

Review

NTP-CERHR Expert Panel report on the reproductive
and developmental toxicity of methanol
Center for the Evaluation of Risks to Human Reproduction[☆]

Preface

The National Toxicology Program (NTP) and the National Institute of Environmental Health Sciences (NIEHS) established the NTP Center for the Evaluation of Risks to Human Reproduction (CERHR) in June 1998. The purpose of the Center is to provide timely, unbiased, scientifically sound evaluations of human and experimental evidence for adverse effects on reproduction, including development, caused by agents to which humans may be exposed.

Methanol was selected for evaluation by the CERHR based on high production volume, extent of human exposure, and published evidence of reproductive or developmental toxicity. Methanol is used in chemical syntheses and as an industrial solvent. It is a natural component of the human diet and is found in consumer products such as paints, antifreeze, cleaning solutions, and adhesives. It is used in race car fuels and there is potential for expanded use as an automobile fuel.

This evaluation is the result of a 10-month effort by a 12-member panel of government and non-government scientists that culminated in a public Expert Panel meeting. This report has been reviewed by CERHR staff scientists, and by members of the Methanol Expert Panel. Copies have been provided to the CERHR Core Committee, which is made up of representatives of NTP-participating agencies. This report is a product of the Expert Panel and is intended to (1) interpret the strength of scientific evidence that a given exposure or exposure circumstance may pose a hazard to reproduction and the health and welfare of children; (2) provide objective and scientifically thorough assessments of the scientific evidence that adverse reproductive/development health effects are associated with exposure to specific chemicals or classes of chemicals, including descriptions of any uncertainties that would diminish confidence in assessment of risks; and (3) identify knowledge gaps to help establish research and testing priorities.

The expert panel report becomes a central part of the subsequent NTP-CERHR Monograph. Each monograph includes the NTP Brief on the chemical under evaluation, the expert panel report, and all public comments on the expert panel report. The NTP Brief contains the NTP's conclusions on the potential for exposure to result in adverse effects on human development and reproduction. It is based on the expert panel report, public comments on the report, and relevant data published after the expert panel report was completed. NTP-CERHR Monographs are publicly available and are transmitted to appropriate health and regulatory agencies.

The NTP-CERHR is headquartered at NIEHS, Research Triangle Park, NC and is staffed and administered by scientists and support personnel at NIEHS and at Sciences International, Inc., Alexandria, Virginia.

Reports can be obtained from the website (<http://cerhr.niehs.nih.gov/>) **or from:** Michael D. Shelby, Ph.D., NIEHS EC-32, P.O. Box 12233, Research Triangle Park, NC 27709, USA. Tel.: +1-919-541-3455; e-mail: shelby@niehs.nih.gov

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A report of the CERHR Methanol Expert Panel

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*These panel members did not fully concur with Section 5 of this report, primarily because they felt (1) the Overall Conclusions did not adequately address uncertainties regarding susceptible subpopulations and total population exposures, and (2) the Critical Data Needs should include studies on female reproductive function.

Abbreviations: ACGIH, American Conference of Governmental Industrial Hygienists; ADH, alcohol dehydrogenase; AF&PA, American Forest & Paper Association; AMI, American Methanol Institute; ANOVA, analysis of variance; apABGlu, *p*-acetamidobenzoylglutamate; Asp, aspartame; AUC, area under curve; BEI, biological exposure index; BMD₀₅, benchmark dose, 5% effect level; bw, body weight; C, Celsius; C1, 2, 5, 7, cervical vertebra 1, 2, 5, 7; cm², centimeters squared; C_{max}, peak concentration; C-section, Cesarean section; CAS RN, Chemical Abstracts Service Registry Number; CERHR, Center for the Evaluation of Risks to Human Reproduction; CI, confidence interval; CL ± P, cleft lip and/or palate; CNS, central nervous system; d, day; DCR, decidual cell response; DMDC, dimethyl dicarbonate; DNA, deoxyribonucleic acid; DOE, Department of Energy; EEG, electroencephalogram; EPA, Environmental Protection Agency; EX, exencephaly; F, female; FA, folic acid; FDA, Food and Drug Administration; FR, fixed ratio; FSH, follicle stimulating hormone; g, gram; GC, gas chromatography; gd, gestation day; h, hour; HEI, Health Effects Institute; HPLC, high pressure liquid chromatography; HSDB, Hazardous Substances Data Bank; IPCS, International Programme on Chemical Safety; IV, intravenous; kg, kilogram; K_m, Michaelis constant; K_{ow}, octanol–water partition coefficient; kPa, kilopascal; l, liter; LD₅₀, lethal dose, 50% mortality; LH, luteinizing hormone; LOAEL, lowest observed adverse effect level; M, male; m³, meters cubed; mg, milligram; min, minute; mM, millimolar; ml, milliliter; MLE, maximum likelihood estimates; mmol, millimole; 4-MP, 4-methylpyrazole; MRC, Medical Research Council; MRCA, Market Research Corporation of America; MTBE, methyl tertiary butyl ether; MV, multivitamin; *n*, number; NCAM, neural cell adhesion molecule; NE, no effect; NEDO, New Energy Development Organization; ng, nanogram; NHANES, National Health and Nutrition Examination Survey; NIEHS, National Institute of Environmental Health Sciences; NIOSH, National Institute of Occupational Safety and Health; nmol, nanomol; NOAEL, no observed adverse effect level; NS, not specified; NTD, neural tube defect; NTP, National Toxicology Program; OR, odds ratios; OSHA, Occupational Safety and Health Administration; pABGlu, *p*-aminobenzoylglutamate; PBPK, physiologically-based pharmacokinetic model; PEL, permissible exposure limit; pnd, postnatal day; ppm, parts per million; QA/QC, quality assurance/quality control; RBC, red blood cell; RDA, recommended daily allowance; RIA, radioimmunoassay; RR, relative risk; SCE, sister chromatid exchange; S.D., standard deviation; S.E., standard error; T, testosterone; TAS-DIET, Technical Assessment System-International Diet Research System; THF, tetrahydrofolate; TLV, threshold limit value; TRI, Toxic Release Inventory; TWA, time weighted average; USDA, United States Department of Agriculture; VOC, volatile organic compound; V_{max}, maximal velocity of metabolism; wk, week; µg, microgram

Note to reader: This report is prepared according to the Guidelines for CERHR Panel Members established by NTP/NIEHS. The guidelines are available from the CERHR web site (<http://cerhr.niehs.nih.gov/>). The format for Expert Panel Reports includes synopses of studies reviewed, followed by an evaluation of the strengths/weaknesses and utility (adequacy) of the study for a CERHR evaluation. Statements and conclusions made under strengths/weaknesses and utility evaluations are those of the Expert Panel and are prepared according to the NTP/NIEHS guidelines. In addition, the Panel often makes comments or notes limitations in the synopses of the study. Bold, square brackets are used to enclose such statements. As discussed

in the guidelines, square brackets are used to enclose key items of information not provided in a publication, limitations noted in the study, conclusions that differ from authors, and conversions or analyses of data conducted by the panel.

1. Chemistry, use, and human exposure

Much of the information in this section was obtained from reviews, especially IPCS [1] and Kavet and Nauss [2]. The Kavet and Nauss [2] paper is the published version of a Health Effects Institute [3] report. Because the Kavet and

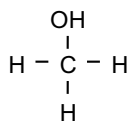


Fig. 1. Chemical structure of methanol.

Nauss paper is more readily available to the public, it is cited instead of the HEI version.

1.1. Chemistry

1.1.1. Nomenclature

The CAS Registry Number for methanol is 67-56-1. Synonyms of methanol include: methyl alcohol, wood alcohol; Carbinol; Methylol; colonial spirit; columbian spirit; methyl hydroxide; monohydroxymethane; pyroxylic spirit; wood naphtha; and wood spirit [4].

1.1.2. Formula and molecular mass

The chemical formula is: CH₃OH (Fig. 1). The molecular mass is 32.04.

1.1.3. Chemical and physical properties

The chemical and physical properties of methanol are given in Table 1.

1.1.4. Technical products and impurities

According to IPCS [1] and HSDB [5], sales grade methanol in the US must meet the following specifications:

Methanol content (wt.%) minimum	99.85
Acetone and aldehydes (ppm) maximum	30
Acid (as acetic acid) (ppm) maximum	30
Water content (ppm) maximum	1500
Specific gravity	0.77928
Permanganate time, minimum	30
Odor	Characteristic
Distillation range at 101 kPa	1 °C, must include 64.6 °C

Table 1
Physicochemical properties of methanol

Property	Value
Vapor pressure	160 mmHg at 30 °C
Melting point	−98 °C
Boiling point	64.7 °C
Specific gravity	0.7866 (25 °C)
Solubility in water	Miscible
log <i>K</i> _{ow}	−0.82 to −0.68

IPCS [1]; Chemfinder [4].

Color, platinum-cobalt scale, maximum	5
Appearance	Clear-colorless
Residual on evaporation (g/100 ml)	0.001
Carbonizable impurities, color	30

There are no known trade names for methanol. Past or present US manufacturers of methanol include: Air Products and Chemicals; Ashland Oil, Inc.; Atlantic Richfield Co.; Borden Chemicals and Plastics Partnership; E I du Pont de Nemours and Company, Inc.; Eastman Kodak Co.; Georgia Gulf Corporation; Hoechst Celanese Corp.; Quantum Chemical Corp.; Tenneco Inc.; and Texaco Inc. [5].

1.2. Use and human exposure

1.2.1. Production

In the past, methanol was produced from the dry distillation of wood. Today methanol is primarily made from steam reformed natural gas and carbon dioxide [6]. It can also be produced from biomass by the catalytic conversion of pressurized synthesis gas (hydrogen, carbon monoxide, and carbon dioxide) in the presence of metallic heterogeneous catalysts [1].

Methanol is among the highest-ranking production volume chemicals. Methanol production volume in the 1990–1992 time period was approximately 8–8.7 million pounds [5]. In 1998, US methanol production capacity totaled more than 2.2 billion gallons (14 billion pounds), which was approximately 75% of the US demand [6]. The remainder was imported, principally from Canada, for a total of approximately 3 billion gallons (19.7 billion pounds).

1.2.2. Use

About 70% of methanol manufactured worldwide is used as feedstock for the production of chemicals such as formaldehyde, methyl tertiary butyl ether (MTBE), acetic acid, methyl methacrylate, and dimethyl terephthalate [1]. Methanol is widely used in a variety of consumer products, as described below. It is also used in the treatment of wastewater and sewage. About 70% of methanol in sewage systems is biodegraded within 5 days [1].

1.2.3. Occurrence

There is a high potential for release of methanol to the environment as a result of its large production volume, widespread use, and physicochemical properties [1]. Methanol releases usually occur from usage of methanol-containing solvents and products, methanol production, end-product manufacturing, and storage and handling losses. The 1998 Toxic Release Inventory (TRI) Data Release for methanol presented a total on- and off-site release of close to 215 million pounds [7]. According to the TRI [8], methanol ranked second to hydrogen chloride in both total air emissions and total on- and off-site releases in 1999.

Persistence, bioconcentration, or bioaccumulation of methanol in the environment are not expected due to its low adsorptive properties in soil and its rapid degradation in water, soil, and air. Methanol is readily degraded by photooxidation and the half-life for reaction with hydroxyl radicals is 7–18 days. Methanol is biodegradable under aerobic and anaerobic conditions [1].

Humans are also exposed to methanol through natural sources. Natural emission sources of methanol include volcanic gasses, vegetation, microbes, and insects. Methanol occurs naturally in humans and animals, and can be found in blood, urine, saliva, expired air, and mother's milk [1]. Methanol is a natural component of fruits, vegetables, and fermented spirits. Ingestion of the food additives aspartame and dimethyl dicarbonate (DMDC) can also result in exposure to methanol.

1.2.4. Human exposure

1.2.4.1. General population exposure. The general population can be exposed to methanol through environmental sources such as air and water and contact with methanol-containing consumer products. Dietary sources including fruits, fruit juices, aspartame, DMDC, and alcoholic beverages are thought to be the primary sources of current exposure in the general population.

Consumer exposure to methanol can occur during use of methanol-containing products such as varnishes, shellacs, paints, windshield washer fluid, antifreeze, adhesives, deicers, and Sterno™ heaters. Methanol vapor may also be present in cigarette smoke at a level of 180 µg per cigarette [1]. While much of the potential human exposure to methanol from the above uses is expected to be through inhalation, important exposure routes also include ingestion and dermal absorption. For oral ingestion, the consumption of adulterated alcoholic beverages or fermented spirits containing wood alcohol, as well as accidental or intentional consumption of pure methanol, are major sources of exposure. In the year 2000, 2474 incidents of methanol poisoning were reported to poison control centers with 613 of those incidents involving children under 6 years of age [9]. The incidents frequently involve young children who ingest methanol in consumer products. Dermal contact with methanol solutions can also lead to rapid absorption and manifestations of toxicity or lethality [1].

The general public is exposed to methanol through diet (Table 2). Methanol occurs naturally in fresh fruits and vegetables as either free alcohol, methyl esters of fatty acids, or methoxyl groups on polysaccharides. Lindinger et al. [10] noted an increase in breath methanol levels in four males who ate 1 kg apples and drank 75 g of 40% ethanol in water. Fruit juices contain methanol or methanol precursors and a range of 12–640 mg methanol/l in juice with a mean of 140 mg/l has been widely quoted [1,2,11]. Methanol has also been detected in beans, split peas, and lentils at levels

Table 2
Methanol levels in foods and beverages

Sample	Methanol level	Reference
Fresh and canned fruit juices (orange and grapefruit juices)	1–43 mg/l	IPCS [1]
	11–80 mg/l	Lund et al. [169]
	12–640 mg/l (average of 140 mg/l)	IPCS [1]
Neutral spirits	≤1.5 g/l	IPCS [1]
Beer	6–27 mg/l	Greizerstein [13]
Wines	96–329 mg/l	Greizerstein [13]
Distilled spirits	16–220 mg/l	Greizerstein [13]
Bourbon	55 mg/l	Monte [170]
50% Grain alcohol	1 mg/l	IPCS [1]
Concentrations permitted in brandies in the USA, Canada, and Italy	6000–7000 mg/l ethanol	IPCS [1]
Beans	1.5–7.9 mg/kg	IPCS [1]
Split peas	3.6 mg/kg	IPCS [1]
Lentils	4.4 mg/kg	IPCS [1]
Carbonated beverages	~56 mg/l	Stegink et al. [11]

ranging from 1.5 to 7.9 mg/kg [1]. Though concentrations were not reported, methanol has been found in roasted filberts, brussel sprouts, carrots, celery, onions, parsnips, peas, and potatoes [1]. In addition to free methanol in fruits and vegetables, more methanol is likely to be released following ingestion due to breakdown of pectins in the gastrointestinal tract [12].

Alcoholic beverages contain methanol at concentrations ranging from 6 to 27 mg/l in beer, 96 to 329 mg/l in wine [1,13], and up to 1500 mg/l in some neutral spirits [1]. Taucher et al. [14] demonstrated an increase in the breath methanol levels of subjects consuming 100 ml brandy; however, the Panel notes that the study does not provide useful information since the correlation between breath and blood methanol was not determined.

In addition to natural sources of methanol in the diet, the public is also exposed to methanol through two direct food additives: aspartame and DMDC. Aspartame (L-aspartyl-L-phenylalanine methyl ester) is an artificial sweetener. It is a dipeptide that is primarily comprised of phenylalanine and aspartic acid [15]. When ingested, about 10% by weight of aspartame is hydrolyzed to free methanol, which is then available for absorption [1]. DMDC is a yeast inhibitor used in tea beverages, sports drinks, fruit or juice sparklers, wines, and wine substitutes [16–18]. DMDC is unstable in aqueous solutions (beverages) and primarily breaks down to methanol and carbon dioxide [16]. Theoretically, full hydrolysis of one mole of DMDC yields two moles of methanol and two moles of carbon dioxide. On a weight basis, 100 mg of DMDC in a beverage would theoretically produce 48 mg methanol.

Estimates of aspartame consumption were reported by Butchko and Kotsonis [19] and were based on a menu census survey conducted by the Market Research Corporation of America (MRCA) in over 2000 US households with 5000

Table 3
Estimates of methanol intake through ingestion of aspartame, Butchko and Kotsonis [19]

Population	90th percentile methanol intake (mg/kg bw per day) ^a	99th percentile methanol intake (mg/kg bw per day) ^a
General population	0.16–0.30	0.64
Children of all age groups	0.26–0.52	0.52–0.85
Diabetics	0.21–0.34	0.82
Dieters	0.16–0.33	0.58
Women of childbearing age	0.2–0.42	0.87
Pregnant women	0.13–0.27	0.27

^a Based on reported intakes of aspartame and assumption that 10% of aspartame by weight is converted to methanol.

Table 4
Estimates of methanol intake through dietary sources and food additives

Source	90th percentile estimate (mg/person per day) ^a	90th percentile estimate for 60 kg adult (mg/kg bw per day)
Fruit juice and wine	48	0.80
DMDC	11	0.18
Fruit juice, wine and DMDC	59	0.98

^a DiNovi [20].

people a year from 1984 to 1992. Those estimates include intake by children, pregnant women, diabetics, and individuals on weight loss programs. Table 3 lists 90th and 99th percentile estimates of methanol intake resulting from aspartame ingestion by various subgroups of the population. A table in the Butchko and Kotsonis [19] report outlines 90th percentile exposures by age group and indicates that the highest exposures occur in children ages 0–5 years. The 90th percentile estimates by Butchko and Kotsonis are about one order of magnitude lower than FDA [15] pre-marketing aspartame intake estimates (resulting in estimated methanol intake of 0.8–3.4 mg/kg bw per day), while the 99th percentile estimates are within the lower range of pre-marketing estimates.

An unpublished and unreviewed FDA analysis estimated mean and 90th percentile exposures to methanol resulting from intake of untreated fruit juice and wine and use of DMDC (Table 4) [20]. Methanol exposures were esti-

mated using the 1989–1992 U.S. Department of Agriculture (USDA) Continuing Survey of Food Intake and the Technical Assessment Systems (TAS) International Diet Research System (TAS-DIET) software. The methanol level in untreated fruit juice and wine was reported to be 140 ppm (mg/l).

Environmental methanol concentrations are outlined in Tables 5 and 6. Most environmental exposures to methanol vapor are orders of magnitude below the occupational time-weighted average threshold limit value of 200 ppm (260 mg/m³) for an 8-h day and 40-h week [21]. Typical rural exposures below 0.0008 ppm (0.001 mg/m³) and typical urban exposures approaching 0.03 ppm (0.04 mg/m³) have been reported [1]. In an unpublished analysis, the American Forest and Paper Association (AF&PA) [22,23] used data from the TRI database and other sources to model average 24-h ambient methanol concentrations from some of the largest methanol-emitting facilities in the US. Maximum 24-h “fence line” concentrations were predicted to be below 4 mg/m³ (3 ppm). There is no known quantitative information about methanol concentrations in drinking water, but IPCS does report levels of methanol in wastewater samples (Table 6).

A potential source of general population exposure to methanol involves motor vehicle fuels. Methanol is currently used to a limited extent as an alternative fuel, primarily in a mixture of 85% methanol and 15% gasoline known as M85. Because of a lack of infrastructure support for such fuels, M85 use is generally limited to fleet vehicles in certain areas. According to the Department of Energy [24], approximately 18,000 vehicles capable of operating on M85 fuel were in use in 2000. These vehicles are typically equipped with “flexible fuel” engines that can run on mixtures ranging from 85% methanol/15% gasoline to 100% gasoline. It is difficult to ascertain the actual frequency of usage of M85 in the population of flex-fuel vehicles. According to DOE estimates [25], approximately 1 million gallons of M85 was used in the United States in 2000, compared to about 125 billion gallons of gasoline. Methanol also receives considerable attention as a potential fuel for fuel cells in motor vehicles. Fuel cell technology appears to be developing rapidly, but it remains to be seen whether methanol will become a major contender in the fuels market.

Table 5
Methanol levels in air samples

Sample	Methanol level	Reference
Mean ambient concentration in Tucson, Arizona in 1982	0.010 mg/m ³ , (0.0079 ppm)	[167]
Mean ambient concentration in two remote locations in Arizona in 1982	0.003 mg/m ³ , (0.0026 ppm)	[167]
Concentrations in Arctic air from Point Barrow, Alaska in 1967	0.00065–0.0018 mg/m ³ (0.0005–0.0012 ppm), (average 0.00077 ppm methanol plus ethanol)	[1]
Concentrations in urban air	0.0105–0.131 mg/m ³ , (0.005–0.1 ppm)	[168]
Concentrations at dense traffic sites in Stockholm, Sweden	0.00059–0.094 mg/m ³ , (0.00045–0.072 ppm)	[1]
Average ambient concentrations at five sites in and around Stockholm	0.005–0.030 mg/m ³ , (0.00383–0.0267 ppm)	[1]
Median levels found in 52 samples from three US locations (Boston, Houston, and Lima, Ohio)	0.006–0.060 mg/m ³	[1]

Table 6
Methanol levels in water samples

Sample	Methanol level	Reference
Rainwater collected during a thunderstorm in Arizona in 1982	0.022 mg/l	IPCS [1]
Wastewater effluents from a Massachusetts specialty chemicals manufacturing facility ^a	17–80 mg/l, (17–80 ppm)	IPCS [1]
Leachate from the Love Canal in Niagara Falls, New York	42.4 mg/l	IPCS [1]
Condensate waters discharged from a coal gasification plant in North Dakota	1050 mg/l	IPCS [1]

^a There was no methanol detected in the river water or sediments associated with the facility.

Given the limited and as yet unknown potential for future growth in the use of methanol fuels, population exposure to methanol in relation to mobile sources cannot be characterized at present. However, some estimates and limited measurements of methanol air concentrations associated with methanol fuel usage in conventional vehicles provide a perspective on potential individual exposures to methanol vapors. Early estimates of “worst case” exposure levels for methanol vapor concentrations in residential garages spanned a broad range of values, up to 200 ppm and possibly higher [2]. These estimates varied greatly for different scenarios, e.g. whether the engine met emission standards or was malfunctioning, or whether the engine was idling or in a “hot soak” condition (evaporation from a hot engine after it had been turned off). Additional estimates have assumed a vehicle under “hot soak” conditions with a malfunctioning emission control device. More recently, empirical measurements of evaporative emissions from such a vehicle were made by Tsai and Weisel [26]. The authors measured methanol and other volatile organic compounds (VOCs) in a garage and attached home as a function of several variables. A vehicle was operated on M85 until fully warmed up and then parked in an attached garage with the garage door closed, the door between the garage and the adjacent room in the house closed, and the door between the adjacent room and the remainder of the house closed. Among the variables manipulated was the emissions control device on the vehicle, namely the charcoal canister hose connection, which was left either connected or disconnected to simulate a malfunctioning device. The highest methanol levels were measured in the garage when the canister hose had been disconnected. Under those conditions, the mean concentration was 0.99 ppm, and the maximum measured concentration was 1.3 ppm. With the hose in place, the mean concentration was 0.50 ppm and the maximum was 0.75 ppm. With the hose disconnected, levels in the adjacent room were 0.12 ppm (mean) and 0.23 ppm (maximum), and were somewhat lower in the remainder of the home (mean: 0.056 ppm; maximum: 0.11 ppm).

Streicher [27] measured methanol vapor concentrations from the fuel system of a vehicle with a malfunctioning emission control device (methanol-saturated canister). In this study, M100 (100% methanol) fuel was used and the measurements were made in a sealed chamber approximately 2/3 the volume of a one-car garage. After 6 h (the maximum interval of the study) the methanol vapor con-

centration was about 270 ppm at 94 °F and about 97 ppm at 75 °F. Using a model based on these and other data, Streicher [27] estimated that a methanol concentration of approximately 230 ppm could occur in a well-sealed one-car garage, given “cold-soak” conditions for 6 h at 100 °F ambient temperature.

The above estimates and measurements cannot be considered representative of potential population exposure levels that would occur under a much wider range of conditions. Also, a more complete exposure assessment would take into consideration the potential for inhalation of vapors during refueling. Other less common scenarios that are part of general population exposures include the use of such fuels as solvents (e.g. by do-it-yourself mechanics) and accidental spillage. Each of the latter scenarios could involve dermal as well as inhalation exposures. No estimate of potential integrated exposure exists at present for these situations that are presumably at the high end of a distribution of population exposure levels.

One type of potential accidental exposure to methanol warrants special note. Each year, several thousand cases of accidental ingestion of gasoline are reported to US poison control centers. Litovitz [28] analyzed 1987 data from US poison control centers and found that 39% of accidental ingestions involved teenage and young adult males (15–29 years old), and almost all of them occurred during the course of siphoning to transfer fuel from one container to another. Nearly as many cases (36%) involved children under 6 years old. Most of the latter cases occurred when the children found a used beverage container in which gasoline was stored. With gasoline, the primary toxicity hazard lies in the possibility of regurgitating the fuel and aspirating the vomitus, which can induce chemical pneumonitis. However, if M85 were substituted for gasoline in these situations, methanol would considerably increase the potential for serious morbidity or mortality. Litovitz [28] noted that ingestion of as little as 5 ml (about a teaspoonful) of M85 fuel by a 10 kg 1-year-old child could require invasive treatment (hemodialysis) and as little as 12 ml (less than a tablespoonful) could result in death. Allowing for unreported cases and extrapolating from 1987 US poison control centers data, Litovitz estimated an annual incidence of 35,000 accidental ingestions of gasoline in the US and 52,000 cases of gasoline poisonings by any route. The actual number of gasoline poisonings reported to poison control centers in 2000 was 20,003 with 5859 of

those cases occurring in children less than 6 years of age [9].

1.2.4.2. Occupational exposure. Occupational exposure to methanol may occur during its production or result from its presence in refrigeration systems, as an inhibitor of hydrate formation at natural gas pipeline pumping stations, and as a component in the production of formaldehyde, MTBE, acetic acid, and other industrial chemicals [1]. Methanol's proposed use as a substitute for petroleum fuels may result in greater environmental releases to the air through vehicle emissions and at fueling stations. One report indicated that concentrations measured during refueling of methanol-powered transit buses were "generally less than 10 ppm" in the breathing zone of the workers [29]. Air concentrations for mechanics who were changing fuel filters for these buses averaged approximately 50 ppm during the 2-min procedure, during which levels reached as high as 2200 ppm.

From the 1950s to the 1980s, teacher aides and clerical workers were exposed to methanol concentrations ranging from 362 to 3052 ppm (475–4000 mg/m³) during the operation of "spirit" duplicator machines [1]. Those workers experienced symptoms of methanol intoxication as described in Section 2.2.1.

Currently the OSHA permissible exposure limit (PEL) and ACGIH threshold limit value (TLV) are set at 200 ppm (260 mg/m³) [5,21]. The ACGIH short term exposure level for methanol is 250 ppm [21]. Assuming worker exposure levels within the TLV and PEL, an 8-h work day, an inhalation rate of 20 m³ per day [30], and a 70 kg body weight, CERHR estimated worker exposures to methanol to be below 25 mg/kg bw per day:

$$\begin{aligned} < (260 \text{ mg/m}^3) \times (20 \text{ m}^3 \text{ per day}) \times (8 \text{ h}/24 \text{ h}) \times (1/70 \text{ kg}) \\ = < 25 \text{ mg/kg bw per day} \end{aligned}$$

The biological exposure index (BEI) for urinary methanol at the end of an 8-h shift is 15 mg/l [21].

1.3. Utility of data

Statistics on acute methanol poisonings are available, but the magnitude of exposures is usually poorly documented. The data on dietary exposure to methanol are judged limited at present. Although information is available on the distribution of population exposures to methanol from dietary sources (e.g. aspartame, fruits, vegetables, fermented spirits), data on the potential contribution from other additives (i.e. DMDC) or other sources (e.g. drinking water) were scant. Federal Register notices on final rules permitting specific uses of DMDC cited that methanol exposure was a factor considered in assessing safety of the permitted uses. The Expert Panel did not review the scientific data that underpin the FDA conclusions. The data on occupational exposure to methanol are judged to be limited. Data on total methanol exposure from all sources are judged insufficient.

Blood methanol levels are useful biomarkers of exposure (discussed in Section 2.1.1), but population data on blood methanol levels are limited.

1.4. Summary of human exposure

Methanol is produced naturally in the human body and is found in expired air and body fluids. Humans are also exposed to methanol through contact with anthropogenic and natural sources. Methanol is a constituent in consumer products such as varnishes, paints, windshield washer fluids, antifreeze, adhesives, deicers, and SternoTM heaters. It is used in the manufacture of other chemicals and is one of the highest production volume chemicals in the US. According to the EPA TRI [8], methanol is among the highest ranking chemicals in terms of environmental releases. The use of methanol in gasoline is currently limited, but increased use of alternative fuels and developments in fuel cell technology could result in much greater use of methanol in the future. Humans are exposed to methanol through foods and beverages. Natural sources of methanol include fruits and vegetables and fermented spirits. Methanol is also released during the metabolism of food additives such as the artificial sweetener, aspartame, and DMDC, a yeast inhibitor added to a variety of beverages.

