis* 12 monkeys/group	0, 200, 600, or 1,800 ppm, 2.5 hr/day, 7 days/wk, during premating, mating and gestation	ND	ND [b]	Shortened period of gestation; may be related to exposure (no dose-response), neurotoxicological deficits including reduced performance in the VDR test; may be related to premature births.	Burbacher et al. (2004b; 2004a; 1999b; 1999a)

[a]Effects in the brain and other organs were noted at exposures as low as 100 ppm (131 mg/m^3), but due to substantial uncertainties associated with these results, EPA was not able to identify a NOAEL or LOAEL from this study.

[b]The shortened gestation period was noted dams exposed to as low as 200 ppm (263 mg/m^3) and signs of possible developmental neurotoxicity were noted in the offspring of dams exposed to as low as 600 ppm (789 mg/m^3). However, because of uncertainties associated with these results, including the lack of a clear dose-response, EPA was not able to identify a NOAEL or LOAEL from this study.

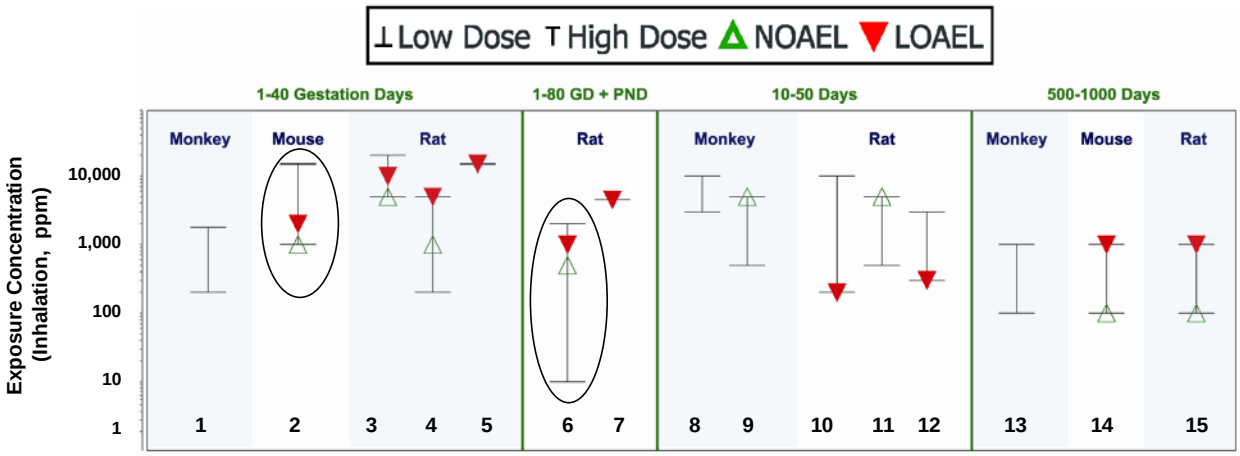
ND = Not determined due to study limitations such as small number of animals /time point/ exposure level

NA = Not applicable.



1= developmental neurobehavioral deficits (Infurna and Weiss, 1986)
2 = resorbed litters, fetal death, cleft palate, exencephaly (Rogers et al., 1993b)
3 = reduced brain weight (TRL, 1986)
4 = increased liver weight and serum activity of ALT/AP (TRL, 1986)
5 = parencymal cell necrosis (Apaja, 1980)
6 = (cancer study): no noncancer effects were reported (Soffritti et al., 2002)

Figure 4-1 Exposure response array for noncancer effects reported in animals from repeat exposure and developmental studies of methanol (Oral).



Note: Oval shapes in the array indicate principal studies used in reference value determinations.
Effects (bolded effects from principal studies were used in reference value determinations):
1= reproductive (shortened gestation) and developmental neurotoxicity (delayed VDR) (Burbacher et al., 2004; Burbacher et al., 1999a); N(L)OAELs not determined
2 = extra cervical ribs, cleft palate, excencephaly, reduced fetal weight & pup survival, delayed ossification (Rogers and Mole, 1997; Rogers et al., 1993b);
3 = reduced fetal weight, visceral and skeletal abnormalities, including rudimentary and extra cervical ribs (Nelson et al., 1985);
4 = late-term resorptions, reduced fetal viability, fetal malformations, variations and delayed ossifications (NEDO, 1987);
5 = reduced pup weight (Stanton et al., 1995)
6 = reduced weight of brain, pituitary, and thymus at 3, 6, 8, 16 and 24 wk postnatal in F1 and at 8 wk in F2 generation (NEDO, 1987)
7 = subtle cognitive deficits (Weiss et al., 1996)
8 = clinical signs of toxicity, CNS changes in bilateral putamen, caudate nucleus, and claustrum. edema of cerebral white matter (NEDO, 1987)
9 = no methanol-related effects (Andrews et al., 1987)
10 = transient reduction in plasma testosterone levels (Cameron et al., 1985; Cameron et al., 1984)
11 = no methanol-related effects (Andrews et al., 1987)
12 = reduction in size of thyroid follicles (Poon et al., 1995; Poon et al., 1994)
13 = limited fibrosis of the liver, possible myocardial and renal effects; fibrosis of responsive stellate cells in the brain (NEDO, 1987)
14 = increased kidney, and decreased testes and spleen weight (NEDO, 1987)
15 = fluctuations in a number of urinalysis, hematology, and clinical chemistry parameters (NEDO, 1987)

Figure 4-2 Exposure response array for noncancer effects reported in animals from repeat exposure and developmental studies of methanol (Inhalation).

4.6.1.1. Oral

There have been very few subchronic, chronic, or in utero experimental studies of oral methanol toxicity. In one such experiment, an EPA-sponsored 90-day gavage study in Sprague-Dawley rats suggested a possible effect of the compound on the liver (TRL, 1986). In the absence of gross or histopathologic evidence of toxicity, fluctuations on some clinical chemistry markers of liver biochemistry and increases in liver weights at the highest administered dose (2,500 mg/kg-day) justify the selection of the mid-dose level (500 mg/kg-day) as a NOAEL for this effect under the operative experimental conditions. That the bolus effect may have been important in the induction of those few effects that were apparent in the subchronic study is suggested by the outcome of lifetime drinking water study of methanol that was carried out in

Sprague-Dawley rats by Soffritti et al. (2002). According to the authors, no noncancer toxicological effects of methanol were observed at drinking water concentrations of up to 20,000 ppm (v/v). Based on default assumptions on drinking water consumption and body weight gain assumptions, the high concentration was equivalent to a dose of 1,780 mg/kg-day in males and 2,177 mg/kg-day in females. In the stated absence of any changes to parameters reflective of liver toxicity in the Soffritti et al. (2002) study, the slight impacts to the liver observed in the subchronic study (TRL, 1986) at 2,500 mg/kg-day suggest the latter dose to be a minimal LOAEL. Logically, the true but unknown threshold would at the high end of the range from 500 (the default NOAEL) to 2,500 mg/kg-day for liver toxicity via oral gavage.

Two studies have pointed to the likelihood that oral exposure to methanol is associated with developmental neurotoxicity or developmental deficits. When Infurna and Weiss (1986) exposed pregnant Long-Evans rats to 2% methanol in drinking water (providing a dose of approximately 2,500 mg/kg-day), they observed no reproductive or developmental sequelae other than from 2 tests within a battery of fetal behavioral tests (deficits in suckling ability and homing behavior). In the oral section of the Rogers et al. (1993b) study, such teratological effects as cleft palate and exencephaly and skeletal malformations were observed in fetuses of pregnant female mice exposed to daily gavage doses of 4,000 mg/kg methanol during GD6-GD15. Likewise, an increase in totally resorbed litters and a decrease in the number of live fetuses/litter appear likely to have been an effect of the compound. Similar skeletal malformations were observed by Rogers and Mole (1997), Rogers et al.(1993b), and Nelson et al. (1985) following inhalation exposure.

4.6.1.2. Inhalation

Some clinical signs, gross pathology, and histopathological effects of methanol have been seen in experimental animals including adult nonhuman primates exposed to methanol vapor. Results from an unpublished study (NEDO, 1987) of *M. fascicularis* monkeys, chronically exposed to concentrations as low as 10 ppm for up to 29 months, resulted in histopathological effects in the liver, kidney, brain and peripheral nervous system. These results were generally reported as subtle or transient. However, brain effects, such as responsive stellate cells in cerebral white matter, were observed as many as 11 months after the cessation of exposure. Confidence in the methanol-induced findings of effects in adult nonhuman primates is limited because this study utilized a small number (2-3) of animals/dose level/time of sacrifice and inadequately reporting of results (e.g., limited details on materials and methods, lack of clear documentation of a concurrent control group). Due to these concerns NOAEL and LOAEL

values could not be identified and the NEDO (1987) monkey studies have limited utility in derivation of an RfC.

A number of studies have examined the potential toxicity of methanol to the male reproductive system (Lee et al., 1991; Cameron et al., 1985; Cameron et al., 1984). The data from Cameron et al. (1985; 1984) showed a transient but not necessarily dose-related decrease in serum testosterone levels of male Sprague-Dawley rats. Lee et al. (1994) reported the appearance of testicular lesions in 18-month-old male Long-Evans rats that were exposed to methanol for 13 weeks and maintained on folate-deficient diets. Taken together, the Lee et al. (1994) and Cameron et al. (1985; 1984) study results could indicate chemically-related strain on the rat system as it attempts to maintain hormone homeostasis. However, the available data are insufficient to definitively characterize methanol as a toxicant to the male reproductive system.

When Sprague-Dawley rats were exposed to methanol, 6 hours/day for 4 weeks, there were some signs of irritation to the eyes and nose. Mild changes to the upper respiratory tract were also described in Sprague-Dawley rats that were exposed for 4 weeks to up to 300 ppm methanol (Poon et al., 1995). Other possible effects of methanol in rats included a reduction in size of thyroid follicles (Poon et al., 1994), panlobular vacuolation of the liver, and a decrease in spleen weight (Poon et al., 1995). NEDO (1987) reported dose-related increases in moderate fatty degeneration in hepatocytes of male mice exposed via inhalation for 12 months, but this finding was not observed in the NEDO (1987) 18-month mouse inhalation study. Nodes reported in the liver of mice from the 18-month study may have been precancerous, but the 18-month study duration was not of sufficient duration to make a determination.

One of the most definitive and quantifiable toxicological impacts of methanol when administered to experimental animals via inhalation is related to the induction of developmental abnormalities in fetuses exposed to the compound in utero. Developmental effects have been demonstrated in a number of species, including monkeys, but particularly rats and mice. Most developmental teratological effects appear to be more severe in the latter species. For example, in the study of Rogers et al. (1993b) in which pregnant female CD-1 mice were exposed to methanol vapors on GD6-GD15 at a range of concentrations, reproductive and fetal effects included an increase in the number of resorbed litters, a reduction in the number of live pups, and increased incidence of exencephaly, cleft palate, and the number of cervical ribs. While the biological significance of the cervical rib effect has been the subject of much debate (See discussion of Chernoff and Rogers (2004) in Section 5), it appears to be the most sensitive indicator of developmental toxicity from this study, with a NOAEL of 1,000 ppm (1,310 mg/m^3). In rats, however, the most sensitive developmental effect, as reported in the NEDO (1987) two-generation inhalation studies, was a postnatal reduction in brain weight at 3, 6 and 8 weeks postnatally, which was significantly lower than controls when pups and their dams were exposed

to 1,000 ppm (1,310 mg/m^3) during gestation and throughout lactation. The NOAEL reported in this study was 500 ppm (655 mg/m^3).

Rogers and Mole (1997) addressed the question of which period of gestation was most critical for the adverse developmental effects of methanol in CD-1 rats. Such malformations and anomalies as cleft palate, exencephaly, and a range of skeletal defects, appeared to be induced with a greater incidence when the dams were exposed on or around GD6. These findings were taken to indicate that methanol is most toxic to embryos during gastrulation and in the early stages of organogenesis. However, NEDO (1987) gestation-only and two-generation studies showed that significant reductions in brain weight were observed at a lower exposure levels when pups and their dams were exposed during lactation as well as gestation, indicating that exposure during the later stages of organogenesis, including postnatal development, can significantly contribute to the severity of the effects in this late-developing organ system.

In comparing the toxicity (NOAELs and LOAELs) for the onset of developmental effects in mice and rats exposed in utero, there is suggestive evidence from the above studies that mice may be more susceptible to methanol than rats. Supporting evidence for this proposition has come from in vitro studies in which rat and mouse embryos were exposed to methanol in culture (Andrews et al., 1993). Further evidence for species-by-species variations in the susceptibility of experimental animals to methanol during organogenesis has come from experiments on monkeys (Burbacher et al., 2004b; 2004a; 1999b; 1999a). In these studies, exposure of monkeys to methanol during premating, mating, and throughout gestation resulted in a shorter period of gestation in dams exposed to as low as 200 ppm (263 mg/m^3). Though statistically significant, the finding of a shortened gestation length may be of limited biological significance. Gestational age, birth weight and infant size observations in all exposure groups were within normal ranges for *M. fascicularis* monkeys, and other "signs of possible difficulty in the maintenance of pregnancy" reported , such as vaginal bleeding, are considered normal within 1-4 days of delivery and do not necessarily imply a risk to the fetus [as cited in CERHR (2004)]. As discussed in Section 4.4.2, there is also evidence from this study that methanol caused neurobehavioral effects in exposed monkey infants that may be related to the gestational exposure. However, the data are not conclusive, and a dose-response trend is not robust. There is insufficient evidence to determine if the primate fetus is more or less sensitive than rodents to methanol teratogenesis. The use of a cohort design necessitated by the complexity of this study may have limited its power to detect effects. Because of the uncertainties associated with these results, including the lack of a clear dose-response for decreased in gestational length and neurological effects, EPA was not able to identify a definitive NOAEL or LOAEL from this study. This study does support the weight of evidence for developmental neurotoxicity in the hazard characterization of low-level methanol exposure.

Weiss et al. (1996) and Stanton et al. (1995) evaluated the developmental and developmental neurotoxicological effects of methanol exposure on pregnant female Long-Evans rats and their progeny. In the latter study, exposure of dams to 15,000 ppm (19,656 mg/m^3), 7 hours/day on GD7-GD19 resulted in reduced weight gain in pups, but produced little other evidence of adverse developmental effects. The authors subjected the pups to a number of neurobehavioral tests that gave little if any indication of compound-related changes. This study, while using high exposure levels, was limited in its power to detect effects due to the small number of animals used. In the Weiss et al. (1996) study, exposure of pregnant female Long-Evans rats to 0 or 4,500 (0 and 5,897 mg/m^3) methanol from GD6 to PND21 likewise provided fluctuating and inconsistent results in a number of neurobehavioral tests that did not necessarily indicate any compound-related impacts. The finding of this study indicated subtle cognitive defects not on the learning of an operant task but in the reversal learning. This study also reported exposure-related changes in neurodevelopmental markers of NCAMs on PND4. NCAMs are a family of glycoproteins that is needed for migration, axonal outgrowth, and establishment of the pattern for mature neuronal function.

Taking all of these findings into consideration reinforces the conclusion that the most appropriate endpoints for use in the derivation of an inhalation RfC for methanol are associated with developmental neurotoxicity and developmental toxicity. Among an array of findings indicating developmental neurotoxicity and developmental malformations and anomalies that have been observed in the fetuses and pups of exposed dams, an increase in the incidence of cervical ribs of gestationally exposed mice (Rogers et al., 1993b) and a decrease in the brain weights of gestationally and lactationally exposed rats (NEDO, 1987) appear to be the most robust and most sensitive effects.

4.7. Noncancer MOA Information

There is controversy over the possible roles of the parent compound, metabolites, reactive oxygen species (from methanol metabolism competitively inhibiting other catalase activity) and folate deficiency (potentially associated with methanol metabolism) in the developmental toxicity of methanol. Experiments that have attempted to address these issues are reviewed in the following paragraphs.

4.7.1. Role of Methanol and Metabolites in the Developmental Toxicity of Methanol

Dorman et al. (1995) conducted a series of in vitro and in vivo studies that provide information for identifying the proximate teratogen associated with developmental toxicity in CD-1 mice. The studies used CD-1 ICR BR (CD-1) mice, HPLC grade methanol, and appropriate controls. PK and developmental toxicity parameters were measured in mice exposed to sodium formate (750 mg/kg by gavage), a 6-hour methanol inhalation (10,000 or 15,000 ppm), or methanol gavage (1.5 g/kg). In the in vivo inhalation study, 12-14 dams/ group were exposed to 10,000 ppm methanol for 6 hours on GD8,[47] with and without the administration of fomepizole to inhibit the metabolism of methanol by ADH1. Dams were sacrificed on GD10, and fetuses were examined for neural tube patency. As shown in Table 4-22, the incidence of fetuses with open neural tubes was significantly increased in the methanol group (9.65% in treated versus 0 in control) and numerically but not significantly increased in the group treated with methanol and fomepizole (7.21% in treated versus 0 in controls). Rodents metabolize methanol via both ADH1 and CAT (as discussed in Section 3.1) which, when coupled with the Dorman et al. (1995) observation that maternal formate levels in blood and decidual swellings (swelling of the uterine lining) did not differ in dams exposed to methanol alone or methanol and fomepizole, suggest that the role of ADH1 relative to CAT and nonenzymatic methanol clearance is not of great significance in adult rodents.

[47] Dorman et al. (1995) state that GD8 was chosen because it encompasses the period of murine neurulation and the time of greatest vulnerability to methanol-induced neural tube defects.

Table 4-22 Developmental outcome on GD10 following a 6-hour 10,000 ppm (13,104 mg/m^3) methanol inhalation by CD-mice or formate gavage (750 mg/kg) on GD8

Treatment	No. of litters	Open neural tubes (%)	Head length (mm)	Body length (mm)
Air	14	2.29 ± 1.01	3.15 ± 0.03	5.89 ± 0.07
Air/fomepizole	14	2.69 ± 1.19	3.20 ± 0.05	5.95 ± 0.09
Methanol	12	9.65 ± 3.13[a]	3.05 ± 0.07	5.69 ± 0.13
Methanol/fomepizole	12	7.21 ± 2.65	3.01 ± 0.05	5.61 ± 0.11
Water	10	0	3.01 ± 0.07	5.64 ± 0.11
Formate	14	2.02 ± 1.08	2.91 ± 0.08	5.49 ± 0.12

[a]$p < 0.05$, as calculated by the authors.
Values are means ± S.D.