Humans can be exposed to methanol by inhalation, oral intake, and dermal contact. Reported concentrations of methanol in ambient air have generally been well below 0.1 ppm in the US [1]. Unpublished modeling data indicate that maximum 24-h "fence line" concentrations from the largest methanol-emitting facilities in the US are predicted to be lower than 4 mg/m³ (3 ppm) [23]. Data reporting methanol vapor concentrations in excess of the OSHA 8-h time-weighted average permissible exposure limit of 200 ppm (260 mg/m³) or short term exposure limit of 250 ppm [21] are limited to case studies or anecdotal reports, and therefore provide no basis for estimating average or typical occupational exposure levels. However, an international review noted that instances of methanol concentration in thousands of ppm for various occupational settings and conditions have been reported [1].

US dietary survey data indicate that 99th percentile 14-day average intakes of methanol from aspartame use were as high as approximately 0.8–0.9 mg/kg bw per day for children of all ages, diabetics, and women of childbearing age [19]. Children from 0 to 5 years of age appear to have even higher intakes (based on 90th percentile data), but 99th percentile data for these ages were not reported. For the entire general population of aspartame users, the 99th percentile intake of methanol was approximately 0.6 mg/kg bw per day. Comparable data are not available for the additive DMDC, except for an unpublished and unreviewed FDA analysis [20]. This FDA analysis concluded that 90th percentile methanol exposure from natural sources in fruit juice and wine, along with DMDC use in beverages, would be approximately 1 mg/kg bw per day. Data on the occur-

rence of methanol in drinking water are limited. At present, it is not possible to estimate 99th percentile methanol intake from all dietary sources based on the limited information currently available to the Panel.

Dermal exposure to methanol can result in significant, even lethal, exposures under some conditions [1]. Although dermal contact with methanol can be anticipated among the general public as well as occupational groups, population exposures to methanol by the dermal route have not been described quantitatively.

Thousands of incidents of methanol poisoning are reported to poison control centers every year [9]. These incidents frequently involve young children who ingest methanol in consumer products. Many more incidents of accidental ingestion of gasoline are reported annually, which suggests that the addition or substitution of methanol to gasoline could result in greater potential for accidental methanol exposures.

The distribution of total daily population exposures to methanol has not been characterized. Although air concentrations and dietary levels of methanol have sometimes been reported as “typical” or presented in ranges from low to high, such data generally do not provide an adequate basis for judging the overall distribution of exposures, especially in the upper tail of the distribution. Even when distributional data are available, e.g. dietary methanol exposures based on a menu census survey of a probabilistic sample, these data have not reflected total exposure from all sources. An adequate characterization of the population distribution of total daily exposures to methanol is needed in order to judge the potential public health implications of methanol. Blood methanol levels are a useful biomarker of exposure (discussed in Section 2.1.1), but population data on blood methanol levels are limited.

The data on dietary exposure to methanol are judged limited at present. Although information is available on the distribution of population exposures to methanol from dietary sources (e.g. aspartame, fruits, vegetables, fermented spirits), data on the potential contribution from other additives (i.e. DMDC) or other sources (e.g. drinking water)

were scant. Federal Register notices on final rules permitting specific uses of DMDC, specifically cited that methanol exposure was a factor considered in assessing safety of the permitted uses. The Expert Panel did not review the scientific data that underpin the FDA conclusions.

2. General toxicology and biological effects

2.1. Toxicokinetics and metabolism

The majority of information in this section was obtained from reviews. Because quality reviews have already been conducted, CERHR is basing the toxicokinetics evaluation on those reviews instead of starting de novo. There were some cases where the primary paper was reviewed, for example more recent and key papers. The primary reviews utilized in this section were IPCS [1] and Kavet and Nauss [2]. The Kavet and Nauss paper is, in the main, the published version of an HEI [3] report. Because the Kavet and Nauss paper is more readily available to the public, it is being cited.

2.1.1. Absorption

2.1.1.1. Humans. Methanol is rapidly absorbed following inhalation, ingestion, and dermal contact, and the absorption capabilities do not appear to differ substantially across mammalian species [1]. Several recent studies have measured background blood methanol levels in humans and those values are summarized in Table 7. A mean pre-exposure blood methanol level of 0.6 mg/l was observed in a study of 12 healthy males after 12 h on a restricted diet (no alcohol, diet foods or drinks, fruit or fruit juices, and coffee) [31]; Chuwers et al. [32] reported background serum methanol levels in 26 volunteers after 24 h on a restricted diet (no coffee, vegetables, fruit, alcohol, or aspartame) to be 1.8 ± 2.6 mg/l (mean \pm standard deviation). Lee et al. [33] reported mean endogenous blood methanol levels of 1.82–1.93 mg/l in five subjects who were allowed to eat a breakfast consisting of non-aspartame containing cereal and no fruit juices. In

Table 7
Background blood methanol and formate levels in humans

Subjects	Methanol mean \pm S.D. (mg/l) (range in mg/l)	Formate mean \pm S.D. in mg/l (range in mg/l)	Reference
Twelve males on restricted diet (no methanol containing or methanol producing foods) for 12 h	0.570 \pm 0.305 (0.25–1.4)	3.8 \pm 1.1 (2.2–6.6)	[31]
Twenty-two adults on restricted diet (no methanol-containing or methanol-producing foods) for 24 h	1.8 \pm 2.6 (no range data)	11.2 \pm 9.1 (no range data)	[32], [40]
Three males who ate a breakfast with no aspartame-containing cereals and no juice	1.82 \pm 1.21 (0.57–3.57)	9.08 \pm 1.26 (7.31–10.57)	[33]
Five males who ate a breakfast with no aspartame-containing cereals and no juice. (second experiment)	1.93 \pm 0.93 (0.54–3.15)	8.78 \pm 1.82 (5.36–10.83)	[33]
Adults who drank no alcohol for 24 h	1.8 \pm 0.7 (no range data)	No data	[34]
Twelve adults who drank no alcohol for 24 h	1.7 \pm 0.9 (0.4–4.7)	No data	[35]
Thirty fasted adults	<4 (no range data)	19.1 (no range data)	[11]
Twenty-four fasted infants	<3.5 (no range data)	No data	[37]

studies where alcohol intake was restricted in subjects for 24 h, Batterman et al. [34], Batterman and Franzblau [35], and Franzblau et al. [36] reported mean background methanol blood levels of 1.7–2.6 mg/l. The Panel notes that widely cited studies by Stegink et al. [11,37] used an analytical method for methanol in blood with limits of detection of 4.0 and 3.5 mg/l, respectively. Those detection limits are approximately 10-fold greater than methods used in studies over the last 15 years.

Oral exposure. A study monitored the blood disposition of methanol in fasted human adults given 34, 100, 150, or 200 mg/kg aspartame in 300 ml orange juice [11]. The size of the lowest dose group was six males and six females, while that of each of the other groups was three males and three females. In the 34 mg/kg group, the blood methanol concentrations were below the detection limit (4.0 mg/l) in all subjects. At doses of 100 mg/kg aspartame and higher, dose-related increases in blood methanol and urinary formate were observed. No significant increases in levels of blood formate were seen at the highest dose. Mean peak blood methanol concentrations (\pm standard error) were 12.7 ± 4.8 , 21.4 ± 3.5 , and 25.8 ± 7.8 mg/l at 100, 150, and 200 mg/kg aspartame, respectively, and were achieved at 1–2 h post-exposure. The area under the blood methanol concentration–time curve (indicative of cumulative methanol exposure) increased proportionally to aspartame dose (4.19 ± 1.12 , 8.71 ± 1.41 , and 9.51 ± 1.69 units, respectively). Eight hours after dosing, blood methanol levels returned to pre-exposure levels in the 100 mg/kg group. Twenty-four hours after dosing, levels returned to pre-exposure levels in all groups. In the 200 mg/kg group, urinary formate excretion was significantly increased up to 8 h post-exposure (34 ± 22 , 101 ± 30 , 81 ± 22 , and 38 ± 12 μ g/mg creatinine in pre-exposure, 0–4, 4–8, and 8–24 h post-exposure samples, respectively). No significant effects on blood chemistry parameters were observed.

Strengths/weaknesses: This is a carefully conducted study with proper controls, adequate number of subjects ($n = 30$), and attention paid to dietary factors. The limit of detection for blood methanol was ten-fold greater than for methods used in more recent studies. As a result, the time course of blood serum values at the lowest dose tested (an aspartame dose equivalent to 3.4 mg/kg methanol) is limited.

Utility (adequacy) for CERHR evaluation process: This aspartame study demonstrates that blood methanol concentrations increased in a dose-related manner, and that there was no increase in blood formate, even at the highest challenge dose equivalent to a methanol exposure of 20 mg/kg. This study will be useful in the evaluation of methanol.

A study in 24 one-year-old infants [37] measured blood methanol concentrations after oral exposure to aspartame. In a series of studies, 10 infants were exposed to 34 mg/kg aspartame (the estimated pre-marketing 99th percentile of adult daily ingestion), 6 infants were exposed to 50 mg/kg (termed a very high dose), and 8 infants received 100 mg/kg (described as an “abusive” dose). Methanol is a hydrolytic

metabolite of aspartame accounting for 10% of aspartame consumed. Thus, these authors estimated the aspartame doses studied to be equivalent to ingestion of 3.4, 5, and 10 mg/kg bw methanol. Aspartame was administered via a cherry-flavored beverage. A fasting blood sample and three subsequent samples were obtained from each subject. The authors observed a positive correlation between aspartame dose and blood methanol level in the infants that was similar to that observed in a previous study of similar design and dose in adults [11]. Mean blood methanol levels were at the limit of detection (3.5 mg/l) in infants administered 34 mg/kg aspartame. Infants administered aspartame at 50 mg/kg had peak blood methanol values of 3.0 ± 1.0 mg/l 30–90 min after aspartame dosing. These values were essentially the same as those seen in adults, 3.4 ± 1.2 mg/l, receiving an equivalent dose. The eight infants administered the 100 mg/kg aspartame dose had a peak mean blood methanol value of 10.2 ± 2.8 mg/l 90 min post dosing. In comparison, the mean blood methanol concentrations in six adults administered an equivalent dose of aspartame was 12.7 ± 2.0 mg/l 60 min after dosing. While the responses in infants and adults at this dose were similar, the serum levels peaked earlier in adults and appeared to persist longer when one compared the area-under-the-curve throughout a 2.5-h sampling period.

Strengths/weaknesses: A strength is the total number of subjects tested ($n = 24$) and an ability to compare these results with adult values that used similar dosing and experimental methods. A weakness is the lack of raw data; one has to obtain blood methanol levels from the figures. Further, the analytical detection limit in this study is ten-fold less sensitive than methods used by many other authors, which prevents critical comparison of response of infant and adult at the lowest doses tested.

Utility (adequacy) for CERHR evaluation process: The Stegink et al. [37] study provides a useful comparison of blood methanol levels in 1-year-old infants and adults. Blood levels observed following high doses were not significantly different from those in adults receiving similar doses indicating that aspartame is metabolized to methanol in a similar manner.

Table 8 includes blood levels of methanol and formate as measured by Stegink et al. [11,37].

Leon et al. [38] monitored the general health of 53 adults (23 males and 30 females) who received an oral dose of 75 mg/kg bw per day aspartame (divided into three doses) for 24 weeks. No differences in health parameters were reported between this group and a group of 55 adults (28 males and 27 females) that received a placebo; both groups were examined every 3 weeks during the study. Blood and formate levels were measured at baseline (within 1 week of study initiation) and then every 6 weeks. Serum folate levels were measured at baseline and at week 24. Blood methanol levels were below the detection limit (0.31 mmol/l = 9.9 mg/l) for most subjects in both groups. There was no significant difference between the aspartame and placebo groups in the

Table 8
Human blood methanol and formate levels reported following methanol exposure

Subjects; type of sample collected ^{a,b}	Exposure route	Exposure duration or condition	Methanol exposure concentration	Blood methanol level (mg/l)	Blood formate level (mg/l)	Reference
Males; post exposure samples	Inhalation	75 min	0	0.570	3.8	[31]
			191 ppm	1.881	3.6	
Males and females; post exposure serum levels	Inhalation	4 h	0	1.8	11.2	[40]
			200 ppm	6.5	14.3	
Males without exercise; post exposure blood methanol and plasma formate	Inhalation	6 h	0	1.82	9.08	[33]
			200 ppm	6.97	8.70	
Males with exercise; post exposure blood methanol and plasma formate	Inhalation	6 h	0	1.93	8.78	[33]
			200 ppm	8.13	9.52	
Females; post exposure samples	Inhalation	8 h	0	1.8	No data	[34]
			800 ppm	30.7		
Adult males and females administered aspartame; peak methanol level and range of formate levels up to 24 h after dosing	Oral	One dose in juice	0		19.1	[11]
			3.4 mg/kg bw ^c	<4	No data	
			10 mg/kg bw ^c	12.7	No data	
			15 mg/kg bw ^c	21.4	No data	
Infants administered aspartame; peak exposure level	Oral	One dose in beverage	0	0	No data	[37]
			3.4 mg/kg bw ^c	<3.5		
			5 mg/kg bw ^c	3.0		
			10 mg/kg bw ^c	10.2		
Adult males administered aspartame; range of peak serum methanol levels in all subjects	Oral	1 dose in water	0	1.4–2.6	No data	[39]
			0.6–0.87 mg/kg bw ^c	2.4–3.6		

^a Unless otherwise specified, it is assumed that whole blood was used for measurements.

^b Information about dietary restrictions is included in Table 7.2-A.

^c Methanol doses resulting from intake of aspartame.

number of individuals with blood methanol levels above the detection limit at each examination period. The highest individual blood methanol levels were 1.0 and 0.84 mmol/kg (32 and 27 mg/kg bw) in the aspartame and placebo group, respectively. There was no significant increase in blood formate level in the aspartame group. No significant changes in mean serum folate levels were observed between groups or within groups when baseline levels were compared to those at week 24. (*Neither the blood formate nor serum folate values were reported*). Twenty-four hour, creatinine-adjusted urine formate values were measured at baseline and weeks 6, 12, and 24. The authors reported no statistically significant differences in urinary formate levels between groups or within groups over the time courses of the study.

Strengths/weaknesses: The study was adequately designed with use of randomized double-blinding, placebo control, and parallel groups. Therefore, the Panel is confident that blood methanol levels are representative of a healthy adult male and female population. Weaknesses of the study include an insensitive detection limit for methanol and no reporting of specific blood methanol, blood formate, or serum folate values. Blood methanol data are only portrayed in a histogram as percent of samples that were above limits of detection.

Utility (adequacy) for CERHR evaluation process: The study has utility in demonstrating no consistent elevation in blood methanol levels above 10 mg/l in adult humans

ingesting aspartame for 24 weeks at a level equating to a methanol dose of 7.5 mg/kg bw per day.

Davoli et al. [39] also administered aspartame to humans and measured methanol levels in blood with a method that results in a lower detection limit (0.012 mg/l). Four healthy adult males fasted for 8 h, drank no alcoholic beverages for 24 h, and consumed no fruit juices or fruits or vegetables for 18 h prior to the study. Blood methanol levels were measured by gas chromatography prior to exposure and at 0, 30, 45, 60, 90, 120, and 180 min following ingestion of 500 mg aspartame in 100 ml tap water. According to the authors, that dose of aspartame is equivalent to 6–8.7 mg/kg bw for a 58–80 kg person and is within the range of average daily intake for aspartame if it replaced all sucrose in the diet. Blood methanol in the subjects prior to exposure was 1.4–2.6 mg/l. Following aspartame administration, blood methanol levels were significantly increased at 30, 45, and 90 min. The peak exposure occurred at 45 min post-exposure, with a mean incremental increase of just below 1.0 mg/l. Methanol levels dropped at 1 h, rose at 90 min, and then consistently declined through the remainder of the experiment. The authors noted that the incremental increase of methanol was within the same order of magnitude for variations in endogenous methanol levels. They also stated that when aspartame is divided into a number of small doses, the incremental increase in methanol levels would not be detectable or significant.

Strengths/weaknesses: The strengths of the Davoli et al. [39] study are that it describes a sensitive method for methanol detection and demonstrates that increases in serum methanol can be detected following administration of aspartame at a dose estimated by FDA to be equivalent to the daily intake of all sugar in the diet, if administered at one time. Weaknesses of the study are the small number of subjects ($n = 4$) and administration of only a single dose level.

Utility (adequacy) for CERHR evaluation process: Davoli et al. [39] is important because it demonstrates that aspartame consumption by adults at a dose equivalent to the daily intake of sugar results in methanol levels similar to endogenous levels. Further, the authors speculate that, unless administered as a single bolus, this dose would not significantly raise the level of methanol in blood.

Inhalation. Experiments in which human volunteers were exposed to moderate levels of methanol vapor have occasionally demonstrated increases in blood and urine methanol concentration. However, as is seen with oral exposure to methanol, levels of plasma formate are not increased following inhalation exposure to approximately 200 ppm methanol. Methanol blood levels obtained during various exposure scenarios are outlined in Table 8.

In a pilot study designed to assess neurobehavioral effects, 12 male volunteers were exposed in a chamber to 250 mg/m³ (191 ppm) methanol for 75 min [31]. A more complete summary of the study is found in Section 2.2.1. Following methanol exposure, subjects exhibited no change in plasma formate concentration, which remained at a mean of 0.08 mmol/l (3.8 mg/l). These same subjects exhibited increases in mean plasma and urine methanol concentrations of about 3.3- and 2.5-fold, respectively.

Strengths/weaknesses: The Cook et al. [31] study was a rigorously controlled double blind study that used dietary controls, up-to-date carefully validated methods for measuring blood methanol and formate levels, and appropriate QA/QC and statistical procedures. The exposure dose is most relevant to occupational exposure, as the dose studied was the current threshold limit value (TLV). The report was well documented. The number of subjects is adequate to note statistically significant differences if they exist. The Panel has a great deal of confidence in the quality and accuracy of the data.

This was a pilot study with a primary objective of exploring possible neurobehavioral effects. It utilized a single exposure dose of methanol and a single exposure period, which was relatively short (75 min). Therefore, it was not possible to construct dose–response information. In addition, kinetic studies were not done.

Utility (adequacy) for CERHR evaluation process: The Cook et al. [31] study provides very useful information on blood and urinary levels of methanol and formate in human subjects before and after a 75-min exposure to either 250 mg/m³ of methanol vapors or filtered air. Given the limited information available on human exposures to methanol

and the quality of this study, the blood methanol and formate data are useful to the Panel. Pre-exposure levels of methanol in blood are given as approximately 0.6 mg/l. This work demonstrates that when humans are exposed to TLV levels of methanol, formate does not accumulate above background levels in blood.

Osterloh et al. [40] and Chuwers et al. [32] reported the methanol concentrations in a randomized, double-blind study of the potential neurobehavioral effects of methanol on a group of 26 volunteers (15 male, 11 female) exposed to 200 ppm (262 mg/m³) methanol for 4 h in an exposure chamber. This study is described in Section 2.2.1 under Chuwers et al. [32]. Each subject was exposed twice: once to methanol and once to water vapor. In each instance, blood samples were collected before exposure, every 15 min for the first hour, every 30 min for the next 3 h, and every hour for 4 h post-exposure. Urine samples were collected before exposure (hour 0), at the end of exposure (hour 4), and 4 h after the end of exposure (hour 8). Outlier analysis resulted in the removal of four subjects from the final results, due to the removal of four or more data time points; thus, the results were presented for 22 subjects.

Pre-exposure serum values for the water vapor (control) and methanol phases of the study were 1.0 ± 0.6 and 1.8 ± 2.6 mg/l, respectively. Peak methanol concentration in blood serum (6.5 ± 2.7 mg/l) occurred at the end of the 4-h exposure, then declined during the 4-h post-exposure period, although not to pre-exposure levels. All levels measured at various exposure and post-exposure times were significantly increased (by at least four times at the peak levels) compared to controls. Serum and urine formate levels were not significantly increased at any point during exposure or post-exposure (pre-exposure serum formate values for control and methanol phases of the study were 10.3 ± 5.5 and 11.2 ± 9.1 mg/l, respectively). Serum methanol concentrations from hour 0 to 8 were adequately described by either a biphasic linear or logarithmic function. No covariance of methanol concentrations with age, sex, weight, or folate level was seen.

Strengths/weaknesses: This is a well designed and reported study with appropriate controls. Strengths of the study include: appropriate dietary restriction; large number of subjects ($n = 26$); up-to-date procedures for measuring methanol and formate; and multiple sampling times.

Only one dose of methanol was used, therefore no dose–response can be calculated. However, the authors did report some kinetic data. Under these exposure conditions, 200 ppm for 4 h, serum and urinary formate levels did not increase.

Utility (adequacy) for CERHR evaluation process: This study is highly useful because it provides reliable information on serum and urinary methanol and formate levels following a well-controlled exposure to 200 ppm methanol vapor for 4 h.

In an experiment by Lee et al. [33], six male volunteers (29–55 years old) were exposed to 200 ppm (262 mg/m³)

methanol vapor in a chamber for 6 h. During this period, subjects were either at rest or under physical exercise (six alternating 20-min periods on a stationary bicycle followed by a 20-min period of rest). This exercise was calculated to increase respiratory rate such that methanol inhalation was increased 1.8 times. Blood was collected pre-exposure and post-exposure, and methanol levels were measured using an analytical method with a detection limit of 0.4 mg/l. On each day of the experiment, subjects could eat cereal with no aspartame, but could not drink fruit juice. Five pre-exposure blood methanol concentrations were given for three subjects. The mean and S.D. were 1.82 ± 1.21 mg/l; the range was 0.57–3.57 mg/l. After a 6-h exposure at rest, blood methanol levels had increased from a mean of 1.82 to 6.97 mg/l; after a 6-h exposure with exercise, blood methanol levels had increased from a mean of 1.9 to 8.1 mg/l. When mean blood methanol concentration of the exercise group was compared with that of the at-rest group, no statistically significant difference was seen, even though pulmonary ventilation had increased 1.8 times (10.5–18.6 l/min). While blood methanol levels had increased, no statistically significant differences in pre- or post-exposure blood formate concentrations were seen in volunteers exposed to methanol vapor under either a resting or exercise regimen. Pre-exposure mean blood formate levels were 9.08 ± 1.26 mg/l, the post-exposure mean level was 8.70 mg/l in the group at rest; with exercise, the mean blood formate level was 8.78 mg/l pre-exposure versus 9.52 mg/l post-exposure.

Strengths/weaknesses: The strengths and weaknesses of the Lee et al. [33] study are similar to those discussed above for Cook et al. [31]. There were fewer subjects in this study ($n = 6$), but the exposure period was longer (6 h). The study did indicate that 6-h exposure to 200 ppm methanol elevated blood methanol levels approximately three- to four-fold without any accompanying increase in blood formate.

Utility (adequacy) for CERHR evaluation process: The study is a useful source of data on background blood methanol and formate levels and also provides data on blood and formate levels after exposures relevant to the workplace, i.e., 6-h exposure at 200 ppm, the current TLV.

Batterman et al. [34] conducted studies to determine the relationship between methanol concentrations in blood, urine, and breath in volunteers exposed to methanol vapors. There were two groups studied. The core group consisted of four female volunteers (ages 41–60 years) exposed to 800 ppm (1048 mg/m³) methanol for 30, 60, and 120 min (two replicates for each, plus a third replicate for 120 min) in an exposure chamber. Total number of exposure sessions were 25 (4 subjects \times 3 durations \times 2 replicates + 1 with a third exposure). The second group consisted of three additional females and 12 males who were exposed to 800 ppm methanol during 8-h sessions and 12 control sessions. Periodic breath, blood, and urine samples were collected. No volunteers had occupational or avocational exposure to methanol. Baseline or endogenous concentrations of methanol in blood averaged 1.8 ± 0.7 mg/l. The half-life of

methanol in blood was determined from the 30- and 120-min exposures to be 1.44 ± 0.33 h. Breath and urine data were also used to estimate half-life, compensating for mucous membrane desorption and voiding time. Results were similar to blood but more variable results were obtained. Data adequately fit a first-order model, with the exception of post-exposure times of 0, 15, and 30 min. The first-order model and the estimated half-life suggested that methanol concentrations in blood do not increase linearly with exposure duration, but asymptotically approach steady-state level. Breath data were fit better with a three-compartment (fast and slow desorption from mucous membranes and end-expired or alveolar air) than a two-compartment model.

Strengths/weaknesses: The strengths of the Batterman et al. [34] study are the well-controlled exposures and sampling procedures. The use of multiple exposure times and the comparative information on blood, urine, and breath methanol are also positive features. There are some weaknesses in the study design. It appears that different subjects were used for the first set of exposures (0–120 min) and the second set (8 h). Alcoholic beverages were restricted 24 h prior to testing but there were no other dietary restrictions. The inhalation exposure dose (800 ppm) greatly exceeded the TLV and is unlikely to be encountered.

Utility (adequacy) for CERHR evaluation process: Despite some limitations, the Batterman et al. [34] study provides useful information on blood, breath, and urine methanol levels under very high exposure conditions. Useful kinetic data—again under these exposure conditions—were also provided.

Franzblau et al. [36] conducted a study to determine if methanol in breath is a useful indicator of blood levels following oral or dermal exposure. Study volunteers were instructed to abstain from alcohol intake for 24 h prior to and during the experiment and were determined to have no occupational or avocational exposure to methanol, formic acid, or formaldehyde. In the inhalation portion of the experiment, mean pre-exposure blood and breath methanol concentrations were measured at 2.65 mg/l and 1.3 ppm, respectively, in four subjects (three males and one female, age 31–55 years). Each subject was exposed to 0, 100, 200, 400, and 800 ppm methanol vapors [**purity not specified**] for 8 h, twice while at rest or exercising. Methanol concentrations inside chambers were monitored by an infrared analyzer. Following 6 and 8 h of exposure, four blood and breath samples were taken at 5-min intervals. Results were only reported for the 400 ppm exposure concentration under sedentary conditions; the pattern of results was reported to be similar with the other methanol concentrations with or without exercise. Blood and breath levels of methanol were significantly increased at 6 and 8 h. Peak blood levels were 11.1 and 13.4 mg/l at each respective time period. Breath concentrations were highest immediately after the 6- and 8-h exposure (71.7 and 76.9 ppm, respectively), but rapidly declined within 15 min of breathing clean air (3.5

and 3.3 ppm). The authors suggested that the initial high concentration of breath methanol reflected absorption and desorption of methanol from airways. Therefore, the authors concluded that methanol breath levels would be useful for estimating blood concentrations only after 10–15 min of breathing clean air because that is the time needed for desorption of methanol from airways.

Volunteers in the dermal exposure portion of the experiment by Franzblau et al. [36] consisted of the four subjects who participated in the inhalation study and four additional male subjects (age 26–33 years). Mean pre-exposure blood and breath methanol levels were measured at 1.2 mg/l and 0.2 ppm, respectively. One hand from each volunteer was placed in a beaker containing neat methanol (99.8% purity) for time periods of 0, 2, 4, 8, and 16 min. Blood and breath methanol samples were taken immediately after exposure and at 12 additional time points for 8 h following exposure. Results were reported only for the 16-min exposure; the authors reported that similar temporal patterns were observed for the shorter exposure durations. Blood and breath methanol concentrations peaked at about 45 and 15 min following exposure and were measured at 11.3 mg/l and 9.3 ppm, respectively. Authors noted that exposure to one hand (<3% of body surface area) for 16 min resulted in a blood methanol concentration that is about equal to that achieved by breathing 400 ppm methanol vapors for 8 h. It was speculated by study authors that the rapid rise in breath, compared to blood methanol levels, occurs because methanol is first transported to the central circulation and lungs prior to becoming equally distributed throughout all body water. The study authors estimated that following a dermal exposure, 2 h would need to pass before methanol blood concentrations could be estimated from breath levels.

Strengths/weaknesses: The study design attempted to control for methanol exposure from alcohol consumption but not from diet. Only some data are presented; the rest are only verbally summarized.

Utility (adequacy) for CERHR evaluation process: This study provides another source of background blood methanol levels in a limited number of healthy adults. It also identifies magnitude of increase in blood methanol levels after specific periods of either dermal or inhalation exposure to methanol. The study provides data on the period of time that must elapse post-exposure for breath to serve as a reliable indicator of blood methanol concentrations, i.e., “washout” from airways.

Heinrich and Angerer [41] examined blood and urinary levels of methanol in workers at a pesticide manufacturing plant, but was excluded by the Panel from this document due to errors in the reporting of concentration units.

Inhalation studies with humans have shown a net absorption of methanol of 60–85% [1]. In a group of 22 volunteers exposed to 200 ppm (262 mg/m³) methanol for 4 h, the mean apparent absorption half-life was 0.80 ± 0.55 h [40]. Lung retention of inhaled methanol does not vary significantly with exposure concentration or ventilation rate.