Source: Dorman et al. (1995) (adapted).

The data in Table 4-22 suggest that the formate metabolite is not responsible for the observed increase in open neural tubes in CD-1 mice following methanol exposure. Formate administered by gavage (750 mg/kg) did not increase this effect despite the observation that this formate dose produced the same toxicokinetic profile as a 6-hour exposure to 10,000 ppm methanol vapors (48.33 mg/L formate in maternal blood and 2.0 mM formate/kg in decidual swellings). However, the data are consistent with the hypotheses that the formaldehyde metabolite of methanol may play a role. Both CAT and ADH1 activity are immature at days past conception (DPC)8 (Table 4-23). If fetal ADH1 is more mature than fetal CAT, it is conceivable that the decrease in the open neural tube response observed for methanol combined with fomepizole (Table 4-22) may be due to fomepizole having a greater effect on the metabolism of fetal methanol to formaldehyde than is observed in adult rats. Unfortunately, the toxicity studies were carried out during a period of development where ADH1 expression and activity are just starting to develop (Table 4-23); therefore, it is uncertain whether any ADH1 was present in the fetus to be inhibited by fomepizole.

Table 4-23 Summary of ontogeny of relevant enzymes in CD-1 mice and humans

	CD-1 Mouse					Human		
	Days Past Conception (DPC)					Trimesters		
	6.5	7.5	8.5		9.5	1	2	3
Somites			(8-12)	(13-20)	(21-29)			
CAT								
mRNA						N/A	N/A	N/A
activity[a]								
embryo			1	10	20			
VYS			10	15	20			
ADH1								
mRNA	–	–	–			+	+	+
activity[a]								
embryo			320	460	450			
VYS			240	280	290			
ADH3								
mRNA	+	+	+			–	–	+
activity[a]								
embryo			300	490	550			
VYS			500	500	550			

[a]Activity of CAT and ADH1 are expressed as nmol/minute/mg and pmol/minute/mg, respectively.

Source: Harris et al. (2003).

Dorman et al. (1995) provide additional support for their hypothesis that methanol's developmental effects in CD-1 mice are not caused by formate in an in vitro study involving the incubation of GD8 whole CD-1 mouse embryos with increasing concentrations of methanol or formate. Developmental anomalies were observed on GD9, including cephalic dysraphism, asymmetry and hypoplasia of the prosencephalon, reductions of brachial arches I and II, scoliosis, vesicles on the walls of the mesencephalon, and hydropericardium (Table 4-24). The concentrations of methanol used for embryo incubation (0-375 mM or 0-12,000 mg/L) were chosen to be broadly equivalent to the peak methanol levels in plasma that have been observed (approximately 100 mM or 3,200 mg/L) after a single 6-hour inhalation exposure to 10,000 ppm (13,104 mg/m^3). As discussed above, these exposure conditions induced an increased incidence of open neural tubes on GD10 embryos when pregnant female CD-1 mice were exposed on GD8. (Table 4-22). Embryonic lesions such as cephalic dysraphism, prosencephalic lesions, and brachial arch hypoplasia were observed with 250 mM (8,000 mg/L) methanol and 40 mM (1,840 mg/L) formate. The study authors noted that a formate concentration of 40 mM

(1,840 mg/L) greatly exceeds blood formate levels in mice inhaling 15,000 ppm methanol (0.75 mM = 35 mg/L), a teratogenic dose.

Table 4-24 Dysmorphogenic effect of methanol and formate in neurulating CD-1 mouse embryos in culture (GD8)

		Live embryos		Cephalic dysraphism		Prosencephalic lesions				Brachial arch hypoplasia
Treatment	Concentration (mg/L)	Total	No. abnormal	Severe	Mode-rate	Total	Hypoplasia	Asymmetry	Total	
Vehicle		20	3	0	2	2	2	0	2	0
Methanol	1984	13	1	0	0	0	1	0	1	0
	4000	14	5	1	0	2	2	2	4	1
	5984	13	7	2	4	6	3	1	4	1
	8000	15	7	2	5	7	7[a]	1	8	6[a]
	12000	12	7	6[a]	5	11[a]	9[a]	1	10[a]	8[a]
Formate	184	12	2	0	0	0	2	0	2	1
	368	13	5	1	5	6	4	2	6	0
	552	9	5	0	5	5	1	2	3	0
	920	16	7	2	5	7	2	1	3	1
	1840	16	14[a]	10[a]	4	14[a]	3	5[a]	8	13[a]

[a]$p < 0.05$, as calculated by the authors.

Source: Dorman et al. (1995) (adapted).

As discussed in Section 4.3.3, a series of studies by Harris et al. (2004; 2003) also provide evidence as to the moieties that may be responsible for methanol-induced developmental toxicity. Harris et al. (2004) have shown that among methanol and its metabolites, viability of cultured rodent embryos is most affected by formate. In contrast, teratogenic endpoints (of interest to this assessment) in cultured rodent embryos are more sensitive to methanol and formaldehyde than formate. Data from these studies indicate that developmental toxicity may be more related to formaldehyde than methanol, as formaldehyde-induced teratogenicity occurs at several orders of magnitude lower than methanol (Table 4-12) (Hansen et al., 2005; Harris et al., 2004). It should also be noted that CAT, ADH1, and ADH3 activities are present in both the rat embryo and VYS at stages as early as 6-12 somites (Harris et al., 2003); thus, it is presumable that in these ex vivo studies methanol is metabolized to formaldehyde and formaldehyde is subsequently metabolized to S-formylglutathione.

Studies involving GSH depletion have been offered as support for the hypothesis that formaldehyde is a key proximal teratogen, and for the role of ROS (see Section 4.7.3). Inhibition

of GSH synthesis with butathione sulfoximine (BSO) has little effect on developmental toxicity endpoints, yet treatment with BSO and methanol or formaldehyde increases developmental toxicity (Harris et al., 2004). Among the enzymes involved in methanol metabolism, only ADH3-mediated metabolism of formaldehyde is GSH dependent. While "depletion of GSH, as the major cellular antioxidant, will also increase the accumulation of reactive oxygen species (ROS)."This hypothesis that ADH3-mediated metabolism of formaldehyde is important for the amelioration of methanol's developmental toxicity is also supported by the diminished ADH3 activity in the mouse versus rat embryos, which is consistent with the greater sensitivity of the mouse to methanol developmental toxicity (Harris et al., 2003) (Section 4.3.3).

Without positive identification of the actual moiety responsible for methanol-induced teratogenicity, MOA remains unclear. If the moiety is methanol, then it is possible that generation of NADH during methanol oxidation creates an imbalance in other enzymatic reactions. Studies have shown that ethanol intake leads to a >100-fold increase in cellular NADH, presumably due to ADH1-mediated reduction of the cofactor NAD^+ to NADH (Cronholm, 1987; Smith and Newman, 1959). This is of potential importance because, for example, ethanol intake has been shown to increase the in vivo and in vitro enzymatic reduction of other endogenous compounds (e.g., serotonin) in humans (Svensson et al., 1999; Davis et al., 1967). In rodents, CAT-mediated methanol metabolism may obviate this effect; in humans, however, methanol is primarily metabolized by ADH1.

If the teratogenic moiety of methanol is formaldehyde, then reactivity with protein sulfhydryls and nonprotein sulfhydryls (e.g., GSH) or DNA protein cross-links may be involved. Metabolic roles ascribed to ADH3, particularly regulation of S-nitrosothiol biology (Foster and Stamler, 2004), could also be involved in the MOA. Recently, Staab et al. (2008) have shown that formaldehyde alters other ADH3-mediated reactions through cofactor recycling and that formaldehyde alters levels of cellular S-nitrosothiol, which plays a key role in cellular signaling and many cellular functions and pathways (Hess et al., 2005).

Studies such as those by Harris et al. (2004; 2003) and Dorman et al. (1995) suggest that formate is not the metabolite responsible for methanol's teratogenic effects. The former researchers suggest that formaldehyde is the proximate teratogen, and provide evidence in support of that hypothesis. However, questions remain. As has been discussed, the capacity for the metabolism of methanol to formaldehyde is likely lower in the fetus and neonate versus adults (Section 3.3). Further, researchers in this area have not yet reported using a sufficient array of enzyme inhibitors to conclusively identify formaldehyde as the proximate teratogen. Studies involving other inhibitors or toxicity studies carried out in genetically engineered mice, while not devoid of confounders, might further inform regarding the methanol MOA for developmental toxicity.

Even if formaldehyde is ultimately identified as the proximate teratogen, methanol would likely play a prominent role, at least in terms of transport to the target tissue. The high reactivity of formaldehyde would limit its unbound and unaltered transport as free formaldehyde from maternal to fetal blood (Thrasher and Kilburn, 2001). However, methanol can be metabolized to formaldehye *in situ* by multiple organ systems (Jelski et al., 2006; Motavkin et al., 1988; Bühler et al., 1983) and dose-dependent increases of formaldehyde DNA adducts derived from exogenous methanol exposure have been observed in multiple tissues such as liver, lung, spleen, thymus, bone marrow, kidney, and WBC (exogenous adduct levels were less than 10% of endogenous adduct levels for most organ systems; embryonic tissue was not examined) of rats (Lu et al., 2012).

4.7.2. Role of Folate Deficiency in the Developmental Toxicity of Methanol

As discussed in Sections 3.1 and 4.1, humans and other primates are susceptible to the effects of methanol exposure associated with formate accumulation because they have lower levels of hepatic tetrahydrofolate-dependent enzymes that help in formate oxidation. Tetrahydrofolate-dependent enzymes and critical pathways that depend on folate, such as purine and pyrimidine synthesis, may also play a role in the developmental toxicity of methanol. Studies of rats and mice fed folate-deficient diets have identified adverse effects on reproductive performance, implantation, fetal growth and developmental defects, and the inhibition of folate cellular transport has been associated with several developmental abnormalities, ranging from neural tube defects to neurocristopathies such as cleft-lip and cleft-palate, cardiac septal defects, and eye defects (Antony, 2007). Folate deficiency has been shown to exacerbate some aspects of the developmental toxicity of methanol in mice (see discussion of (Fu et al., 1996), and (Sakanashi et al., 1996), in Section 4.3.1) and rats (see discussion of (Aziz et al., 2002), in Section 4.4.1).

The studies in mice focused on the influence of FAD on the reproductive and skeletal malformation effects of methanol. Sakanashi et al. (1996) showed that dams exposed to 5 g/kg-day methanol on GD6-GD15 experienced a threefold increase in the percentage of litters affected by cleft palate and a 10-fold increase in the percentage of litters affected by exencephaly when fed a FAD (resulting in a 50% decrease in liver folate) versus a FAS diet. They speculated that the increased methanol effect from FAD diet could have been due to an increase in tissue formate or a critical reduction in conceptus folate concentration immediately following the methanol exposure. The latter appears more likely, given the high levels of formate needed to cause embryotoxicity (Section 4.3.3) and the decrease in conceptus folate that is observed within 2 hours of GD8 methanol exposure (Dorman et al., 1995). Fu et al. (1996) confirmed the findings

of Sakanashi et al. (1996) and also determined that the maternal FAD diet had a much greater impact on fetal liver folate than maternal liver folate levels.

The rat study of Aziz et al. (2002) focused on the influence of FAD on the developmental neurotoxicity of methanol. Experiments by Aziz et al. (2002) involving Wistar rat dams and pups exposed to methanol during lactation provide evidence that methanol exposure during this postnatal period affects the developing brain. These effects (increased spontaneous locomotor activity, decreased conditioned avoidance response, disturbances in dopaminergic and cholinergic receptors and increased expression of GAP-43 in the hippocampal region) were more pronounced in FAD as compared to FAS rats. This suggests that folic acid may play a role in methanol-induced neurotoxicity. These results do not implicate any particular proximate teratogen, as folate deficiency can increase levels of both methanol, formaldehyde and formate (Medinsky et al., 1997). Further, folic acid is used in a number of critical pathways such as purine and pyrimidine synthesis. Thus, alterations in available folic acid, particularly to the conceptus, could have significant impacts on the developing fetus apart from the influence it is presumed to have on formate removal.

Another problem with the hypothesized folate deficiency MOA is that an explanation for this greater mouse sensitivity is not readily apparent. Mouse livers actually have considerably higher hepatic tetrahydrofolate and total folate than rat or monkey liver (Johlin et al., 1987).

4.7.3. Methanol-Induced Formation of Free Radicals, Lipid Peroxidation, and Protein Modifications

Oxidative stress in mother and offspring has been suggested to be part of the teratogenic mechanism of a related alcohol, ethanol. Certain reproductive and developmental effects (e.g., resorptions and malformation rates) observed in Sprague-Dawley rats following ethanol exposure were reported to be ameliorated by antioxidant (Vitamin E) treatment (Wentzel et al., 2006; Wentzel and Eriksson, 2006). A number of studies have examined markers of oxidative stress associated with methanol exposure.

McCallum et al. (2011a; 2011b) treated adult male CD-1 mice, DNA repair deficient oxoguanine glycosylase (Ogg1) knockout mice, NZW rabbits and cynomolgus monkeys (*Macaca fascicularis*) with a single i.p. injection of 2 g/kg methanol and measured 8-hydroxy-2'-deoxyguanosine (8-oxodG), as an indicator of tissue oxidative DNA damage, 6 hours post-injection in the lung, liver, kidney, bone marrow and spleen. They also examined these organs for 8-oxodG in adult male CD-1 mice injected daily for 15 days with 2 g/kg methanol. They reported no evidence of methanol-dependent increases in 8-oxodG in any of the species and organ systems tested.

Miller and Wells (2011) exposed mouse embryos expressing human catalase (hCat) or their wild-type controls, and acatalasemic (aCat) mouse embryos or their wild-type controls for 24 hours to 4 mg/mL methanol or vehicle on gestational day 9. They observed higher methanol-induced teratogenicity in catalase deficient embryos, and interpreted this as an indication that ROS is involved in the embryopathic mechanism of methanol. However, contradictory results were obtained from subsequent in-vivo studies performed by the same laboratory using the same mouse strains. Siu et al. (2013) treated pregnant hCat and aCat mice and their wild-type (WT) controls with 4 g/kg of methanol (ip) or saline on GD 8. Although catalase activities were confirmed to be substantially increased in the hCat maternal livers and embryos, increases in fetal ophthalmic abnormalities and cleft palate, similar to those reported for C57BL/6J mice by Rogers et al. (2004), were observed in methanol-exposed hCat mice and their WT controls but not in methanol-exposed aCat mice or their WT controls. The authors indicated that the relative resistance of aCat mice to the embryotoxic effects of methanol could not be explained by differences in methanol metabolism because similar peak and AUC levels of methanol and its formic acid metabolite were observed for male aCat and hCat mice and their WT controls, but this would need to be verified with pharmacokinetic data for the female mice and their affected embryos. Siu et al. (2013) suggest that the apparent discrepancy between their in-vivo results and the Miller and Wells (2011) in-vitro results could be due to yet to be determined maternal factors associated with metabolism and membrane transport and/or a requirement for high catalase activity in the hCat mice, but acknowledge that it may also be an indication that ROS does not play an important embryopathic role in vivo.

Skrzydlewska et al. (2005) provided inferential evidence for the effects of methanol on free radical formation, lipid peroxidation, and protein modifications, by studying the protective effects of N-acetyl cysteine and the Vitamin E derivative, U83836E, in the liver of male Wistar rats exposed to the compound via gavage. Forty-two rats/group received a single oral gavage dose of either saline or 50% methanol. This provided a dose of approximately 6,000 mg/kg, as calculated by the authors. Other groups of rats received the same concentration of methanol, but were also injected intraperitoneally with either N-acetylcysteine or U-83836E. N-acetylcysteine and U-83836E controls were also included in the study design. Animals in each group were sacrificed after 6, 14, and 24 hours or after 2, 5, or 7 days. Livers were rapidly excised for electron spin resonance (ESR) analysis, and 10,000 × g supernatants were used to measure GSH, malondialdehyde, a range of protein parameters, including free amino and sulfhydryl groups, protein carbonyls, tryptophan, tyrosine, and bityrosine, and the activity of cathepsin B. They reported (1) an ESR signal (thought to be indicative of free radical formation) at g = 2.003 in livers harvested 6 and 12 hours after methanol exposure, (2) a significant decrease in GSH levels that was most evident in rats sacrificed 12 and 24 hours after exposure; (3) increased

concentrations in the lipid peroxidation product, malondialdehyde (by a maximum of 44% in the livers of animals sacrificed 2 days after exposure); (4) increased specific concentrations of protein carbonyl groups and bityrosine; but (5) reductions in the specific level of tryptophan. Given the ability of N-acetylcysteine and U83836E to oppose these changes, at least in part, the authors speculated that methanol-induced free radical formation and lipid peroxidation are involved. However, it is unclear whether or not the metabolites of methanol, formaldehyde, and/or formate, were involved in any of these changes.

Rajamani et al. (Rajamani et al., 2006) examined several oxidative stress parameters in male Wistar rats following methotrexate-induced folate deficiency. Compared to controls, the levels of free radical scavengers SOD, CAT, GSH peroxidase, oxidized GSH, protein carbonyls, and lipid peroxidation were elevated in several regions of the brain, with greater increases observed in the MTX-methanol-treated animals than in the MTX-alone group. The level of GSH and protein thiols was decreased in all regions of the brain, with a greater decrease observed in the MTX-methanol-treated animals than MTX-treated animals.

Dudka (2006) measured the total antioxidant status (TAS) in the brain of male Wistar rats exposed to a single oral gavage dose of methanol at 3 g/kg. The animals were kept in a nitrous oxide atmosphere (N_2O/O_2) throughout the experiment to reduce intrinsic folate levels, and various levels of ethanol and/or fomepizole (as ADH antidotes) were administered i.p. after 4 hours. Animals were sacrificed after 16 hours, the brains homogenized, and the TAS determined spectrophotometrically. As illustrated graphically by the author, methanol administration reduced TAS in brain irrespective of the presence of ADH antidotes. The author speculated that, while most methanol is metabolized in the liver, some may also reach the brain. Metabolism to formate might then alter the $NADH/NAD^+$ ratio resulting in an increase in xanthine oxidase activity and the formation of the superoxide anion.

Parthasarathy et al. (2006a) investigated the extent of methanol-induced oxidative stress in rat lymphoid organs. Six male Wistar rats/group received 2,370 mg/kg methanol (mixed 1:1 with saline) injected i.p. for 1, 15 or 30 days. A control group received a daily i.p. injection of saline for 30 days. At term, lymphoid organs such as the spleen, thymus, lymph nodes, and bone marrow were excised, perfused with saline, then homogenized to obtain supernatants in which such indices of lipid peroxidation as malondialdehyde, and the activities of CAT, SOD, and GSH peroxidase were measured. Parthasarathy et al. (2006a) also measured the concentrations of GSH and ascorbic acid (nonenzymatic antioxidants) and the serum concentrations of a number of indicators of liver and kidney function, such as ALT, AST, blood urea nitrogen (BUN), and creatinine.

Table 4-25 shows the time-dependent changes in serum liver and kidney function indicators that resulted from methanol administration. Treatment with methanol for increasing

durations resulted in increased serum ALT and AST activities and the concentrations of BUN and creatinine.

Table 4-25 Time-dependent effects of methanol administration on serum liver and kidney function, serum ALT, AST, BUN, and creatinine in control and experimental groups of male Wistar rats

Parameters	Methanol administration (2,370 mg/kg)			
	Control	Single dose	15 days	30 days
ALT (µmoles/min-mg)	29.0 ± 2.5	31.4 ± 3.3	53.1 ± 2.3[a]	60.4 ± 2.8[a]
AST (µmoles/min-mg)	5.8 ± 0.4	6.4 ± 0.3	9.0 ± 1.2[a]	13.7 ± 1.2[a]
BUN (mg/L)	301 ± 36	332 ± 29	436 ± 35[a]	513 ± 32[a]
Creatinine (mg/L)	4.6 ± 0.3	4.8 ± 0.3	5.6 ± 0.2[a]	7.0 ± 0.4[a]

[a]$p < 0.05$ versus controls.
Values are means ± S.D. of 6 animals.

Source: Parthasarathy et al. (2006a) (adapted).

Table 4-26 Effect of methanol administration on male Wistar rats on malondialdehyde concentration in the lymphoid organs of experimental and control groups and the effect of methanol on antioxidants in spleen

Parameters	Methanol administration (2,370 mg/kg)			
	Control	Single dose	15 days	30 days
Malondialdehyde in lymphoid organs				
Spleen	2.62 ± 0.19	4.14 ± 0.25[a]	7.22 ± 0.31[a]	9.72 ± 0.52[a]
Thymus	3.58 ± 0.35	5.76 ± 0.36[a]	9.23 ± 0.57[a]	11.6 ± 0.33[a]
Lymph nodes	3.15 ± 0.25	5.08 ± 0.24[a]	8.77 ± 0.57[a]	9.17 ± 0.67[a]
Bone marrow	3.14 ± 0.33	4.47 ± 0.18[a]	7.20 ± 0.42[a]	9.75 ± 0.56[a]
Antioxidant levels in spleen				
SOD (units/mg protein)	2.40 ± 0.16	4.06 ± 0.19[a]	1.76 ± 0.09[a]	1.00 ± 0.07[a]
CAT (µmoles H_2O_2 consumed/min-mg protein	35.8 ± 2.77	52.5 ± 3.86[a]	19.1 ± 1.55[a]	10.8 ± 1.10[a]
GPx (µg GSH consumed/min-mg protein)	11.2 ± 0.60	20.0 ± 1.0[a]	7.07 ± 0.83[a]	5.18 ± 0.45[a]
GSH (µg/mg protein)	2.11 ± 0.11	3.75 ± 0.15[a]	1.66 ± 0.09[a]	0.89 ± 0.04[a]
Vit C (µg/mg protein)	0.45 ± 0.04	0.73 ± 0.05[a]	0.34 ± 0.18[a]	0.11 ± 0.03[a]

[a]$p < 0.05$, versus controls.
Values are means ± S.D. of six animals.

Source: Parthasarathy et al. (2006a) (adapted).

Table 4-26 gives the concentration of malondialdehyde in the lymphoid organs of control and experimental groups, and, as an example of all tissue sites examined, the levels of enzymatic and nonenzymatic antioxidants in spleen. The results show that malondialdehyde concentrations were time-dependently increased at each tissue site and that, in spleen as an example of all the lymphoid tissues examined, increasing methanol administration resulted in lower levels of all antioxidants examined compared to controls. Parthasarathy et al. (2006a) concluded that exposure to methanol may cause oxidative stress by altering the oxidant/antioxidant balance in lymphoid organs in the rat.

4.7.4. Exogenous Formate Dehydrogenase as a Means of Detoxifying the Formic Acid that Results from Methanol Exposure

In companion reports, Muthuvel et al. (2006a; 2006b) used 6 male Wistar rats/group to test the ability of exogenously-administered formate dehydrogenase (FD) to reduce the serum levels of formate that were formed when 3 g/kg methanol was administered i.p. to rats in saline.

In the first experiment, purified FD (from *Candida boitinii*) was administered by i.v. conjugated to the N-hydroxysuccinimidyl ester of monomethoxy polyethylene glycol propionic acid (PEG-FD) (Muthuvel et al., 2006b). In the second, rats were administered FD-loaded erythrocytes (Muthuvel et al., 2006a). In the former case, some groups of rats were made folate deficient by means of a folate-depleted diet; in the latter, folate deficiency was brought about by i.p. administration of methotrexate. In some groups, the rats received an infusion of an equimolar mixture of carbonate and bicarbonate (each at 0.33 mol/L) to correct the formate-induced acidosis. As illustrated by the authors, methanol-exposed rats receiving a folate-deficient diet showed significantly higher levels of serum formate than those receiving a folate-sufficient diet. However, administration of native or PEG-FD reduced serum formate in methanol-receiving folate-deficient rats to levels seen in animals receiving methanol and the folate-sufficient diet.

In the second report, Muthuvel et al. (2006a) carried out some preliminary experiments to show that hematological parameters of normal, reconstituted but unloaded, and reconstituted and FD-loaded erythrocytes, were similar. In addition, they showed that formate levels of serum were reduced in vitro in the presence of FD-loaded erythrocytes. Expressing blood formate concentration in mmol/L at the 1-hour time point after carbonate/bicarbonate and enzyme-loaded erythrocyte infusion via the tail vein, the concentration was reduced from 10.63 ± 1.3 (mean ± S.D.) in methanol and methotrexate-receiving controls to 5.83 ± 0.97 (n = 6). This difference was statistically significant at the $p < 0.05$ level. However, FD-loaded erythrocytes were less efficient at removing formate in the absence of carbonate/bicarbonate. Effective elimination of formate appears to require an optimum pH for the FD activity in the enzyme-loaded erythrocytes.

4.7.5. Summary and Conclusions Regarding MOA for Developmental Toxicity

Data from experiments carried out by Dorman et al. (1995) indicate that formate is not the probable proximate teratogen in pregnant CD-1 mice exposed to high concentrations of methanol vapor. This conclusion is based on the observation that there appeared to be little, if any, accumulation of formate in the blood of methanol-exposed mice, and exencephaly did not occur until formate levels were grossly elevated. In addition, treatment of pregnant mice with a high oral dose of formate did not induce neural tube closure defects at media concentrations comparable to those observed in uterine decidual swelling after maternal exposure to methanol. Lastly, methanol- but not formate- induced neural tube closure defects in mouse embryos in vitro at media concentrations comparable to the levels of methanol detected in blood after a teratogenic exposure.

Harris and colleagues (Hansen et al., 2005; Harris et al., 2004; Harris et al., 2003) carried out a series of physiological and biochemical experiments on mouse and rat embryos exposed to methanol, formaldehyde and formate, concluding that the etiologically important substance for embryo dysmorphogenesis and embryolethality was likely to be formaldehyde rather than the parent compound or formate. Specific activities for enzymes involved in methanol metabolism were determined in rat and mouse embryos during the organogenesis period of 8-25 somites (Harris et al., 2003). The experiment was based on the concept that differences in the metabolism of methanol to formaldehyde and formic acid by the enzymes ADH1, ADH3, and CAT may contribute to hypothesized differences in species sensitivity that were apparent in toxicological studies. A key finding was that the activity of ADH3 (converting formaldehyde to formate) was lower in mouse VYS than that of rats throughout organogenesis, consistent with the greater sensitivity of the mouse to the developmental effects of methanol exposure. Another study (Harris et al., 2004) which showed that the inhibition of GSH synthesis increases the developmental toxicity of methanol also lends support to this hypothesis because ADH3-mediated metabolism of formaldehyde is the only enzyme involved in methanol metabolism that is GSH-dependent. These findings provide inferential evidence for the proposition that formaldehyde may be the ultimate teratogen through diminished ADH3 activity. This concept is further supported by the demonstration that the LOAELs for the embryotoxic effects of formaldehyde in rat and mouse embryos were much lower than those for formate and methanol (Hansen et al., 2005). The findings from both sets of experiments (Hansen et al., 2005; Harris et al., 2004; Harris et al., 2003) suggest that the lower capacity of mouse embryos to transform formaldehyde to formate (by ADH3) could explain the increased susceptibility of mouse versus rat embryos to the toxic effects of methanol.

Recent studies suggest that mouse embryo tissue may have a high sensitivity to oxidative damage relative to other species following methanol exposure (Miller and Wells, 2011; Sweeting et al., 2011). Sweeting et al. (2011) postulated that one possible explanation for this sensitivity may be a strong reliance of mice on catalase over ADH to metabolize embryonic methanol. A low ADH activity in mouse embryo relative to rats [(Harris et al., 2003), Section 4.3.3], combined with a preference of catalase to metabolize methanol over hydrogen peroxide (Sweeting et al., 2011), could lead to a reduction in catalase activity and a higher level of ROS in mouse versus rat embryos, partially explaining the higher sensitivity of mice to the embryotoxic effects of methanol. If an appreciable portion of methanol's teratogenicity in sensitive mouse strains can be explained by this mode of action, and if this mode of action is not applicable to human fetuses, then sensitive mouse strains may not adequately reflect human risk. However, the evidence for this mode of action remains limited. Further, as discussed in Section 3.3, there is

reason to believe that human infants can metabolize methanol via a mechanism other than ADH, and that this alternative mechanism could involve catalase (Tran et al., 2007).

While studies such as those by Harris et al. (2004; 2003) and Dorman and colleagues (Dorman and Welsch, 1996; Dorman et al., 1995) strongly suggest that formate is not the metabolite responsible for methanol's teratogenic effects, there are still questions regarding the relative involvement of parent methanol, formaldehyde and ROS. However, both the proposed formaldehyde and ROS MOAs require methanol to be present at the target site. Methanol can be metabolized to formaldehye *in situ* by multiple organ systems and the high reactivity of formaldehyde would limit its unbound and unaltered transport as free formaldehyde (see discussion in Section 4.7.1), and the ROS MOA would require the presence of methanol to alter embryonic catalase activity.

4.8. Evaluation of Carcinogenicity

Carcinogenicity will be addressed in a separate document.

4.9. Susceptible Populations and Life Stages

4.9.1. Possible Childhood Susceptibility

Studies in animals have identified the fetus as being more sensitive than adults to the toxic effects of methanol; the greatest susceptibility occurs during gastrulation and early organogenesis (CERHR, 2004). Table 4-23 summarizes some of the data regarding the relative ontogeny of CAT, ADH1, and ADH3 in humans and mice. Human fetuses have limited ability to metabolize methanol as ADH1 activity in 2-month-old and 4-5 month-old fetuses is 3-4% and 10% of adult activity, respectively (Pikkarainen and Raiha, 1967). ADH1 activity in 9-22 week old fetal livers was found to be 30% of adult activity (Smith et al., 1971). Likewise, ADH1 activity is ~20-50% of adult activity during infancy (Smith et al., 1971; Pikkarainen and Raiha, 1967). Activity continues to increase until reaching adult levels at 5 years of age (Pikkarainen and Raiha, 1967). However, no difference between blood methanol levels in 1-year-old infants and adults was observed following ingesting the same doses of aspartame, which releases 10% methanol by weight during metabolism (Stegink et al., 1983). Given that the exposure was aspartame as opposed to methanol, it is difficult to draw any conclusions from this study vis-à-vis ontogeny data and potential influences of age differences in aspartame disposition. With regard to inhalation exposure, increased breathing rates relative to adults may result in higher

blood methanol levels in children compared to adults (CERHR, 2004). It is also possible that metabolic variations resulting in increased methanol blood levels in pregnant women could increase the fetus' risk from exposure to methanol. In all, unresolved issues regarding the identification of the toxic moiety increase the uncertainty with regards to the extent and pathologic basis for early life susceptibility to methanol exposure.

The prevalence of folic acid deficiency has decreased since the United States and Canada introduced a mandatory folic acid food fortification program in November 1998. However, folate deficiency is still a concern among pregnant and lactating women, and factors such as smoking, a poor quality diet, alcohol intake, and folic antagonist medications can enhance deficiency (CERHR, 2004). Folate deficiency could affect a pregnant woman's ability to clear formate, which has also been demonstrated to produce developmental toxicity in rodent in in vitro studies at high-doses (Dorman et al., 1995). It is not known if folate-deficient humans have higher levels of blood formate than individuals with adequate folate levels. A limited study in folate-deficient monkeys demonstrated no formate accumulation following an endotracheal exposure of anesthetized monkeys to 900 ppm methanol for 2 hours (Dorman et al., 1994). The situation is obscured by noting that folic acid deficiency during pregnancy by itself is thought to contribute to the development of severe congenital malformations (Pitkin, 2007).

4.9.2. Possible Gender Differences

There is limited information on potential differences in susceptibility to the toxic effects of methanol according to gender. One study (n=12) reported a higher background blood methanol level in human females versus males (Batterman and Franzblau, 1997), but a larger study (n=35) did not observe gender differences (Sarkola and Eriksson, 2001). In rodents, fetuses exposed in utero were found to be the most sensitive subpopulation. One study suggested a possible increased sensitivity of male versus female rat fetuses and pups. When rats were exposed to methanol pre- and postnatally, 6- and 8-week-old male progeny had significantly lower brain weights at 1,000 ppm, compared to those in females that demonstrated the same effect only at 2,000 ppm (NEDO, 1987). In general, there is little evidence for substantial disparity in the level or degree of toxic response to methanol in male versus female experimental animals or humans. However, it is possible that the compound-related deficits in fetal brain weight that were evident in the pups of F_1 generation Sprague-Dawley rats exposed to methanol in the NEDO (1987) study may reflect a threshold neurotoxicological response to methanol. It is currently unknown whether higher levels of exposure would result in brain sequelae comparable to those observed in acutely exposed humans.

4.9.3. Genetic Susceptibility

Polymorphisms in enzymes involved in methanol metabolism may affect the sensitivity of some individuals to methanol. For example, as discussed in Section 3, data summarized in reviews by Agarwal (2001), Burnell et al. (1989), Bosron and Li (1986), and Pietruszko (1980) discuss genetic polymorphisms for ADH. Class I ADH, the primary ADH in human liver, is a dimer composed of randomly associated polypeptide units encoded by three genetic loci (ADH1A, ADH1B, and ADH1C). Polymorphisms are observed at the ADH1B (ADH1B*2, ADH1B*3) and ADH1C (ADH1C*2) loci. The ADH1B*2 phenotype is estimated to occur in ~15% of Caucasians of European descent, 85% of Asians, and less that 5% of African Americans. Fifteen percent of African Americans have the ADH1B*3 phenotype, while it is found in less than 5% of Caucasian Europeans and Asians. The only reported polymorphisms in ADH3 occur in the promoter region, one of which reduces the transcriptional activity in vitro nearly twofold (Hedberg et al., 2001). While polymorphisms in ADH3 are described in more than one report (Cichoz-Lach et al., 2007; Hedberg et al., 2001), the functional consequence(s) for these polymorphisms remains unclear.

5. DOSE-RESPONSE ASSESSMENTS AND CHARACTERIZATION

5.1. Inhalation Reference Concentration (RfC)[48]

In general, the RfC is an estimate (with uncertainty spanning perhaps an order of magnitude) of a continuous inhalation exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. It is derived from a POD, generally an estimated 95 percent lower confidence limit on the BMD (i.e., BMDL), with uncertainty factors applied to reflect limitations of the data used. The inhalation RfC considers toxic effects for both the respiratory system (portal-of-entry) effects and systems peripheral to the respiratory system (extra-respiratory or systemic effects). It is generally expressed in mg/m^3.

This assessment uses BMD methoding to identify the POD.[49] The suitability of these methods to derive a POD is dependent on the nature of the toxicity database for a specific chemical. Details of the BMD analyses are found in Appendix D. The use of the BMD approach for identifying the POD is preferred over the NOAEL/LOAEL approach because the BMD approach includes consideration of the shape of the dose-response curve, is less dependent onexperimental dose selection, and estimates uncertainty pertaining to themodeled dose response. However, the methanol database still has limitations and uncertainties associated with it, in particular, uncertainties associated with human variability, animal-to-human differences, and limitations in the database that influence derivation of the RfC.

5.1.1. Choice of Principal Study and Critical Effect(s)

5.1.1.1. Key Inhalation Studies

While a substantial body of information exists on the toxicological consequences to humans exposed to high concentations of methanol, no human studies exist that would allow for quantification of subchronic, chronic, or in utero effects of methanol exposure. Table 4-21 summarizes available experimental animal inhalation studies of methanol. Several of these

[48] The RfC discussion precedes the RfD discussion in this assessment because the inhalation database ultimately serves as the basis for the RfD. The RfD development would be difficult to follow without prior discussion of inhalation database and PK models used for the route-to-route extrapolation.

[49] Use of BMD modeling involves fitting mathematical models to dose-response data and using the results to estimate a POD that is associated with a selected benchmark response (BMR), such as a percentage increase in the incidence of a particular lesion or a percentage decrease in body weight gain (see Section 5.1.2.2).

studies, including monkey chronic (NEDO, 1987) and developmental (Burbacher et al., 2004b; 2004a; 1999b; 1999a) studies, male rat reproductive studies (Lee et al., 1991; Cameron et al., 1985; Cameron et al., 1984), and 4-week rat studies (Poon et al., 1994), are lacking in key attributes (e.g., documented dose response, documented controls, and duration of exposure) necessary for use in the derivation of a chronic RfC. The inhalation reproductive or developmental studies that were adequately documented and are of appropriate size and design for use in the derivation of an RfC are summarized in Table 5-1 below.