Table 9
Mean percent lung retention of inspired methanol in human male subjects, Sedivec et al. [42]

Methanol concentration in air (mg/m ³)	Experimental subject				
	1	2	3	4	5
103	56.4	–	54.4	61.2	60.7
194	56.6	–	53.4	60.5	59.6
195	56.7	56.2	57.6	60.5	–
205	54.2	–	55.0	60.6	60.4
284	56.4	57.0	54.0	61.3	–
Mean resting ventilation rate (l/min)	10.3	9.7	10.9	11.2	10.4

Five healthy men, exposed for 8 h to methanol concentrations of 103–284 mg/m³, had mean ventilation rates of 9.7–11.2 l/min; lung retention, as determined from methanol concentration in inspired and expired air, ranged from 53.4 to 61.3% (Table 9) [42]. During exercise, the ventilation rate of the subjects increased by 2.5-fold, but the lung retention of methanol did not change significantly.

Dermal exposure. Methanol is readily absorbed through the skin. Upon direct skin contact with pure methanol, absorption is rapid, and cases of methanol poisoning in children exposed dermally have been reported [43].

Dutkiewicz et al. [44] compared the amount of unchanged methanol excreted after administration of identical doses through skin or by mouth. Six human volunteers were exposed dermally to methanol by attaching a flat glass applicator containing methanol onto an 11.2 cm² surface area of the forearm. Absorption periods of 15–60 min were used. The absorbed dose was calculated from the amount applied to the skin and the amount of methanol recovered from the skin after the exposure period. Methanol levels in urine (every hour for 8 h) and exhaled air (every 30 min until hour 2.5, then at hours 4 and 5) were also measured after a 20-min immersion of the hand (435–445 cm² surface area) in methanol. Three subjects were also given oral doses of methanol (1.67 g); urine and exhaled air samples were then taken. The authors estimated that immersion of one hand in liquid methanol for 2 min would result in a body burden of up to 170 mg, which is similar to that resulting from inhaling approximately 40 ppm methanol for 8 h. The mean calculated absorption rate of methanol through human skin resulting from 22 experiments in six subjects was 0.192 mg/cm²/min. The absorption rate peaked at 30 min post-exposure. Excretion also peaked at 30 min post-exposure in the oral and hand immersion experiments.

Strengths/weaknesses: This is an older (1980) study and the analytical methodology procedures are only briefly described. There was no direct measure of methanol absorbed, i.e., concentration in blood.

Utility (adequacy) for CERHR evaluation process: This study demonstrates the importance of the dermal route of exposure. There is limited confidence in the absolute values presented.

Batterman and Franzblau [35] reported on a study of dermal exposure to neat methanol in human volunteers. Seven men (ages 22–54) and five women (ages 41–63) were the study subjects for a total of 65 sessions and had no occupational or avocational exposure to methanol, formic acid, or formaldehyde. All refrained from alcohol consumption during the 24-h period prior to a session. Two males were smokers. Methanol exposure occurred by immersing one hand for 0–16 min in a vessel containing neat methanol. Exposure sessions for each volunteer were spaced at least 1 week apart. Blood samples were taken 10 and 15 min prior to exposure and at 0, 15, 30, and 45 min, and 1, 1.5, 2, 3, 4, 5, 6, and 7 h following the exposure. A two-compartment model was used to derive absorption rates and delivery kinetics. The mean background concentration of methanol in blood for all subjects was 1.7 ± 0.9 mg/l. The authors noted that average baseline values among the 12 subjects differed significantly and means ranged from 0.9 to 2.9 mg/l. The average baseline for females (2.4 ± 0.8 mg/l) was significantly higher than that for males (1.3 ± 0.8 mg/l). Methanol delivery into the blood began during or immediately after exposure and reached a maximum rate 1/2 h after the exposure. The area-under-the-curve (AUC) correlated highly with duration of exposure and blood concentration maximums. The average derived dermal absorption rate was 8.1 ± 3.7 mg/cm²/h. The authors noted that their absorption rates (from hands) were similar to those reported by Dutkiewicz et al. [44] for forearms. They further noted that these in vivo derived data were at least six times greater than those derived from in vitro results.

According to Batterman and Franzblau [35], EPA's 1992 guidance on dermal exposure assessment recommends using a methanol absorption rate of 1.27 mg/cm²/h. However, this rate was six times less than that derived in vivo in the current study (8.1 mg/cm²/h), and almost 10 times less than that measured in vivo by Dutkiewicz et al. [44] (11.7 mg/cm²/h).

Strengths/weaknesses: This is a well conducted study with good methodology, data were thoroughly presented, and appropriate statistical analyses were performed. The study did not control for dietary sources of methanol exposure. They did, however, subtract individual background levels from data obtained.

Utility (adequacy) for CERHR evaluation process: These data provide a reliable estimate of dermal exposure. The similarity of results with the Dutkiewicz et al. [44] study provides a basis for greater confidence in the absorption estimate from that older study. The data also reveal the variability of background methanol blood values across time with individuals and between individuals. These values are also greater than those given as the endogenous or background levels for the general population.

2.1.1.2. Animals. Methanol blood levels have been measured under various exposure scenarios in monkeys, mice, and rats and are summarized in Tables 10–12, respectively.

Inhalation exposure. The major objective of the multi-experiment study reported by Pollack and Brouwer [45] was to determine the distribution of methanol in female Sprague–Dawley rats (Hilltop Laboratories) and Crl:CD-1 mice [ages not specified] at different stages of gestation. Baseline studies were performed on non-pregnant animals after exposure by the intravenous (IV) or oral routes (dose range 100–2500 mg/kg). The disposition of methanol was studied in pregnant rats on gestation days (gd) 7, 14, and 20 and in pregnant CD-1 mice on gd 9 and 18. Pesticide-grade methanol was used, which is 99.8% pure according to Tedia [46]. In these studies, exposure was by the oral, IV and inhalation routes (1000–20,000 ppm for 8 h). Saline was the vehicle for oral and IV exposure. Three to five animals were examined per dose and exposure condition. Methanol concentrations were measured in blood, urine, and amniotic fluid by gas chromatography (GC). Dose-dependent differences in kinetic parameters and influences of gestational stage were analyzed by analysis of variance (ANOVA). Differences in venous and arterial blood methanol concentrations were analyzed by paired Student's *t*-test. The authors developed major conclusions from their studies that are presented below.

- Methanol absorption is rapid and essentially complete following oral exposure.
- Over the methanol inhalation concentrations used in the study, decreasing absorption was seen in rats and mice.

Table 10
Monkey blood methanol and formate levels reported following methanol exposure

Species-sex	Exposure route	Exposure duration	Methanol exposure concentration	Blood methanol level in mg/l	Blood formate level (mg/l)	Reference
Cynomolgus monkey-female; mean blood methanol and range of plasma formate at 30 min post daily exposure during premating, mating, and pregnancy	Inhalation	2.5 h per day, 7 days per week during premating, mating, and gestation (~348 days)	0	2.4	8.7	Burbacher et al. [52]
			200 ppm	5	8.7	
			600 ppm	11	8.7	
			1800 ppm	35	10	
Rhesus monkey-male; post-exposure blood level at all doses	Inhalation	6 h	200 ppm	3.9	5.4–13.2	Horton et al. [53]
			1200 ppm	37.6		
			2000 ppm	64.4		

FR: Folate-reduced.

Table 11
 Mouse blood methanol and formate levels reported following methanol exposure

Strain	Exposure route	Exposure duration	Methanol exposure concentration	Blood methanol level (mg/l)	Blood formate level (mg/l)	Reference
CD-1 female; post-exposure plasma methanol and peak formate level	Inhalation	6 h on gd 8	10,000 ppm	2,080	28.5	[66]
			10,000 ppm + 4-MP	2,400	23	
			15,000 pp	7,140	34.5	
CD-1 mice-female; post-exposure blood methanol level	Inhalation	8 h	2500 ppm	1,883	No data	[62], [45]
			5000 ppm	3,580		
			10,000 ppm	6,028		
			15,000 ppm	11,165		
CD-1 female; mean post-exposure plasma methanol level	Inhalation	7 h per day on gd 6–15	0	1.6	No data	[96]
			1000 ppm	97		
			2000 ppm	537		
			5000 ppm	1,650		
			7500 ppm	3,178		
			15,000 ppm	7,330		
CD-1 female; plasma level 1 h post-dosing	Oral-Gavage	Gd 6–15	4000 mg/kg bw	3,856	No data	[96]
CD-1 female; peak plasma level	Oral-Gavage	Gd 8	1500 mg/kg bw	1,610	35	[66]
			1500 mg/kg bw +4-MP	1,450	43	

4-MP: 4-methylpyrazole.

Table 12
 Rat blood methanol and formate levels reported following methanol exposure

Strain-sex: type of sample collected	Exposure route	Exposure duration	Methanol exposure concentration	Blood methanol level (mg/l)	Blood formate level (mg/l)	Reference
Sprague–Dawley rat-female; post-exposure blood methanol level on 3 days	Inhalation	7 h per day for 19 days	5000 ppm	1000–2170	No data	[98]
			10,000 ppm	1840–2240		
			20,000 ppm	5250–8650		
Rat-female; post-exposure blood methanol level	Inhalation	8 h	1000 ppm	83	No data	[62], [45]
			5000 ppm	1047		
			10,000 ppm	1656		
			15,000 ppm	2667		
			20,000 ppm	3916		
Long-Evans–female; post-exposure plasma level on gd 7–12	Inhalation	7 h per day on gd 7–19	0	2.7–1.8	No data	[100]
			15,000 ppm	3826–3169		
Long-Evans–female; 1 h post-exposure blood level	Inhalation	6 h per day on gd 6–pnd 21	4500 ppm	555	No data	[95]
Long-Evans–pups; 1 h post-exposure blood level	Inhalation	6 h per day on pnd 1–21	4500 ppm	1260	No data	[95]
Fischer-344–male; post-exposure blood level	Inhalation	6 h	200 ppm	3.1	5.4–13.2 at all doses	[53]
			1200 ppm	26.6		
			2000 ppm	79.7		
			2000 ppm	79.7		
Long-Evans–male; post-exposure serum level	Inhalation	6 h	200 ppm	7.4	No data	[101]
			5000 ppm	680–873		
			10,000 ppm	1468		
Long-Evans–male; peak blood formate level	Inhalation	6 h	0 FS	No data	8.3	[50]
			0 FS		10.1	
			1200 ppm–FS		8.3	
			1200 ppm–FR		46	
			2000 ppm–FS		8.3	
			2000 ppm–FR		83	
			2000 ppm–FR		83	
Long-Evans–male; peak blood methanol and formate	Oral-Gavage	Single dose	3500 mg/kg bw–FS	4800	Baseline level	[50]
			3500 mg/kg bw–FP	4800	382	
			3500 mg/kg bw–FR	4800	860	
			3000 mg/kg bw per day–FS	No data	9.2	
			3000 mg/kg bw per day FR		718	
			2000 mg/kg bw per day FS		9.2	
			2000 mg/kg bw per day FR		538	

FS: Folate-sufficient; FR: folate-Reduced; FP: Folate Pared.

This is attributed to a decreased rate of breathing and a parallel lowering of absorption efficiency from the upper respiratory tract.

- Under the high exposure conditions used in the rodent studies, disposition is nonlinear in female rats and mice for all three routes of exposure. There are linear and nonlinear pathways for elimination of methanol; the relevant contribution of each pathway is concentration-dependent. The saturable nonlinear pathway seen at the 100 and 500 mg/kg doses involves metabolism of methanol to formaldehyde and then to formic acid. A parallel linear route for elimination of methanol was observed that accounted for an increasingly significant fraction of total elimination as systemic concentration increased. This pathway is characteristic of passive-diffusion and, at the highest dose (2500 mg/kg), accounted for nearly 90% of methanol elimination, with pulmonary and urinary clearance occurring in equal amounts.
- The rate of methanol accumulation in the mouse was two- to three-fold greater than that in the rat. This difference persisted notwithstanding the two-fold higher rate of elimination seen in the mouse. Plausible explanations put forth by the authors were the more rapid rate of respiration and more complete absorption in the nasal cavity in the mouse. They believe this may account for the greater sensitivity in this species to the teratogenic effects observed by others.
- Examining the bioavailability data as a whole, the authors concluded that systemic availability of orally administered methanol was similar in pregnant and non-pregnant animals. Minor changes in volume of distribution were noted, possibly related to re-compartmentalization of total body water as gestation progressed.
- Penetration of methanol from maternal blood to the fetal compartment appeared to be inversely proportional to maternal blood methanol concentration. The authors believe this is consistent with a possible decrease in blood flow to the fetal compartment.

Strengths/weaknesses: This was a well conducted study. Appropriate procedures were used to generate methanol, measure respiratory parameters, and analyze blood methanol concentrations. The QA/QC procedures were excellent. The grade of methanol used was reported and chamber concentrations were monitored. The investigators chose inhalation exposure levels to approximate those of previous animal studies in which teratogenic effects of methanol had been demonstrated; however, these levels are orders of magnitude higher than those experienced in occupational or ambient settings. This is the major weakness of the study.

The authors do not comment on the fact that the increased absorption observed in the mouse may have been due to the fact that, in addition to respiration rates, the mucus membranes in the nasal area are significantly thinner in mice than in rats. This fact is critical to any extrapolation of these data to humans. Decreased absorption with increasing respiration

rates and thickness of the nasal mucosa are consistent with the observation of Sedivec et al. [42], who reported the retention of inhaled methanol in humans to be 58%. Lastly, it was not reported if assignment to groups was random.

Utility (adequacy) for CERHR evaluation process: The results are very useful for comparing the two rodent species, but only for the high-level exposure conditions that were used. The results have not been validated for ambient exposure situations. Any interpretation of this study should include this limitation.

2.1.2. Distribution

Methanol distributes rapidly and uniformly to all organs and tissues in direct relation to their water content, with an overall volume of distribution of approximately 0.6l/kg [1,3].

2.1.3. Metabolism

An understanding of the metabolism of methanol is important since the toxic properties of acute methanol poisonings are associated with intermediate metabolites rather than with the alcohol per se. There is an extensive database on the metabolism of methanol with good reviews provided by IPCS [1], Kavet and Nauss [2], and Liesivuori and Savolainen [47]. The narrative in this document is drawn from these reviews. However, Panel members did review the primary sources cited in these reviews to ensure that key statements are consistent with the primary literature.

In mammals, methanol is metabolized in a series of oxidation steps to sequentially form formaldehyde, formate, and carbon dioxide. A schematic illustration of the overall metabolism of methanol in primates and rodents is represented in Fig. 2.

Methanol can be oxidized to formaldehyde through three different pathways within the liver, although two are of primary importance. In primates, alcohol dehydrogenase catalyzes the metabolism of methanol to formaldehyde, whereas in rodents, the catalase pathway performs this function. Despite this difference, this first metabolic step proceeds at similar rates in non-human primates and rats. Formaldehyde is rapidly oxidized (half-life ~1 min) to formic acid (formate + H⁺) and does not accumulate in animals or humans exposed to methanol. Formaldehyde dehydrogenase is found in liver, brain, and erythrocytes and catalyzes a reaction of formaldehyde with reduced glutathione to form S-formyl glutathione, which subsequently hydrolyzes in the presence of glutathione thiolase to formic acid and reduced glutathione. Formate is primarily oxidized to carbon dioxide and water in mammals through a tetrahydrofolate-dependent pathway that is presented in Fig. 3.

- (1) Formyl THF Synthetase
- (2) Formyl THF Dehydrogenase

Formate combines with tetrahydrofolate enzymatically to form 10-formyl tetrahydrofolate. Through another enzyme

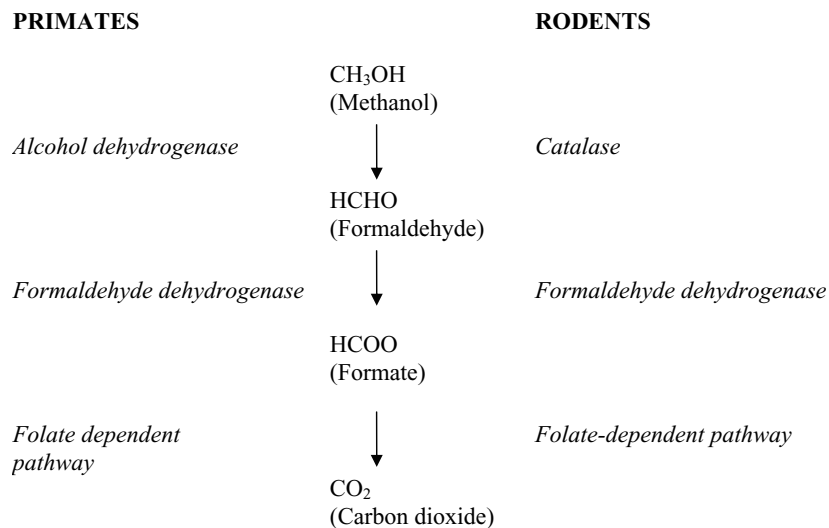


Fig. 2. Metabolic pathways and primary catalysts for methanol oxidation in primates and rodents.

reaction, 10-formyl tetrahydrofolate is oxidized to carbon dioxide and tetrahydrofolate. The availability of tetrahydrofolate, derived from folic acid in the diet, is the major determinant of the rate of formate metabolism. In primates, the folate-mediated oxidation of formate proceeds at one-half the rate observed in rats. The rate of formate oxidation in rats exceeds the maximal rate at which methanol is converted to formate: 1.6 versus 0.9 mmol/kg/h, respectively [2]. In contrast, when primates receive moderately high doses of methanol, the formation of formate can exceed the oxidation of formate: approximately 1.5 versus 0.75 mmol/kg/h, respectively. A calculated estimate of the methanol concentration that saturates the human folate pathway is 11 mM or 210 mg/kg [2]. There is substantial evidence that formic acid, which readily dissociates to formate and hydrogen ion, is the metabolite responsible for the visual and metabolic poisoning seen in primates. In studies where severely toxic or lethal doses were administered, the development of acidosis coincided with the accumulation of formic acid in blood with a parallel decrease of bicarbonate in plasma. In monkeys, it has been demonstrated that inhibition of tetrahydrofolate generation specifically affects formate oxidation, but not methanol disappearance. Decrease in the folate metabolic pool prolongs blood levels of formate by decreasing the rate at which formate combines with tetrahydrofolate. Tables 13 and 14

compare levels and activities of folate and folate enzymes in various species.

IPCS [1] stated that endogenous formate is generally present in human blood at levels of 0.07–0.4 mM (3.2–18.4 mg/l). These levels do not appear to be affected by methanol exposures within the range of those expected to be experienced by the general population (see Section 1). The background blood formate values from several recent studies are presented in Table 7. Values from selected methanol exposures are included in Table 8.

In a study of 12 men exposed in a chamber to 250 mg/m³ (191 ppm) methanol for 75 min, no increase in mean plasma formate concentration was observed (\approx 0.08 mM (3.8 mg/l) before and immediately after exposure), indicating that elimination pathways were not saturated [31]. In support of this hypothesis, mean urinary-methanol concentration increased from 0.9 mg/l pre-exposure to 2.2 mg/l immediately post-exposure, and remained at that level when measured 1 h later. Osterloh et al. [40] and Chuwers et al. [32] observed no significant increase in blood formate levels following inhalation exposure of 26 volunteers to 200 ppm methanol for 4 h (11.2 mg/l pre-exposure and 14.3 mg/l post-exposure). Urine formate levels were only slightly higher at 0–4 h post exposure compared to unexposed controls (2.2 mg/4 h versus 1.7 mg/4 h, respectively). Lee et al. [33] observed

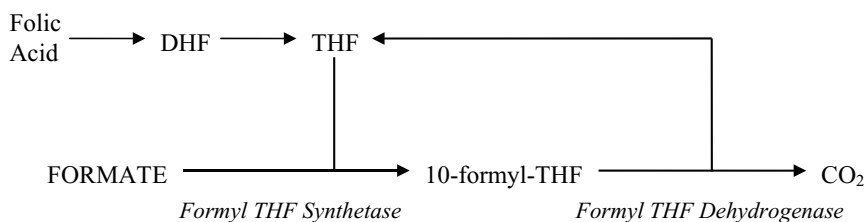


Fig. 3. Metabolism of formate through the folate pathway. IPCS [1].

Table 13

Mean levels of hepatic folate and folate co-enzymes (nmol/g liver \pm standard error (S.E.) in various species

	Species			
	Mouse	Rat	Human	Monkey
Formyltetrahydrofolate	6.4 \pm 0.6	4.6 \pm 1.3 5.0 \pm 1.2*	3.3 \pm 0.5	10.5 \pm 0.8*
Tetrahydrofolate	42.9 \pm 1.2	11.4 \pm 0.8 12.6 \pm 1.1*	6.5 \pm 0.3	7.4 \pm 0.8*
5-Methyltetrahydrofolate	11.6 \pm 0.4	9.3 \pm 0.6 9.4 \pm 1.5*	6.0 \pm 0.7	7.6 \pm 1.1*
Total folate	60.9 \pm 2.1	25.3 \pm 0.9 26.9 \pm 3.3*	15.8 \pm 0.8	25.5 \pm 1.2*

N = 4–7 subjects per group.

Data are from Johlin et al. [48] or *Black et al. [49].

no significant increase in blood formate levels following inhalation exposure of five subjects to 200 ppm methanol for 6 h; mean formate levels ranged from 8.7 to 9.52 mg/l both prior to and following exposure. In the inhalation studies, volunteers were subjected to various levels of dietary restriction that are discussed in Section 2.1.1.1. In an oral exposure study, Stegink et al. [11] noted that blood formate levels did not increase significantly in six adults administered 200 mg/kg bw aspartame (equivalent to 20 mg/kg methanol); mean blood formate levels were 19.1 mg/l prior to exposure and ranged from 8.4 to 22.8 mg/l during the 24-h period after exposure. However, urinary levels of formate were significantly increased from background levels (34 μ g/mg creatinine) at 0–4 h (101 μ g/mg creatinine) and 4–8 h (81 μ g/mg creatinine) after exposure, thus demonstrating metabolism of methanol to formate without saturation of metabolic capacity.

Studies in monkeys, mice, and rats have measured blood formate levels following various exposure scenarios and these values are listed in Tables 10–12, respectively.

A study by Lee et al. [50] illustrates the effects of folate deprivation on methanol disposition and toxicity in rats. Lee et al. [50] reported that controlled dietary folate permitted the development of a rodent model whose toxicological response to methanol mimicked that seen in primates. Groups of five 4-week-old male Crl:Long Evans rats were fed one of three diets for at least 18 weeks that the authors designated as folate-sufficient, folate-pared, or folate-reduced (a folate-pared diet with 1% succinylsulfathiazole added to inhibit endogenous production of formate by gut flora). Body weights were measured weekly and liver samples were periodically taken for folate analysis. The authors stated that the rate of bodyweight gain was similar across all three groups. No differences in bodyweight changes were seen. Liver folate levels increased with time in the folate-sufficient group, but decreased in the folate-pared group to a steady-state level, and declined to an even lower steady-state level in the folate-reduced group to 10–30% of the control level. After a single gavage dose of 3500 mg/kg methanol in water (purity not specified), blood methanol

Table 14

Mean activities of hepatic folate-dependent enzymes (nmol/min/mg protein \pm S.E.) in various species

	Species		
	Rat	Human	Monkey
10-Formyltetrahydrofolate synthetase	65.9 \pm 5.0 41 \pm 3*	75.0 \pm 8.7	142 \pm 16 184 \pm 14*
10-Formyltetrahydrofolate dehydrogenase	88.3 \pm 11.7 26.0 \pm 1.0*	23.0 \pm 2.2	33.0 \pm 4.0 52.6 \pm 2.3*
Serine hydroxymethyltransferase	10.8 \pm 0.6 9.4 \pm 1.1*	18.5 \pm 0.7	17.1 \pm 0.7*
Tetrahydrofolate reductase	19.8 \pm 1.3 20.3 \pm 2.2*	0.74 \pm 0.17	4.1 \pm 0.7*
5,10-Methylenetetrahydrofolate reductase	1.21 \pm 0.07 1.00 \pm 0.05*	0.42 \pm 0.07	0.22 \pm 0.02*
Methionine synthase	0.09 \pm 0.007 0.08 \pm 0.014*	0.10 \pm 0.008	0.09 \pm 0.012*

N = 3–9 subjects per group.

Data are from Johlin et al. [48] or *Black et al. [49].

and formate levels were measured by gas chromatography (GC) in five rats per group. It appears that the dose was selected based on doses in monkey studies by McMartin et al. [51]. Statistical significance between experimental groups was evaluated by the Dunnett's *t*-test. A peak blood level of about 150 mmol/l (4800 mg/l) methanol was seen in all groups, followed by a similar pattern of decline over 48 h. Blood formate profiles differed significantly, however, with no accumulation in the folate-sufficient group, accumulation in the folate-paired group (8.3 mmol/l (382 mg/l) after 48 h), and even greater accumulation in the folate-reduced group (18.7 mmol/l (860 mg/l) after 48 h). Following a gavage dose of either 3000 or 2000 mg/kg methanol, a dose-related increase in blood formate was seen in folate-reduced, but not in folate-sufficient rats, to 9.2 mmol/l (423 mg/l) at 24 h, and 15.6 mmol/l (718 mg/l) at 48 h. The authors compared their results with published results in monkeys, in which oral exposure to 3000 mg/kg produced a peak blood formate concentration of 7.4 mmol/l (340 mg/l) after 12 h [51]. Oral exposure to 2000 mg/kg methanol produced a peak blood formate level at 24 h post-exposure of 6.5 mmol/l (299 mg/l) and 8.1 mmol/l (373 mg/l) in the monkey and folate-reduced rats, respectively. Formate level returned to normal by 48 h post-exposure in the monkey, whereas the level in folate-reduced rats was 11.7 mmol/l (538 mg/l) at 48 h, and at normal level at 72 h. Folate reduction increased sensitivity to methanol as noted by death in 8/11 folate-reduced rats after 4 days of exposure to 3000 ppm for 20 h per day; there were no deaths in folate-sufficient rats after 14 days of exposure. The study authors concluded that rats on their folate-reduced diet regimen were more sensitive than monkeys to methanol poisoning because they accumulated more formate than did monkeys at an equivalent dose.

Strengths/weaknesses: The strengths of this study were the development of a rodent model that would be useful for studying methanol toxicity and the fact that a variety of inhalation and oral exposure scenarios were used. Another strength of this study was that chamber concentrations of methanol were monitored. A weakness of this study is that the purity of methanol was not reported. It was not stated if animals were randomly assigned to exposure groups. Comparisons between vitamin-deficient and normal animals usually include pair-fed controls that were not part of this study. However, Lee et al. [50] did state that bodyweight gain was generally similar across all groups. The study does indirectly support the belief that the tetrahydrofolate pathway is critical to the disposition of formate.

Utility (adequacy) for CERHR evaluation process: This study provides information about a rodent animal model for folate deficiency that has not been physiologically characterized.

Several studies are presented below that provide insight into the metabolism and excretion of methanol in the non-human primate. The study by Burbacher et al. [52] was published subsequent to the reviews from which this summary was developed.

An extensive methanol study was conducted in the non-human primate *Macaca fascicularis* [52]. Toxicokinetic objectives were to assess whether repeated exposure to methanol changes methanol disposition kinetics, whether repeat exposure results in accumulation of blood formate, and whether methanol metabolism and disposition changes during pregnancy. In addition, the study assessed whether chronic methanol exposure at levels of 200–1800 ppm was associated with overt adult toxicity, female reproductive toxicity, or both, and whether in utero exposure to methanol affects offspring development. The reproductive and developmental portions of the study are found in Section 3.2.2 of this report.

A two-cohort study design utilized 48 adult females. See Section 3.2.2 for details about animal ages and sources. For each cohort, 24 females were randomly assigned to one of four exposure groups and, after a baseline period of approximately 4 months, were exposed to 0, 200, 600 or 1800 ppm methanol vapors (99.9% purity) for 2.5 h per day, 7 days per week. Doses were selected to produce blood methanol concentrations from just above background to just below levels resulting in non-linear clearance kinetics. Controls were exposed to air only in chambers. Methanol exposure occurred daily through an initial 4-month methanol exposure period, breeding, and pregnancy. Six-hour methanol clearance studies were performed after the initial exposure to methanol and after approximately 3 months of exposure; two additional clearance studies were performed during pregnancy. Blood methanol, formate, and folate concentrations were measured in 11–12 monkeys per group by GC, a colorimetric enzymatic assay, and radioimmunoassay, respectively. Statistical significance was evaluated using standard and repeated measures ANOVA models. Results (means \pm S.E. in mg/l) of the biweekly monitoring of blood methanol concentrations are presented below.

The authors reported that endogenous blood methanol levels in female cynomolgus monkeys ranged from 2.2 to 2.4 mg/l (Table 15). As can be seen, there were no material differences in blood methanol values as a result of pregnancy. Values were \sim 2.4 (control), 5.0 (200 ppm group), 11.0 (600 ppm group), and 35 mg/l (1800 group). Burbacher et al. [52] noted a disproportionate blood concentration-to-exposure-level dose relationship when they compared mean, dose-normalized, and net blood methanol concentration-time profiles for the 600 and 1800 ppm groups. This finding suggests saturation of the metabolism-dependent (hepatic alcohol dehydrogenase) process reported by others. Methanol clearance rates increased with time.

Results of the biweekly monitoring of plasma formate concentrations are presented in Table 16. There were no differences in formate concentrations among the groups during the baseline period. There were significant differences (ANOVA; $P = 0.005$) between baseline and pre-breeding and from pre-breeding to pregnancy (ANOVA; $P = 0.0001$). These changes were not dose-dependent. Serum folate levels were reported to be within the normal range of values

Table 15
Blood Methanol Concentrations in *M. fascicularis*^a

Exposure group	Baseline	Pre-breeding	Breeding	Pregnancy
Control (<i>n</i> = 9)	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.7 ± 0.1
200 ppm (<i>n</i> = 12)	2.2 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	5.5 ± 0.2
600 ppm (<i>n</i> = 11)	2.4 ± 0.1	10.5 ± 0.3	10.9 ± 0.2	11.0 ± 0.2
1800 ppm (<i>n</i> = 12)	2.4 ± 0.1	35.6 ± 1.0	35.7 ± 0.9	35.5 ± 0.9

^a Data presented as mean ± S.E. in mg/l.

Table 16
Plasma formate concentrations in *M. fascicularis*^a

Exposure group	Baseline	Pre-breeding	Breeding	Pregnancy
Control (<i>n</i> = 11)	8.3	7.8	10	8.3
200 ppm (<i>n</i> = 12)	7.4	8.3	9.7	7.8
600 ppm (<i>n</i> = 11)	6.9	7.8	9.2	8.7
1800 ppm (<i>n</i> = 12)	6.4	8.7	11	10

^a Values presented as mean in mg/l (converted from mM by CERHR).

for macaques; values during the baseline and pre-breeding phase were ~12–15 µg/l (Table 17). There were slight but significant changes in folate levels when the baseline and pre-breeding periods were compared as well as when pregnancy values were compared to those obtained prior to pregnancy. These differences were not dose-dependent.

Net blood methanol concentration–time data for the 600 and 1800 ppm groups were fitted to a linear, one-compartment first-order model or a saturable one-compartment Michaelis–Menten model. In these models, allometrically estimated ventilation rates, assumed ventilation rate, and fractional absorption were constant across exposure concentrations, and methanol uptake in the lung was constant throughout the exposure period. The data from the 600 ppm group adequately fit the linear model, while the majority of the data sets from the 1800 ppm group better fit the Michaelis–Menten model. These findings suggest saturation of methanol metabolism at high doses and are consistent with the findings of others who studied non-human primates [53]. The half-life for blood methanol estimated from the linear model for the 600 ppm group ranged from 55.4 to 90.7 min in the four exposure scenarios, while the half-life for the 1800 ppm

group from the Michaelis–Menten fit ranged from 56.6 to 77.6 min.

Strengths/weaknesses: Burbacher et al. [52] is one of the best studies of methanol disposition in non-human primates available. The strengths of the study are:

- It was conducted in macaque monkeys—a species similar to humans in its sensitivity to methanol. The animals were first separated into groups based on age, size, and parity, then randomly assigned to exposure groups.
- All procedures were carefully controlled and validated. Methanol concentrations in chambers were monitored and reported. Therefore, the Panel has a high degree of confidence in the absolute values reported.
- Inhalation exposure was to environmentally relevant doses of methanol vapors as well as to one dose that approached a toxic level. The methanol purity was reported.
- The study provides information on blood methanol and plasma formate levels following acute and chronic exposures.
- Blood values were determined in the same monkeys prior to and during pregnancy.

A possible weakness was the authors' presumption that formate alone is the only toxic metabolite of methanol. In addition, there is a presumption that maternal blood methanol and formate levels are reliable predictors of what the fetus experiences; there are no empirical data from this study on placental or fetal tissue levels of methanol or formate.

Utility (adequacy) for CERHR evaluation process: The biochemical data in this study are highly relevant for the CERHR process because of the high quality of the study, the relevance of the animal model, the use of environmentally

Table 17
Serum folate concentrations for baseline and exposure periods in *M. fascicularis*^a

Exposure group	Baseline	Exposure period			
		Pre-pregnancy ^b		Pregnancy ^{b,c}	
		70 Days	98 Days	55 Days	113 Days
Control (<i>n</i> = 11)	14.4 ± 1.0	14.0 ± 1.2	13.4 ± 1.2	16.0 ± 1.1	15.6 ± 1.1
200 ppm (<i>n</i> = 12)	11.9 ± 1.3	13.2 ± 1.6	12.9 ± 1.3	15.5 ± 1.5	13.4 ± 1.3
600 ppm (<i>n</i> = 11)	12.5 ± 1.4	15.4 ± 1.2	13.4 ± 1.0	14.8 ± 1.1	16.4 ± 1.0
1800 ppm (<i>n</i> = 12)	12.6 ± 0.7	14.8 ± 1.2	15.3 ± 1.1	15.9 ± 1.2	15.7 ± 1.0

^a Data presented as mean ± S.E. in µg/l.

^b Number of days exposed to methanol.

^c *n* = 9 for control and 600 ppm-exposure groups; *n* = 10 for 200 ppm and 1800 ppm exposure groups.

relevant doses of methanol and routes of exposure, and the availability of dose–response and kinetic information.

Medinsky et al. [54] and Dorman et al. [55] examined the pharmacokinetics of [^{14}C]methanol and [^{14}C]formate in normal and folate-deficient cynomolgus monkeys, *Macaca fascicularis*, following inhalation of environmentally relevant concentrations of [^{14}C]methanol while anesthetized. Four normal female 12-year-old cynomolgus monkeys were initially exposed for 2 h to each of four different concentrations of [^{14}C]methanol vapors (>98% purity): 10, 45, 200, and 900 ppm (13, 60, 260, and 1200 mg/m³) with each exposure separated by at least 2 months. The doses were based on likely exposure scenarios resulting from use of methanol as an automotive fuel and one higher dose. After this series of experiments, monkeys were fed a folate-deficient diet supplemented with 1% succinylsulfathiazole for 6–8 weeks to reduce serum folate concentration to <3 ng/ml serum and <120 ng/ml erythrocytes. The monkeys were then exposed to 900 ppm [^{14}C]methanol for 2 h. Folate deficiency did not affect hematocrit, red blood cell (RBC) count, mean corpuscular volume, or mean corpuscular hemoglobin concentration. In each experiment, methanol was administered via an endotracheal tube while the animals were under general anesthesia. Blood samples were collected at 0, 0.25, 0.5, 1, 1.5, and 2 h into the exposure period, and at 3, 4.5, 6, and 7.5 h post-exposure. Urine was collected during exposure and until 48 h post-exposure. Methanol and formate levels in blood and urine were measured by high pressure liquid chromatography (HPLC). The Student's *t*-test was used to determine statistical significance between results obtained under folate-sufficient and deficient conditions.

Blood methanol level peaked at the end of each 2-h exposure and then declined to undetectable levels at 8–10.5 h post-exposure. End-of-exposure methanol concentration, methanol area-under-the-curve (AUC), and total amounts of [^{14}C]methanol and [^{14}C]carbon dioxide exhaled were linearly and significantly related to inhaled methanol concentration. The elimination half-life of methanol (<1 h) was not significantly affected by inhaled methanol concentration. Urinary excretion of methanol was <0.01% absorbed dose at all doses, and no significant difference was seen in methanol urinary excretion or exhalation between folate-deficient and folate-sufficient monkeys exposed to 900 ppm methanol. The linear relation between inhaled methanol dose and blood methanol concentration AUC indicate that dose-dependent methanol metabolism and pharmacokinetics did not occur. Dorman et al. [55] found no significant formate accumulation at any dose in folate-sufficient animals. Peak [^{14}C]formate levels were significantly higher in folate-deficient versus folate-sufficient animals exposed to 900 ppm methanol. However, the blood [^{14}C]formate concentrations in all exposure groups were 10–1000-fold lower than reported endogenous blood formate concentrations of 0.1–0.2 mmol/l (4.6–9.2 mg/l). This suggests that exposure to methanol vapor at low, yet environmentally relevant, doses does not result in elevation of formate levels.

Strengths/weaknesses: Strengths of the study are that it used a primate model, had an excellent exposure system, measured respiratory parameters, reported methanol purity, measured and reported methanol concentrations in test atmosphere, and used state-of-the-art procedures for measuring methanol metabolites and quantifying exhaled and excreted radiolabeled methanol.

Limitations in extrapolation noted by an HEI Review Committee [54] included: exposure was via an endotracheal tube, thus bypassing the nose; exposures were conducted under general anesthesia, thus, the delivered doses of methanol are probably not comparable to those in animals breathing normally; and there was substantial variation among monkeys, and the statistical analysis may not have been optimal to account for this variation.

It should be noted that although [^{14}C]formate concentrations increased in the blood of folate-deficient monkeys exposed to 900 ppm methanol vapors, this represents only a small fraction of the total blood formate (estimated to be about 1%).

Utility (adequacy) for CERHR evaluation process: The Dorman et al. [55] study is highly relevant to the consideration of toxicokinetics, pharmacokinetic models, and mechanisms. However, because the exposure conditions are not the same as those experienced by people, the absolute blood methanol and formate levels should not be directly extrapolated to humans.

The pharmacokinetics of methanol and formate were characterized in male F-344 rats (CDF(F-344)/CrIBR) and three young adult rhesus monkeys (*Macaca mulatta*; from Hazleton Laboratories) (*age not specified for either species*) [53]. Based on data collected over 6-h periods where IV and inhalation exposure occurred, the authors developed a physiologically-based pharmacokinetic (PBPK) model. Two groups of four rats were given 100 mg/kg [^{14}C]methanol (>98% purity) in saline intravenously. One group was used to determine blood concentration–time course and cumulative urinary excretion of [^{14}C]methanol and [^{14}C]formate. The second group was used to determine cumulative exhalation time courses of [^{14}C]methanol and $^{14}\text{CO}_2$. Four rats per concentration were exposed to methanol vapor (>99.9% purity) concentrations of 0, 200, 1200, or 2000 ppm (0, 260, 1560, or 2600 mg/m³) for 6 h in a head-only chamber. Monkeys were individually exposed to atmospheres of 0, 50, 200, 1200, and 2000 ppm with 2-week recovery periods between exposures. (*The rationale for doses selected was not discussed*). In the inhalation experiment, blood methanol and formate levels were measured by GC. For the IV experiment, blood and urine [^{14}C]methanol and [^{14}C]formate were measured by HPLC.

The IV studies indicated that 96.6% of methanol clearance was via metabolism with pulmonary and renal clearance accounting for 2.6 and 0.8%, respectively. A total of 1.7% of the dose was eliminated as [^{14}C]formate in the urine. Blood methanol in rats reached a plateau after 1 h of inhalation of 200 ppm methanol but continued to rise in the

1200 and 2000 ppm groups. Blood methanol levels after 6 h exposure were 3.1 ± 0.4 , 26.6 ± 2.0 , and 79.7 ± 6.1 mg/l in the 200, 1200, and 2000 ppm groups, respectively. These end-of-exposure blood concentrations (and AUCs) were not proportional to exposure level, with the non-linearity most pronounced between the 1200 and 2000 ppm dose. Blood methanol concentrations in monkeys at the end of exposure were 3.9 ± 1.0 mg/l, 37.6 ± 8.5 mg/l, and 64.4 ± 10.7 mg/l at the 200, 1200, and 2000 ppm doses, respectively. No significant increase over background was observed at the 50 ppm dose. There was proportionality between exposure dose and blood concentration and AUC between 1200 and 2000 ppm. The peak blood formate concentrations in rats and monkeys ranged from 5.4 to 13.2 mg/l; there were no statistically significant differences between the control and methanol treated groups.

Horton et al. [53] stated that the lack of a discernable increase in blood formate in monkeys was not surprising and was consistent with estimates [3] of dose required to saturate folate-dependent metabolism of formate, i.e. 250 mg/kg. In modeling their monkey data, they noted that after inhalation of low concentrations of methanol the initial step of metabolism was compatible with rodent catalase. They further noted observations by others that high methanol concentrations were necessary to show that methanol was a substrate for rhesus monkey alcohol dehydrogenase. The authors stated that, while dose-dependent pharmacokinetics occurred in monkeys, blood methanol levels decreased in a mono-exponential manner, suggesting that repeated 6-h exposures should not result in an accumulation of methanol in blood. They reported that this hypothesis was corroborated by exposing monkeys to 2000 ppm 6 h per day, 5 days per week for 2 weeks. Blood samples after the end of 1 or 2 weeks exposure showed that neither methanol nor formate had accumulated in the blood.

Strengths/weaknesses: The strengths of this study include:

- Primate model.
- Rigorous monitoring and control of exposures, sampling procedures, and analyses.
- Range of inhaled methanol doses (50–2000 ppm) that included environmentally relevant doses.
- Purity of methanol was reported.
- Use of two species and comparison to human data (not cited in the above paragraph).
- Ability to compare kinetics following IV and inhalation routes of exposure.

The major weakness is the small number of animals (four rats and three monkeys).

Utility (adequacy) for CERHR evaluation process: Very useful for the CERHR process.

Noting that water soluble vapors can be reversibly retained in respiratory airways (and therefore not be available for lung absorption), Fisher et al. [56] quantified the relative respiratory uptake of methanol in the lungs of female *Macaca cynomolgi*. Relative respiratory uptake was

determined using unpublished [^{14}C]methanol breath time-course data from the Dorman et al. [55] study in which anesthetized monkeys were exposed to 10, 45, 200, or 900 ppm [^{14}C]methanol (lung only) for 2 h. Fisher et al. reported relative respiratory uptake values of 0.56 and 0.61 for 200 and 900 ppm lung-only exposures, and noted that these values were in good agreement with the value of 0.65 for male rhesus monkeys reported by Perkins et al. [57]. Using a four-compartment PBPK model, it was predicted that 40–81% of [^{14}C]methanol was bioavailable to the lung for absorption into the systemic circulation following a 2-h exposure of the monkeys. Noting linearity for concentration of methanol and percent absorption from the lung, Fisher et al. [56] concluded that PBPK models can simulate respiratory uptake of methanol by adjusting the inhaled exposure concentration and measuring or estimating the breathing rate. Failure to adjust for the reversible retention of methanol in the respiratory airways will result in models over-predicting the amount of [^{14}C]methanol clearance from the lung. Fisher et al. [56] concluded that it is important to consider fractional uptake of polar substances in risk assessment.

Strengths/weaknesses: This is a well conducted and clearly reported study. A limitation is that only four primates were used.

Utility (adequacy) for CERHR evaluation process: This study clearly identifies the need and feasibility for PBPK models to adjust for the proportion of methanol that is available to the lung for uptake in order to provide a more accurate estimate of dose in risk estimation procedures.

2.1.4. Elimination

Information about methanol elimination was obtained from reviews by IPCS [1], Kavet and Nauss [2], and Liesivuori and Savolainen [47]. After methanol is distributed in the body it is either directly excreted in urine and exhaled breath or metabolized in the liver. Clearance from circulation in humans following low-level exposures follows first-order kinetics with a half-time of ~2.5–3 h. At higher doses the elimination becomes saturated. The kidney appears to exert no active control over urinary methanol concentration. Exhalation levels are proportional to methanol concentration in blood. While excretion by kidney and lung are linear (first-order kinetics), metabolic conversion is not a linear function of concentration. Biotransformation by sequential oxidation in the liver accounts for 96.9% of the elimination, while urinary excretion and exhalation account for the remainder. The presence of ethanol can slow the clearance of methanol from blood through metabolic pathways, a fact that is used in the treatment of methanol poisoning. Formaldehyde, which is formed as the first oxidation step in the metabolism of methanol, is metabolized to formate very rapidly with half-life of ~1 min. The rate of elimination of formate, the oxidation product of formaldehyde, is dose dependent as discussed in Section 2.1.3.

A population of 84 non-occupationally exposed subjects (31 males, 53 females) in Sao Paulo, Brazil were

assessed for urinary methanol in order to establish reference values for occupational biomonitoring [58]. The cohort consisted of non-smokers or smokers of less than 10 cigarettes per day, non-frequent alcohol consumers, and non-users of aspartame. No significant differences in urine methanol levels were seen between males and females. Clinical signs (hemogram, glycosis, urea, creatinine, gamma-glutamyltransferase, alanine aminotransferase, aspartate aminotransferase, total cholesterol, triglycerides, and urine type I) were within the normal range. The mean urinary methanol level was 2.26 ± 1.26 mg/l standard deviation (S.D.). The range of values was 0.50–4.78 mg/l.

Strengths/weaknesses: This is an observational survey that provides some baseline information on urinary methanol levels in the general population. The subjects reportedly did not consume aspartame. Diet was not restricted, with the exception of the exclusion of alcoholic beverages during the 24 h before the urine sampling. Therefore, some subjects may have been exposed to methanol through the consumption of fruits and vegetables. It would have been useful to have some information on ambient methanol levels in the region.

Utility (adequacy) for CERHR evaluation process: For the reasons cited in the section above, this study is of limited use to the Panel.

2.1.5. Pregnancy

A study in humans demonstrated that breakdown and excretion of folate is accelerated during the second and third trimesters of pregnancy [59]. Additional details of the study are included in Section 3.1.

Pikkarainen and Raiha [60] measured in vitro alcohol dehydrogenase (ADH) activity in the livers of human fetuses, children, and adults ($n = 1-3$ per age group) using ethanol as a substrate. The ADH activity in 2-month-old fetal livers was about 3–4% that of adults. In 4–5 months old fetuses, ADH activity was roughly 10% that of adults, and in infancy, activity was about 20% that of adults. ADH activity increased in children with age, and at 5 years of age reached a level that was within the ranges noted for adults. Great variation was noted in adult ADH activity. The observations of ADH activity in fetal livers are qualitatively consistent with those observed in rats and mice by Ward and Pollack [61] and discussed in Section 3.2.3.

Available data in primates indicate little or no differences in methanol pharmacokinetics as a function of pregnancy [52]. In rodents, methanol uptake and elimination was virtually unaffected by pregnancy [45]. Pollack and Brouwer did report a statistically significant decrease in V_{\max} for formaldehyde formation in rat and mouse liver homogenate, a finding they described as relatively minor. Additional details for the Burbacher et al. [52] and Pollack and Brouwer [45] studies are in Sections 2.1.3 and 2.1.1.2, respectively.

2.1.6. Physiologically-based pharmacokinetic models

A number of models have been developed specifically for methanol. PBPK models incorporate species-specific pa-

rameters such as blood flow rates, tissue volumes and relative levels of blood perfusion, and known metabolic mechanisms. Once developed, PBPK models can then be validated using available data on the disposition of the chemical of interest in various species. Based on the validity of the model, a decision can then be made on its use for predicting human risk from chemical exposure. These models are briefly described below.

A one-compartment, “semi-physiologic” PBPK model was developed by Perkins et al. [57,62] to describe methanol disposition in mice and rats. Model predictions for methanol disposition in mice during and after inhalation exposure were compared to those previously determined in the female Sprague–Dawley rat, and the disposition in mice after various exposure routes was also examined.

Using published kinetic parameters determined after IV and oral administration of methanol in humans and other primates, and estimated fraction of absorbed methanol (ϕ) and physiological parameters, Perkins et al. [57] next applied the inhalation pharmacokinetic model for rodents to humans. Data for the IV exposure were modeled with the one-compartment model described in Perkins et al. [62], with saturable elimination that was first-order at low levels of blood methanol. Data for oral exposure were modeled similarly but with a factor for gut absorption. Maintaining the fraction of absorbed methanol (ϕ) as the dependent variable, and using kinetic parameters from the oral or intravenous data, inhalation data were then fitted to the previously determined pharmacokinetic model. Background human blood methanol from both endogenous and exogenous sources was set at 1.0 mg/l for the initial time step. The authors estimated that following an 8-h exposure to 5000 ppm methanol vapor (6550 mg/m^3), blood methanol concentrations in the mouse would be 13–18-fold higher than in humans, whereas methanol concentrations in the rat would be five-fold higher than the value for humans.

The semi-physiologic model was further applied to methanol disposition in rodents when absorption was confined to the upper respiratory tract, where the majority of methanol absorption occurs [63]. Their research results support the hypothesis that absorption of inhaled methanol takes place entirely in the upper respiratory tract of rodents. Methanol absorption was increased by decreased ventilation, but unaffected by increased ventilation. The semi-physiologic pharmacokinetic model developed by the study authors incorporated the body burden of methanol computed from blood methanol measurements, methanol elimination estimates, ventilation rate, and fractional absorption. Because ventilation rate varies with blood methanol concentration, and fractional absorption varies with environmental methanol concentration and ventilation rate, additional equations were derived to modulate these values using nonlinear least-squares regression.

A two-compartment model for methanol disposition in pregnant rodents that utilized Michaelis–Menten elimination from the maternal compartment was developed [61].

Pregnant Sprague–Dawley rats were given a single dose of 100 or 2500 mg/kg/methanol by gavage or by IV. Pregnant CD-1 mice were also given a single dose of 2500 mg/kg by gavage or IV. Methanol disposition was determined in non-pregnant rats, and at gd 7, 14, and 20 (to approximate three trimesters); in mice, non-pregnant animals and pregnant animals at gd 9 and 18 were examined. Blood samples were taken via jugular vein cannula. Rat concentration–time data were modeled using two-compartment models for each dose; mouse data were modeled with a one-compartment model with Michaelis–Menten elimination. Blood methanol levels after oral exposure rose more rapidly in pregnant than non-pregnant rats, but the opposite was true for mice [61]. Peak blood levels in rats were higher during pregnancy. V_{\max} for elimination in near-term rats and mice was 65–80% of that in non-pregnant animals. Mice eliminated methanol twice as quickly as rats. Qualitatively, the disposition between pregnant and non-pregnant animals was similar, with the same model, incorporating different parameter estimates, adequately fitting both conditions. In vitro studies showed that adult near-term livers have a V_{\max} for methanol metabolism of 85% that in livers from non-pregnant rodents. Mouse liver homogenates metabolized methanol twice as fast as rat liver homogenates. Fetal rodent livers had a V_{\max} less than 5% that of adults.

A PBPK model was developed by Ward et al. [64] for the disposition of methanol in rat and mouse dams and the conceptus. The model was validated by exposing rats on gd 14 and 20, and mice on gd 18, via injection into the jugular cannula, and using intrauterine microdialysis to measure transplacental methanol toxicokinetics.

The conceptal/maternal diffusion constant ratio consistently decreased with increasing dose in pregnant rats and mice, consistent with earlier observations that methanol limits its own delivery to the conceptus [65]. The validated model described methanol elimination as occurring primarily in the liver by a saturable, first-order metabolic process, as has been demonstrated in other studies [45,53]. Methanol tended to partition to tissues with high water content. Peak methanol concentration (C_{\max}) increased slightly but non-significantly in maternal blood as gestation progressed, consistent with the decrease in V_{\max} for methanol elimination described by Ward and Pollack [61]. The conceptal/maternal ratio of AUCs decreased with dose and gestation progression; at low doses conceptal AUC exceeded maternal, but at higher doses, maternal exceeded conceptal AUC. Pregnant mice data from the Dorman et al. [66] study were also used to validate the model; at 10,000 ppm the conceptal methanol AUC exceeded maternal blood AUC by 10%, while at 15,000 ppm, the maternal blood methanol AUC exceeded the conceptal AUC by 30%.

A disadvantage of the microdialysate procedure is the need to keep animals anesthetized. Urethane was used in this study for anesthesia, and it may have had some effect on pharmacokinetic parameters. However, the parameters

obtained here fit well with those obtained from other studies with non-anesthetized animals.

The Panel concluded that the PBPK studies described above represent an extensive series of carefully conducted experiments to develop pharmacokinetic models for rodents exposed to methanol and to begin to apply the results to humans. The strengths of these studies are the use of appropriate techniques to measure blood methanol, good study design, and justification of the models. This work has the most utility for understanding rodent toxicity studies.

As discussed earlier in Section 2.1.3, Horton et al. [53] developed a four-compartment PBPK model that does not include a fractional absorption parameter (ϕ). The model utilized a double pathway for metabolism to formaldehyde in the liver: one pathway using rodent catalase K_m and V_{\max} , and one using smaller K_m and V_{\max} values to simulate an enzyme with higher affinity and lower capacity. The compartments were richly perfused tissue (adrenals, brain, gastrointestinal tract), slowly perfused tissue (muscle, fat), kidney, and liver (the major metabolizing compartment). The model was scaled up for humans using the 0.74 power of body weight.

Horton et al.'s [53] is a careful attempt to develop PBPK models for methanol in rats, monkeys, and humans. The Horton models differ from those discussed in the preceding section in that they include more compartments but do not account for fractional absorption. Another important difference is that Horton et al. used a much lower range of methanol exposure conditions for the rodent studies, therefore there is more confidence extrapolating the results to humans. The inclusion of data on primates that was developed in the same laboratory, using the same techniques, is a plus.

As discussed earlier in Section 2.1.3, the publication of Fisher et al. [56] quantitatively estimated relative respiratory uptake of methanol, demonstrated the linearity of uptake over a range of doses, and proposed that correction for uptake can be readily incorporated in PBPK models.

Environ [67] performed a comparative analysis of the Perkins et al. [57,62] and Horton et al. [53] models on behalf of the American Forest and Paper Association (AF&PA). The analysis included the presentation of the exact algebraic forms of the models' mathematical relationships, and the application of these relationships to the prediction of human, monkey, and rodent blood methanol levels following exposure to low (83 mg/m³) and higher (260, 1300, and 2600 mg/m³) levels of methanol vapor. Both models produced similar results for steady-state blood methanol levels at various exposures, with the exception of the failure of the Perkins et al. model to achieve steady state at the highest exposure concentration (2600 mg/m³) in mice and rats. Because the Perkins et al. [57,62] model exhibited consistently smaller initial rates of methanol uptake across species, the Horton et al. [53] model predicts higher blood methanol levels prior to achieving steady state. This difference may be due to the fact that the Horton et al. model does not incorporate a fractional absorption parameter (ϕ). The Perkins

et al. [57,62] model, however, incorporates only a single metabolic compartment, and does not consider lung or kidney elimination, resulting in its inability to reach steady state at high methanol vapor concentrations. Environ [67] concluded that both models support a similar, predicted result. The Environ [67] analysis also provides additional insights and explanation of the models used in the above studies.

2.2. General toxicity

The majority of information in this section was obtained from reviews. Because quality reviews have already been conducted, CERHR is basing the general toxicity evaluation on those reviews instead of starting *de novo*. There were some cases where the primary paper was reviewed, for example more recent studies and key papers. The primary reviews utilized in this section were IPCS [1] and Kavet and Nauss [2]. The Kavet and Nauss paper is in the main the published version of an HEI [3] report. Because the Kavet and Nauss paper is more readily available to the public, it is being cited instead of the HEI report.

2.2.1. Human data

2.2.1.1. Laboratory studies. Two controlled studies examined the neurotoxic effects associated with methanol inhalation in humans and were evaluated by the Expert Panel.

Cook et al. [31] conducted a pilot study to obtain information about effects of acute methanol exposure on neurobehavioral function and methanol and formate levels in blood. Twelve healthy young men (22–32 years of age) were trained on tests for neurobehavioral function. They were then randomly exposed to air or methanol at 250 mg/m³ (191 ppm) for 75 min in a double blind study. Each subject served as his own control and was exposed twice to both methanol and air at the same time of the morning. For 12 h prior to exposure, the subjects were instructed to eliminate alcohol, diet foods and drinks, fruit and fruit juices, and coffee from their diets. The methanol exposures resulted in an increase in blood methanol but not blood formate levels, as discussed in Section 2.1.1.1. Subjects were tested for a battery of neurobehavioral endpoints that are widely used to identify effects of environmental pollutant exposure. The majority of results were negative. Statistically significant effects and trends were found for brainwave patterns in response to light flashes and sounds (P-200 and N1-P2 component of event-related potentials), performance on the Sternberg memory task, and subjective measures of fatigue and concentration. The study authors noted that effects were mild and did not exceed normal ranges. However, they noted some limitations in their study such as small sample size, use of only one exposure concentration and duration, and an inability to completely mask the odor of methanol from subjects and experimenters. The authors recommended that steps be taken to eliminate those limitations in future studies.

Strengths/weaknesses: There are a number of experimental design strengths in this study.

- The use of each subject as his own control,
- random assignment to exposure condition in order to control for potential order effects,
- double-blinding to exposure condition,
- monitoring of blood methanol and formate levels,
- multiple neurobehavioral testing consisting of validated outcome measures that pertain to everyday tasks,
- careful attention to calibration of instruments, and
- strict statistical design of study protocol and data analysis to take repeated measurements and multiple comparisons into account.

Although the sample size was small ($n = 12$), the selection of sample size was based on consideration of statistical power (to the extent possible).