5.1.1.2. Selection of Critical Effect(s)

5.1.1.2.1. Skeletal Development

Skeletal effects have been observed in developmental studies of rats (Weiss et al., 1996; NEDO, 1987; Nelson et al., 1985) and mice (Bolon et al., 1993; Rogers et al., 1993b). The findings of Bolon et al. (1993) and Rogers and Mole (1997) indicate that methanol is toxic to mouse embryos in the early stages of organogenesis, on or around GD7. In the study of Rogers et al. (1993b), in which pregnant female CD-1 mice were exposed to methanol vapors (1,000, 2,000, and 5,000 ppm) on GD6-GD15, reproductive and fetal effects included an increase in the number of resorbed litters, a reduction in the number of live pups, and increased incidences of exencephaly, cleft palate, and the number of cervical ribs. They reported a NOAEL for extra cervical ribs at 1,000 ppm (1,310 mg/m^3) and a LOAEL of 2,000 ppm (2,620 mg/m^3, 49.6% per litter versus 28.0% per litter in the control group). Increased incidence of cervical ribs was also observed in the rat organogenesis study (NEDO, 1987) in the 5,000 ppm dose group (65.2% per litter versus 0% in the control group), indicating that the endpoint is significant across species.

The biological significance of the cervical rib endpoint has been the subject of much debate (Chernoff and Rogers, 2004). Previous studies have classified this endpoint as either a malformation (birth defect of major importance) or a variation (morphological alternation of minor significance). There is evidence that incidence of supernumerary ribs (including cervical ribs) is not just the addition of extraneous, single ribs but rather is related to a general alteration in the development and architecture of the axial skeleton as a whole. In CD-1 mice exposed during gestation to various types of stress, food and water deprivation, and the herbicide dinoseb, supernumerary ribs were consistently associated with increases in length of the 13th rib (Branch et al., 1996). This relationship was present in all fetal ages examined in the study. The authors concluded that these findings are consistent with supernumerary ribs being one manifestation of a basic alteration in the differentiation of the thoraco-lumbar border of the axial skeleton. The biological significance of this endpoint is further strengthened by the association of

supernumerary ribs with adverse health effects in humans. The most common effect produced by the presence of cervical ribs is thoracic outlet disease (Nguyen et al., 1997; Fernandez Noda et al., 1996; Henderson, 1914). Thoracic outlet disease is characterized by numbness and/or pain in the shoulder, arm, or hands. Vascular effects associated with this syndrome include cerebral and distal embolism (Bearn et al., 1993; Connell et al., 1980; Short, 1975), while neurological symptoms include extreme pain, migraine, and symptoms similar to Parkinson's (Evans, 1999; Saxton et al., 1999; Fernandez Noda et al., 1996). Schumacher et al. (1992) observed 242 rib anomalies in 218 children with tumors (21.8%) and 11 (5.5%) in children without malignancy, a statistically significant ($p < 0.001$) difference that indicates a strong association between the presence of cervical ribs and childhood cancers. Thus, the mouse cervical rib endpoint is considered potentially relevant to humans and appropriate for use in the derivation of an RfC or RfD.

5.1.1.2.2. Developmental Neurotoxicity

NEDO (1987) reported reduced brain, pituitary, and thymus weights in F_1 and F_2 generation Sprague-Dawley rats at 1,000 ppm methanol. In a follow-up study of the F_1 generation brain weight effects, NEDO (1987) reported decreased brain, cerebellum, and cerebrum weights in F_1 males exposed at 1,000 ppm methanol from GD0 through the F_1 generation.[50] The exposure levels used in these studies are difficult to interpret because dams were exposed prior to gestation, and dams and pups were exposed during gestation and lactation. However, it is clear that postnatal exposure increases the severity of brain weight reduction. In another experiment in which NEDO (1987) exposed rats only during organogenesis (GD7-GD17), the observed decreases in brain weights in offspring at 8 weeks of age were less severe than in the studies for which exposure was continued postnatally. This finding is not unexpected, given that the brain undergoes tremendous growth beginning early in gestation and continuing in the postnatal period. Rats are considered altricial (i.e., born at relatively underdeveloped stages), and many of their neurogenic events occur postnatally (Clancy et al., 2007). Brain effects from postnatal exposure are also relevant to humans given that, in humans, gross measures of brain growth increase for at least 2-3 years after birth, with the growth rate peaking approximately 4 months after birth (Rice and Barone, 2000).

A change in brain weight is considered to be a biologically significant effect (U.S. EPA, 1998a). This is true regardless of changes in body weight because brain weight is generally conserved during malnutrition or weight loss, unlike many other organs or tissues (U.S. EPA,

[50] For the interpretation of the dose-response data, EPA did not rely on the statistics reported by NEDO (1987) which were based on inappropriate t-test methods but, instead, relied on the results of the benchmark dose analyses described in Appendix D.

1998a). Thus, change in absolute brain weight is an appropriate measure of effects on this critical organ system. Decreases in brain weight have been associated with simultaneous deficits in neurobehavioral and cognitive parameters in animals exposed during gestation to various solvents, including toluene and ethanol (Gibson et al., 2000; Coleman et al., 1999; Hass et al., 1995). NEDO (1987) reports that brain, cerebellum, and cerebrum weights decrease in a dose-dependant manner in male rats exposed to methanol throughout gestation and the F_1 generation. While brain weight reduction has not been observed in other developmental bioassays, it has been observed in adult rats exposed to methanol (TRL, 1986), and it was not an endpoint that has been extensively measured in other developmental studies of methanol [e.g., the Rogers et al. (1993b) mouse studies].

Developmental neurobehavioral effects associated with methanol inhalation exposure have been investigated in monkeys. Burbacher et al. (2004b; 2004a; 1999b; 1999a) exposed *M. fascicularis* monkeys to 0, 200, 600, or 1,800 ppm (0, 262, 786, and 2,359 mg/m^3) methanol, 2.5 hours/day, 7 days/week during premating/mating and throughout gestation (approximately 168 days). There appeared to be neurotoxicological deficits in methanol-exposed offspring. VDR was significantly reduced in the 600 ppm (786 mg/m^3) methanol group for males and in the 1,800 ppm (2,359 mg/m^3) methanol group for both sexes. However, a dose-response trend for this endpoint was only exhibited for females. In fact, the VDR response in females is the only effect reported in the Burbacher et al. (2004b; 2004a; 1999b; 1999a) studies for which a significant dose-response trend is evident. As discussed in Section 4.4.2, confidence may have been increased by statistical analyses to adjust for multiple comparisons (CERHR, 2004). Yet, it is worth noting that the dose-response trend for VDR in females remained significant with (p = 0.009) and without (p = 0.0265) an adjustment for the shortened gestational periods, and it is a measure of functional deficits in sensorimotor development that is consistent with early developmental CNS effects (brain weight changes discussed above) that have been observed in rats.

Another test, the Fagan test of infant intelligence, indicated small but not significant deficits of performance (time spent looking at novel faces versus familiar faces) in treated monkeys. Although not statistically significant and not quantifiable, the results of this test need to be considered, in conjunction with VDR test results and brain weight changes noted in the NEDO (1987) rat study, as a possible indication of CNS effects. As discussed in Section 4.6.1.2, the monkey data are not conclusive, and there is insufficient evidence to determine if the primate fetus is more or less sensitive than rodents to methanol teratogenesis. Taken together, however, the NEDO (1987) rat study and the Burbacher et al. (2004b; 2004a; 1999b; 1999a) monkey study suggest that prenatal exposure to methanol can result in adverse effects on developmental neurology pathology and function, which can be exacerbated by continued postnatal exposure.

5.1.1.2.3. Reproductive Effects

In the Burbacher et al. (2004b; 2004a; 1999b; 1999a) studies, exposure of monkeys to up to 1,800 ppm (2,359 mg/m^3) methanol during premating, mating, and throughout gestation resulted in no changes in reproductive parameters other than a shorter period of gestation in all exposure groups that did not appear to be dose related. As discussed in Section 4.6.1.2, though statistically significant, the shortened gestation finding may be of limited biological significance given questions concerning its relation to the methanol exposure. Developmental parameters, such as fetal crown-rump length and head circumference, were unaffected.

A number of studies described in Section 4.3.2 and summarized in Section 4.6.1.2 have examined the potential toxicity of methanol to the male reproductive system (Lee et al., 1991; Cameron et al., 1985; Cameron et al., 1984). Some of the observed effects, including a transient decrease in testosterone levels, could be the result of chemically related strain on the rat hormonal system. However, the data are insufficient to definitively characterize methanol as a toxicant to the male reproductive system.

5.1.1.2.4. Chosen Critical Effects

The studies considered for use in the derivation of an RfC are summarized in Table 5-1. As discussed in Sections 5.1.3.1 and 5.3.1, there is uncertainty associated with the selection of an effect endpoint from the methanol database for use in the derivation of an RfC. Though monkeys may represent the more relevant species, the available monkey studies are not adequate for dose-response analysis. Taking into account the advantages and limitations of the studies available for quantification purposes and the relative sensitivities for the effects observed, two developmental effect endpoints were chosen as candidate critical effects for the purposes of this dose-response assessment, cervical rib anomalies in fetal CD-1 mice (Rogers et al., 1993b) and decreased brain weight in male Sprague-Dawley rats exposed throughout gestation and lactation (NEDO, 1987). These endpoints can be reliably quantified and represent adverse effects in two separate sensitive organ systems at key periods of their development. RfC derivations for these endpoints using various derivation options are summarized in Appendix D.

Table 5-1 Summary of studies considered most appropriate for use in derivation of an RfC

Reference	Species (strain)	Number/ dose group	Exposure Duration	Critical Effect	NOAEL (ppm)	LOAEL (ppm)
NEDO (1987) Teratology study	Rat Sprague-Dawley	10-12/sex/ group	GD7-GD17	Reduced brain, pituitary, thyroid, thymus, and testis weights at 8 wk postnatal.	1,000	5,000
NEDO (1987) Two-generation study	Rat Sprague-Dawley	Not specified - F_1 and F_2 generation	F_1-Birth to end of mating (M) or weaning (F); F_2-birth to 8 wk	Reduced weight of brain, pituitary, and thymus at 8, 16, and 24 wk postnatal in F_1 and at 8 wk in F_2	100	1,000
NEDO (1987) Follow-up study, F_1 generation		10-14/ sex/ group- F_1 generation	GD0 through F_1 generation	Reduced brain weight at 3 wk and 6 wk (males only). Reduced brain and cerebrum weight at 8 wk (males only)	500	1,000
Rogers et al. (1993b)	Mouse CD-1	30-114 pregnant dams/ group	GD6-GD15	Increased incidence of extra cervical ribs, cleft palate, exencephaly; reduced fetal weight and pup survival, delayed ossification	1,000	2,000
Burbacher et al. (2004b; 2004a; 1999b; 1999a)	*M. fascicularis*	12 pregnant monkeys/group	2.5 hr/day, 7 days/wk, during premating, mating and gestation	Shortened period of gestation; may be related to exposure (no dose response), neurotox. deficits including reduced performance in the VDR test	–	–[a]

[a]Gestational exposure resulted in a shorter period of gestation in dams exposed to as low as 200 ppm (263 mg/m^3). However, because of uncertainties associated with these results, including the lack of a clear dose-response, EPA was not able to identify a definitive NOAEL or LOAEL from this study.

5.1.2. Methods of Analysis for the POD—Application of PBPK and BMD Models

Potential PODs for the RfC derivation, described in Appendix D, have been calculated via the use of PBPK models, described in Section 3.4 and to a greater extent in Appendix B. First, the doses used in an experimental bioassay were converted to an internal dose metric that is most appropriate for the endpoint being assessed. The PBPK models are capable of calculating several measures of dose for methanol, including the following:

- C_{max} – The peak concentration of methanol in the blood during the exposure period;
- AUC – Area under the curve, which represents the cumulative product of concentration and time for methanol in the blood; and
- Total metabolism – The production of metabolites of methanol, namely formaldehyde and formate.

Although there remains uncertainty surrounding the identification of the proximate teratogen of importance (methanol, formaldehyde, or ROS), the dose metric chosen for derivation of an RfC was based on blood methanol levels. This decision was primarily based on evidence that the toxic moiety for developmental effects is not likely to be the formate metabolite of methanol (CERHR, 2004) and evidence that levels of the formaldehyde metabolite following methanol maternal and/or neonate exposure would be much lower in the fetus and neonate than in adults. While recent in vitro evidence indicates that formaldehyde is more embryotoxic than methanol and formate, the high reactivity of formaldehyde would limit its unbound and unaltered transport as free formaldehyde from maternal to fetal blood (Thrasher and Kilburn, 2001), and the capacity for the metabolism of methanol to formaldehyde is likely lower in the fetus and neonate versus adults (see discussion in Section 3.3). Thus, even if formaldehyde is identified as the proximate teratogen, methanol would likely play a prominent role, at least in terms of transport to the target tissue. Further discussions of methanol metabolism, dose metric selection, and MOA issues are covered in Sections 3.3 and 4.7.

A BMDL was then estimated in terms of the internal dose metric utilized. Finally, after application of UFs (see Section 5.1.3.2), the BMDL values were converted to HECs via the use of a PBPK model parameterized for humans. The next section describes the rationale for and application of the benchmark modeling methodology for the RfC derivation.

5.1.2.1. Application of the BMD/BMDL Approach

Several developments over the past few years impact the derivation of the RfC: (1) EPA has developed BMD assessment methods (U.S. EPA, 2012a, 1995) and supporting software (U.S. EPA, 2011a) to improve upon the previous NOAEL/LOAEL approach; (2) MOA studies have been carried out that can give more insight into methanol toxicity; and (3) EPA has refined PBPK models for methanol on the basis of the work of Ward et al. (1997) (see Appendix B for a description of the EPA models). The EPA PBPK models provide estimates of HECs from test animal exposures that are supported by pharmacokinetic information available for rodents, monkeys and humans. The following sections describe how the BMD/BMDL approach, along

with the EPA PBPK models, are used to obtain PODs for use in the derivation of an RfC and RfD for methanol consistent with current BMD technical guidance (U.S. EPA, 2012a).

The BMD approach attempts to fit models to the dose-response data for a given endpoint. It has the advantage of taking more of the dose-response data into account when determining the POD, as well as estimating the dose for which an effect may have a specific probability of occurring. The BMD approach also accounts, in part, for the quality of the study by estimating a BMDL, the 95% lower confidence limit on the BMD. Larger studies (i.e.,with more test subjects) and studies with a low background response (i.e., with more test subjects for which a relationship between dose and response can be evaluated) generally yield narrower confidence intervals and higher BMDLS than smaller studies and studies with a high background response. For this reason and because the BMDL approach will account, in part, for a study's power, dose spacing, and the steepness of the dose-response curve, it is generally preferred over the NOAEL/LOAEL approach.

Use of the BMD approach has uncertainty associated with it. An element of the BMD approach is the use of several models to determine which best fits the data.[51] In the absence of an established MOA or a theoretical basis for why one model should be used over another, model selection is based on best fit to the experimental data selection. Model fit fit is evaluated through use of model goodness-of-fit diagnostics (i.e., AIC and χ^2 residuals of individual dose groups) and visual inspection as recommended by EPA guidance (U.S. EPA, 2012a).[52]

When performing a BMD analyses, it is important to choose a reliably measured or estimated dose metric that has a close relationship to the health effects under consideration. For the BMD analyses of the mouse cervical rib endpoint, peak (C_{max}) internal methanol blood concentrations reported by Rogers et al.(1993b) for the dams of each dose group at day 6 of gestation were used as the modeled dose metric. For the BMD analyses of the rat brain weight endpoint following gestational only (GD7-GD17) exposure, PBPK model estimates of C_{max} methanol in blood for the dams of each dose group were used as the modeled dose metric. C_{max} of methanol in blood (mg/L) was chosen as the appropriate internal dose metric for these two gestational exposure studies because the magnitude of exposure is believed to be more important

[51]USEPA's BMDS 2.2 (U.S. EPA, 2011a) was used for this assessment as it provides data management tools for running multiple models on the same dose-response data set. At this time, BMDS offers over 30 different models that are appropriate for the analysis of dichotomous, continuous, nested dichotomous and time-dependent toxicological data. Results from all models include a reiteration of the model formula and model run options chosen by the user, goodness-of-fit information, the BMD, and the estimate of the lower-bound confidence limit on the BMD (BMDL).

[52]Akaike's Information Criterion (AIC) (Akaike, 1973) is used for model selection and is defined as -2L + 2P where L is the log-likelihood at the maximum likelihood estimates for the parameters and P is the number of model degrees of freedom.

than the duration of exposure, particularly for the cervical rib endpoint, which has been shown to have a small gestational window of susceptibility (Rogers and Mole, 1997; Bolon et al., 1993)

For the BMD analyses of the rat brain weight endpoint following gestational and lactational exposure, PBPK model estimates of AUC methanol in blood for the dams of each dose group were used as the modeled dose metric. While the results of NEDO (1987), described in Section 4.4.2 and shown in Table 4-13, indicate that there is not an obvious cumulative effect of ongoing exposure on brain-weight decrements in rats exposed postnatally; i.e., the dose response in terms of percent of control is about the same at 3 weeks postnatal as at 8 weeks postnatal in rats exposed throughout gestation and the F_1 generation, there does appear to be a greater brain-weight effect in rats exposed postnatally versus rats exposed only during organogenesis (GD7-GD17). In male rats exposed during organogenesis only, there is no statistically significant decrease in brain weight at 8 weeks after birth at the 1,000 ppm exposure level. Conversely, in male rats exposed to the same level of methanol throughout gestation and the F_1 generation, there was an approximately 5% decrease in brain weights (statistically significant at the $p < 0.01$ level). Also, male rats exposed to 5,000 ppm methanol only during organogenesis experienced a smaller decrease in brain weight at 8 weeks postnatal than male rats exposed to 2,000 ppm methanol throughout gestation and the 8 week postnatal period (10% versus 13%). Further, brain weight reductions have been observed in adult rats that were exposed for 90 days beginning no earlier than 30 days of age (TRL, 1986). That brain weight is susceptible to both the level and duration of exposure suggests that a dose metric that incorporates a time component would be the most appropriate metric to use. For these reasons, and because it is more typically used in internal-dose-based assessments and better reflects total exposure within a given day, daily AUC (measured for 22 hours exposure/day) was chosen as the most appropriate dose metric for modeling the effects of methanol exposure on brain weights in rats exposed throughout gestation and continuing into the F_1 generation.