The design also imposes limitations on the interpretation of results. Notable are the single-dose design that precluded assessment of potential dose response and the short duration of exposure (75 min). Another possible weakness is an apparent failure to completely blind subjects to exposure conditions. However, subjects who were most accurate at guessing conditions did not necessarily demonstrate the greatest exposure-related changes in test scores, suggesting that their hunches did not affect their performance.

The Expert Panel noted that although the authors concluded that the results were essentially negative, the differences seen all tended to be in the direction favoring the control condition over the methanol condition (self-ratings of vigor, concentration, and fatigue; reaction time, slope and intercept measures on the Sternberg memory task; P200 latency and N1-P2 interval on the auditory event-related potential task). Moreover, the results of the regression analyses indicated that chamber methanol concentration, blood methanol concentration post exposure, and blood methanol change contributed to the prediction of a variety of test scores. As the authors recognized, if more than one methanol concentration, or more than one exposure duration had been included in the experimental design, “meaningful dose-relationships might be found even at levels of methanol exposure expected as a result of its use as a motor fuel” [31]. The *P*-values associated with some of the trends reported might have reached statistical significance if sample size were only modestly increased. The authors minimized the importance of the neurobehavioral effects seen, noting that they were still “within the normal range.” While perhaps true, the test battery did not assess or rule out effects with more serious implications for daily life. As the authors suggest, the use of more difficult tasks, such as those that model more closely complex, demanding behaviors such as driving, might reveal larger methanol-associated changes in performance.

Utility (adequacy) for CERHR evaluation process: The use of a young, healthy population limits the utility of this study. The generalizability of the findings might be limited as

other populations such as the elderly, children, or individuals with lung disease, could potentially be more susceptible to methanol effects than healthy young males. This study suggests that short-term exposure (75-min) to methanol at a concentration of 250 mg/m³ might be associated with a variety of mild neurobehavioral changes. Although effects on P300 and the Sternberg test were weak, the Expert Panel notes similar observations at similar exposure levels as Chuwers et al. [32]. The study raises the possibility of more serious findings or effects at lower exposure level in possibly sensitive subpopulations. However, the Panel could not ascribe a level of confidence to the neurobehavioral findings due to the small magnitude of response and the fact that the single dose design of the study does not allow an assessment of a possible dose–response relationship. At best, neurobehavioral test performance at 250 mg/m³ suggests either a free-standing NOAEL or LOAEL for minimal effects close to a NOAEL. The study provides many useful suggestions about future directions for research.

Chuwers et al. [32] also studied the neurotoxic effects of acute methanol inhalation in human subjects exposed to the occupational threshold limit value of 200 ppm for 4 h. In a randomized double-blind study design, 15 men and 11 women (healthy, ages 26–51 years) served as their own controls and were exposed 1 time each to water or methanol vapors for 4 h. Subjects were trained on neurobehavioral tests prior to exposures. The exposures were conducted at the same time of the morning and were separated by 4 weeks in women to minimize hormonal effects. Subjects were instructed to eliminate coffee, vegetables, fruit or fruit juices, fermented drinks, and aspartame from their diet for 24 h prior to exposure. In addition, they were told not to take vitamin C for 3 days prior to exposure because it interferes with folate measurements. Exposures increased blood and urine concentrations of methanol but not formate, as discussed under Osterloh et al. [40] in Section 2.1.1.1. Most study results were negative. There were no significant effects on visual, neurophysiological, or neurobehavioral endpoints, with the exception of some between-subject variables. Slight effects on P-300 amplitude (brain waves in response to sensory stimuli) and Symbol Digit testing (information processing and psychomotor skills) were noted. Between subject variables for P-300 included alcohol consumption and smoking and the between subject variable in the symbol digit test was age. Double blinding was not completely effective because some experimenters and subjects were able to correctly guess when the methanol exposures occurred. The study authors concluded that methanol exposure at this concentration had little effect on neurobehavioral performance.

Strengths/weaknesses: In many respects, this is a very strong study methodologically with strict statistical design. Although the subjects were a convenience sample, care was taken to eliminate individuals with potentially confounding conditions such as liver or CNS (e.g. visual) disorders. The design included using subjects as their own controls (pre-testing and post-testing within both methanol and control

exposure conditions), randomizing the order of exposure to methanol and control (double-blind), providing training on the neurobehavioral tests to reduce learning effects and anxiety, administering the tests at the same hour each day, and 4-week separation of testing in women to reduce hormonal effects. The selection of the neurobehavioral tests included in the battery was based on prior literature on solvent exposures. A number of sensitive neurobiological endpoints were examined, and the endpoints were sensitive to the types of findings expected from environmental exposure. For the most part, the tests were well standardized and appropriate for repeated administration over short periods of time. Good quality control procedures were implemented for both biological and neurobehavioral measurements.

The study has some important weaknesses. First, the sample size was small, so that the statistical power for hypothesis testing was adequate only for detecting rather substantial differences (0.8 standard deviations). It might not be reasonable to expect that exposure to methanol at the concentrations used would have effects of this magnitude. In fact, only slight effects were noted on P300 and Symbol Digit Testing with the performance of multiple tests. Second, despite the QA/QC procedures, a surprising amount of data had to be discarded because of apparent experimenter error (Symbol-Digit) or technical problem (seven of 26 P-300 waveforms unacceptable, 5% contamination of serum methanol levels). Third, blinding apparently failed insofar as the primary investigator was correct 100% of the time in guessing whether an exposure was methanol or control. Subjects correctly identified exposure conditions 18 of 26 times. This could easily have affected subjects' test performance. Fourth, the manner in which the statistical analyses are reported is confusing, making it difficult to understand exactly what the findings were. The authors suggest that factors such as alcohol use, smoking, and folate status might alter susceptibility, although it is not clear whether the appropriate interaction terms for testing such hypotheses were included in the regression analyses. It appears that they were included as main effect terms, which would not address the issue of effect modification which this study would have had very low power to evaluate.

Utility (adequacy) for CERHR evaluation process: This study essentially found that if 4 h of exposure to a methanol concentration of 200 ppm has effects on neurobehavioral functioning, the effects are likely to be smaller than a 0.8 standard deviation in magnitude. The study is uninformative on the issue of whether or not this is actually the case. However, this is a well-designed study with double blinding and exposed subjects serving as their own controls. It has a strict statistical design and examines a number of relevant neurobehavioral endpoints that are sensitive to the types of findings expected from environmental exposure. It has limited ability to draw conclusions relevant to reproductive effects. As the LOAEL observed is for very mild effects it is likely very close to a no effects level. The findings in this study are similar to findings in the Cook et al. [31] study.

However, confidence in neurobehavioral findings is uncertain due to the small magnitude of response. The single acute exposure design is not relevant for chronic exposure to the general public. Results from a single dose in healthy young adults may not predict effects in sensitive populations.

Kavet and Nauss [2] reviewed Russian studies that reported effects in visual, olfactory, and reflex thresholds in humans following exposure to <9 ppm methanol vapors. However, Kavet and Nauss noted limitations such as inadequate reporting of details and the fact that some of the effects occurred at levels that would not impact background levels of methanol.

2.2.1.2. General population case studies. Information on methanol toxicity in the general population is available for acute and repeated exposure. The information provides no insight on effects to the reproductive system. This summary of general population effects is based on reviews by Kavet and Nauss [2] and IPCS [1].

Case studies describing effects of acute methanol exposure in humans date back to the early 1900s. The majority of human methanol poisonings have resulted from consumption of adulterated alcohol beverages [1]. However, acute methanol toxicity has been noted in adults and children following percutaneous or inhalation exposures, and symptoms have been equivalent to those observed with oral exposure. The progression of methanol-induced toxicity in humans has been well characterized in reviews by Kavet and Nauss [2] and IPCS [1]. The first symptom of acute methanol poisoning is a transient, mild central nervous system depression that is followed by an asymptomatic period usually lasting from 12 to 24 h. After the asymptomatic period, metabolic acidosis develops in parallel with toxicity to the eye. Symptoms during this time period include headache, dizziness, nausea, and vomiting. Visual symptoms may include blurred vision, altered visual fields, impaired pupil response to light, and permanent or temporary blindness. In patients with visual toxicity, examination by ophthalmoscope may initially reveal hyperemia of the optic disc followed by the development of peripapillary edema. Edema, which may persist for up to 2 months, occurs along the major blood vessels and seems to be found primarily in the nerve fiber layer of the retina. Optic disc pallor may occur 1–2 months after poisoning and is a sign of irreversible eye damage.

In severe cases of acute methanol poisoning, abdominal pain and difficulty breathing may occur and progress to coma and death, usually from respiratory distress [1,2]. Autopsies conducted on victims of methanol poisoning revealed gross pathological effects consisting of edematous, hemorrhagic, and degenerative changes in visceral organs, liver, kidneys, lungs, and central nervous system (CNS). The part of the brain most affected by methanol poisoning is the basal ganglia, especially the putamen. Survivors of severe methanol intoxication may suffer from motor disorders associated with damage to the putamen. It has been reported that 300–1000 mg/kg bw methanol is the minimum lethal

dose in untreated victims. Blood levels ≥ 500 mg/l may be obtained after ingestion of 0.4 ml/kg bw (315 mg/kg bw) and patients with that blood level generally require treatment by hemodialysis. However, doses producing toxicity, the types of symptoms developing, and the time course of symptom development vary widely among members of the population. Sensitivity to methanol poisoning may be affected by concurrent ingestion of ethanol which may increase the latency period. Inadequate dietary folate intake may result in compromised metabolism and increased sensitivity to methanol.

Use of methanol in gasoline is a potential source of acute methanol exposure and data on accidental ingestion of gasoline is discussed in Section 1.2.4.

Kavet and Nauss [2] describe case studies involving repeated exposure to methanol. Most case studies provide no information about levels and duration of exposure. However, they do demonstrate effects that are consistent with acute intake such as visual toxicity, headache, and vomiting. Those symptoms were noted after inhalation, oral, and dermal exposure.

2.2.1.3. Occupational epidemiological studies. A series of epidemiological studies addressed methanol exposure in occupational settings. Four studies were reviewed by both IPCS [1] and Kavet and Nauss [2]. The studies were also reviewed by CERHR to verify the information reported in Kavet and Nauss and IPCS. A study by Frederick et al. [68] of NIOSH was considered by Kavet and Nauss to be the most definitive. In that study, headaches, dizziness, blurred vision, and nausea/upset stomach were reported by teacher aids working near spirit duplicators using a 99% methanol fluid for 1 h per day for 1 day per week or 8 h per day for 5 days per week over a period of 3 years. Methanol levels in air ranged from 365 to 3080 ppm. A study by Kingsley and Hirsch [69] reported headaches in clerical personnel working near duplicating equipment using methanol-based fluids. Methanol air levels near the equipment were measured at up to 375 ppm. In a second study by NIOSH [70] it was reported that 45% of spirit duplicating machine operators at the University of Washington experienced symptoms such as blurred vision, headache, nausea, dizziness, and eye irritation; the average methanol concentration in the area was measured at 1025 ppm. Greenberg et al. [71] reported no visual or CNS symptoms in 19 workers manufacturing fused collars who were exposed to 22–25 ppm methanol vapors from 9 months to 2 years.

A study by Kawai et al. [72] examined subjective complaints and clinical findings in workers exposed to methanol for 0.3–7.8 years and utilized methanol in urine as a biological indicator of exposure. Regression analysis estimated that an 8-h exposure to 200 ppm methanol would result in a mean urinary methanol level of 42 mg/l. The most common complaints in workers exposed to a mean methanol concentration of 459 ppm included nasal irritation, headache, forgetfulness, and increased skin sensitivity. A complaint of dimmed vision was found to be due to methanol vapors in air

and not retinal toxicity. In three workers exposed to ranges of 953–1626, 1058–1585, and 119–3577 ppm methanol, pupil response to light was slow in two workers and a third worker had dilated pupils. However, the optic disc was unaffected and there was no indication of permanent eye damage.

2.2.2. Animal data

Studies examining methanol-induced toxicity in animals following acute- and repeat-dose toxicity are available. The majority of the acute studies provide no insight on methanol-induced toxicity to the reproductive system. Therefore, most of the information about methanol induced systemic toxicity was summarized from reviews by Kavet and Nauss [2] and IPCS [1].

Acute toxicity has been examined in rats, mice, rabbits, dogs, and monkeys. Kavet and Nauss [2] discussed the relevancy of different animal models for evaluating acute methanol toxicity in humans. They noted that the majority of laboratory animals do not develop acidosis and visual toxicity as noted in human methanol poisonings. Kavet and Nauss discuss a landmark paper published in 1955 by Gilger and Potts that established the non-human primate as the model of choice for evaluation of acute toxicity. In the Gilger and Potts [73] paper, oral acute methanol toxicity was examined in rats, rabbits, dogs, and rhesus macaque monkeys. The two main findings of Gilger and Potts were: the lethal dose in non-primates was two to three times higher than the 3000 mg/kg bw lethal dose reported for monkeys and 6–10 times higher than lethal doses reported for humans (Table 18); and only the non-human primates experienced symptoms similar to humans: intoxication, a 1-day latency period and then development of acidosis with some ocular toxicity prior to death. In the non-primates, acidosis did not develop and symptoms consisted of narcosis that was sometimes followed by death. Kavet and Nauss [2] concluded that the legitimacy of the non-human primate has been confirmed but also stated that “. . . non-primates may remain appropriate models in studies that seek to understand the direct alcoholic effects of methanol.”

Additional studies of acute toxicity in primate and non-primate species were reviewed by IPCS [1] and the results following oral or inhalation dosing were consistent with those described by Kavet and Nauss [2]. IPCS also reviewed a study by Dorman et al. [74] that reported intoxication, but a lack of optic nerve lesions, formate accumulation, and metabolic acidosis in minipigs gavaged with a single dose of methanol up to 5000 mg/kg bw. The Panel noted that the

histological examination by Dorman et al. [74] did not include reproductive organs.

An acute study by Youssef et al. [75] was reviewed by the Panel because it examined neurobehavioral toxicity, an effect evaluated in some developmental toxicity studies. The study was designed to examine methanol-induced effects at levels that do not produce overt toxicity. The study used rats, a model considered appropriate by authors because formate levels in humans are not elevated at low-to-moderate doses of methanol. Eleven adult (*age not specified*) male Crl: Long-Evans rats served as their own controls and were gavaged with water and 1000, 2000, and 3000 mg/kg bw methanol in water (50% solution) on different days. HPLC-grade methanol was used, which has a purity of 99.93% [76]. The doses represented 10, 20, and 30% of the methanol LD₅₀. The experiment was conducted twice at each dose. Ten min after dosing, the animals were subjected to the fixed wheel running ratio test to assess operant running. The test required the animal to run inside a wheel and rotate it under a fixed ratio of 20 times (FR20) in order to receive a food reward. Data were evaluated by conducting repeated measures analysis of variance (ANOVA), determining linear trend, correcting for degrees of freedom, and performing analysis of residuals to identify outliers and skewed distribution. The rats displayed no signs of overt intoxication such as gait disturbance, but a significant, dose-related reduction in FR20 response was observed with methanol treatment.

Strengths/weaknesses: The study by Youssef et al. [75] has many strengths. Chemical grade of methanol was reported. The doses were not expected to form significant formate levels in rats and dose–response relationships were identified. The operant-running test is very sensitive to alterations in complex motor performance and is able to identify responses in a more sensitive manner than observational studies. Another strength of this study was that a stable baseline, within-subject approach was used, generating great confidence in the dose effects.

Weaknesses include the use of 50% methanol by gavage, clearly an irritating dose, and the lack of a control for the volume of the highest dose. In addition, methanol concentrations in dosing solutions were not verified. A minor weakness was the failure to test even higher doses, as the statistics did not indicate whether the highest dose would have resulted in a statistically significant effect alone (i.e. what was the LOAEL?).

Utility (adequacy) for CERHR evaluation process: This study demonstrated a monotonic dose-effect relationship, with about a 40% decrease in responding with the highest dose of methanol. The study contributes to our discussion in that it is one of the few to produce clear dose-related effects. It also contributes to the discussion of whether ‘other’ effects should be included in risk assessments for methanol exposure. The Panel believes the study is valuable because it identifies a relevant endpoint in a particularly sensitive fashion. It indicates that effects occur at exposures below those identified in observational studies. However, despite

Table 18
Minimal lethal doses of methanol in humans and animals

Species	Minimal lethal dose (mg/kg bw)	Reference
Human	300–1000	[1]
Rhesus monkey	3000	[73]
Sprague–Dawley Rat	9500	[73]
Albino rabbit	7000	[73]

the sensitivity of testing methods used, studies correlating the relationship of the test protocol to human function are needed. While relevance to reproductive consequences is in question, it does indicate the need to use sensitive neurobehavioral testing during times of rapid brain growth and integration (in fetal and postnatal exposures) and in chronic exposure scenarios. The article also included an interesting analysis in the attempt to determine whether methanol's effects on behavior were motivational or motoric. This issue continues to plague behavioral research, but is not germane to the discussion. One limitation to utility was that the study only provided information about acute exposures and doses were greater than those expected from environmental exposure. In addition only adult males were examined and a NOAEL was not identified. Confidence is moderate with the limitations noted.

Numerous repeat dose studies were reviewed by Kavet and Nauss [2] and IPCS [1]. The majority of those studies provided no information on effects to reproductive organs or other endpoints of interest, but did identify the primary organs affected by methanol exposure. Studies in rats, dogs, and rabbits primarily noted toxicity to the eye, brain, and liver. Russian studies by Chao and Ubaydullayev (reviewed in Kavet and Nauss [2]) reported changes in chronaxy ratio (minimum time for a stimulus twice the intensity of the absolute threshold to induce a response) following exposure of rats to ≤ 38 ppm methanol vapors for 90 days. Kavet and Nauss concluded that the studies do not provide sufficient evidence of an association between low-level methanol exposure and neurobehavioral effects in rats due to limitations such as inadequate reporting of details and unknown biological significance.

Kavet and Nauss [2] and IPCS [1] reviewed methanol toxicity studies by the Japanese New Energy Development Organization (NEDO). In a study to evaluate non-carcinogenic effects, 20 Fischer-344 rats/sex per group and 30 B6C3F1 mice/sex per group were exposed to 10, 100, or 1000 ppm methanol vapors for 20 h per day for 12 months. Mild effects were only observed at the highest dose for rats and mice. Effects in rats included reduced weight gain in males and females and a non-significant increase in relative liver and spleen weight in females. In mice, bodyweights were increased in males at 6 months and females at 9 months and fatty degeneration of hepatocytes was enhanced. Clinical analysis resulted in no treatment related effects. Kavet and Nauss [2] noted that a critical review of the NEDO studies and results was not possible because the reports did not contain sufficient amounts of technical data or histopathological results.

One recent study provided information about sensitivity in folate-reduced rats, and a limited number of studies included a histological examination of the reproductive system. These studies were reviewed by the Expert Panel and are discussed below. Because methanol has been proposed for use as an additive in gasoline, some studies have been conducted to examine the toxicity of gasoline/methanol blends. This doc-

ument will only focus on studies that provide information on the toxicity of methanol alone or on the interaction between methanol and gasoline.

In a series of experiments, Lee et al. [50] demonstrated that the toxic response in rats fed a folate-reduced diet and exposed to 3000 ppm methanol vapor included death, elevated blood formate level, and metabolic acidosis. These effects were similar to those reported in the literature for non-human primates. The details of this study are presented in Section 2.1.3.

Andrews et al. [77] conducted a subchronic inhalation study in rats and monkeys. The monkey study is discussed later in this section. Five male and female Crl: CD (Sprague-Dawley) rats per group (50 days old) were exposed to methanol vapors (99.85% purity) at 0, 500, 2000, and 5000 ppm for 6 h per day, 5 days per week for 4 weeks. (*The rationale for dose selection was not discussed.*) Controls were exposed to house-supply air only. Statistical evaluation of data is discussed below in the synopses of the primate study conducted by Andrews et al. [77]. The only clinical sign observed was nasal and ocular discharge in methanol-treated rats. Weekly measurement of bodyweight revealed no differences between control and treated animals. At necropsy, organ weights were measured and the organs assessed included testes and epididymides and ovaries, apparently in all male and female animals, respectively. Relative spleen weight was significantly increased in female rats exposed to 2000 ppm methanol, but the study authors did not consider the effect to be of biological significance. Thyroids were not examined histologically and it is not certain if a histopathological examination of reproductive tissues was conducted. The authors stated that testes, epididymides, and eyes were among the tissues preserved in Bouin's solution for microscopic examination. However, of those three organs, only the eye from control and high-dose animals was said to be prepared in slides and examined microscopically. Gross and histopathological examination revealed no effects in organs examined. No ocular abnormalities were noted in an ophthalmoscopic exam. The study authors concluded that the study identified no target organs of effect.

Strengths/weaknesses: See summary under the discussion of primate effects later in chapter.

Utility (adequacy) for CERHR evaluation process: See summary under the discussion of primate effects later in chapter.

Poon et al. [78] studied the toxicity associated with methanol exposure alone or in combination with toluene, a component of gasoline. Groups of 10 Crl:Sprague Dawley rats/sex per group (~4 weeks old) were exposed to filtered room air or vapors of methanol (300 or 3000 ppm), toluene (30 or 300 ppm), or methanol/toluene (300/30, 300/300, 3000/30 or 3000/300;ppm) for 6 h per day, 5 days per week, for 4 weeks. Purity of both methanol and toluene was >99.7%. (*No rationale for dose selection was discussed.*) Ten animals/dose/sex were evaluated in all methanol-containing groups at the end of exposure. Statistical signif-

ificance was determined by one-way ANOVA and Duncan's range test. Methanol treatment alone did not result in clinical signs of toxicity, reduced growth rate, or effects on serum chemistry or hematology. A limited number of organs were weighed at necropsy, but the reproductive organs were not. Methanol exposure alone had no effect on organ weights. The pituitary gland and reproductive organs were among the organs fixed in 10% buffered formalin and examined histologically in 5–6 animals per group/sex. However, effects on reproductive organs were not reported. The authors stated that a mild-to-moderate reduction in thyroid follicle size was noted in female rats treated with both doses of methanol only. Although the authors stated in the text that thyroid changes in males were not as apparent, the tables reported a higher incidence and greater severity of thyroid effects in control males and males exposed to methanol. Mild histological effects in nasal passages were noted for both males and females exposed to both dose levels of methanol. The incidence of nasal lesions was increased in rats exposed to mixtures of methanol and toluene compared to exposure to either compound alone. Other effects noted in rats exposed to toluene or methanol/toluene mixtures included mild thyroid and liver effects. The authors concluded that "there were no apparent interactive effects observed."

Strengths/weaknesses: The strengths of this study included use of a large study population (100 animals, 10/sex per group) that was randomly assigned to exposure groups, evaluation of blood chemistry and liver P450 level, reporting of the methanol purity, monitoring of chamber methanol concentrations, and considerations of interactive effects with toluene. A limitation of study design was that histopathological evaluation was only conducted in about half the animals.

Utility (adequacy) for CERHR evaluation process: This study raises the question of thyroid as a possible target organ for methanol. However, the Expert Panel concluded that thyroid findings were questionable. They noted that control males experienced a reduction in follicle size. No substantial or consistent thyroid findings were noted in this study and the thyroid findings were not confirmed by Poon et al. [79] (discussed below). The thyroid findings were mild and half of the animals were not examined histopathologically, resulting in examination of small numbers. The nasal respiratory findings require careful consideration due to anatomic differences between rats and humans and because rats are obligate nose breathers. No significant toxicological effects were identified by this study. No information is given regarding possible structural or functional findings in the reproductive organs. The study is of limited utility in evaluating reproductive hazards.

Poon et al. [79] studied the toxicity associated with exposure to methanol, gasoline, and methanol/gasoline blends. Groups of 15 Crl: Sprague Dawley rats/sex per group (4–5 weeks old) were exposed to filtered room air or vapors of 2500 ppm methanol, 3200 ppm gasoline, 2500/3200 ppm methanol/gasoline, or 570/3200 ppm methanol/gasoline

for 6 h per day, 5 days per week, for 4 weeks. Methanol purity was >99%. (*The rationale for dose selection was not discussed.*) Effects were evaluated in 10 rats/sex per group. Statistical significance was evaluated by one-way analysis of variance and Duncan's multiple range test. No clinical signs were observed and methanol had no effect on bodyweight gain. Mild histological changes were noted in nasal passages following exposure to methanol. A lack of significant changes in protein concentrations and enzyme activities in bronchoalveolar lavage fluid indicated that lung injury did not occur with methanol exposure. Serum chemistry and hematological analyses were conducted and the only effect noted was a significant decrease in serum sodium levels in females treated with methanol. At necropsy it was noted that two males exposed to methanol had collapsed left eyes. Measurement of organ weights included the left testis weight in which effects were not observed. A significant decrease in relative spleen weight was noted in the methanol-exposed females. Histopathological examination included reproductive organs, the pituitary, and thyroid preserved in 10% buffered formalin. The only histological effect noted was mild hepatic panlobular vacuolation in females exposed to methanol. It is interesting to note that this study failed to replicate the thyroid effects seen in the earlier study by Poon et al. [78]. Effects noted with exposure to gasoline or methanol/gasoline mixtures included decreased bodyweight gain, liver effects, reduced hemoglobin levels, and suppressed uterine eosinophilia. The study authors concluded that there were "no apparent interactive effects between methanol and gasoline."

Strengths/weaknesses: The strengths of this study included a large study population (15/sex per group) that was randomly assigned to exposure groups, gross and histopathological examination of male and female reproductive organs, reporting of methanol purity, control of and reporting of chamber conditions (i.e. vapor concentrations), and broad-spectrum of measures such as serum chemistry and hepatic enzyme activity.

Some weaknesses were noted from the point of view of a reproduction assessment. For example, there was limited measurement of reproductive organ weights. Also, the testis and ovary were inappropriately fixed and stained, thus reducing the confidence that the authors would be able to find the inhibited spermiation lesion characteristic of reduced testosterone levels. Additionally, formalin fixation prior to paraffin embedment makes for greater variability in the quality of testis sections. That means that subtle changes in cell associations (which could portend larger changes with further exposure) could easily be overwhelmed by shrinkage artifact. Lastly, there was no evaluation of female reproductive cycling, and no ovarian morphometry. Therefore, there is no information about any change in follicle dynamics that underlie female fertility.

Utility (adequacy) for CERHR evaluation process: This well-designed study demonstrated neither significant findings attributable to the methanol components nor an inter-

active effect with gasoline. It would appear that the minimal nasal histological changes at 2500 ppm represent a finding close to a NOAEL for this endpoint. However, as discussed previously, rat nasal findings require careful interpretation. There is a high level of confidence for this study within the limits noted above. The Panel notes that uterine histopathology was specifically reported and the study demonstrated no myometrial eosinophilic changes attributed to methanol exposure alone. However, the Panel found these studies of modest utility in assuring a lack of methanol effect on rodent reproduction. The Panel is confident that the authors would have found major lesions or massive cell loss from the gonads and associated reproductive tissues due to methanol exposure had they occurred. The Panel was less confident in the ability of these methods to accurately identify and characterize modest lesions in reproductive organs.

The utility of the general toxicity data set would improve if a chronic exposure study was available.

Andrews et al. [77] conducted a subchronic inhalation study in monkeys. Three male and three female *Cynomolgus* (*Macaca fascicularis*) monkeys per group (from Primate Imports, age not specified) were exposed to house-supply air or methanol vapors (99.85% purity) at 500, 2000, and 5000 ppm for 6 h per day, 5 days per week for 4 weeks. (The rationale for dose selection was not discussed.) Body and organ weight data were first analyzed by Bartlett's test and if variances were equal, parametric procedures were used (one-way ANOVA). Non-parametric procedures (Kruskal–Wallis test and summed-rank test) were applied if variances were not equal. Dose-trend tests were also conducted. Weekly measurement of bodyweight revealed no differences between control and treated animals. At necropsy, organ weights were measured and the organs assessed included testes, epididymides, and ovaries. Absolute adrenal weight was significantly decreased in female monkeys of the 5000 ppm group, but the effect was not considered to be of biological significance by authors. Thyroids were not examined histologically and it is not certain if a histopathological examination of reproductive tissues was conducted. The authors stated that testes, epididymides, and eyes were among the tissues preserved in Bouin's solution for microscopic examination. However, of these three or-

gans, only the eye from control and high-dose animals was said to be prepared in slides and examined microscopically. Gross and histopathological examination revealed no effects in organs examined. No ocular abnormalities were noted in an ophthalmoscopic exam. The study authors concluded that the study identified no target organs of effect.

Strengths/weaknesses: The strengths of the Andrews et al. [77] study in rats (discussed above) and monkeys included the examination of repeated exposures via inhalation (few other studies looked at this presumed common environmental pathway). The range of exposures was large (0–5000 ppm), the purity of methanol was noted, and chamber concentrations of methanol was verified and reported. Lastly both rats and monkeys were used (rat study described above).

Limitations in study design included no report of histopathological evaluation of reproductive organs, small group sizes ($n = 5$ rats/sex per group and 3 monkeys/sex per group), a lack of hematological and blood chemistry analysis, and no measurement of formate levels. The authors did not state if assignment to exposure groups was random.

The Expert Panel notes one questionable finding. Female monkeys had a statistically significant decrease in adrenal weights and an increase in splenic weights. These findings are discounted by the authors as “not of biologic significance.”

Utility (adequacy) for CERHR evaluation process: This study is of limited utility to the CERHR process as specific mention of pathological examination of reproductive organs is missing. The small number of non-human primates limits statistical significance.

2.3. Genetic toxicity

Because the IPCS [1] already conducted a thorough review of genetic toxicity information, the Expert Panel summarized the main findings of the review in Tables 19 and 20. The majority of findings were negative, but some positive results were obtained. The IPCS [1] stated that “The structure of methanol (by analogy with ethanol) does not suggest that it would be genotoxic.”

IPCS [1] also reported negative findings in the Ames test, cultured cell mutation assay in CH-V79 cells, chromosome

Table 19
In vivo genotoxicity results

Species or assay type	Dose	Endpoint	Result	Reference
Mouse	≤4000 ppm	Micronuclei in blood or lung cells, SCE, Chromosomal aberrations in lung cells, and Synaptonemal complex damage in spermatocytes	Negative	[1]
Mouse fed adequate or marginal folate diet	5000 mg/kg bw per day	Micronuclei	Negative	[80]
Mouse urine	5000 mg/kg bw total	Mutagenic activity	Negative	[1]
Mouse	1000 mg/kg bw	Chromosomal aberration (aneuploidy and SCE) and micronuclei in erythrocytes	Positive	[1]
Mouse	≤300 mg/kg	Structural chromosome aberrations in bone marrow	Positive	[1]

SCE: Sister chromatid exchange.

Table 20
In vitro genotoxicity results

Species (strain)	Concentration	Endpoint	Result without activation	Result with activation	Reference
<i>S. typhimurium</i> (TA98, TA100, TA1535, TA1537, TA1538)	NS	Mutation	Negative	Negative	[1]
<i>E. coli</i> (WP 2, WP 67, CM 871)	NS	DNA repair	Negative	Negative ^a	[1]
<i>A. nidulans</i> (diploid strain P1)	6.0% (v/v)	Chromosomal malsegregation	Positive ^a	^a	[1]
<i>S. pombe</i> ade 6 locus	NS	Mutation	Negative	Negative	[1]
<i>N. crassa</i>	NS	Mutation (<i>n</i> + 1 aneuploidy)	Negative ^a	^a	[1]
Chinese hamster cells	0.1%	SCE	Negative ^a	^a	[1]
L5178Y mouse lymphoma cells	7.9 mg/ml	Mutation	Negative	Positive	[1]
Syrian hamster embryo clonal system	NS	Cell transformation	Negative ^a	^a	[1]
Rausher leukemia virus-infected rat embryo cells	NS	Cell transformation	Negative ^a	^a	[1]

NS: not specified; SCE: Sister chromatid exchange.

^a It was not stated if a metabolic activation system was used.

aberrations, SCEs and the micronucleus test performed by NEDO.

The following study was not included in the IPCS review:

Fu et al. [80] examined micronuclei formation in reticulocytes of pregnant CD-1 mice fed diets with adequate or marginal levels of folic acid (1200 nmol and 400 nmol/kg, respectively) and gavaged with methanol in water at 0 or 5000 mg/kg bw per day on gd 6–10. Neither methanol nor reduced folic acid intake increased the frequency of micronucleated cells. Additional details of this study are included under Section 3.2.3.

2.4. Carcinogenicity

Kavet and Nauss [2] and IPCS [1] reviewed methanol studies by the Japanese New Energy Development Organization (NEDO). Rats and mice were exposed to 10, 100, or 1000 ppm methanol vapors for 20 h per day for 24 and 18 months, respectively. A non-statistically significant increased incidence of papillary adenomas and adrenal pheochromocytomas were observed at the highest dose, but NEDO concluded that there was no evidence of cancer. NEDO also exposed eight female *Macaca fascicularis* monkeys per group to 10, 100, or 1000 ppm methanol vapors for 22 h per day for up to 29 months and reported a non-dose- and time-related hyperplasia of “reactive astrocytes” in the nervous system. Methanol exposure had no effect on bodyweight or hematological or pathological parameters. Kavet and Nauss [2] noted that a critical review of the NEDO studies and results was not possible because the reports did not contain sufficient amounts of technical data and histopathological results.

2.5. Potentially sensitive sub-populations and children’s susceptibility

2.5.1. Folate deficiencies

Studies suggest an increased sensitivity to developmental toxicity in folate deficient states. Several factors predispose

humans to folate deficiencies or decreases in folate activity from methanol. These include pregnancy and lactation, gastrointestinal disorders (including celiac disease, Crohn’s disease, adult gluten enteropathy), chronic alcoholism, smoking, psychiatric disorders (including depression), and pernicious anemia [54,81]. Medications that are folic acid antagonists include dihydrofolate reductase inhibitors (including methotrexate, sulfasalazine, and trimethoprim) and drugs such as various antiepileptics that affect other enzymes involved in folate metabolism [82]. Several demographic groups are known to have higher than average rates of folate deficiency. These include Hispanic and Black women, the low income elderly and the mentally ill elderly.

2.5.2. Genetic factors

The methylenetetrahydrofolate reductase (see Fig. 2.2) polymorphism 677T mutation which decreases folate activity is common. Homozygosity is found in 21% of Hispanics in California and 12% of US Whites [83]. Genetic differences in folate receptor activity and in enzymes involved in folic acid metabolism are, at this time, theoretical causes of a heritable functional folate deficiency [84]. Inborn errors of folate metabolism are rare genetic disorders resulting in defective folate absorption, interconversion, or utilization [85].

The mechanisms underlying varying susceptibility to methanol [1] may also be related to genetic differences in ethanol metabolism through polymorphisms in the alcohol dehydrogenase (ADH2*2) [86,87] (Fig. 3) and P450 2E1 (CYP2E1) genes [88,89]. Population studies reveal significant ethnic differences in these genes with greater ethanol susceptibility in Asian and Native American populations. Given that methanol metabolism in humans is similar to ethanol, these polymorphisms in the alcohol dehydrogenase allele may lead to greater susceptibility to methanol toxicity. This would result from decreases in metabolism leading to higher peak-blood levels.

2.5.3. Children

Children may receive higher doses than adults when exposed to the same concentrations of any air pollu-

tants. This is because of their higher baseline breathing rates and their greater physical activity. Children's surface area/bodyweight ratio is greater than adults, making dermal absorption potentially greater. Hand-to-mouth behaviors as well as indiscriminate ingestions increase childhood risk by the oral route [90,91]. Alcohol dehydrogenase activity is 3–4% of adult levels in the 2-month old fetus and increases linearly until reaching adult values at about 5 years of age [92]. This lower enzyme activity may provide a level of protection against acute poisoning because it may reduce the rate of formate production [93]. However, as noted above, susceptibility to the effects of methanol itself may be enhanced.

Given that methanol is believed to be the proximate toxicant for teratogenesis in experimental animals and because methanol and ethanol metabolism are similar in humans, there is legitimate concern about potentially similar adverse neurodevelopmental outcomes. The current ethanol data set is robust for neurodevelopmental findings on altered cell proliferation, migration, differentiation, and apoptosis. These endpoints have only had limited assessment in experimental animals following developmental methanol exposure. The current methanol literature does not adequately address these more mechanistic endpoints. There is some limited support for the hypothesis that the mode of action of methanol and ethanol has some overlap. This evidence is supported by effects on cell proliferation [94] and neural markers associated with migration and differentiation (NCAM) [95].

2.6. Summary of general toxicological and biological parameters

2.6.1. Toxicokinetics

Methanol is not foreign to the bodies of mammals, including man, as it occurs naturally as a product of endogenous biochemical processes. As described in Section 1, methanol is a natural constituent in fruits, vegetables, and fermented drinks common in the American diet. Human exposure to methanol also results from consumption of liquids that contain the direct food additives aspartame and DMDC. Thus, methanol is present in human blood; mean background blood levels are somewhat variable and may range from 0.6 [31] to 2.6 mg/l [36]. Although gender differences have not been routinely evaluated, at least one study has reported higher baseline blood levels of methanol in females than males [35].

The absorption, distribution, metabolism, and excretion of methanol are generally understood in humans, monkeys, rats, and mice [1,2]. There are sufficient data from human studies and other species to demonstrate rapid absorption following exposure by inhalation, dermal, and oral routes. Following absorption, methanol distributes rapidly and uniformly to all organs and tissues in direct relation to their water content. Methanol elimination in expired air and urine is somewhat proportional to methanol concentration in blood, but accounts for a minor portion (3.1%) of the dose at con-

centrations that do not saturate metabolic pathways. At saturating doses these routes of elimination may become more significant [45]. In mammals, methanol is eliminated primarily by metabolism through a series of oxidation steps to sequentially form formaldehyde, formate, and carbon dioxide (Fig. 2).

The disposition and metabolism of methanol appear to be similar regardless of the route of administration (oral, dermal, or inhaled). However, due to the fact that respiration rates are the inverse of size, smaller species are predicted to accumulate higher blood methanol concentrations than larger species when exposed to similar methanol concentrations [45]. As noted in Table 21, this projection is confirmed by data obtained following inhalation exposures to high concentrations of methanol ($\geq 10,000$ ppm) where blood methanol concentrations observed in mice were two to five times higher than those of rats exposed to the same concentrations. Species differences are less obvious at lower exposure levels as noted in Table 21. At 5000 ppm the differences between blood methanol levels in rats and mice were generally two-fold or less; at 1000 ppm rat and mouse blood levels were similar. The limited data indicate that at 200 ppm rat, monkey, and human blood methanol levels were similar.

The fate of methanol in pregnant animals has been subject to limited research. Available data indicate little or no difference in methanol toxicokinetics as a function of pregnancy in non-human primates [52]. In pregnant mice and rats there was an indication that penetration of methanol to the fetal compartment decreased in inverse proportion to higher dose, possibly as a result of decreased blood flow [45].

There are marked species differences in the rate of methanol metabolism and these differences are important in interpreting methanol toxicity data. Although metabolism of methanol to formaldehyde utilizes different enzymatic pathways, this step occurs at similar rates in primates and rodents [1]. Formaldehyde is rapidly oxidized (half-life of ~ 1 min) to formate in all species. It is the rate at which formate is oxidized to CO_2 that accounts for the pronounced species difference in the toxicity of methanol (primates are more sensitive than rodents to the acute effects of methanol exposure). In rodents the catalase-peroxide system and enzymes utilizing folate as a co-enzyme provide considerable capacity to catalyze this reaction whereas primates depend heavily on the pathway involving folate. Because primates naturally have lower folate concentrations than do rodents they have considerably less capacity to metabolize formate. Formate is oxidized to CO_2 in rodents at twice the rate seen in primates. As a result, the rate of formate oxidation in rats exceeds the maximal rate at which methanol is converted to formate: 1.6 mmol/kg/h versus 0.9 mmol/kg/h, respectively [2]. In contrast, when primates receive moderately high doses of methanol, the formation of formate can exceed the oxidation of formate: ~ 1.5 mmol/kg/h versus 0.75 mmol/kg/h, respectively. The net result is that primates may accumulate levels of formate that exert toxicologi-

Table 21
Interspecies comparisons of blood methanol and formate levels

Estimated doses in mg/kg bw ^{a,b}				Blood/plasma methanol (mg/l) (range as reported in multiple studies) ^b				Blood formate (mg/l) (range as reported in multiple studies) ^b				
Background levels	Mouse	Rat	Monkey	Human	Mouse	Rat	Monkey	Human	Mouse	Rat	Monkey	Human
	0	0	0	0	1.6 ^c	1.8–3 ^{d,e}	2.4 ^f	0.6–2.6 ^{g,h,i,j,k,l}	No data	8.3 ^m	8.7 ^f	4–11 ^{g,h,i} (one value was 19) ⁿ
Inhaled dose (ppm–h)												
191–1.25				3.8				1.9 ^g				3.6 ^g
200–2.5			11				5 ^f				8.7 ^f	
200–4				19				6.5 ^h				14.3 ^h
200–6		11	31	27		3.1–7.4 ^{o,p}	3.9 ^o	7.0–8.1 ⁱ	5.4–13.2 ^o		5.4–13.2 ^o	8.7–9.5 ⁱ
400–8				74				13.4 ^l				
600–2.5			33				11 ^f				8.7 ^f	
800–8				133				31 ^q				
1000–7	819			97 ^c (NOAEL)								
1000–8		428					83 ^f					
1200–6		385	184				27 ^o		5.4–13.2 ^{o,i}		5.4–13.2 ^o	
1800–2.5			98					35 ^f			10 ^f	
2000–6		642	308	308			80 ^o	64 ^o	5.4–13.2 ^{o,i}		5.4–13.2 ^o	
2000–7	1,638				537 ^c (LOAEL)							
2500–8	2,340				1883 ^f							
3000–21			1375				80 ^d				30 ^d	
4500–6		1444					555–1260 ^s					
5000–6							680–873 ^p					
5000–7	4,095	1869			1650 ^c		1000–2170 ^t (NOAEL)					
5000–8	4,680	2139			3580 ^f		1047 ^r					
5000–21			2293					5250 ^d			1210 ^d	
7500–7	6,143				3178 ^c							
10,000–6	7,020				1468–2080 ^{u,p}							
10,000–7	8,190	3738			4204 ^c		1840–2240 ^t (LOAEL)					
10,000–8	9,360	4280			6028 ^f		1656 ^t					
15,000–6	10,530				7140 ^u							
15,000–7	12,285	5616			7330 ^c		3169–3826 ^e					
15,000–8	14,040	6420			11,165 ^f		2667 ^t					
20,000–7		7476					5250–8650 ^t					
20,000–8		8560					3916 ^t					
Oral dosing												
Lethal dose—bolus		9500 ^v	3000 ^v									
Human lethal dose				300–1000								
6.0–9.0 mg/kg Asp				6.0–9.0 ^w				2.4–3.6 ^w				
34 mg/kg Asp				3.4 ^e				≤4 ⁿ				
100 mg/kg Asp				10 ⁿ				12.7 ⁿ				
150 mg/kg Asp				15 ⁿ				21.4 ⁿ				
200 mg/kg Asp ^{x,y}				20 ^p				25.8 ⁿ				8–22 ⁿ

Asp: Aspartame.

^a Inhalation doses in mg/kg bw were estimated by the Methanol Institute [102] and verified by CERHR to ensure that calculations were accurate and reasonable assumptions were used.

^b Blank cells in tables signify no known information for a particular dose and species.

^c Rogers et al. [96].

^d NEDO [99].

^e Stanton et al. [100].

^f Burbacher et al. [52].

^g Cook et al. [31].

^h Osterloh et al. [40].

ⁱ Lee et al. [33].

^j Batterman et al. [34].

^k Batterman and Franzblau [35].

^l Franzblau et al. [36].

^m Lee et al. [50].

ⁿ Stegink et al. [11].

^o Horton et al. [53].

^p Cooper et al. [101].

^q Batterman et al. [34].

^r Perkins et al. [62].

^s Weiss et al. [95].

^t Nelson et al. [98].

^u Dorman et al. [66].

^v Gilger and Potts [73].

^w Davoli et al. [39].

^x Pollack and Brouwer [45].

^y Stern et al. [97].

cal consequences at doses far lower than those needed to produce equivalent effects in rodents.

A calculated estimate of the methanol concentration that saturates the human folate pathway is 11 mM or 210 mg/kg [2]. It should be noted, that whereas exposure of healthy humans to up to 200 ppm methanol for varying periods of time demonstrates time and concentration-dependent increases in blood methanol, no increases in blood formate were detected [31,33,40]. Short-term exposures of non-human primates to concentrations of methanol ranging from 200 to 1800 ppm resulted in increases in the levels of blood methanol from approximately 2.4 mg/l prior to exposure to 35 mg/l following exposure to 1800 ppm. There was no increase in blood formate at any dose in these studies.

There is limited information on the effects of chronic methanol exposure on toxicokinetics in humans. Leon et al. [38] reported there were no significant increases of blood methanol levels above 10 mg/l in 53 healthy adults who for 24 weeks consumed daily doses of aspartame that yielded a methanol equivalent dose of ~7.5 mg/kg. Information from non-human primates [52] indicates that long-term exposure (exposure for 2.5 h each day for ~300 days) resulted in an increase in methanol clearance rates with no increase in blood formate at exposure levels up to 1800 ppm. From these data it is reasonable to conclude that inhalation of methanol at doses up to 1800 ppm is unlikely to result in elevated blood formate levels in healthy humans. However, there are no toxicokinetic data on chronic methanol exposures in humans with marginal folate tissue concentrations—a condition that is of concern for susceptible populations. There are limited data to indicate that a single 2-h exposure of folate deficient non-human primates to 900 ppm methanol vapor did not increase blood formate levels [54].

Finally, it is to be noted that several pharmacokinetic models have been developed for the extrapolation of methanol data [45,57,62]. These models are of value in better understanding the dose and metabolite effects of high doses of methanol in rat and mouse studies. The Horton et al. [53] model is a careful attempt to develop PBPK models for methanol in rats, monkeys, and humans. The authors included some lower methanol exposure conditions for the rodent studies, which increases confidence in extrapolating results to humans. The importance of having models account for relative respiratory uptake so as not to overestimate lung absorption was reported by Fisher et al. [56]. The Panel notes that Environ [67] performed a comparative analysis of the Perkins et al. [57] and Horton et al. [53] models that provides insights as to the model features and differences. The Expert Panel concludes that the existing pharmacokinetic models may be useful for future quantitative or semi-quantitative assessments of the risks posed by methanol exposure. However, such modeling was outside the scope of this Panel and would require further evaluation of the strengths and limitations of the models.

The Panel concluded that the toxicokinetic data pertaining to methanol are of sufficient breadth, depth, and quality

to contribute in a material way to evaluating the potential for methanol to pose a risk to human reproduction. There is convincing evidence that formate is the metabolite responsible for methanol toxicity associated with systemic clinical signs, metabolic acidosis, and ophthalmic effects. Since humans and other primates oxidize formate less efficiently than rodents and other laboratory animal species, they accumulate formate at lower doses of methanol than do other species.

2.6.2. General toxicity

The primary sources of information used by the Panel on the general toxicity of methanol were the reviews of IPCS [1] and Kavet and Nauss [2].

2.6.2.1. Human data. Information about methanol toxicity in humans from high levels of exposure is available from acute intoxications (poisonings) in the general population, occupational exposures, and laboratory studies. The minimal lethal dose for methanol in untreated humans has been reported as a range of 300–1000 mg/kg bw [1]. Typical findings in acute methanol toxicity are temporary mild central nervous system depression followed by an asymptomatic period with a duration of 12–24 h that is followed by metabolic acidosis. Ocular toxicity also develops in parallel with these effects. In severe poisonings, abdominal pain and difficulty breathing can occur and progress to coma and death due to respiratory failure. Five epidemiological studies reported symptoms such as headaches, dizziness, blurred vision, nausea, and/or eye irritation in workers exposed to methanol at concentrations exceeding the occupational limit of 200 ppm [1,2]. Two well controlled studies exposed healthy adults to 200 ppm methanol for 75 min, leading to a blood methanol of 1.9 mg/l [31], or 4 h leading to a blood level of 6.5 mg/l [32], and performed a variety of neurophysiologic and neurobehavioral tests. Most results were negative. However, small effects were seen with some evoked potentials and cognitive measures in both studies. The Expert Panel was unable to develop a level of confidence that the effects were methanol related due to the low magnitude of the responses and because the single dose designs did not allow an assessment of dose response.

2.6.2.2. Experimental animal studies. Studies in animals have examined methanol toxicity following acute or repeat dosing. The lethal dose in rats and rabbits was reported to be 2–3 times higher than the lethal dose reported for monkeys and 6–10 times higher than the lethal dose reported for humans (See Table 18). Although primates, including humans, experience acidosis and adverse visual effects following acute exposure to methanol, those effects do not occur in most laboratory animals such as rats, mice, rabbits, dogs, and minipigs. For this reason, non-human primates are the most relevant animal models for studying the acute effects of methanol exposure, which are generally thought to be due to formate-induced toxicity. However, non-primate species

may be appropriate animal models for studies that examine the direct alcoholic effects of methanol. A number of studies identified the eye, brain, and liver as target organs in rats, dogs, and rabbits. The Expert Panel reviewed three short-term studies in which Sprague–Dawley rats were exposed with methanol vapors at concentrations up to 5000 ppm for 6 h per day for 4 weeks [77–79]. These authors reported nasal irritation but no consistent signs of systemic toxicity. Histological examination inconsistently revealed thyroid and liver effects in rats exposed to 300 and 2500 ppm methanol, respectively; reproductive organ lesions were not observed. No signs of systemic toxicity or histological abnormalities were observed in *Macaca fascicularis* monkeys exposed with up to 5000 ppm methanol vapors for 6 h per day for 4 weeks, but it does not appear that reproductive organs were examined [77]. No effects on weight gain or overt toxicity were noted in female *M. fascicularis* monkeys exposed to up to 1800 ppm methanol vapors for about 11 months [52].

2.6.2.3. Sufficiency statement. The Panel concluded there are sufficient data to characterize the general toxicity of methanol in humans and laboratory animals, including non-human primates. The general toxicity of methanol has been characterized in humans exposed to low doses in the laboratory and through observation of individuals accidentally or deliberately exposed to high doses. These data confirm that humans and other primates, in contrast to other species, are uniquely sensitive to the toxic effects of methanol at lower doses as a result of formate toxicity and metabolic acidosis that result from a slow rate of formate metabolism and clearance. In comparison to non-primate species, the accumulation of formate and resulting acidosis effectively limit the methanol dose tolerated by humans.

2.6.3. Genetic toxicity

Results of *in vivo* genetic toxicity assays in mice have been mixed, with both negative and positive results in micronuclei formation and chromosomal aberration assays and negative results in SCE and urine mutagenicity assays [1,80]. Negative results were obtained in the majority of *in vitro* assays that examined mutations in bacteria and yeast, DNA repair in bacteria, and SCE and cell transformation in mammalian cells; positive results were obtained in a chromosomal malsegregation assay in yeast only in the absence of metabolic activation and in a mutation assay in mammalian cells only with metabolic activation [1]. IPCS concluded that “The structure of methanol (by analogy with ethanol) does not suggest that it would be genotoxic.”

2.6.4. Carcinogenicity

There are no reliable data for evaluating carcinogenicity.

3. Developmental toxicity data

This section contains evaluations of original studies.

3.1. Human data

Hantson et al. [103] reported a case of a 26-year-old woman who ingested 250–500 ml of methanol in the 38th week of pregnancy. Five hours after methanol ingestion, the woman was slightly acidotic and had a serum methanol level of 2300 mg/l and a formic acid concentration of 336 mg/l. Treatment consisted of ethanol and bicarbonate administration together with hemodialysis. Six days later, the woman gave birth to an infant with no signs of distress; Apgar scores were 9/10 and 10/10 at 1 and 5 min, respectively. At the time of birth, the blood formic acid level was 2.4 mg/l in the mother and was below the detection level in the infant. A 10-year follow-up of the child revealed no visual disturbances.

Strengths/weaknesses: This is a report of clinical findings and the outcome of a single patient with methanol poisoning. There appears to be a discrepancy in the units used by authors for expressing methanol concentrations in mass versus molarity; based on the high level of intake, it appears that the unit of mass is correct. Case reports by their nature provide anecdotal information that sometimes is of value in formulating or revising research hypotheses.

Utility (adequacy) for CERHR evaluation process: Very limited.

Lorente et al. [104] investigated the role of maternal occupational exposure in occurrence of cleft lip and palate. Data from the study was obtained from a multicenter European case-referent study utilizing six congenital malformation registers between 1989 and 1992. Occupational exposures during the first trimester were studied in 851 women; 100 cases had infants with oral clefts and 751 referents had infants without oral clefts. The subjects were interviewed to determine occupational history and the types of products used on the job. An industrial hygienist reviewed interview responses to determine the probability of chemical exposures. Confounding factors considered included maternal age, socioeconomic status, residence, urbanization, country of origin, and medical history. Subjects were interviewed about smoking, and alcohol intake but it is not clear if the analyses considered those factors. Data were analyzed by estimating an adjusted odds ratio for each type of exposure and then conducting a stepwise logistic regression on all exposures with $P \leq 20\%$. Analyses determined that at least 10% of the subjects were likely exposed to methanol during the first trimester of pregnancy. Odds ratios of 3.61 (95% CI: 0.91–14.4) and 3.77 (95% CI: 0.65–21.8) were calculated for methanol exposure and occurrence of cleft palate only and cleft lip with or without cleft palate, respectively. Although these ratios are elevated, they are consistent with the null hypothesis of no increased risk for orofacial clefts after occupational exposure to methanol. The authors reported no association between methanol exposure and oral clefts. Associations were reported for aliphatic aldehydes, glycol ethers, biocides, lead compounds, antineoplastic drugs, trichloroethylene, and aliphatic acids. Authors concluded that caution is required in the interpretation of these

results due to the small numbers of subjects studied, but emphasized that some of these compounds are known or suspected reproductive toxicants.

Strengths/weaknesses: This study is unique in that it examines methanol exposure in humans and developmental outcomes.

Several weaknesses were noted in the study design. The study was not designed to look specifically at methanol. Presumably most subjects were exposed to mixtures of chemicals. Exposure assessments were conducted according to occupation without individual measurements of chemical exposures. Methanol exposures were highly correlated with aliphatic alcohols in general. For methanol exposure as a subgroup, the numbers are too small to reach statistical significance (only two with cleft palate and four with cleft lip with or without cleft palate exposed). Statistical procedures were not clearly defined. For example, Table 5 in the study includes 11 significant exposures but these are apparently lumped together in the table into the three chemical families. Criteria for exclusion in the backward stepwise regression were not stated. There was no analysis of respondent/nonrespondent comparison.

Utility (adequacy) for CERHR evaluation process: If true, the odds ratios of 3.61 and 3.77 reported in this study between maternal methanol exposure and the risk of cleft lip and palate in offspring are substantial. Several factors limit the confidence that can be placed in a causal interpretation of these data, however. First, the confidence intervals around these point estimates are wide, and fail to exclude the number one, indicating that the *P*-value associated with the odds ratio is not statistically significant. Thus, the null hypothesis of no association cannot be formally rejected. Second, exposure was classified simply as “yes” or “no” on the basis of job title, with no information available on an individual’s exposure. Therefore, the study provides no information that is useful in establishing dose–response relationships. Third, many of the 96 chemical exposures were highly correlated with one another, although the authors attempted to reduce the resulting confounders by considering “only the broader exposure, representative of the chemical family. . .” and by selecting one exposure to be representative of a particular occupation (e.g. hairdressers). Backwards logistic regression analyses were conducted in which several candidate chemical exposures were included as predictors, but to the extent that the exposures were confounded, the resulting coefficients might be biased. In any case, for neither endpoint was the variable representing the general class of aliphatic alcohols retained in the final model, indicating that exposure to this class of chemicals was not associated with excess risk.

This study was not designed to look specifically at methanol and no individual exposure measurements were made. This limits any utility for the Panel. Due to the small numbers, the high correlation with other aliphatic alcohol exposure, and the resulting lack of statistical significance found, the Panel has low level of confidence in this study to

provide elucidation of any link between methanol and the outcomes that were investigated.

Because methanol is metabolized by a folate-dependent pathway, the Expert Panel reviewed a limited number of epidemiological studies that examined folate supplementation and birth defects such as neural tube abnormalities, cleft lip, and cleft palate. A comprehensive literature search and review was not conducted since that is beyond the scope of this Panel. The majority of the studies reviewed were selected from the bibliographies of two animal studies addressing this issue [80,105]. The intent of the Panel was to briefly review some human studies addressing the issue of folate supplementation during pregnancy in order to obtain an understanding of effects observed, limitations commonly associated with these types of studies, and the relevancy to methanol toxicity in humans.

Numerous other studies have been conducted to address the issue of folate acid supplementation and oral clefts or neural tube defects and are summarized in recent reviews by Hartridge et al. [106] and Kalter [107], respectively. The studies discussed in the two reviews are presented in Tables 22–25 in order to provide the reader with information about the size of the database and the overall findings. The majority of the studies in the tables were not reviewed by the Panel. The Panel did review studies by Peer et al. [108], Tolarova and Harris [109], Shaw et al. [110], Czeizel and Dudas [111], MRC [112], and Hernandez-Diaz et al. [82].

Peer et al. [108] conducted a study to determine the effects of vitamin B₆ and folic acid supplementation in women who had previously given birth to at least one child with cleft lip and/or palate. Of the 594 women in the study, 418 did not receive vitamins and 176 were given vitamins containing 5 mg folic acid and 10 mg vitamin B₆ during the first trimester of pregnancy. The percentages of children with cleft/lip and/or palate were 4.7% in the group without vitamin supplementation and 2.2% in the group that received vitamins. The authors believed their study to be suggestive but not statistically significant. This group continued to study this issue and the complete findings are listed in Table 23 under Briggs and Peer.