5.1.2.2. BMD Approach Applied to Brain Weight Data in Rats

The developmental study performed as a supplement to the NEDO (1987) two generation rat study reported decreases in brain weights in developing rats exposed during gestation only (GD7-GD17) or during gestation and the postnatal period, up to 8 weeks (see Section 4.4.2). Because of the biological significance of decreases in absolute brain weight as an endpoint in the developing rat and because this endpoint was not evaluated in other peer-reviewed studies, BMD analysis was performed using these data. For the purposes of deriving an RfC for methanol from developmental endpoints using the BMD method and rat data, decreases in brain weight at 6 weeks of age in the more sensitive gender, males, exposed throughout gestation and continuing

into the F_1 generation (both through lactation and inhalation routes) were utilized. Decreases in brain weight at 6 weeks (gestational and postnatal exposure), rather than those seen at 3 and 8 weeks, were chosen as the basis for the RfC derivation because they resulted in lower estimated BMDs and BMDLs. Decreased brain weights in male rats at 8 weeks of age after gestation-only exposure were not utilized because they were lower in magnitude at the same dose level (1,000 ppm) compared to gestation and postnatal exposure.

The first step in the current BMD analysis is to convert the inhalation doses, given as ppm values from the studies, to an internal dose metric using the EPA PBPK model (see Appendix B for a detailed description of the PBPK models developed for methanol). Application of the EPA methanol PBPK model is complicated by the exposure regimen used in the NEDO (1987) developmental studies. The neonatal rats in the developmental study performed as a supplement to the NEDO (1987) two generation rat study were exposed to methanol gestationally before parturition (as well as lactationally and inhalationally after parturition). Because data on lactational transfer and early postnatal inhalation exposures are limited, the PBPK model developed by EPA only estimates internal dose metrics for methanol exposure in non-pregnant adult rats. Experimental data indicate that inhalation-route blood methanol kinetics in non-pregnant mice and pregnant mice on GD6-GD10 are similar (Dorman et al., 1995; Perkins et al., 1995b; Rogers et al., 1993a; Rogers et al., 1993b). In addition, experimental data indicate that the maternal blood:fetal partition coefficient for mice and rats is approximately 1 up to GD 20 (see Sections 3.2 and 3.4.1.2). Assuming that these findings apply for rats later in pregnancy, the data indicate that PBPK estimates of PK and blood dose metrics for NP rats are better predictors of fetal exposure during gestation than would be obtained from default extrapolations from external exposure concentrations. However, as is discussed in Section 5.1.3.2.2, the additional routes of exposure presented to the pups in this study (lactation and inhalation routes) present uncertainties in that the average blood levels in pups are likely to be greater than those of their dams. The assumption made in this assessment is that, if such differences exist between human mothers and their offspring, they are not significantly greater than that which has been postulated for rats. Assuming this is true, the PBPK model-estimated adult blood methanol level is considered to be an appropriate dose metric for the purpose of this analysis and the estimation of a human equivalent concentration (HEC).

Predicted AUC values above background for methanol in the blood of rats are summarized in Table 5-2. These AUC values are then used as the dose metric for the BMD analysis of response data shown in Table 5-2 for decreased brain weight at 6 weeks in male rats following gestational and postnatal exposure.[53] The full details of this analysis are reported in

[53]All BMD assessments in this review were performed using BMDS version 2.2 (U.S. EPA, 2011a).

Appendix D. More details concerning the PBPK modeling are presented in Section 3.4 and Appendix B.

Table 5-2 The EPA PBPK model estimates of methanol blood levels (AUC) above background (control) levels[a] in rat dams following inhalation exposures and reported brain weights of 6 week old male pups

Exposure level (ppm)	Blood methanol AUC – control AUC (mg-hr/L)[a] in rat dams	Mean male rat (F_1 generation) brain weight at 6 weeks[b]	N
0	0	1.78 ± 0.07	12
500	547	1.74 ± 0.09	12
1,000	2,310	1.69 ± 0.06[c]	11
2,000	17,500	1.52 ± 0.07[d]	14

[a]AUCs were obtained by simulating 22 hr/day exposures for 5 days and calculated for the last 24 hours of that period; AUCs above background were obtained by subtracting the estimated AUC for controls of 72 mg-hr/L.
[b]Exposed throughout gestation and F_1 generation. Values are means ± S.D.
[c]$p < 0.01$
[d]$p < 0.001$, as calculated by the authors.

Source: NEDO (1987).

The current BMD technical guidance (U.S. EPA, 2012a) suggests that, in the absence of knowledge as to what level of response to consider adverse, a change in the mean equal to one standard deviation (SD) from the control mean can be used as a benchmark response (BMR) for continuous endpoints. However, it has been suggested that other BMRs, such as 5% change relative to estimated control mean, are also appropriate when performing BMD analyses on fetal weight change as a developmental endpoint (Kavlock et al., 1995). Therefore, both a one SD change from the control mean and a 5% change relative to estimated control mean were considered (see Appendix D for RfC derivations using alternative BMRs).

As described in Appendix D, consistent with criteria described in EPA BMD Technical Guidance (U.S. EPA, 2012a), the BMDL from the Hill model, is selected as the most appropriate basis for an RfC derivation because it results in the lowest BMDL from among a broad range of BMDLs and provides a superior fit in the low dose region nearest the BMD. The Hill model dose-response curve for decreased brain weight in male rats is presented in Figure 5-1, with response plotted against the chosen internal dose metric of AUC above background of methanol in rats. The $BMDL_{1SD}$ was estimated to be 858 mg-hr/L expressed in terms of the AUC above background for methanol in blood.

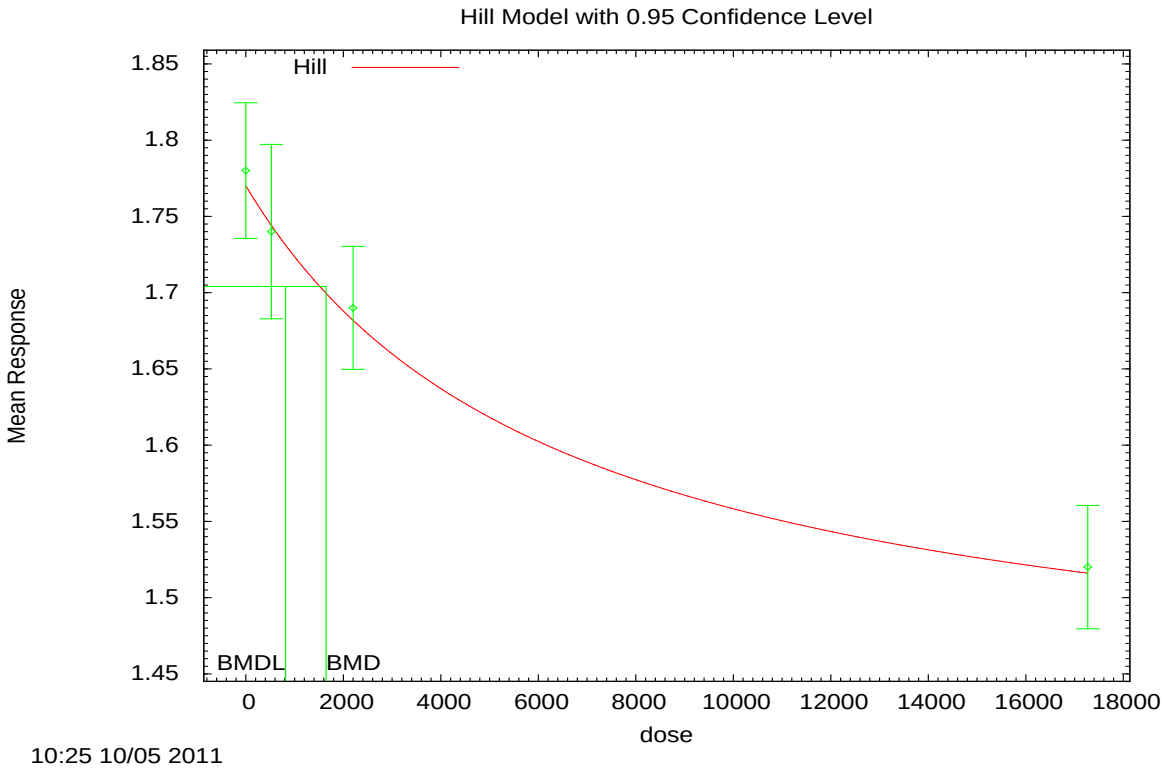


Figure 5-1 Hill model BMD plot of decreased brain weight in male rats at 6 weeks age using modeled AUC above background of methanol in blood as the dose metric, 1 control mean S.D.

5.1.2.3. BMD Approach Applied to Cervical Rib Data in Mice

For the purposes of deriving an RfC for methanol from developmental endpoints using the BMD method and mouse data, cervical rib incidence data were evaluated from Rogers et al. (1993b). Although the teratology portion of the NEDO study (1987) also reported increases in cervical rib incidence in Sprague-Dawley rats, the Rogers et al. (1993b) study was chosen for dose-response modeling because effects were seen at lower doses, it was peer-reviewed and published in the open literature, and data on individual animals were available for a more statistically robust analysis utilizing nested models available in BMDS 2.2 (U.S. EPA, 2011a).

For cervical rib anomalies, C_{max} of methanol in blood (mg/L) is chosen as the appropriate internal dose metric because studies that indicate a small gestational window of susceptibility (Rogers and Mole, 1997; Bolon et al., 1993) suggest that the level of exposure is more important than the duration of exposure. Because the critical window for methanol induction of cervical rib malformations in CD-1 mice is between GD6 and GD7 (Rogers and Mole, 1997; Rogers et al., 1993a), the measured C_{max} plasma methanol levels for gestation day 6 from the Rogers study are

used with background levels (1.6 mg/L) subtracted. C_{max} values for methanol in the blood of mice are summarized in Table 5-3. These C_{max} values are then used as the dose metric for the BMD analysis of the litter-specific cervical rib response. The overall cervical rib/litter (%) reported by Rogers et al. (1993b) is shown in Table 5-3, but litter-specific response data from this study (170 litters) obtained from John Rogers (personal communication) was used for the nested BMD analysis described in Appendix D. Due to high mortality, the high (15,000 ppm) dose group (5 litters) was excluded from the analysis. The individual animal response data for the four dose groups shown in Table 5-3 are displayed in the Appendix D model output file.

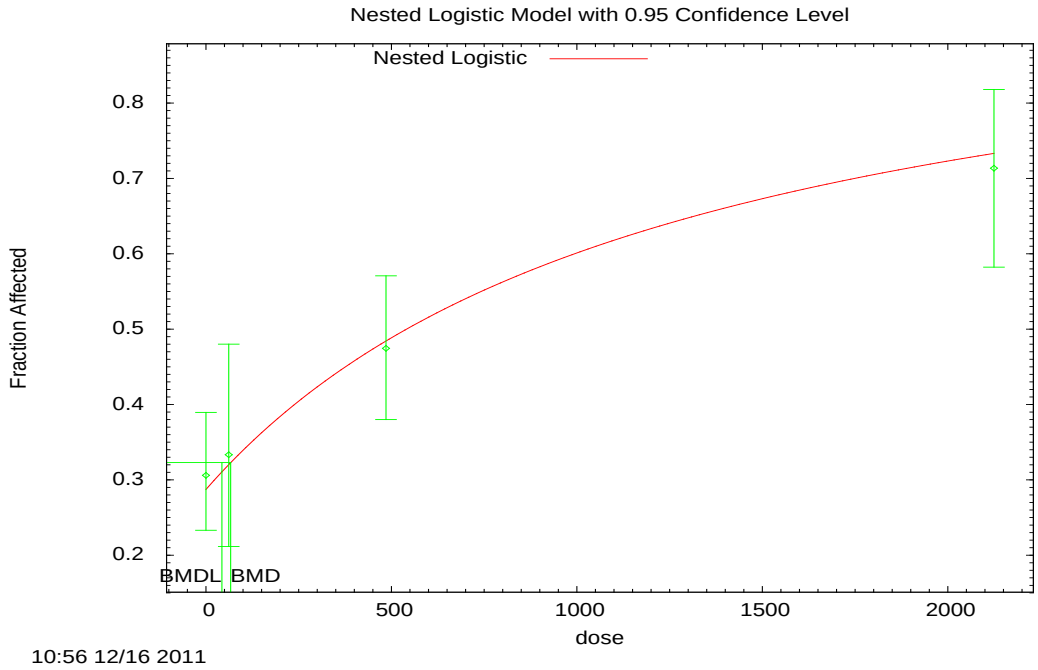
Table 5-3 Methanol blood levels (Cmax) above background (control) levels in mice following inhalation exposures

Exposure (ppm)	Blood methanol C_{max} – control C_{max} (mg/L)[a] in mouse dams	Cervical Rib/Litter (%)
0	0	28
1,000	61.4	33.6
2,000	485	49.6
5,000	2,120	74.4

[a]C_{max} above background was obtained by subtracting the C_{max} for controls reported by Rogers et al. (1993b) of 1.6 mg/L.

Source: Rogers et al. (1993b)

Both 10% and 5% extra risk BMRs were considered for this endpoint. A 10% extra risk BMR is adequate for most traditional bioassays using 50 animals per dose group. A smaller BMR of 5% extra risk is sometimes justified for developmental studies such as Rogers et al. (Rogers et al., 1993b) depending on the size of the study and the severity of the effects observed. As described in Appendix D, the best model fit to these data (from visual inspection and comparison of AIC values) was obtained using the NLogistic model. The NLogistic model dose-response curve for increased cervical ribs in fetal mice is presented in Figure 5-2, The $BMDL_{05}$ was estimated to be 43.10 mg/L expressed in terms of the C_{max} above background for methanol in blood (Rogers et al., 1993b).



Source: Rogers et al. (1993b).

Figure 5-2 Nested logistic model, 0.05 extra risk - Incidence of cervical rib in mice versus C_{max} above background of methanol, GD6-GD15 inhalation study.

5.1.3. RfC Derivation – Including Application of Uncertainty Factors

5.1.3.1. Selected Endpoints and BMDL Modeling Approaches

A summary of the PODs for the candidate developmental endpoints and BMD modeling approaches considered for the derivation of an RfC (see Appendix D for details), applied UFs (see Section 5.1.3.2 for details) and the estimated candidate RfCs (obtained from PBPK models described in Appendix B) are presented in Table 5-4. Information is presented that compares the use of different endpoints (i.e., cervical rib and decreased brain weight) and different methods (i.e., different BMR levels) for estimating the POD. Each approach considered for the determination of the POD has strengths and limitations, but when considered together for comparative purposes they allow for a more informed determination for the POD for the methanol RfC.

As described in Section 5.1.3.2 and Table 5-4, the internal BMDL ($POD_{internal}$) values are divided by a total UF of 100 (UF_H of 10, UF_A of 3 and a UF_D of 3) to yield an $RfC_{internal}$, which is

converted to a candidate RfC using the human PBPK model described in Appendix B.[54] Candidate RfCs estimated from the Rogers et al. (1993b) study for cervical rib incidence in mice using C_{max} as the dose metric were 43.7 and 20.9 mg/m^3 using $BMDL_{10}$ and $BMDL_{05}$ PODs, respectively. Candidate RfCs estimated from the NEDO (1987) study for decreases in brain weight at 6 weeks of age in male rats exposed during gestation and throughout the F_1 generation using AUC as the dose metric were 23.9 and 17.4 mg/m^3 for BMRs of 5% change relative to control mean and one S.D. from the control mean, respectively. Because a one S.D. decrease in brain weight in male rats at 6 weeks (postnatal) resulted in the lowest of the candidate RfC estimates and, therefore, the most likely to be protective against other effects of methanol exposure, it was chosen as the critical endpoint for use in the RfC derivation.

$$\text{RfC} = 858 \text{ mg-hr/L} \div 100 = 8.58 \text{ mg-hr/L} \Rightarrow \text{PBPK} \Rightarrow \mathbf{2\times10^1 \text{ mg/m}^3}$$

(rounded to 1 significant figure)

Table 5-4 Summary of PODs for critical endpoints, application of UFs and conversion to candidate RfCs using PBPK modeling

	Rogers et al. (1993b) mouse cervical rib C_{max}		NEDO (1987) rat brain weight AUC	
	$BMDL_{10}$	**$BMDL_{05}$**	**$BMDL_{05}$**	**$BMDL_{1SD}$**
BMDL = $POD_{internal}$	90.9 mg/L	43.1 mg/L	1,183 mg-hr/L	**858 mg-hr/L**
$RfC_{internal}$ = $POD_{internal}$/UFs[a]	0.909 mg/L	0.43 mg/L	11.85 mg-hr/L	**8.58 mg-hr/L**
RfC (mg/m^3)[b]	43.7	20.9	23.9	**17.4**

[a]UF_A =3; UF_D = 3; UF_H = 10; UF_S = 1; UF_L = 1; product of all UFs = 100; see Section 5.1.3.2 below for details.

[b]Each candidate RfC is the inhalation exposure concentration predicted to yield a blood concentration equal to its corresponding $RfC_{internal}$, using the human PBPK model; the final RfC is rounded to one significant figure.