Strengths/weaknesses: Several limitations in study design were noted. The value of this study is limited by its apparent ad hoc nature. The authors claim that the study “was begun to determine the effects of a prenatal vitamin capsule, supplemented with 5 mg of folic acid and 10 mg of B₆, administered during the first trimester to women who had previously given birth to one or more cleft lip and or cleft palate children.” Numerous methodological details are poorly reported. A total of 594 women were involved, although the source(s) of these patients, as well as the inclusion and exclusion criteria applied, are not clearly presented. The reasons why only 176 took the vitamin supplement are also not explained, although women who became nauseated when taking them were advised to discontinue and were “dropped from the study.” How the compliance of the 176 women was ascertained is not described, nor are

Table 22

Summary of case-control retrospective studies addressing folic acid and oral clefting, Hartridge et al. [106]

Reference	Study design	Cases	Controls	Results
[113]	Mothers asked about periconceptional diet and FA use	Mothers of 59 infants with midline birth defects	Mothers of 115 infants without defects	No association between defects and diet or FA use
[114]	Medical records reviewed and mothers asked about FA use	Mothers of 17,300 infants with defects	Mothers of 30,663 infants without defects	FA significantly protected against oral clefts, cardiovascular defects and NTD
[115]	Mothers asked about periconceptional vitamin use	Mothers of 146 infants with oral clefts	Mothers of 90 infants with genetically-related diseases	No significant differences in vitamin use between groups
[116]	Mothers asked about periconceptional diet and FA use	Mothers of 303 infants with oral clefts	Mothers of 1167 infants with defects other than oral clefts, NTD, or other midline defect	FA did not significantly protect against oral clefts
[117]	Medical records reviewed for preconceptional drug history and prescribed vitamins	Mothers of 676 infants with oral clefts	Mothers of 676 infants without defects	FA did not significantly protect against oral clefts
[118]	Mothers asked about post conception drug and vitamin use	Mothers of 599 infants with oral clefts	Mothers of 599 infants without defects	Vitamins and iron did not significantly protect against oral clefts
[110]	Mothers asked about periconceptional diet, vitamin, and FA use	Mothers of 731 infants with oral clefts	Mothers of 734 infants without malformations	Significant reduction in cleft lip/palate with FA use

FA: Folic acid; NTD: neural tube defect.

Table 23

Summary of prospective supplement trials addressing folic acid and oral clefts [106]

Reference	Treatment	Number of infants or fetuses evaluated: treated/control	Percentage of oral clefts: treated vs. control
Conway [119]	MV with 0.5 mg FA	59/78	0% vs. 5.1% ^a
Fraser and Warburton [115]	Vitamins	156/383	1.9% vs. 5.7% ^b
Briggs [120]	MV with 5 mg FA	348/417	3.2% vs. 4.8% ^a
Tolarova and Harris [109]	MV with 10 mg FA	214/1901	1.4% vs. 4.0% ^c
Czeizel [121]	MV with 0.8 mg FA	2471/2391	0.16% vs. 0.21% ^b

MV: Multivitamin; FA: folic acid.

^a Statistical significance was not determined.^b Results were not statistically significant.^c Results were statistically significant.

Table 24

Summary of case-control retrospective studies addressing folic acid and neural tube defects [107]

Reference	Study design	Cases	Controls	Results
[123]	Periconceptional use of MV examined	Mothers of 347 infants with NTD	Mothers of 2829 infants without defects or with defects other than NTD	MV appeared to protect against NTD
[124]	Mothers asked about periconceptional MV or FA	Mothers of 571 infants or fetuses with NTD	Mothers of 573 normal infants and mothers of 546 infants with defects other than NTD or experiencing pregnancy complications	No apparent protective effect of MV or FA
[125]	Mothers asked about diet and vitamin use before and during pregnancy	Mothers of 77 infants with NTD	Mothers of 154 normal infants and 77 infants with defects other than NTD	No association between vitamin use and NTD
[126], [127]	Mothers asked about periconceptional diet and vitamin use with and without FA	Mothers of 436 infants or fetuses with NTD	Mothers of 2615 infants without NTD or oral clefts	MV significantly decreased NTD (relative risk = 0.2–0.6); possible dose-related decreased by dietary FA
[128]	Mothers asked about periconceptional diet and MV use	Mothers of 549 infants with NTD	Mothers of 540 normal infants	Vitamin use protected against NTD and dietary FA intake appeared to decrease NTD in dose-related manner

FA: Folic acid; NTD: neural tube defects; MV: multivitamins.

Table 25
Summary of prospective supplement trials addressing folic acid and neural tube defects [107]

Reference	Treatment	Number of infants or fetuses evaluated: treated/control	Percentage of NTD: treated vs. control
[129]	MV with 0.36 mg FA	187/320	0.5% vs. 5.6% ^a
[130]	2 mg FA	44/51	0% vs. 7.8% ^b
[112]	4 mg FA or 4 mg FA and MV	593/602	1.0% vs. 3.5% ^a
[131]	0.36 mg FA or 0.36 mg FA and MV	89/172	0% vs. 0.58% ^c
[132]	Mothers undergoing prenatal screening asked about using MV with FA (0.10–1.0 mg)	7261/3157	0.12% vs. 0.35% ^b
[133]	0.4 mg FA taken periconceptionally	Northern Chinese Province: (high risk area): 13,012/13,369 Southern Chinese Province (low risk area): 58,638/104,320	Northern Chinese Province (high risk area): 0.13% vs. 0.65%, risk ratio = 0.10–0.43 Southern Chinese Province (low risk area): 0.07% vs. 0.08%, risk ratio = 0.36–0.97

MV: Multivitamin; FA: folic acid; NTD: neural tube defects.

^a Results were statistically significant.

^b Statistical significance was not discussed in review.

^c Results were not statistically significant.

the procedures for confirming the presence or absence of cleft lip and/or palate in offspring. No statistical analyses of the data are reported. At the end of the report, the authors request that “additional colleagues will. . . send their data to the senior author,” apparently to be added to the database. It is not clear whether study procedures to be followed have been shared with these colleagues, however, bringing into question whether such data will be useful for comparison.

Utility (adequacy) for CERHR evaluation process: Overall, this study is of little use.

Tolarova and Harris [109] conducted a study to determine if periconceptional multivitamin and folic acid supplementation reduces the risk of giving birth to an infant with cleft lip and/or palate in high risk groups. The subjects for this study were obtained from a registry in the Czech Republic. Subjects consisted of women who previously gave birth to a child with cleft lip and/or palate (CL ± P) between 1970 and 1982, women with cleft lip or palate, or women married to someone with cleft lip or palate born between 1930 and 1962. Subjects with syndromic or familial cases of cleft palate were excluded from the study. The supplementation group consisted of 221 women who agreed to take multivitamins containing 10 mg folic acid for at least 2 months prior to conceiving and during the first 3 months of pregnancy. The control group consisted of 1901 women who either refused to participate in the study, began taking the multivitamin after the embryonic period, or stopped taking the vitamin before or during the embryonic period. Women participating in the study were given physical exams that included a gynecological evaluation and blood work. Unless affected by a seasonal affliction such as allergy, the women were urged to plan pregnancies for late spring or summer when more fresh produce is available. Statistical significance of findings were determined by chi-square tests and Fisher’s exact test. Of the 214 informative pregnancies in the supplemental group, three infants were born with oral cleft defects. One female infant had bilateral cleft lip and palate, one female had unilateral cleft

lip and palate, and one male had a unilateral cleft lip. The authors noted a 65.4% decrease in clefts compared to expected values since the expected value was 8.7 cases with cleft based on family history ($P = 0.031$). The incidence of cases of clefts in the supplemental group (1.4%) was lower than the incidence in the control group (4.05%). Authors noted that supplementation is most effective in preventing unilateral cleft (82.6% decrease, $P = 0.024$) and in males versus females. The authors concluded that further studies are needed to determine whether the effective agent in periconceptional vitamin supplementation is folic acid, multivitamins, or both.

Strengths/weaknesses: The strengths of this study, conducted in the Czech Republic from 1976 to 1980, include the great care taken to assemble a homogeneous sample of women at increased risk of producing offspring with cleft lip and/or palate. A detailed 10-step protocol was implemented in order to identify other medical causes of reproductive morbidity, as well as to eliminate syndromic and familial cases of CL ± P.

The weaknesses of the study are similar to those of other studies on this topic. First, women were not randomized to the folate supplementation and comparison groups, leaving open the possibility of residual confounding by some factors associated with a woman’s choice to supplement. Second, the supplementation consisted not only of folate but of a variety of vitamins as well, precluding the certain attribution of a beneficial effect of supplementation solely to folic acid. Third, the analyses, particularly those involving subgroups are limited by the small numbers of cases. For example, only three cases of CL ± P occurred among the 214 infants born to supplemented women, yet the authors draw fairly strong inferences, without any statistical basis, of the beneficial effect of multivitamin supplementation on male probands with unilateral cleft. The occurrence of one additional child with clefting in one or another of the subgroups would have changed the results (e.g. the percentage reduction in occurrence) dramatically.

Utility (adequacy) for CERHR evaluation process: The results of this study are consistent with those of several other experimental and observational studies in suggesting a reduction in oral clefting among women who took multivitamin supplements, but a causal interpretation is difficult to support.

Shaw et al. [110] conducted a population-based case-control study to investigate if maternal multivitamin use reduced the risk of cleft palates in infants. California birth defect records from 1987 to 1989 were reviewed to identify infants or fetuses with cleft palate and/or cleft lip. Interviews were conducted in 731 cases and 734 controls to determine types and frequency of maternal supplement and cereal intake from 1 month before conception to 3 months afterwards. The information was used to estimate maternal folate intake levels. Confounding factors that were controlled for included race, ethnicity, education, age, gravidity, smoking, and alcohol use. Use of folate-antagonistic medications and family history of oral facial clefts and epilepsy or diabetes were also considered. It was found that women who took folic acid-containing multivitamins periconceptionally have a 25–50% decreased risk of having children with orofacial clefts (odds ratios ranged from 0.50 with 95% CI of 0.36–0.68 to 0.73 with 95% CI of 0.46–1.2, depending on cleft phenotype). The risk of oral clefts was also reduced in women who did not take vitamins but ate folate-supplemented cereals. The authors cautioned that the association may not be due to folic acid but to other factors correlated with vitamin use such as another vitamin or mineral found in supplements or healthy behavior.

Strengths/weaknesses: The care with which this case-control study was conducted and reported provides a strong basis for confidence in its results. It is a population-based study, using the data of the California Birth Defects Monitoring Program. A large case series was assembled from births within a well-defined geographic/temporal setting, and controls were randomly selected. The case definitions applied were explicit and rigorously applied and verified. The statistical analyses were sophisticated, addressing the importance of a variety of potential confounders, including use of known folate antagonist medications, as well as of potential effect modifiers.

A weakness of this study is its retrospective design. Women were not randomly assigned to receive folate or non-folate preparations, reducing the confidence that can be placed in conclusions drawn about the causal role of folate in reducing the occurrence of orofacial clefts, compared to other behaviors correlated with use of multivitamins containing folic acid. In addition, as the authors note, constituents of the multivitamin supplements other than folic acid might have been responsible for the beneficial effects. Folate use during pregnancy was ascertained by interviews conducted an average of 3.5 years after delivery. At that time, women were asked about consumption habits during the 1-month period preceding conception and the 3-month period following conception. At worst, this could introduce

recall bias and at best, imprecision of recall regarding folate dose. The authors do provide persuasive evidence against the occurrence of recall bias, however. The folate dose had to be reconstructed based on the assumed folate contents of different vitamin preparations and cereals that the women reported using, creating the possibility of exposure misclassification and reduced precision of effect estimates. This might explain why no dose–response relationship was seen, with the odds ratios (OR) associated with different folate doses being more or less equivalent in magnitude. For most phenotypes and most doses, the 95% CIs for the estimated odds ratios included 1, meaning that the odds ratios were not significantly different from 1. The ORs for isolated cleft lip and/or palate did not include 1 for “any use of multivitamins with folic acid” or with the two lower doses (0–0.4 mg per day, 0.4–0.9).

Utility (adequacy) for CERHR evaluation process: In summary, the results of this very well-conducted study are consistent with the hypothesis that use of folic-acid containing multivitamin supplements in early pregnancy are associated with reduced occurrence of orofacial clefting, and, furthermore, that concurrent alcohol use acts synergistically in producing this protective effect.

As noted above, Hartridge et al. [106] reviewed studies examining the issue of folic acid supplementation and oral cleft defects. A summary of the retrospective and prospective studies reviewed by Hartridge et al. are included in Tables 22 and 23, respectively.

The Panel noted the relevancy of a case-control study conducted by Hernandez-Diaz et al. [82] to determine if prenatal exposure to folic acid antagonist drugs increases the risk of oral clefts or heart and urinary tract defects and if those risks are reduced by folic acid supplementation. Two types of folate antagonistic drugs were evaluated: those classified as dihydrofolate reductase inhibitors and anti-epileptic drugs which affect folate through other mechanisms. The study was based on interviews conducted in mothers of live-born infants in Boston, Philadelphia, and Toronto from 1976 to 1998 and in Iowa from 1983 to 1985. Cases included 3870 infants with non-syndromic cardiovascular defects, 1962 infants with oral clefts, and 1100 infants with urinary tract defects. Controls included 8387 infants with malformations other than NTD or those described for case infants. Within 6 months after giving birth, mothers of the infants were asked about medication use (including vitamins and minerals), demographic characteristics, medical history, habits, and occupations. An unconditional logistic-regression analysis was used to determine relative risk (RR) and 95% confidence intervals (CI). Confounding effects considered in the analysis included time period of interview, geographic region, infections during pregnancy, education level, smoking, alcohol intake, previous affected pregnancies, family history of birth defects, infant’s birth order, planning of pregnancy, diabetes mellitus in mother, and maternal age, race, and weight. Exposure to dihydrofolate inhibitors during the second or third months after the last menstrual period

was associated with increased risk of cardiovascular defects (RR = 3.4, 95% CI = 1.8–6.4) and oral clefts (RR = 2.6, 95% CI = 1.1–6.1). Intake of anti-epileptic drugs during the second or third month after the last menstrual period was associated with increased risk of cardiovascular defects (RR = 2.2, 95% CI = 1.4–3.5), oral clefts (RR = 2.5, 95% CI = 1.5–4.2), and urinary tract defects (RR = 2.5, 95% CI = 1.2–5.0). Stratification of results according to drug use and intake of folic acid-containing vitamins suggested that folic acid intake reduced the risks associated with dihydrofolate reductase inhibitors but not anti-epileptic drugs. The study authors concluded that folic acid antagonist use in early pregnancy increases the risks of some birth defects and that folic acid found in multivitamins may reduce these risks.

Strengths/weaknesses: This is a well-designed, very large, multi-center case-controlled epidemiological study. The study utilizes data that have been collected in a variety of diverse geographical communities. Exposure was to prescribed medications. Most of the medications are taken for prolonged periods (i.e. antiepileptics), which aids in exposure classification. An attempt to limit recall bias was made by defining controls as babies with malformations other than those of interest (i.e. oral clefts). Known confounders were accounted for and statistical methods were appropriate. While confounding, as a result of the disease states that resulted in the prescriptions for medication, could not be entirely controlled for, several medications that interfere with folate were evaluated and show consistent associations—even though the conditions that they are given for are very different. Folic acid supplementation was found to diminish these effects.

Utility (adequacy) for CERHR evaluation process: The Panel has a high level of confidence in the findings of this study. The study demonstrates an association between prenatal exposure to folic acid antagonists and development of oral clefts, cardiovascular defects, and urinary tract defects. An associated study by the same authors, not reviewed by the Panel, shows a similar association between the same medications and NTDs [122]. This study adds the observation that folic acid antagonists are associated with these birth defects to several studies that have shown that folic acid supplementation may reduce the occurrence of the defects. To the degree that methanol may interfere with folic acid metabolism, there is an as yet undefined potential to be associated with these defects.

A study was conducted in Hungary in 1984 to determine if periconceptional vitamin supplementation could reduce the incidence of first occurrence neural-tube defects [111]. Nulliparous women younger than 35 years of age were randomly administered either a multivitamin supplement containing 0.8 mg folic acid or a trace element supplement containing only copper, manganese, zinc, and low levels of vitamin C. The women were instructed to take the supplement daily for at least 1 month before attempting to become pregnant, while trying to conceive, and throughout the first 3 months

of pregnancy. Confounding factors taken into consideration included demographics, intake of valproic acid or other teratogens, and family history of NTDs. Vitamin intake was confirmed by questioning the subjects and counting unused tablets returned by the subject. Statistical significance was determined by two-tailed chi-square and Fisher's exact tests. There were no NTD's in the 2104 informative pregnancies in the vitamin supplementation group. Six cases of NTDs were observed among the 2052 informative pregnancies of the trace supplement group and the difference between the supplementation group was statistically significant. It was concluded by authors that these study results indicate that periconceptional vitamin supplementation reduced the incidence of first occurrence NTDs.

Strengths/weaknesses: The confidence that can be placed on the results of this study is enhanced by several of its characteristics. First, it is a randomized controlled trial rather than an observational study, thereby providing a stronger basis for drawing causal inferences. Second, systematic procedures were in place to ascertain compliance with the assigned treatment and to follow-up all pregnancies, including review of hospital and autopsy records, and investigation of all reports of a neural tube defect (NTD). Third, outcome definitions were clear and external oversight was provided to confirm case status. Fourth, the follow-up rate was very high, with pregnancy outcome ascertained in 99% of women who became pregnant.

Some limitations in study design were also noted. Although a significantly higher rate of NTDs was found in the trace-element group (6/2052 versus 0/2104 in the vitamin-supplement group), the numbers of cases was clearly quite small, and the significance of the treatment group differences would be changed dramatically if one case were to have occurred in the vitamin group, or one fewer case had occurred in the trace-element group. The rate in the trace-element group was exactly what would be expected to occur in Hungary, so this small number of occurrences could have been predicted and incorporated into the study design to ensure a large enough number of cases. It is presumed by the authors that it was the folate in the vitamin supplement that was responsible for the lower NTD rate, but the treatment differed in many respects. This presumption is not unreasonable based on other literature, but the complexity of the intervention represented by vitamin supplementation nevertheless leaves this possibility.

Utility (adequacy) for CERHR evaluation process: Overall, this study suggests that 0.8 mg of folate daily reduces the risk of congenital malformations, and specifically, NTDs in offspring.

The MRC Vitamin Study Research Group conducted a randomized double-blind study to determine if recurrence of NTDs (anencephaly, spina bifida, and encephalocele) could be prevented by periconceptional supplementation with folic acid and/or a mixture of vitamins [112]. The subjects of this study were 1817 women who previously had an infant or fetus affected with NTD that was not associated with the ge-

netic disorder Meckel's syndrome. The study was conducted from July of 1983 through April of 1991 with subjects from the United Kingdom, Hungary, Israel, Australia, Canada, the Soviet Union, and France. Subjects were randomly divided into four groups ($n = 449$ – 461 per group) that received capsules containing: (1) 4 mg of folic acid; (2) 4 mg folic acid and vitamins A, D, B₁, B₂, B₆, C, and nicotinamide; (3) vitamins only; or (4) no folic acid or vitamins (ferrous sulfate/dicalcium phosphate control). Capsules were taken daily from the period prior to conception through the twelfth week of pregnancy. All groups were similar in regards to members' age and outcomes of prior pregnancies. Social classes were similar between groups for subjects from the United Kingdom. A total of 298–302 informative pregnancies were evaluated in each group. Six infants or fetuses with NTDs were observed in the folic acid groups, while 21 were observed in the groups that did not receive folic acid. Therefore, folic acid supplementation reduced the risk of NTD by 72%. The relative risk for folic acid versus no folic acid supplementation was 0.28 (95% CI of 0.12–0.71). The other vitamins did not demonstrate a protective effect.

Strengths/weaknesses: Strengths of the study include the fact that it was a large trial, that women were randomly allocated to a treatment group, that the trial was double-blinded, that the specific role of folic acid rather than multi-vitamins was examined, and that an attempt was made to determine if women complied with the treatment by counting pills and collecting serum samples for folate levels. These strengths addressed many of the concerns raised over previous trials in which the role of folic acid in NTDs had been examined.

Weaknesses of the study include that this study was conducted in women who had already had a child with a NTD. It is possible that the underlying mechanism for recurrence of NTDs may be different from that for occurrence of such defects. Another weakness was the large dose of folic acid administered in this trial. The administered dose was 10 times the RDA, and it is not clear if a lower dose (one which may be more in line with normal human exposures) would have also been protective.

Utility (adequacy) for CERHR evaluation process: This trial is the strongest study to demonstrate that folic acid could prevent the recurrence of NTDs. However, the high dose of folate used remains problematic. Therefore, the Panel's confidence in these data is moderate-to-high.

As mentioned previously, Kalter [107] reviewed studies examining the issue of folic acid supplementation and NTDs. A summary of the retrospective and prospective studies reviewed by Kalter are included in Tables 24 and 25, respectively. The tables include additional details that were obtained from the original studies by the Expert Panel.

McPartlin et al. [59] studied the breakdown and excretion of folic acid in pregnant women. At one time point during each trimester of pregnancy and postpartum, six pregnant women were administered a nutritionally complete liquid enteral diet for 42 h. During the last 24 h of receiving the special diet, urine was collected and assayed for the breakdown

products of folate, *p*-aminobenzoylglutamate (pABGlu) and *p*-acetamidobenzoylglutamate (apABGlu). Six non-pregnant women of similar ages underwent the same procedure. The breakdown and excretion of folate during the first trimester was equivalent to the non-pregnant controls. During the second and third trimesters, breakdown and excretion of folate was significantly higher than in the non-pregnant controls and were highest during the second trimester. Postpartum breakdown and excretion of folate was not statistically different from the non-pregnant women.

Strengths/weaknesses: Although the group numbers were small, the study was well conducted.

Utility (adequacy) for CERHR evaluation process: This small but well-conducted metabolic study ($n = 6$) demonstrates that folate metabolism changes over the course of pregnancy, accelerating in the second trimester and remaining elevated even into the postpartum period. The findings suggest that increased folic acid intake throughout pregnancy, but especially in the second (660 μ g per day) and third trimesters (470 μ g per day), is necessary. It appeared that folate catabolism was similar in the first trimester pregnant women and the non-pregnant women, however, suggesting a need for 280 μ g per day during the period of organogenesis.

In 1996 the FDA mandated the fortification of all enriched cereal grain products with folic acid [134]. To assess the effects of folate fortification, the Centers for Disease Control compared serum and red blood cell folate levels in women of childbearing age (15–44 years) who participated in National Health and Nutrition Examination Surveys (NHANES) in 1988–1994 ($n = 5254$ – 5261) versus 1999 ($n = 658$ – 663). Mean serum folate levels rose from 6.3 ng/ml in 1988–1994 to 16.2 ng/ml in 1999. In red blood cells, which provide a better measure of long-term folate status, the respective folate levels increased from 181 to 315 ng/ml. The increases in folate levels occurred whether or not the women used vitamin/mineral supplements. Authors noted that the national health objective for 2010 is to increase the median red blood cell folate level to 220 ng/ml in non-pregnant women of childbearing age.

3.1.1. Conclusions for folate studies

These studies are generally consistent with the hypothesis that periconceptional supplementation with vitamin preparations that include folate in varying amounts is associated with a reduction in the risk of birth defects including NTDs and orofacial clefts. The Hernandez-Diaz et al. [82] study is consistent with an association between toxicant interference with folate status and the development of oral clefts and NTDs. Due to the greater folate level in rats compared to humans, such an alteration could have a significant effect if it were to occur in humans. The reduced risk of NTDs observed in human studies after multivitamin or folate supplementation suggests that women with low folate status may be more sensitive to methanol exposure since they would be less able to metabolize methanol. Studies in mice [80,105] have provided evidence of increased NTDs and cleft palates in

offspring of methanol-exposed folate-deficient dams. However, several factors need to be considered in a comparison of the human and mouse effects. Folate levels in the mouse are far greater than in primates, and relevant enzyme activity in metabolism of formate may be different. Artificially folate-deficient mice may be deficient in other relevant nutritional components. Relatively large embryotoxic doses of methanol were used to induce these defects in the rodents. Neither liver, plasma, nor RBC folate activity was significantly impacted by methanol exposure in the mice. Therefore, evidence to date suggests that women of low folate status may be more susceptible to the adverse developmental effects of methanol, but further work is needed to clarify this point.

3.2. Experimental animal data

3.2.1. Prenatal development

As part of an effort to assess teratogenic effects of industrial alcohols, Nelson et al. [98] studied the effects of prenatal methanol exposure in Crl: Sprague–Dawley rats. Nelson et al. exposed 15 pregnant rats per group to 0, 5000, 10,000, or 20,000 ppm methanol (99.1% purity; nominal concentrations) in air for 7 h per day (Table 26). The two lower dose groups were exposed on gd 1–19 whereas the 20,000 ppm group was exposed on gd 7–15. (*It appears that some doses were evaluated in separate experiments; the rationale for dose selection was not discussed.*) Two groups of 15 control rats (one for the 10,000 and 20,000 ppm group and one for the 5000 ppm groups) were exposed to air only. Blood methanol levels in concurrently-exposed, non-pregnant rats

on days 1, 10, and 19 of exposure were measured by GC at 1000–2170, 1840–2240, and 5250–8650 mg/l in the low- to high-dose group, respectively. Background levels of blood methanol were not provided. The study authors assumed that blood methanol levels in pregnant rats were similar to those determined in non-pregnant rats. Maternal toxicity was evidenced by a slightly unsteady gait only in the high dose group during the first few days of exposure; there were no effects on bodyweight or food intake at any dose. The number of litters evaluated included 30 in the control group, 13 in the low dose group, and 15 in the two highest dose groups. Statistical analysis of fetal data included analysis of variance (ANOVA) for weight effects, the Kruskal–Wallis test for parameters such as litter size and percent alive/litter, and Fisher's exact test for malformations. For examination of skeletal effects, half the fetuses were fixed in 80% ethanol, macerated in 1.5% KOH, and stained with alizarin red S. The other half of fetuses were fixed in Bouin's solution and examined for visceral effects. Statistically significant and dose-related reductions in fetal weight were observed in the two highest dose groups. The increased number of litters with skeletal or visceral malformations was statistically significant at the 20,000 ppm dose. A range of visceral malformations were observed including exencephaly and encephalocele. Rudimentary and extra cervical ribs were the skeletal effects observed at the greatest frequency at the 20,000 ppm dose. The authors concluded that methanol was a definite teratogen at 20,000 ppm, a developmental toxicant (decreased fetal weight) and possible teratogen (numerical elevation of some malformations) at 10,000 ppm, with a fetal no effect level of 5000 ppm. (A

Table 26
Summary of developmental toxicity study in rats [98]

Experimental regimen	Number ^a	Dose in ppm (mg/l blood level)	Maternal effects	Fetal effects
Prenatal toxicity study. Nelson et al. [98] exposed Crl:Sprague–Dawley rats in control and 2 lowest dose groups to methanol vapors on gd 1–19. The highest dose group was exposed on gd 7–15. Methanol concentrations were measured inside chambers. Food and water intakes and bodyweights were measured weekly in dams. The dams were sacrificed on gd 20 and examined for implantation sites and resorptions. Corpora lutea were measured in controls and two lowest dose groups. Fetuses were examined, sexed, and weighed. One half of fetuses were examined for skeletal malformations and the other half for visceral malformations	15 15 15	0 5,000 (1,000–2,170) 10,000 (1,840–2,240) 20,000 (5,250–8,650)	NE NOAEL Unsteady gait during initial exposure NE on food intake or bodyweight gain	NOAEL ↓Fetal weight (7%) ↓Fetal weight (12–16%) ↑ Litters with abnormal fetuses (93% vs. 0%) ↓Normal fetuses (46% vs. 100%) ↑Litters with skeletal malformations (14 vs. 0 litters with 79% fetuses affected) ^b ↑Litters with visceral malformations (10 vs. 0 litters with 29% of fetuses affected) ^b ↑Exencephaly (4 fetuses in 3 litters vs. 0) ↑Encephaloceles (3 fetuses in 2 litters vs. 0)

NE: No effects.

↑: Statistically Significant increase.

↓: Statistically significant decrease.

^a Number of pregnant dams and litters evaluated.

^b Malformations noted in cranium, vertebrae, ribs, eye, brain, and cardiovascular and urinary systems.

maternal NOAEL of 10,000 ppm was noted by the Expert Panel.)

Strengths/weaknesses: This is a prenatal developmental toxicity study of standard design with the number of animals per dose group ($n = 15$) considered adequate at the time the study was performed. Endpoints observed were appropriate for a prenatal toxicity study. There was an effort to determine blood methanol concentrations. The purity of methanol was reported, chamber methanol concentrations were monitored, and adequate statistical analyses were conducted.

A limitation was the measurement of blood methanol concentrations in non-pregnant instead of pregnant females. Although a different (shorter) duration of exposure was used for the 20,000 ppm group, the limiting effect is minor given that this dose was clearly a developmental toxicant and teratogen.