5.1.3.2. Application of UFs

UFs are applied to account for recognized uncertainties in extrapolation from experimental conditions to the assumed human scenario (i.e., chronic exposure over a lifetime). According to EPA recommendations (U.S. EPA, 2002, 1994b), UFs are generally applied to HEC estimates. However, as described in Appendix B (Section B.2.7, Table B-6), the human PBPK model developed for methanol is considered uncertain above inhalation concentrations of 500

[54] An algebraic equation provided near the end of Appendix B approximates the PBPK model predicted relationship between methanol AUC and C_{max} blood levels above background and the HEC in ppm.

ppm (655 mg/m^3) or oral ingestions of 50 mg/kg-day, since the blood levels predicted rise above those for which there are model calibration data. The HEC values (1,042 to 1,604 mg/m^3) and HED values (133 to 220 mg/kg-day) predicted by the human PBPK model for BMDLs from the candidate principal studies are well above these exposure levels. Consequently, the standard EPA practice of applying a human PBPK model to derive HEC values prior to dividing by UFs (U.S. EPA, 2002, 1994b) would engender considerable model uncertainty. In order to avoid the uncertainty associated with applying the model to exposure levels that are above the levels for which the model was calibrated and to account for possible non-linearities in the external versus internal dose relationships at high doses, EPA has applied the UFs to the internal BMDL ($POD_{internal}$) prior to HEC derivation to obtain an $RfC_{internal}$. This approach results in more scientifically reliable model predictions by lowering the BMDLs to within the more linear, calibrated range of the human PBPK model.

5.1.3.2.1. Interindividual variation UF_H

A factor of 10 was applied to account for variation in sensitivity within the human population (UF_H). The UF_H of 10 is commonly considered to be appropriate in the absence of convincing data to the contrary. The data from which to determine the potential extent of variation in how humans respond to chronic exposure to methanol are limited, given the complex nature of the developmental endpoint employed and uncertainties surrounding the importance of metabolism to the observed teratogenic effects. Susceptibility to methanol is likely to involve intrinsic and extrinsic factors. Some factors may include alteration of the body burden of methanol or its metabolites, sensitization of an individual to methanol effects, or augmentation of underlying conditions or changes in processes that share common features with methanol effects. Additionally, inherent differences in an individual's genetic make-up, diet, gender, age, or disease state may affect the pharmacokinetics and pharmacodynamics of methanol, influencing susceptibility intrinsically. Co-exposure to a pollutant that alters metabolism or other clearance processes, or that adds to background levels of metabolites may also affect the pharmacokinetics and pharmacodynamics of methanol, influencing susceptibility extrinsically (see Section 4.9). The determination of the UF for human variation is supported by several types of information, including information concerning background levels of methanol in humans, variation in pharmacokinetics revealed through human studies and from PBPK modeling, variation of methanol metabolism in human tissues, and information on physiologic factors (including gender and age), or acquired factors (including diet and environment) that may affect methanol exposure and toxicity.

Sensitivity analyses of the human PBPK models were performed (see Appendix B), and the results suggest that parameter variability is not likely to result in methanol blood level estimates that vary more than 3-fold, the toxicokinetic portion of the 10-fold UF_H. However, one needs to also consider the variation in endogenous levels of methanol (Table 3-1), because that can be a factor governing the impact of an exogenous methanol exposure. From the data in Table 3-1, EPA has estimated an average methanol blood level in humans of 1.5 ± 0.7 mg/L. According to EPA's PBPK model, a 10-fold UF_H reduces the RfC and RfD to levels that would increase methanol blood levels by a daily maximum of 0.86 mg/L and a daily average of 0.59 mg/L in individuals receiving both an RfC and RfD exposure. These increases are comparable to the 0.7 mg/L standard deviation estimated for the average methanol blood levels in humans (see Section 5.3.6), indicating that the estimated increase in blood levels of methanol from exogenous exposures at the level of the RfD or the RfC (or from the RfC + RfD) are distinguishable from natural background variation.

The candidate effects for RfC derivation have been observed in a potentially susceptible and sensitive fetal/neonatal subpopulation. However, there is also variability across fetuses and neonates that need to be taken into account. Children vary their ability to metabolize and eliminate methanol and in their sensitivity to methanol's toxic teratogenic effects. There is information on PK and pharmacodynamic factors suggesting that children can have differential susceptibility to methanol toxicity (see Section 4.9.1). Thus, there is uncertainty in children's responses to methanol that should be taken into consideration for derivation of the UF for human variation that is not available from either measured human data or PBPK modeling analyses. The enzyme primarily responsible for metabolism of methanol in humans, ADH, has been reported to be reduced in activity in newborns. Differences in pharmacokinetics include potentially greater pollutant intake due to greater ventilation rates, activity, and greater intake of liquids in children. In terms of differences in susceptibility to methanol due to pharmacodynamic considerations, the substantial anatomical, physiologic, and biochemical changes that occur during infancy, childhood, and puberty suggest that there are developmental periods in which the endocrine, reproductive, immune, audiovisual, nervous, and other organ systems may be especially sensitive.

There are some limited data from short-term exposure studies in humans and animal experiments that suggest differential susceptibility to methanol on the basis of gender. Gender can provide not only different potential targets for methanol toxicity but also differences in methanol pharmacokinetics and pharmacodynamics. NEDO (1987) reported that in rats exposed to methanol pre- and postnatally, 6- and 8-week-old male progeny had significantly lower brain weights at 1,000 ppm, whereas females only showed decreases at 2,000 ppm. In general, gender-related differences in distribution and clearance of methanol may result from the greater muscle

mass, larger body size, decreased body fat, and increased volumes of distribution in males compared to females.

5.1.3.2.2. Animal-to-human Extrapolation UF_A

A factor of 3 was applied to account for uncertainties in extrapolating from rodents to humans (UF_A). Application of a full UF of 10 would depend on two areas of uncertainty: toxicokinetic and toxicodynamic uncertainty. The rodent-to-human toxicodynamic uncertainty is addressed by a factor of 3, as is the practice for deriving RfCs (U.S. EPA, 1994b). In this assessment, the toxicokinetic component is addressed by the determination of a HEC through the use of PBPK modeling. Use of PBPK-estimated maternal blood methanol levels for the estimation of HECs allows for the use of data-derived extrapolations rather than standard methods for extrapolations from external exposure levels. Though PBPK model uncertainties exist, for reasons discussed below, the toxicokinetics uncertainty is reduced to a value of 1 for both of the candidate principal studies.

There is uncertainty surrounding the identification of the proximate teratogen of importance (methanol, formaldehyde, or formate) for PBPK modeling, but it is not considered to be substantial enough to warrant an additional uncertainty factor. A review of the reproductive and developmental toxicity of methanol by a panel of experts concluded that methanol, not its metabolite formate, is likely to be the proximate teratogen and that blood methanol level is a useful biomarker of exposure (CERHR, 2004; Dorman et al., 1995). The CERHR Expert Panel based their assessment of potential methanol toxicity on an assessment of circulating blood levels (CERHR, 2004). EPA has chosen to use blood methanol levels as the dose metric for RfC derivation primarily based on evidence that the toxic moiety is not likely to be the formate metabolite of methanol (CERHR, 2004). While in vitro evidence indicates that formaldehyde is more embryotoxic than methanol and formate (Harris et al., 2004; 2003), the high reactivity of formaldehyde would limit its unbound and unaltered transport as free formaldehyde from maternal to fetal blood (Thrasher and Kilburn, 2001) (see discussion in Section 3.3). Thus, even if formaldehyde is ultimately identified as the proximate teratogen, methanol would likely play a prominent role, at least in terms of transport to the target tissue. Further discussions of methanol metabolism, dose metric selection, and MOA issues are in Sections 3.3 and 4.7.

There is uncertainty regarding whether the rat and human PBPK models adequately characterize species differences. However, given the chosen dose metrics (AUC or C_{max} for methanol blood), uncertainties in the PBPK modeling of methanol are not expected to be substantially greater for one species than another. Specifically, the analysis of parameter sensitivity and uncertainty for the PBPK modeling performed for human and rat data gave

similar results as to how well the model fit the available data (Appendix B). Thus, the human and rat PBPK model performed similarly using these dose metrics for comparisons between species.

HEC predictions from the models can vary depending on the dose metric (e.g., AUC or C_{max}), but this is not a major source of uncertainty. In the case of the mouse cervical rib endpoint, the choice of the C_{max} dose metric was well justified based on studies that show a narrow gestational window of susceptibility (Rogers and Mole, 1997; Bolon et al., 1993). In the case of the rat brain weight endpoint, the choice of the AUC dose metric was well justified based on studies which show an exacerbation of the effect from continued exposure beyond gestation (NEDO, 1987; TRL, 1986). Study conditions that involved nearly 24 hours of exposure, resulted in an HEC estimate that was not significantly different (~10% lower) than the HEC estimate that would be obtained using C_{max} as the dose metric.

For estimation of an HEC from the NEDO (1987) rat study, uncertainty that could result in the underestimation of toxicity exists regarding the use of maternal blood levels because of possible species differences in the relation of maternal blood levels estimated by the model to fetal and neonatal blood levels that would be obtained under the (gestational, postnatal and lactational) exposure scenario. Young animals have different metabolic and physiological profiles than adults. This fact, coupled with multiple routes of exposure, complicate the prediction of internal dose to the offspring.[55] However, , it is assumed that the ratio of the difference in blood concentrations between a human infant and mother would be similar to and not significantly greater than the difference between a rat dam and its fetus. This assumption is based largely on the fact that key parameters and factors which determine the ratio of fetal or neonatal versus maternal methanol blood levels in humans either do not change significantly with age (partition coefficients, relative blood flows) or scale in a way that is common across species (allometrically). Further, the health-effects data indicate that most of the effects of concern are due to fetal exposure, with a relatively small influence due to postnatal exposures.

5.1.3.2.3. Database UF_D

A database UF of 3 was applied to account for deficiencies in the toxicity database (UF_D). The database for methanol toxicity is quite extensive: there are chronic and developmental toxicity studies in rats, mice, and monkeys, a two-generation reproductive toxicity study in rats, and neurotoxicity and immunotoxicity studies. As discussed in Section 5.1.1.1, chronic and developmental studies in monkeys, the species most likely to best represent the potential for

[55]Stern et al. (1996) reported that when rat pups and dams were exposed together during lactation to 4,500 ppm methanol in air, methanol blood levels in pups from GD6–PND21 were approximately 2.25 times greater than those of dams. It is reasonable to assume that similar differences in blood methanol levels would be observed in the NEDO (1987) F_1 study, as the exposure scenario is similar to that of Stern et al. (1996).

developmental effects in humans, were considered inadequate or inferior to the candidate principal rodent studies for the purposes of RfC/D derivation. The lack of a quantifiable monkey study is an important data gap given the potential relevance to humans and the uncertainties raised by existing monkey studies regarding this species sensitivity to reproductive effects (e.g shortened pregnancies discussed in Section 4.3.2), CNS degeneration (e.g., stellate cell fibrosis described in Section 4.4.2) and delayed neurobehavioral development (e.g., VDR response described in Section 4.4.2) from methanol exposure. In addition, a full developmental neurotoxicity test (DNT) in rodents has not been performed and is warranted given the critical effect of decreased brain weight in rats and the suggestive (but quantitatively inconclusive) DNT results in monkeys. For these reasons, an UF of 3 was applied to account for deficiencies in the database.

5.1.3.2.4. Extrapolation from subchronic to chronic UFs

A UF of 1 was used for extrapolation from less than chronic results because developmental toxicity (cervical rib and decreased brain weight) was used as the critical effect. The developmental period is recognized as a susceptible lifestage where exposure during certain time windows is more relevant to the induction of developmental effects than lifetime exposure (U.S. EPA, 1991).

5.1.3.2.5. LOAEL-to-NOAEL extrapolation UFs

A UF of 1 was used for LOAEL to NOAEL (UF_L) because the current approach is to address this extrapolation as one of the considerations in selecting a benchmark response (BMR) for BMD modeling. In this case, the endpoint and benchmark response level employed for the RfD/C derivation is appropriate for use in deriving the RfD under the assumption that it represents a minimal biologically significant change.

5.1.3.3. Confidence in the RfC

The confidence in this RfC is medium to high. Confidence in the Rogers et al. (1993b) study is high and confidence in the NEDO (1987) developmental studies is medium. The Rogers et al. (1993b) study was well designed, including large sample sizes, well documented, peer reviewed and published. While there are issues with the lack of detail regarding methods and results in the NEDO (1987) report, the observed effect (brain weight reduction) is a relevant endpoint that has been reproduced in an oral study of adult rats (TRL, 1986), and the exposure regimen involving pre- and postnatal exposures addresses a potentially sensitive human

subpopulation. Confidence in the database is medium. Though skeletal and brain effects have been demonstrated and corroborated in multiple animal studies in rats, mice, and monkeys, some study results were not quantifiable, thus there is uncertainty regarding which is the most relevant test species, and there is limited data regarding reproductive or developmental toxicity of methanol in humans. There is also uncertainty regarding the potential active agent—the parent compound, methanol, formaldehyde, formic acid or some other (e.g., reactive oxygen) species. There are deficiencies in the knowledge of the metabolic pathways of methanol in the human fetus during early organogenesis, when the critical effects can be induced in animals. Thus, the medium-to-high confidence in the critical studies and the medium confidence in the database together warrant an overall confidence descriptor of medium to high.

5.1.4. Previous RfC Assessment

The health effects data for methanol were assessed for the IRIS database in 1991 and were determined to be inadequate for derivation of an RfC.

5.2. Oral Reference Dose (RFD)

In general, the RfD is an estimate of a daily exposure to the human population (including susceptible subgroups) that is likely to be without an appreciable risk of adverse health effects over a lifetime. It is derived from a POD, generally the statistical lower confidence limit on the BMD, with uncertainty/variability factors applied to reflect limitations of the data used. The RfD is expressed in terms of mg/kg-day of exposure to a substance and is derived by a similar methodology as is the RfC. Ideally, studies with the greatest duration of exposure and conducted via the oral route of exposure give the most confidence for derivation of an RfD. For methanol, the oral database is currently more limited than the inhalation database. With the development of PBPK models for methanol, the inhalation database has been used to help bridge data gaps in the oral database to derive an RfD.

5.2.1. Choice of Principal Study and Critical Effect–with Rationale and Justification

No studies have been reported in which humans have been exposed subchronically or chronically to methanol by the oral route of exposure and thus, would be suitable for derivation of an oral RfD. Data exist regarding effects from oral exposure in experimental animals, but they are more limited than data from the inhalation route of exposure (see Sections 4.2, 4.3, and 4.4).

Only two oral studies of 90-days duration or longer in animals have been reported (Soffritti et al., 2002; TRL, 1986) for methanol. U.S. EPA (TRL, 1986) reported that there were no differences in body weight gain, food consumption, or gross or microscopic evaluations in Sprague-Dawley rats gavaged with 100, 500, or 2,500 mg/kg-day versus control animals. Liver weights in both male and female rats were increased, although not significantly, at the 2,500 mg/kg-day dose level, suggesting a treatment-related response despite the absence of histopathologic lesions in the liver. Brain weights of high-dose group males and females were significantly less than control animals at terminal (90 days) sacrifice. The data were not reported in adequate detail for dose-response modeling and BMD estimation. Based primarily on the qualitative findings presented in this study, the 500 mg/kg-day dose was deemed to be a NOAEL.[56]

The only lifetime oral study available was conducted by Soffritti et al. (2002) in Sprague-Dawley rats exposed to 0, 500, 5,000, 20,000 ppm (v/v) methanol, provided ad libitum in drinking water. Based on default, time-weighted average body weight estimates for Sprague-Dawley rats (U.S. EPA, 1988), average daily doses of 0, 46.6, 466, and 1,872 mg/kg-day for males and 0, 52.9, 529, 2,101 mg/kg-day for females were reported by the study authors. All rats were exposed for up to 104 weeks, and then maintained until natural death. The authors report no substantial changes in survival nor was there any pattern of compound-related clinical signs of toxicity. The authors did not report noncancer lesions, and there were no reported compound-related signs of gross pathology or histopathologic lesions indicative of noncancer toxicological effects in response to methanol.

Five oral studies investigated the reproductive and developmental effects of methanol in rodents (Aziz et al., 2002; Fu et al., 1996; Sakanashi et al., 1996; Rogers et al., 1993b; Infurna and Weiss, 1986), including three studies that investigated the influence of FAD diets on the effects of methanol exposures (Aziz et al., 2002; Fu et al., 1996; Sakanashi et al., 1996). Infurna and Weiss (1986) exposed pregnant Long-Evans rats to 2,500 mg/kg-day in drinking water on either GD15-GD17 or GD17-GD19. Litter size, pup birth weight, pup postnatal weight gain, postnatal mortality, and day of eye opening were not different in treated animals versus controls. Mean latency for nipple attachment and homing behavior (ability to detect home nesting material) were different in both methanol treated groups. These differences were significantly different from controls. Rogers et al. (1993b) exposed pregnant CD-1 mice via gavage to 4 g/kg-day methanol, given in 2 equal daily doses. Incidence of cleft palate and exencephaly was increased following maternal exposure to methanol. Also, an increase in totally resorbed litters and a decrease in the number of live fetuses per litter were observed.

[56] U.S. EPA [TRL (1986)] did not report details required for a BMD analysis such as standard deviations for mean responses.

Aziz et al. (2002), Fu et al. (1996), and Sakanashi et al. (1996) investigated the role of folic acid in methanol-induced developmental neurotoxicity. Like Rogers et al. (1993b), the former 2 studies observed that an oral gavage dose of 4–5 g/kg-day during GD6-GD15 or GD6-GD10 resulted in an increase in cleft palate in mice fed sufficient folic acid diets, as well as an increase in resorptions and a decrease in live fetuses per litter. Fu et al. (1996) also observed an increase in exencephaly in the FAS group. Both studies found that an approximately 50% reduction in maternal liver folate concentration resulted in an increase in the percentage of litters affected by cleft palate (as much as threefold) and an increase in the percentage of litters affected by exencephaly (as much as 10-fold). Aziz et al. (2002) exposed rat dams throughout their lactation period to 0, 1, 2, or 4% v/v methanol via the drinking water, equivalent to approximately 480, 960 and 1,920 mg/kg-day.[57] Pups were exposed to methanol via lactation from PND1–PND21. Methanol treatment at 2% and 4% was associated with significant increases in activity (measured as distance traveled in a spontaneous locomotor activity test) in the FAS group (13 and 39%, respectively) and most notably, in the FAD group (33 and 66%, respectively) when compared to their respective controls. At PND45, the CAR in FAD rats exposed to 2% and 4% methanol was significantly decreased by 48% and 52%, respectively, relative to nonexposed controls. In the FAS group, the CAR was only significantly decreased in the 4% methanol-exposed animals and only by 22% as compared to their respective controls.

5.2.1.1. Expansion of the Oral Database by Route-to-Route Extrapolation

Developmental effects are considered the most sensitive effects of methanol exposure (see Section 5.1.1). EPA has derived an RfD by using developmental response data from the candidate principal inhalation studies and route-to-route extrapolation with the aid of the EPA PBPK model (see Sections 3.4 and 5.1). Several factors support use of route-to-route extrapolation for methanol. The oral database has significant limitations, including the limited reporting of noncancer findings in the subchronic (TRL, 1986) and chronic studies (Soffritti et al., 2002) of rats, and the use of high-dose levels in the rodent oral developmental studies. In addition, the limited data from oral studies indicate similar effects as reported via inhalation exposure (e.g., the brain and fetal skeletal system are targets of toxicity). Further, methanol has been shown to be rapidly and well-absorbed by both the oral and inhalation routes of exposure (CERHR, 2004; Kavet and Nauss, 1990). Once absorbed, methanol distributes rapidly to all organs and tissues according to water content, regardless of route of exposure.

[57] Assuming that Wistar rat drinking water consumption is 60 mL/kg-day (Rogers et al., 2002), 1% methanol in drinking water would be equivalent to 1% × 0.8 g/mL × 60 mL/kg-day = 0.48 g/kg-day = 480 mg/kg-day.

As with the species-to-species extrapolation used in the development of the RfC, the dose metric used for species-to-species and route-to-route extrapolation of inhalation data to oral data is the C_{max} (in the case of the mouse cervical rib endpoint) or AUC (in the case of the rat brain weight endpoint) of methanol in blood. Simulations for human oral methanol exposure were conducted using the model parameters as previously described for human inhalation exposures, with human oral kinetic/absorption parameters from Sultatos et al. (2004) (i.e., k_{as} = 0.2, k_{si} = 3.17, and k_{ai} = 3.28). Human oral exposures were assumed to occur during six drinking episodes during the day, at times 0, 3, 5, 8, 11, and 15 hours from the first ingestion of the day. For example, if first ingestion occurred at 7 a.m., these would be at 7 a.m., 10 a.m., 12 noon, 3 p.m., 6 p.m., and 10 p.m. Each ingestion event was treated as occurring over 3 minutes, during which the corresponding fraction of the daily dose was infused into the stomach lumen compartment. The fraction of the total ingested methanol simulated at each of these times was 25%, 10%, 25%, 10%, 25%, and 5%, respectively. Six days of exposure were simulated to allow for any accumulation (visual inspection of plots showed this to be finished by the 2nd or 3rd day), and the results for the last 24 hours were used. Dividing the exposure into more and smaller episodes would decrease the estimated peak concentration but have little effect on AUC. This dose metric was used for dose-response modeling to derive the $POD_{internal}$, expressed as a BMDL.

5.2.2. RfD Derivation–Including Application of Uncertainty Factors

5.2.2.1. Selected Endpoints and BMDL Modeling Approaches

Inhalation studies considered for derivation of the RfC are used to supplement the oral database using the route-to-route extrapolation, as previously described. BMD approaches were applied to the existing inhalation database, and the EPA PBPK model was used for species-to-species extrapolations. Table 5-5 contains a summary of the PODs for the candidate developmental endpoints and BMD modeling approaches considered for the derivation of an RfD (see Appendix D for details), applied UFs (see Section 5.2.2.2) and the estimated candidate RfDs (obtained from PBPK models described in Appendix B). Like the RfC derivation, the internal BMDL ($POD_{internal}$) values are divided by a total UF of 100 (UF_H of 10, UF_A of 3 and a UF_D of 3) to yield an $RfD_{internal}$, which is converted to a candidate RfD using the human PBPK model described in Appendix B.[58] Candidate RfDs estimated from the Rogers et al. (1993b) study for cervical rib incidence in mice using C_{max} as the dose metric were 4.1 and 1.9 mg/kg-day using $BMDL_{10}$ and $BMDL_{05}$ PODs, respectively. Candidate RfDs estimated from the NEDO

[58] An algebraic equation is provided near the end of Appendix B that approximates the PBPK model predicted relationship between methanol AUC above background and the HED in mg/kg-day.

(1987) study for decreases in brain weight at 6 weeks of age in male rats exposed during gestation and throughout the F_1 generation using AUC as the dose metric were 5.4 and 4.0 mg/kg-day for BMRs of 5% change relative to control mean and one S.D. from the control mean, respectively. Because the cervical rib endpoint resulted in the lowest of the candidate RfD estimates it was chosen as the critical endpoint for use in the RfD derivation.

RfD = 43.1 mg/L ÷ 100 = 0.43 mg/L ⇒PBPK⇒ **2 mg/kg-day**
(rounded to 1 significant figure)

Table 5-5 Summary of PODs for critical endpoints, application of UFs and conversion to candidate RfDs using PBPK modeling

	Rogers et al. (1993b) (mouse cervical rib C_{max})		**NEDO (1987)** (rat brain wt. AUC)	
	$BMDL_{10}$	**$BMDL_{05}$**	**$BMDL_{05}$**	**$BMDL_{1SD}$**
BMDL = $POD_{internal}$	90.9 mg/L	**43.1 mg/L**	1,183 mg-hr/L	858 mg-hr/L
$RfD_{internal}$ = $POD_{internal}$/UFs[a]	0.909 mg/L	**0.43 mg/L**	11.83 mg-hr/L	8.58 mg-hr/L
RfD (mg/kg/day)[b]	4.1	**1.9**	5.4	4.0

[a]UF_A =3; UF_D = 3; UF_H = 10; UF_S = 1; UF_L = 1; product of all UFs = 100 ; see Section 5.2.2.2 below for details.
[b]Each candidate RfD is the oral dose predicted to yield a blood concentration equal to its corresponding $RfD_{internal}$, using the human PBPK model described in Appendix B; the final RfC is rounded to one significant figure.

5.2.2.2. Application of UFs

Because the same data set, endpoints, BMD methods and PBPK models used to derive the RfC were also used to calculate the candidate RfDs, the RfD derivation uses the same uncertainty factors as are described for the RfC derivation (Section 5.1.3.2). Consistent with the RfC derivation, in order to avoid the uncertainty associated with applying the human PBPK model to exposure levels that are above the levels for which the model was calibrated and to account for possible non-linearities in the external versus internal dose relationships at high doses, EPA applied the UFs to the internal BMDL ($POD_{internal}$) prior to HED derivation to obtain an $RfD_{internal}$ (see Table 5-5). This approach results in more scientifically reliable model predictions by lowering the BMDLs to within the more linear, calibrated range of the human PBPK model.

5.2.2.3. Confidence in the RfD

The confidence in the RfD is medium to high. Despite the relatively high confidence in the critical studies, all limitations to confidence as presented for the RfC also apply to the RfD. Confidence in the RfD is slightly lower than for the RfC due to the lack of adequate oral studies for the RfD derivation, necessitating a route-to-route extrapolation.

5.2.3. Previous RfD Assessment

The previous IRIS assessment for methanol included an RfD of 0.5 mg/kg-day that was derived from a U.S. EPA [(TRL, 1986)] subchronic oral study in which Sprague-Dawley rats (30/sex/dose) were gavaged daily with 0, 100, 500, or 2,500 mg/kg-day of methanol. There were no differences between dosed animals and controls in body weight gain, food consumption, gross or microscopic evaluations. Elevated levels of serum glutamic pyruvic transaminase (SGPT), serum alkaline phosphatase (SAP), and increased but not statistically significant liver weights in both male and female rats suggest possible treatment-related effects in rats dosed with 2,500 mg methanol/kg-day, despite the absence of supportive histopathologic lesions in the liver. Brain weights of both high-dose group males and females were significantly less than those of the control group. Based on these findings, 500 mg/kg-day of methanol was considered a NOAEL in this rat study. Application of a 1,000-fold UF (interspecies extrapolation, susceptible human subpopulations, and subchronic to chronic extrapolation) yielded an RfD of 0.5 mg/kg-day.

5.3. Uncertainties in the Inhalation RfC and Oral RfD

The following is a more extensive discussion of the uncertainties associated with the RfC and RfD for methanol beyond that which is addressed quantitatively in Sections 5.1.2, 5.1.3, and 5.2.2. A summary of these uncertainties is presented in Table 5-6.

Table 5-6 Summary of uncertainties in methanol noncancer assessment

Consideration	Potential Impact	Decision	Justification
Choice of study/endpoint	Minimal impact. RfD and RfC estimates from candidate principal studies were extremely close to one another.	RfC is based on brain weight reduction in rats NEDO (1987) ; RfD is based on cervical rib anomalies in mice Rogers et al. (1993b)	The chosen endpoints were observed in adequate studies, have been observed in other rodent studies, are considered biological significant and relevant to humans, and were the most sensitive of the quantifiable endpoints for their respective route of exposure.
Choice of model for BMDL derivation	BMDLs from adequately fitting models differed by 5-fold for the RfC, indicating high model dependence, and were within 25% of each other for the RfD, indicating little model dependence.	Hill model was chosen for derivation of the POD for the RfC and NLogistic model was chosen for derivation of the POD for the RfD.	Hill model was chosen because it resulted in the lowest BMDL from among a wide range (>3-fold) of BMDL estimates from adequately fitting models. NLogistic model was the best fitting model in accordance with U.S. EPA (2012a) criteria.
Route-to-Route Extrapolation method	Raises the RfD 7-fold above 1988 methanol RfD of 0.5 mg/kg-day based on oral study by TRL (1986)	Human PBPK model was used to estimate HED from blood levels reported in Roger et al. (1993b) study	Rogers et al. (1993b) study was a quality study, measured a sensitive and relevant endpoint, provided measured blood concentrations that could be converted to oral doses with the EPA human PBPK model.
Statistical uncertainty at POD (sampling variability due to bioassay size)	POD would be ~50% higher if BMD were used	A BMDL was used as the POD	Lower bound is 95% CI of administered exposure
Choice of species/gender	PODs for the RfC and RfD estimates based on rat and mouse data are similar; POD estimates based on monkey data would be ~30-50% lower	RfC and RfD were based on the most sensitive of relevant and quantifiable endpoints in the most sensitive species and, in the case of the RfC, also in the most sensitive gender	Mouse and rat studies gave similar results for RfC/D. Qualitative evidence from NEDO (1987), Burbacher, et al. (2004a) and Burbacher, et al. (2004b) suggest that monkeys may be a sensitive species, but data are not as reliable for quantification. No gender differences were noted by Rogers et al. (1993b), but NEDO (1987) reported slightly greater brain weight changes in male offspring.
Relationship of the RfC and RfD to Endogenous Methanol Blood Levels	RfC and RfD could be deemed unreasonably low if they increase blood levels by much less than normal variation (e.g., 1 SD)	RfD and RfCs are deemed adequately protective and reasonable	Increases in methanol blood levels associated RfD and RfC exposures are projected to be comparable to 1 S.D. of endogenous methanol blood levels of humans. This is deemed adequate to protect sensitive subpopulations but not so low as to be indistinguishable from background variation.

5.3.1. Choice of Study/Endpoint

As discussed in Sections 5.1.1 and 5.2.1, developmental effects observed in two candidate principal studies were considered relevant and quantifiable for the purposes of RfC/D derivation. Brain weight reduction in rats (NEDO, 1987) and cervical rib anomalies in mice (Rogers et al., 1993b) were the most sensitive of the relevant and quantifiable endpoints reported in these studies. Candidate RfCs derived based on these endpoints ranged from 17.4 to 43.7 mg/m^3 (Table 5-4). Potential RfDs derived based on these endpoints ranged from 1.9 to 5.4 mg/kg-day (Table 5-5).

Uncertainty associated with the Rogers (1993b) study results are primarily with respect to the relevance of developmental studies in rodents to humans, which is discussed in Sections 5.1.1.2.1 and 5.3.5. There is less uncertainty associated with the Rogers et al. (1993b) study methods and reporting because it has undergone independent peer review, is well documented, used robust group sizes, reports effects that have been observed by other laboratories, and because additional study details (e.g., individual animal data) were made available by the authors (see Appendix D).

Uncertainties with the NEDO (1987) developmental study are primarily associated with the reproducibility of the brain weight endpoint and the level and quality of study documentation. Neonatal reduction in brain weight is not as well documented across laboratories and across species and strains of test animals as the fetal cervical rib endpoint. However, this is not a major concern given that reduced brain weight following methanol gavage exposure was reported in adult SD rats by another laboratory (TRL, 1986) and in two other NEDO (1987) SD rat developmental inhalation studies, including in another teratogenicity study and in both generations of a two generation study. In addition, CNS effects have been reported in inhalation studies of monkeys, including brain histopathology following chronic exposure (NEDO, 1987) and delayed neurological development following gestational exposure (Burbacher et al., 2004b; 2004a; 1999b; 1999a). Further, the primary reason that the developmental brain weight effect has not been identified in other species could be that it has not been the focus of other laboratory research. The greater uncertainty is associated with the documentation for the NEDO (1987) supplementary developmental study that formed the basis for EPA's benchmark dose analysis. The three primary uncertainties related to the documentation of this study identified during external peer reviews of the NEDO (1987) studies (ERG, 2009) were related to what was or was not reported with respect to (1) the number and health of pregnant dams, (2) the body weight of the offspring and (3) the statistical analysis of response data. While the methods for this supplementary study are not described, the methods for the parent two-generation study are adequately described and it is reasonable to assume that the supplementary study was performed

under the same protocol, starting with a number of F_0 parents appropriate for a one-generation developmental study.[59] While data related to maternal or gestational outcomes in the supplementary study are not given, signs of overt maternal toxicity were not reported in the two-generation study at similar exposure levels and it is reasonable to assume that they did not occur, and would have been reported had they been observed, in the supplementary study. With respect to the second source of documentation-related uncertainty, NEDO only reported means and standard deviations for absolute brain weight change and did not report body weight data for the offspring of the supplementary study. However, body weight data reported for the parent, two-generation study did not indicate a body weight effect in the exposed F_1 or F_2 generation pups. Further, EPA neurotoxicity guidelines (U.S. EPA, 1998a) state that a "change in brain weight is considered to be a biologically significant effect," and further states that "it is inappropriate to express brain weight changes as a ratio of body weight and thereby dismiss changes in absolute brain weight." The third source of documentation-related uncertainty noted by the external peer reviewers of the NEDO studies, was that NEDO did not report the results of a more appropriate (e.g., ANOVA) test for statistical significance. This is not a significant source of uncertainty because EPA did not rely on the NEDO statistical determinations, but performed its own more definitive benchmark dose analysis of the data (see Appendix D). In summary, while there are uncertainties concerning the NEDO (1987) supplementary study that forms the basis of the RfC, particularly with respect to documentation deficiencies, there is sufficient ancillary evidence to offset these concerns and allow for the consideration the this study as a basis for RfC or RfD derivation.

The use of reproductive and neurotoxicity endpoints reported in developmental (Burbacher et al., 2004b; 2004a; 1999b; 1999a) and chronic (NEDO, 1987) monkey studies would potentially result in lower reference values but significant uncertainties associated with those studies and the reported dose-response data preclude their use as the basis for an RfC. Burbacher et al. (2004b; 2004a; 1999b; 1999a) exposed *M. fascicularis* monkeys to 0, 200, 600, or 1,800 ppm (0, 262, 786, and 2,359 mg/m^3) methanol 2.5 hours/day, 7 days/week during premating/mating and throughout gestation (approximately 168 days). They observed a slight but statistically significant gestation period shortening in all exposure groups. As discussed in Sections 4.3.2 and 5.1.1.2, there are questions concerning this effect and its relationship to methanol exposure. Neurobehavioral function was assessed in infants during the first 9 months

[59] The number of F0 parents in the supplemental experiment was not reported, but the number of pups per dose group was and it is reasonable to assume that, consistent with the culling protocol used for the two-generation study (NEDO, 1987 pages 185 and 189), each dose group pup came from a different litter (to avoid "litter correlation" issues). EPA developmental neurotoxicity guidelines (U.S. EPA, 1998b) require that "on postnatal day 11, either 1 male or 1 female pup from each litter (total of 10 males and 10 females per dose group) should be sacrificed." Hence, by examining more than 10 male and 10 female litter-specific pups per dose group at three time points (3, 6 and 8 wks), the NEDO supplementary study would exceed EPA recommendations for this type of study.

of life. Two tests out of nine returned positive results possibly related to methanol exposure. The Fagan test of infant intelligence indicated small but not significant deficits of performance (time spent looking a novel faces versus familiar faces) in treated infants. VDR performance was reduced in all treated male infants, and was significantly reduced in the 1,800 ppm (2,359 mg/m^3) group for both sexes and the 600 ppm (786 mg/m^3) group for males. However, as discussed in Appendix D, an overall dose-response trend for this endpoint was not apparent in males and was only marginally significant in females, which had a larger overall sample size across dose groups than males (21 females versus 13 males). A benchmark dose analysis was done for the VDR effect in female monkeys using C_{max} above background of blood methanol as the dose metric (results detailed in Appendix D.3). The BMDL was estimated to be 19.6 mg/L. While there are significant concerns regarding the quantification of a dose-response for this VDR endpoint, this C_{max} BMDL is consistent with the C_{max} and AUC BMDLs estimated from the more reliable rodent studies and represent a measure of functional deficits in sensorimotor development that is possibly consistent with developmental CNS effects (i.e., brain weight changes) that have been observed in rats (NEDO, 1987). Although the VDR test results suggest that prenatal exposure to methanol can result in neurotoxicity to the offspring, the use of such statistically borderline dose-response data is not warranted in the derivation of the RfC or RfD, given the availability of better dose-response data in other species.

NEDO (1987) also examined the chronic neurotoxicity of methanol in *M. fascicularis* monkeys exposed to 0, 10, 100, or 1,000 ppm (13.1, 131, or 1,310 mg/m^3) for up to 29 months. Multiple effects were noted at 131 mg/ m^3, including slight myocardial effects (negative changes in the T wave on an EKG), degeneration of the inside nucleus of the thalamus, and abnormal pathology within the cerebral white tissue in the brain. The results support the identification of 10 ppm (13.1 mg/m^3) as the NOAEL for neurotoxic effects in monkeys exposed chronically to inhaled methanol. However, as discussed in Section 4.2.2.3, there exists significant uncertainty in the interpretation of these results and their utility in deriving an RfC for methanol. These uncertainties include lack of appropriate control group data and limited nature of the reporting of the neurotoxic effects observed. Thus, while the NEDO (1987) study suggests that monkeys may be a more sensitive species to the neurotoxic effects of chronic methanol exposure than rodents, the substantial deficits in the reporting of data preclude the quantification of data from this study for the derivation of an RfC.

5.3.2. Choice of Model for BMDL Derivations

As described in Appendix D, the Hill model adequately fit the data set for the rat brain weight endpoint used to derive the RfC (goodness-of-fit *p*-value = 0.59). Data points were well predicted near the BMD (scaled residual = 0.18) (see Figure 5-1). There is a 5-fold range of BMDL estimates from adequately fitting models, indicating considerable model dependence. The BMDL from the Hill model was selected as the most appropriate model for derivation of an RfC from this endpoint, in accordance with EPA BMD Technical Guidance (U.S. EPA, 2012a) , because it results in the lowest BMDL from among a broad range of BMDLs and provides a superior fit in the low dose region nearest the BMD. The nested Logistic (NLogistic) model adequately fit the data set for the cervical rib endpoint used to derive the RfD (goodness-of-fit *p*-value = 0.34). Data points were well predicted near the BMD (scaled residual = 0.54) (see Figure 5-2). There is a small, 1.3-fold range of BMDL estimates from adequately fitting models, indicating little model dependence. In accordance with EPA BMD Technical Guidance (U.S. EPA, 2012a), the BMDL from the NLogistic model was selected a as the most appropriate model for derivation of an RfC from this endpoint based on visual inspection, low AIC, and a superior fit in the low dose region nearest the BMD.

5.3.3. Route-to-Route Extrapolation

To estimate an oral dose POD for cervical rib anomalies in mice, a route-to-route extrapolation was performed on the inhalation exposure POD used to derive the RfC. One way to characterize the uncertainty associated with this approach is to compare the responses observed in the critical inhalation study to responses observed in similar oral developmental studies. As discussed in Section 5.2.1, Rogers et al. (1993b) also conducted an oral developmental study in CD-1 mice. Though their oral study involved a higher dose and was not conducive to a dose-response analysis, it did result in effects (cleft palate and exencephaly) consistent with skeletal abnormalities observed in their inhalation developmental studies in CD-1 mice (Rogers and Mole, 1997; Rogers et al., 1993b). In addition, brain weight reductions observed in rats by the other candidate principal developmental study (NEDO, 1987) have been observed in an oral study of adult rats (TRL, 1986).

5.3.4. Statistical Uncertainty at the POD

Parameter uncertainty in the model used to derive the RfC/D can be assessed through confidence intervals. Each description of parameter uncertainty assumes that the underlying model and associated assumptions are valid. For the Hill and NLogistic models applied to the

data for decreased brain weight in rats and cervical rib anomalies in mice, respectively, there is a degree of uncertainty in the BMD estimate at the BMR reflected by a 40-50% difference between the 95% one-sided lower confidence limit (BMDL) and the maximum likelihood estimate of the BMD.

5.3.5. Choice of Species/Gender

Effects considered for the RfC and RfD derivation were decreased brain weight at 6 weeks (postnatal) in male (the gender most sensitive to this effect) SD rats (NEDO, 1987) and cervical rib anomalies in male and female CD-1 mice (Rogers et al., 1993b). If the decreased brain weight in female rats had been used, higher RfCs and RfDs would have been derived (approximately 66% higher than the male derived values). As discussed in Section 5.3.1, while existing developmental and chronic studies suggest that monkeys may be the more sensitive and relevant species, these studies were not chosen for RfC or RfD derivation due to substantial deficits in the NEDO (1987) monkey study and uncertainties in the dose-response data reported in the Burbacher et al. (2004a; 1999a) study.

Researchers at the University of Toronto (Miller and Wells, 2011; Sweeting et al., 2011) have suggested that developmental studies in rodents may not be suitable for assessing human toxicity. Their hypothesis that mouse studies are not relevant to humans is based on several assumptions, including that (1) mouse embryos have a higher reliance on catalase over ADH to metabolize embryonic methanol, (2) catalase has a higher affinity for methanol than reactive oxygen species, (3) due to this affinity, embryonic methanol competitively inhibits catalase antioxidant activity, (4) this competitive inhibition results in an increase in embryonic ROS activity, and (5) this increased embryonic ROS activity is the primary MOA responsible for the teratogenic effects observed in mice following methanol exposure. The first of these assumptions is uncertain given the complexity of enzyme kinetics, the limited knowledge of how a human fetus/infant metabolizes methanol, existing evidence that a human fetus/infant can metabolize methanol via a mechanism(s) other than ADH, and the possibility that this alternative mechanism could involve catalase (Tran et al., 2007). The second assumption is based on published reports of catalase affinity (K_m) for methanol (Perkins et al., 1995a; Ward et al., 1995) and hydrogen peroxide (Vetrano et al., 2005) and merits a greater degree of certainty. However, there is limited, and conflicting evidence for assumptions 3 and 4, (i.e., that catalase affinity for methanol can lead to an increase in embryonic ROS). In order for assumptions 3 and 4 to be true, catalase affinity for methanol would need to be strong enough to overcome catalase's extremely high

reaction rate with ROS[60], and other enzymes (e.g., glutathione and superoxide dismutase) can also protect against ROS. Miller and Wells (2011) point out that methanol radicals have been detected by electron spin resonance spectrometry in methanol intoxicated rats (Skrzydlewska et al., 2000) and methanol derived adducts have been observed in the bile and urine of rats exposed to methanol and a free radical spin trapping agent (Mason and Kadiska, 2003). However, these observations do not answer the question of whether methanol's impact on catalase activity can cause an overall increase in embryonic ROS, and evidence to the contrary exists for adult organ systems. No increase in a general indicator of tissue oxidative DNA damage [8-hydroxy-2'-deoxyguanosine (8-oxodG)] was observed in the lungs, livers, bone marrow and spleen of male CD-1 mice, DNA repair deficient oxoguanine glycosylase (Ogg1) knockout mice, NZW rabbits and cynomolgus monkeys (*Macaca fascicularis*) given a single i.p. injection of 2 g/kg methanol and male CD-1 mice injected daily for 15 days with 2 g/kg methanol (Mccallum et al., 2011a; 2011b). With respect to the fifth assumption, it has been suggested that in-vitro studies that report an enhancement of methanol-induced embryopathies in glutathione-depleted rat embryos (Harris et al., 2004) provide support for a ROS-mediated mode of action for methanol developmental toxicity. However, as discussed in Section 4.7.1, the impact of glutathione depletion on the methanol induced embryopathies has also been attributed to a decreased ability to metabolize formaldehyde (Harris et al., 2004). It has also been suggested that the enhancement of methanol-induced embryopathies in acatalasemic (aCat; low catalase activity) mouse embryos supports a ROS-mediated mode of action (Miller and Wells, 2011). However, in-vivo studies from the same laboratory using the same strains of mice as the Miller and Wells (2011) study observed enhanced fetal effects in the hCat mice similar to those observed in mice by Rogers et al. (2004) and no enhancement of fetal effects in aCat mice (Siu et al., 2013). Siu et al. (2013) acknowledge that their in-vivo results imply no ROS involvement in the embryopathology of methanol-induced fetal effects in mice. While ROS may yet be determined to play a role in the pathological progression of methanol-induced fetal effects in rodents, available information is not consistent or adequate to conclude that the rodent developmental studies are not relevant in the assessment of human toxicity from methanol exposure.

Sweeting et al. (2011) have also suggested that rabbits would be a more appropriate test species than mice and that rabbits are resistant to methanol teratogenicity. A developmental study in rabbits via an appropriate route of exposure would be of interest, particularly if it involved an investigation of effects over a broad set of gestational days. However, more research is needed before it can be stated that rabbit developmental study would be more relevant to humans than rodent studies and that rabbits are resistant to methanol teratogenicity. The identification of

[60] Catalase's interaction rate with hydrogen peroxide (K_{cat}) is roughly 40,000,000/second (Garrett and Grisham, 2010).

species and strain differences in dose-susceptibility is complicated for developmental effects. In particular, test animals may have different gestational windows of susceptibility. The Sweeting et al. (2011) study assumes that the gestational window of susceptibility for developmental effects in rabbits following methanol exposure is at or close to that for mice. While the gestational window of susceptibility for developmental effects in mice is well studied and documented (Degitz et al., 2004a; Degitz et al., 2004b; Rogers et al., 2004; Rogers and Mole, 1997; Dorman and Welsch, 1996; Fu et al., 1996; Dorman et al., 1995; Andrews et al., 1993; Bolon et al., 1993; Rogers et al., 1993a; Rogers et al., 1993b), no studies have been done to identify the gestational window of susceptibility for methanol exposures in rabbits. As mouse studies have shown, missing the true gestational window of susceptibility for a species/strain can make a marked difference in the developmental effect observed (Rogers and Mole, 1997; Bolon et al., 1993).

5.3.6. Relationship of the RfC and RfD to Endogenous Methanol Blood Levels

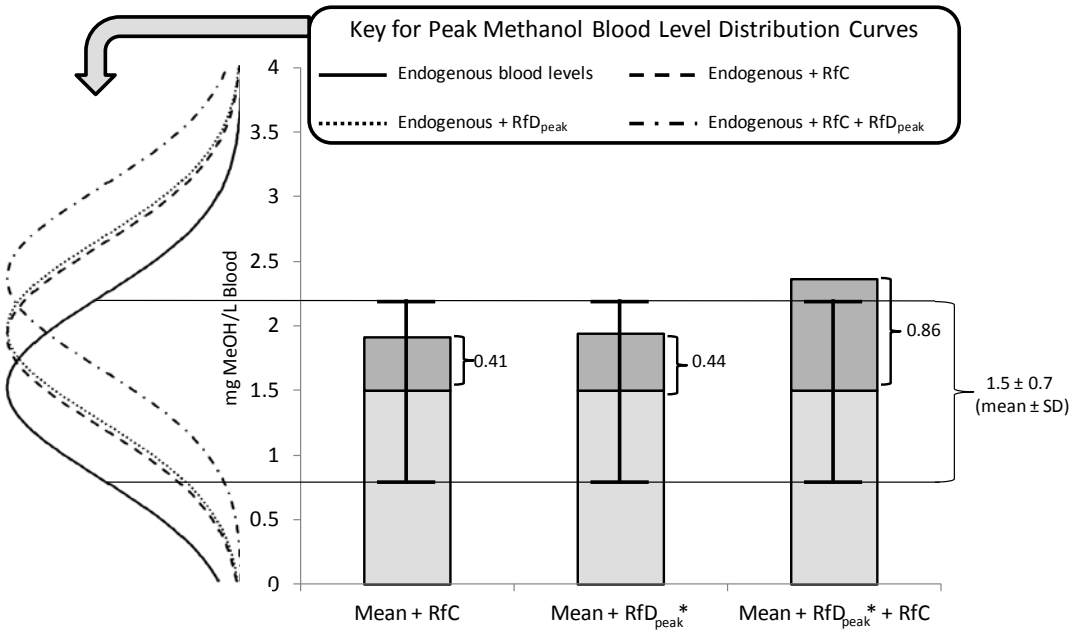
The approach taken by EPA in deriving the RfC and the RfD assumes that endogenous blood levels of methanol in a human population with normal background variation do not elicit adverse health effects. There is currently little evidence, epidemiological or otherwise, to challenge this assumption. Thus, a comparison of the increase in blood levels expected from exposure at the RfC or RfD to the existing range of endogenous levels of methanol observed in human blood is warranted.

Using the mean and standard deviation values reported in Table 3-1 for ten study groups, an approximation of the overall mean and standard deviation of endogenous background blood methanol in humans can be calculated. Simply adding and averaging the mean for each study would assume that the study methods (e.g., dietary restrictions, measuring techniques) and subject group characteristics (e.g., age range, gender proportion, and ethnicity) are similar across the studies. Since this is not likely to be true, a Random-Effects model was used to estimate the amount of heterogeneity (variability) between the sampled subpopulations (Viechtbauer, 2010; Raudenbush, 1994). A significant amount of variability was found between groups. Ninety-five percent of the total variability in the samples was due to variability between groups, as opposed to variability within groups (as measured by the reported standard deviations). Hence, the Random-Effects model was used to estimate a more representative mean and SD of 1.5 mg/L and ± 0.7 mg/L, respectively. [61]

If the increase in methanol blood levels in humans estimated from exposure at the 2×10^{1} mg/m^3 RfC or 2 mg/kg-day RfD (or both combined) was negligible relative to normal variation

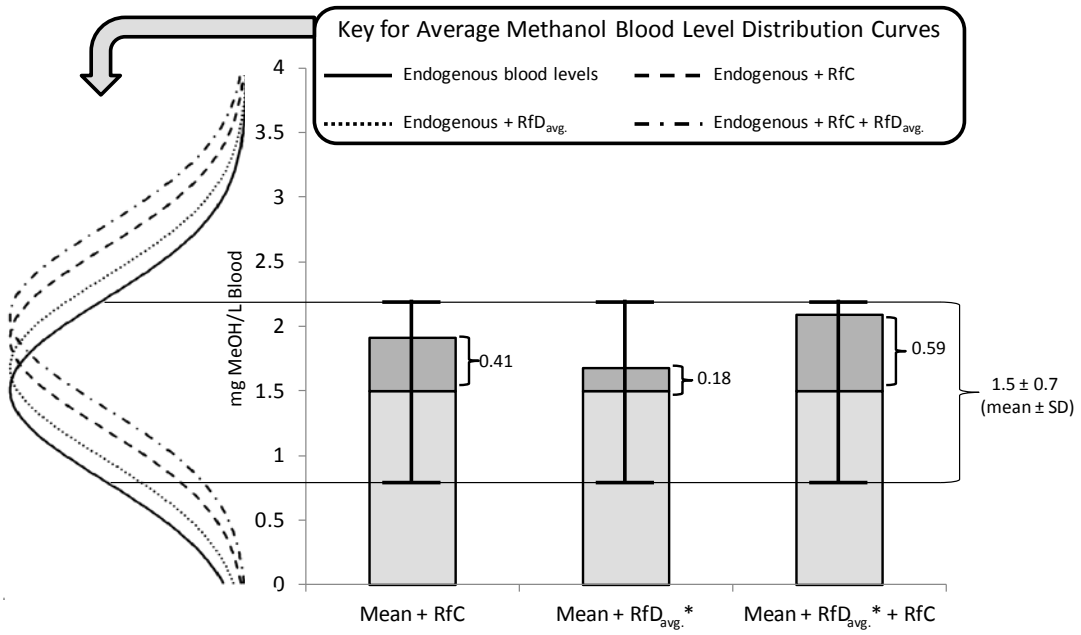
[61] The "Random-Effects Model" was applied by entering the means and variances for the ten study groups for which means and standard deviations were reported (Table 3-1) into the subroutine "rma" in the "metafor" package in R.

in endogenous levels of methanol in blood then one could argue the increase would be indistinguishable from natural variation and toxicologically irrelevant. EPA's PBPK model predicts that a continuous daily methanol exposure equivalent to the RfC would raise an individual's existing methanol blood level by 0.41 mg/L. The model also predicts that a daily exposure to methanol at the RfD (distributed as six bolus ingestions) would raise an individual's existing methanol blood level by a peak value of 0.44 mg/L and by an average value of 0.18 mg/L. Further, the PBPK model estimates that the methanol blood levels of individuals exposed to both an RfC and RfD (via simultaneous inhalation and ingestion, respectively) would increase their blood methanol levels by a daily maximum value of 0.86 mg/L and by a daily average value of 0.59 mg/L. As shown in Figures 5-3, 5-4 and B-17, the estimated increase in blood levels of methanol resulting from exposure to methanol at the RfC alone, at the RfD alone, or at the RfC + RfD combined is comparable to background methanol blood levels in humans, represented as a mean plus standard deviation of 1.5 ± 0.7 mg/L (Table 3-1). From this analysis EPA concludes that the estimated increase in blood levels of methanol from exogenous exposures at the level of the RfD or the RfC (or from the RfC + RfD) are distinguishable from natural background variation.



*For the exposure regimen assumed (Section B.2.7), daily increases for an RfD vary between 0.01 and 0.44 mg/L (Appendix B, Figure B-17).

Figure 5-3 Peak projected daily impact of RfC and RfD exposures on endogenous methanol background blood levels (mg MeOH/Liter [mg/L] blood) in humans.



*For the exposure regimen assumed (Section B.2.7), daily increases for an RfD vary between 0.01 and 0.44 mg/L (Appendix B, Figure B-17).

Figure 5-4 Average projected daily impact of RfC and RfD exposures on endogenous methanol background blood levels (mg MeOH/Liter [mg/L] blood) in humans.

CERHR (2003) has stated in their report that up to 10 mg/L in blood would not be associated with adverse developmental effects in humans, but there is uncertainty associated with this assumption. As discussed in Section 5.1.3.2.3, there is considerable uncertainty as to whether rodents are as sensitive as monkeys and humans to the reproductive and developmental neurotoxic effects of methanol. In the Burbacher et al. (2004a; 1999a) study, statistically significant shortened pregnancy duration was observed in monkeys exposed to 200 ppm and statistically significant VDR delay was observed in male monkey infants exposed to 600 ppm methanol for just 2 hours per day. EPA estimates that these exposures raised the methanol blood levels over endogenous methanol blood levels in these monkeys to peak values of just 3 and 10 mg/L, respectively (see Appendix D, Table D-10), corresponding to total blood levels of approximately 5 and 12 mg/L, respectively. Also, NEDO (1987) observed potential signs of CNS degeneration in histopathology reported for monkeys exposed chronically to 100 ppm for 21 hours per day, which is estimated by EPA's monkey PK model to be associated with an increase in methanol blood levels over endogenous levels of approximately 1 mg/L, corresponding to total methanol blood levels of roughly 3 mg/L (assuming an endogenous background in these monkeys of 2 mg/L).

5.4. Cancer Assessment

A cancer dose-response estimation is not addressed in this document. However, the Agency is currently reviewing the literature and will develop a cancer assessment for methanol at a later date.

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