Utility (adequacy) for CERHR evaluation process: Maternal and developmental NOAELs were identified for this study. The Panel's confidence in the data is high and it has clear utility in defining the broad dose range at which prenatal developmental toxicity is observed in the rat.

Slikker and Gaylor [135] evaluated the developmental toxicity data from the Nelson et al. [98] study using a quantitative dose–response risk assessment model. It was determined that excess risks of 1 in 1000 for reduced fetal weight and increased fetal brain malformations would oc-

cur from exposure to methanol vapors at concentrations of 980 and 1100 ppm, respectively. Slikker and Gaylor [135] concluded that adjustment of the risk values by 10 for interspecies sensitivity (intraspecies sensitivity accounted for in model) would result in values (98 and 110 ppm) comparable to those obtained by adjustment of the NOAEL (5000 ppm) with 100 (50 ppm) for intra- and interspecies variability.

Rogers et al. [96] examined the sensitivity of Crl:CD-1 mice to the developmental toxicity of inhaled methanol (Table 27). In the original three block design, groups of mice were exposed to one of four doses of methanol vapors (Fisher Scientific [136] Optima grade, $\geq 99.9\%$ purity) for 7 h per day on gd 6–15. The nominal doses and numbers of mice per dose (in parentheses) were air-exposed control [114], 1000 [40], 2000 [80], 5000 [79], and 15,000 ppm [44]. A final block of mice was added to fill in intermediate concentrations of 7500 [30], and 10,000 ppm [30]. (*The rationale for dose selection was not discussed.*) During the 7-h inhalation exposure period, treated and air exposed mice were deprived of food but had access to water. An additional set of 88 controls were not handled (remained in their home cage) and fed ad libitum. Another group of 30 control mice remained in their home cage and were food deprived for 7 h per day on gd 6–15. Approximately three pregnant mice per block/treatment group were killed following exposure on gd 6, 10, or 15 and their blood was collected

Table 27
Summary of developmental toxicity study in mice, Rogers et al. [96]

Experimental regimen	Number ^a	Dose in ppm (mg/l blood level)	Maternal effects	Fetal effects
Prenatal toxicity study. Rogers et al. [96] exposed Crl:CD-1 mice to methanol vapors for 7 h per day on gd 6–15. Methanol levels inside inhalation chambers were verified. Dam bodyweights were measured on alternate days and at sacrifice on gd 17. Resorption sites were assessed and all fetuses were examined externally. With the exception of the fetuses in the 7500 and 10,000 ppm groups, half the fetuses were examined for skeletal defects and the other half for soft tissue defects. Fetuses in the 7500 and 10,000 ppm groups were only examined for exencephaly and encephaloceles	70 (59–70)	0 (1.6)		NOAEL
	26 (24–26)	1,000 (97)	NE	↑Cervical ribs/litter (50% vs. 28%)
	41 (41)	2,000 (537)	NE	↑Cleft palate/litter (9% vs. 0.2%)
	40 (39–40)	5,000 (1,650)	NE	↑Exencephaly/litter (7% vs. 0%)
				↑Cervical ribs/litter (74% vs. 28%)
				↑Total skeletal defects/litter (29% vs. 12%)
	15 (15)	7,500 (3,178)	1 Death	↓Live pups/litter (8.6 vs. 9.9)
				↑Cleft palate/litter (47% vs. 0.2%)
	11 (11)	10,000 (4,204)	1 Death	↑Exencephaly/litter (7% vs. 0%) ^b
				↑Complete resorption ($n = 5$ litters vs. 0)
			↓Live pups/litter (7.3 vs. 9.9).	
			↓Fetal weight (13%)	
			↑Cleft palate/litter (53% vs. 0.2%)	
			↑Exencephaly/litter (27% vs. 0%)	
			↑Complete resorption ($n = 14$ litters vs. 0)	
			↓Live pups/litter (2.2 vs. 9.9)	
			↓Fetal weight (42%)	
			↑Cleft palate/litter (48% vs. 0.2%)	
			↑Exencephaly/litter (43% vs. 0%)	
			↑Cervical ribs/litter (60% vs. 28%)	
			↑Total skeletal defects/litter (100% vs. 12%)	

NE: No effects.

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a Number of pregnant dams (litters evaluated).

^b Results not statistically significant.

for plasma methanol analyses by GC. The mean plasma methanol concentrations averaged for the three gestational days were 1.6, 97, 537, 1650, 3178, 4204, and 7330 mg/l in the control to high-dose groups, respectively. Methanol plasma concentrations were dose-related, did not appear to reach saturation, and were not consistently affected by gestation day or previous days of exposure. Analysis of plasma methanol levels was conducted in a few non-pregnant mice and there appeared to be no differences compared to pregnant mice. Rogers et al. [96] noted that plasma levels at a given methanol concentration were lower in non-pregnant rats exposed through a similar protocol by Nelson et al. [98].

Following sacrifice of dams on gd 17, Rogers et al. [96] compared developmental effects in treated groups to effects in the chamber air-exposed control group. Dams and litters were considered the statistical unit and the numbers evaluated are listed under Table 27. Statistical analysis included the General Linear Models procedure and multiple *t*-test of least squares method for continuous variables and the Fisher's exact test for dichotomous variables. The chamber air-exposed control dams gained significantly less weight than both types of cage controls. Methanol exposure did not produce overt intoxication or further reduce weight gain in dams. There was a dose related and statistically significant decrease in the number of live pups per litter in groups exposed to methanol vapor doses of 7500 ppm and higher; there was also a dose-related increase in females with fully resorbed litters at 10,000 ppm and higher. Fetal bodyweights were significantly reduced at 10,000 ppm and higher. The incidence of cleft palate was increased at doses of 5000 ppm and greater. The percent incidence/litter of exencephaly was significantly increased at the 5000, 10,000 and 15,000 ppm doses (not statistically significant at 7500 ppm). Only fetuses from the 1000, 2000, 5000, and 15,000 ppm groups were examined for either skeletal malformations or visceral defects. Skeletal effects were examined in half the fetuses that were fixed in 70% ethanol, macerated with 1% KOH, and stained with Alizarin red S. Visceral effects were examined in the

other half of fetuses that were fixed in Bouin's solution. Delayed ossification effects were commonly observed at the 15,000 ppm dose whereas several skeletal anomalies were seen at doses of 5000 ppm and higher. The lowest dose at which an effect (cervical ribs) was observed was 2000 ppm. Increased cervical ribs at 2000 ppm were statistically significant in a pairwise comparison and showed a dose–response relationship with higher doses.

In this same study by Rogers et al. [96], additional pregnant mice were exposed to methanol by the oral route to determine comparability of effects between exposure routes (Table 28). On gd 6–15, 20 mice were gavaged with methanol twice daily at a dose of 2000 mg/kg for a total dose of 4000 mg/kg per day and eight control pregnant mice were gavaged twice daily with water. The dose was selected to produce blood methanol levels observed in the inhalation study at the higher doses. Twice daily gavage doses of 2000 mg/kg methanol (eight mice) on gd 6–15 gave a pattern of response similar to that seen in the mouse group exposed to 10,000 ppm by inhalation. Mean maternal blood methanol levels 1 h following the second daily exposure (3856 mg/l) were slightly lower than blood levels in dams inhaling 10,000 ppm methanol in a previous experiment (4204 mg/l). Fetal effects in the treated group included decreased fetal weight, increased resorptions, decreased live fetuses, and an increased incidence of fetuses/litter with cleft palate or exencephaly. Statistical significance of effects is indicated in Table 28.

Rogers et al. identified a developmental LOAEL of 2000 ppm and a NOAEL of 1000 ppm. Benchmark doses were also calculated. The benchmark doses for a 5% added risk (BMD₀₅) from the upper 95% confidence limit on the maximum likelihood estimates (MLE) are generally consistent with NOAELs (Table 29).

Strengths/weaknesses: Strengths of this study of prenatal development included wide range of dose levels used, quantification of internal dose through the measurement of plasma methanol levels, achievement of very stable vapor

Table 28
Summary of developmental toxicity study in mice [96]

Experimental regimen	Number ^a (mg/l blood level)	Dose (mg/kg bw per day)	Maternal effects	Fetal effects
Prenatal toxicity study. On gd 6–15, Rogers et al. [96] gavaged Crl:CD-1 mice twice daily with methanol in water at 0 or 2000 mg/kg bw per day. Blood methanol levels were measured in dams 1 h following the second daily exposure. Dam bodyweights were measure from gd 6–17. Weight gain was corrected for gravid uterine weight at sacrifice on gd 17. Resorption sites were examined. Fetuses were weighed, and examined for viability and external malformations	4	0		
	8	4000 (3856)	1 Death No effect on corrected weight gain or clinical signs of toxicity	3 Complete litter resorptions ^b ↓Live fetuses/litter (5.9 vs. 10.5) ^b ↓Fetal weight (17%) ↑Fetuses/litter with cleft palate (43.5% vs. 0%) ↑Fetuses/litter with exencephaly (28.8% vs. 0%) ^b ↑Fetuses/litter with cleft palate or exencephaly (72.3% vs. 0%)

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a Number of pregnant dams.

^b Effect not statistically significant.

Table 29
Developmental NOAELs, MLEs and BMD₀₅s, Rogers et al. [96]

Endpoint	NOAEL in ppm (blood methanol level)	MLE (ppm)	BMD ₀₅ (ppm)
Cleft Palate (CP)	2000 (537mg/l) ^a	4,314	3,398
Exencephaly (EX)	2000 (537 mg/l)	5,169	3,760
CP and EX		3,713	3,142
Resorptions (RES)	5000 (1650 mg/l)	5,650	4,865
CP, EX, and RES		3,667	3,078
Cervical ribs	1000 (97 mg/l)	824	305

^a Mean plasma methanol concentration.

concentrations, use of a sufficient number of pregnant animals for most comparisons, evaluation of appropriate endpoints for a prenatal study, appropriate statistical analyses, and calculation of benchmark doses. The study was well-controlled with the use of cage control mice that were not handled or not handled and food deprived.

Limitations included limited fetal examinations performed at concentrations of 7500 and 10,000 ppm, measurement of plasma methanol levels in only three animals at three time points, and no reporting of number of fetuses and litters with skeletal defects (only litter means reported).

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is high. The data provide adequate expression of prenatal dose-effects over a range of exposure concentrations. The results of the oral gavage study provide a minimal basis for assessing comparability of effect from inhalation and oral gavage exposure and it provides data that support the belief that blood level equivalence is the significant factor rather than route of exposure.

The Japanese New Energy Development Organization [99] sponsored a study to evaluate the effects of prenatal exposure on prenatal and postnatal endpoints in Crl:CD Sprague–Dawley rats. Rats were randomly assigned to groups ($n = 36$ per group) that were exposed to 0, 200, 1000, or 5000 ppm methanol vapors (reagent grade, stated to have <1 ppm vinyl chloride monomer and <3 ppm formaldehyde) on gd 7–17 for an average of 22.7 h per day. The low dose in the study was selected because it is the ACGIH TLV, while higher doses were based upon observations in other studies sponsored by this group. Chamber concentrations of methanol were monitored and reported. Data were analyzed by *t*-test, Mann–Whitney *U*-test, Fisher's exact test and/or Armitage's χ^2 -test.

In the assessment of prenatal development, a total of 19–24 dams per group were sacrificed on gd 20 and examined for implantation sites and number of corpora lutea. Fetuses were assessed for viability, sexed, weighed, and examined for external malformations. Half the fetuses were fixed in Bouin's solution and examined for visceral malformations. Skeletons from the remaining fetuses were stained with alizarin Red S and examined. Dams in the 5000 ppm group experienced a reduction in bodyweight gain and food and water intake (statistical significance not reported) during the first 7 days of methanol exposure; 1 died on gd 19 and another was sacrificed in extremis on gd 18. Significant

fetal effects were only observed at 5000 ppm and included increased late resorptions, reduced numbers of live fetuses, decreased fetal weight, and increased numbers of litters containing fetuses with malformations, variations, and delayed ossification. Malformations noted were ventricular septal defect, while variations were noted in the thymus, vertebrae, and ribs (including cervical ribs).

Twelve dams per group were allowed to deliver and nurse their litters. The dams were sacrificed when pups were weaned and examined for implantation sites. Statistically significant effects noted in the 5000 ppm group included prolonged gestation period (21.9 ± 0.3 days versus 22.6 ± 0.5 days in control and treated group), reduced post-implantation embryo survival ($96.3 \pm 4.2\%$ versus $86.2 \pm 16.2\%$), and number of live pups/litter (15.2 ± 1.6 versus 12.6 ± 2.5). Survival rate on postnatal day (pnd) 4 was significantly reduced (98.9% versus 81.8%). Pups were monitored for survival, growth, and achievement of developmental milestones (eyelid opening, auricle development, incisor eruption, testes descent, vaginal opening). Treatment related effects involving developmental milestones were not present when the delay in parturition was taken into consideration. Several organs (brain, thyroid, thymus, and testes) in animals prenatally exposed to 5000 ppm methanol were decreased in weight at 8 weeks of age; overall bodyweight was not adversely affected by methanol exposure.

An unspecified number of offspring were examined for reflex development and neurobehavioral tests that assessed emotional responses, learning ability, and movement coordination. Some offspring were also necropsied at weaning or later periods. Both the neurobehavioral data and necropsy data were incompletely reported. However, it does seem that treatment-related effects, if any, were confined to the 5000 ppm group. About two offspring/sex group were used in a fertility study, in which results were also incompletely reported.

The authors noted the similarity of fetal abnormality type seen in their study with those reported by Nelson et al. [98]; differences in dose level and duration between the two studies were acknowledged. **[The Expert Panel noted the post-natal component of the experimental design and was of the opinion that the level of data reporting was quite variable for different endpoints. The Expert Panel believed data were reported in sufficient detail to conclude that pregnant rats exposed to 5000 ppm methanol almost con-**

tinuously during gd 7–17 delivered litters with reduced numbers of pups at birth and with reduced survival at pnd 4. Other aspects of the postnatal study were not reported in sufficient detail to be of value to the Panel. The apparent NOAEL as determined by standard fetal examination on gd 20 was 1000 ppm.]

Strengths/weaknesses: The prenatal portion of this study (the Segment II portion) is well-designed, with adequate numbers of animals, rational choice of exposure concentrations, and clearly presented results. Chamber methanol concentrations were monitored and reported as was the purity of the methanol used for the exposures. The postnatal study adds to the confidence in the choice of NOAEL and LOAEL. Both portions of the study clearly indicate that 5000 ppm is the LOAEL and 1000 ppm is the NOAEL. The findings in the fetal examinations generally support those in the Nelson study.

A weakness is that the postnatal portion of the study is not reported with enough detail to evaluate thoroughly, although there are unambiguous positive findings at 5000 ppm. No blood levels are reported for the Segment II study. Further, categorization of fetal morphological observations into categories of malformation and variation is not useful, and should be eliminated. Cervical ribs are not generally considered variations even by those that use this categorization.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in the data is fairly high. Similarity in some of the defects observed in this study compared to the study of Nelson et al. [98] adds confidence to characterization of the developmental toxicity of methanol in the Sprague–Dawley rat. The postnatal study provides additional evidence of toxicity at 5000 ppm, including effects on several organ weights, including the brain.

Another NEDO [99] study reported a lack of teratogenic effects in monkeys that inhaled 1000 ppm methanol vapors for 22 h per day for up to 30 months. ILSI [137] concluded that the NEDO studies were not adequately reported and that findings need to be verified in other laboratories.

Cummings [138] conducted studies in rats to examine reproductive physiology and embryo/fetotoxicity following early pregnancy exposure to methanol (high purity solvent grade). Holtzman rats (from the Small Animal Supply Co.) were gavaged with water or 1600, 2400, or 3200 mg/kg bw per day methanol in water on gd 1–8 (Table 30). Based on conversion factors reported by Mole et al. [139], the author estimated that peak blood methanol levels would be 1875, 2800, and 3700 mg/l in the low- to high-dose dams, respectively. Those blood levels are estimated to equal blood levels resulting from exposure to 10,000, 15,500, or 21,000 ppm methanol vapor, respectively, for 6 h. Eight rats per group were sacrificed on gd 9, 11, and 20. Data was analyzed using general linear models and when a significant ANOVA was detected, data were further analyzed by multiple *t*-tests of least square means. On gd 9, gravid uterine weight was significantly reduced in dams at all doses and a significant decrease in implantation site weight was first noted in the mid dose group. Also noted was a significantly decreased maternal body weight and an increased number of small implantation sites with extravasated blood in the high dose group. Methanol treatment had no effect on the number of implantation sites or corpora lutea, ovarian weight, or serum levels of progesterone, estradiol, luteinizing hormone, and prolactin on the day following the last dose of methanol. An examination of embryonic development on gd 11 revealed no effects on the yolk sac diameter, fetal size, number of somites, viability, or overall development. When lit-

Table 30
Summary of developmental toxicity study in rats, Cummings [138]

Experimental regimen	Number ^a	Dose in mg/kg bw per day	Maternal effects	Offspring effects
Prenatal toxicity study. Cummings [138]	8	0		
gavaged Holtzman rats (from Small Animal Supply, Co.) with methanol in water on gd 1–8. Dams were sacrificed on either gd 9, 11, or 20, and bodyweights and gravid uterine weights were measured. Dam ovaries were weighed and examined for corpora lutea on gd 9 and gd 20. On gd 9, maternal hormone levels were measured and implantation sites were examined. On gd 11, embryos were examined for viability, development, and growth. On gd 20, fetuses were weighed and examined for viability and gross external malformations	8	1600	↓Gravid uterine weight (gd 9)	NE
	8	2400	↓Gravid uterine weight (gd 9)	NE
	8	3200	↓Implantation site weight (gd 9) ↓Bodyweight gain (gd 9)	NE on embryonic viability, yolk sac, crown rump or head length, or number of somites (gd 11)
			↓Gravid uterine weight (gd 9) ↓Implantation site weight (gd 9) ↑Atypical implantation sites ^b NE on serum progesterone, estradiol, luteinizing hormone, or prolactin levels on gd 9 NE on ovarian weight or corpora lutea	NE on resorptions, litter size, fetal weight, or external defects (gd 20)

NE: No effects.

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a Number of pregnant dams/each sacrifice period.

^b Small sites containing extravasated blood.

ters were examined on gd 20 there were no effects noted on litter size, fetal weight, or resorptions. Fetuses were only assessed for external abnormalities and none were observed. Maternal ovary weight and corpora lutea counts were determined in dams sacrificed on gd 9 and 20 and there were no effects noted. In contrast to results obtained on gd 9, methanol did not affect uterine weight on gd 20. Additionally, the decreased maternal body weight observed at gd 9 after the highest dose of methanol was not observed on gd 20. The authors also studied decidual cell response (DCR) in pseudopregnant rats. Results indicated that effects on uterine weight and implantation sites on gd 9 may have resulted from methanol-induced inhibition of the DCR. The author concluded that chemical exposure may cause some impairment of the DCR without necessarily affecting implantation success. (The Expert Panel observed that there was no increase in resorptions on gd 20 at the highest methanol dose used, leading to the question of whether the atypical sites observed on gd 9 represented a significant toxic manifestation. Further, the general lack of response is difficult to interpret given that there are no data in this strain that characterizes the general pattern of developmental toxicity following traditional (gd 6–15) periods of dosing.)

Strengths/weaknesses: The strengths of study design are the use of three doses, reporting of methanol grade, and the examination of endpoints during different dosing periods.

Study limitations included the small number of pregnant rats used in each group and performance of only external gross examinations with no examination of possible visceral or skeletal defects. It was not stated if animals were randomly assigned to treatment groups. It is not clear if the litter or the fetus was used as the experimental unit for sta-

tistical analyses. Hormone levels were measured at only a single time point and it is not clear how much time elapsed between the final methanol dose and the time of sacrifice.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is low due to the weaknesses in the study. Some of these data may have confirmatory value if other studies without these limitations show relevant effects.

Youssef et al. [140] conducted a study to determine toxicity of methanol in rats following oral administration at a single time point (Table 31). On gd 10, 10–12 CrI: Long-Evans rats were gavaged with methanol, HPLC grade, at 1.3, 2.6, or 5.2 ml/kg bw (1023, 2045, or 4090 mg/kg bw according to CERHR calculations). The doses were selected according to guidelines for segment II studies that require one maternally toxic dose equal to 40% of the LD₅₀. The rats were first gavaged with mineral oil to prevent gastric irritation. A control group of nine rats was not gavaged and a control group of four rats was gavaged with mineral oil. Because no differences were found between the two control groups, data were combined into a single control group. Dams were sacrificed and necropsied on gd 20 and 10–13 dams and fetuses were examined per group. Statistical analysis for fetal anomalies and variations included ANOVA, the Fischer PLSD exact test, and determination of dose–response relationships. Both the individual fetus and litter were considered statistical units. Signs of maternal toxicity were limited to the high dose group and included significantly decreased bodyweight gain and food intake. There were no signs of intoxication and a histological evaluation of tissues in two dams per group revealed no effects on liver, spleen, heart, lungs, and kidneys. Fetuses were examined grossly and the heads and

Table 31
Summary of developmental toxicity study in rats [140]

Experimental regimen	Number ^a	Dose (mg/kg bw)	Maternal effects	Fetal effects
Prenatal toxicity study. Youssef et al. [140] gavaged CrI: Long-Evans rats with mineral oil and then methanol on gd 10. Bodyweight and food intake were measured. Dams were sacrificed on gd 20 and examined for implantation sites and resorptions. Fetuses were examined, sexed, and weighed. The head and skeleton were examined for malformations	13	0		
	12	1023	NE	↓Bodyweight (18%) ↑Fetuses with anomalies (3.7% vs. 0.6%) ^b ↑Fetuses with variations (30% vs. 14%) ^b
	11	2045	NE	↓Bodyweight (18%) ↑Fetuses with anomalies (7% vs. 0.6%) ^b ↑Fetuses with variations (34% vs. 14%) ^b
	10	4090	↓Bodyweight gain ↓Food intake No signs of intoxication or histological effects at any dose	↓Bodyweight (8%) ↑Fetuses with anomalies (17% vs. 0.6%) ^b ↑Fetuses with variations (43% vs. 14%) ^b ↑Litters with undescended testes (60% vs. 0%) ↑Fetuses with undescended testes (9% vs. 0%) ↑Litters with eye defects (30% vs. 0%) ↑Fetuses with eye defects (7.5% vs. 0%)

NE: No effects.

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a Number of pregnant dams.

^b Includes hemorrhage, undescended testes, eye defects, and dilated renal pelvis. Incidences of some major effects are listed in the table.

Table 32
Summary of developmental toxicity study in rats, Infurna and Weiss [141]

Experimental regimen	Number ^a	Dose (mg/kg bw per day)	Maternal effects ^b	Offspring effects ^b
Prenatal exposure study with postnatal evaluation of neurobehavioral toxicity. Infurna and Weiss [141] exposed pregnant Long-Evans rats (Blue Spruce Farms) to drinking water with 2% methanol on gd 15–17 or gd 17–19. Dams were monitored for weight gain during third week of pregnancy, daily water intake, duration of pregnancy, and maternal behavior. At birth, pups were examined externally and weighed. Postnatal bodyweight gain was measured weekly and day of eye opening was recorded. Neurobehavioral testing included suckling behavior on pnd 1 and nest seeking behavior on pnd 10	10 20	0 2500	NE on bodyweight gain, fluid intake, gestation length, or maternal behavior	↑Latency to nipple attachment (~85 s vs. 63 s) ↑Time to find home nesting material (~80 s vs. 40 s) NE on litter size, birth weight, postnatal weight gain or mortality, or day of eye opening

NE: No effects.

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a Total number of pregnant rats for both exposure periods.

^b Results for gd 15–17 and gd 17–19 group were virtually identical.

skeleton were examined for malformations according to the Dawson method. Methanol exposure did not increase prenatal fetal mortality. Bodyweights of fetuses were significantly reduced in all treatment groups, but the response was not dose-related. The numbers of fetuses with anomalies or variations was significantly increased at all doses. Dose-related anomalies included undescended testes and eye defects (exophthalmia and anophthalmia) that reached statistical significance in fetuses and litters of the high dose group. Other fetal effects that appeared to be dose related included facial hemorrhage, and dilated renal pelvis. Authors noted that in contrast to previous rodent studies, exencephaly was not observed. According to authors, possible reasons for this discrepancy include differences in day of dosing, dose level, route of administration, or interspecies effect.

Strengths and weaknesses: The strengths of this study are the complete examination of the fetuses (gross, visceral and skeletal) and a thorough analysis of the data. Animals were randomly assigned to treatment groups, a sufficient number of animals were used, and methanol purity was reported.

A weakness in this study design is that treatment occurred on a single day of gestation that is not the day most sensitive to developmental toxicity effects of methanol. Further the effect of mineral oil gavage prior to methanol gavage on absorption kinetics is not known.

Utility (adequacy) for CERHR evaluation process: The utility of these data are limited due to timing of the single dose and lack of understanding of dosing regimen on blood methanol concentrations.

3.2.2. Postnatal development

Infurna and Weiss [141] conducted a study to assess maternal and neonatal behavioral effects in Long-Evans rats (90–120 days old from Blue Spruce Farms) and their offspring when dams were exposed to 2% methanol (purity not specified) in water on either gd 15–17 or gd 17–19

(Table 32). There were 10 dams in the control and each treatment group. The authors reported that water consumption was similar in treated and control groups; the lack of preference for the water versus the 2% methanol solution was the basis for dose selection. The daily consumption of methanol averaged 2500 mg/kg bw per day. Data were analyzed by one-way ANOVA with the litter serving as the unit of observation. There was no effect on gestational length or maternal bodyweight. Maternal behavior was unaffected as judged by the time for the dam to retrieve pups after they were weighed and returned to the cage. Methanol treatment had no effect on litter size, pup birth weight, postnatal weight gain, postnatal mortality, or day of eye opening. Neurobehavioral tests revealed effects in offspring of methanol treated dams in both dose groups. On pnd 1, 3–5 pups/litter were tested for suckling ability and the proportion of pups that successfully attached to nipples was not significantly different across the three groups. However, the mean latency to nipple attachment was significantly longer in the methanol treated groups; there was no statistically significant difference between the methanol treatment groups. On pnd 10, 8 pups per group were tested for homing behavior; specifically, the ability to locate home nesting material within a cage containing four squares of clean shavings and one square with material from the pup's home cage. There were statistically significant differences between the performance of treated pups when compared to controls. It took about twice as long for the treated pups to reach the home area and they took less direct paths than the control pups. There was no difference in performance between the two methanol treated groups. Citing unpublished data, placental transfer of [¹⁴C]methanol was reported to occur in rats exposed overnight to 2% methanol in water. The authors stated that the results of their study indicate that methanol can be defined as a behavioral teratogen in rats.

Strengths/weaknesses: The strengths of this study were a stress-free exposure route (pilot study showed rats chose equally the methanol or water solutions), sensitive measures of neonatal behavior, finding of an effect, random assignment of animals to groups, a sufficient number of animals, and appropriate statistical analyses.

A weakness of this study is that single dose design precluded determination of the existence of a dose-effect response. In addition, the purity of methanol was not reported.

Utility (adequacy) for CERHR evaluation process: The utility of the study may be limited by uncertainty implicit in any toxicological response where there are no dose response data and the inability to place these behavioral effects in the context of other potential positive controls. Specifically, it is not known if any other neurotoxicant have produced the same effects. If ethanol had been included as a positive control, the effects of methanol could have been compared to those of ethanol.

Stanton et al. [100] assessed the postnatal effects of in utero methanol exposure by examining a range of functional, physiological, and behavioral parameters. Those parameters included neonatal mortality and bodyweight, motor activity, olfactory learning/retention, behavioral thermoregulation, *t*-maze delayed alternation learning, acoustic startle response, pubertal development, motor activity, reflex modification audiometry, passive avoidance, and visual evoked potentials. Groups of 6–7 Crl: Long-Evans rats were exposed to air or 15,000 ppm methanol vapors (Fisher Scientific [136] Optima grade, ≥99.9% purity) for 7 h per day on gd 7–19 (Table 33). That dose was chosen because it was

the highest vapor level that could be obtained without producing an aerosol and because it was halfway between doses that were non-teratogenic (10,000 ppm) and teratogenic (20,000 ppm) in the Nelson et al. [98] study. The authors estimated that treated dams received a dose of 6100 mg/kg bw per day. Maternal serum methanol levels were measured after exposure on gd 7, 10, 14, and 18. Methanol concentrations were highest on gd 7 at 3826 mg/l and gradually decreased to a level of 3169 mg/l by gd 12. The only effect noted in dams was lower bodyweight on the first two days of exposure. All but one dam each in the control and treated groups delivered litters. Sacrifice and necropsy of dams on pnd 23 revealed no increase in postimplantation loss. External examination of pups revealed one missing eye in two pups from the same litter in the methanol exposed group. Postnatal bodyweights were modestly but statistically significantly lower in treated pups on pnd 1, 21, and 35, but there was no increase in postnatal mortality. Methanol treatment did not affect the age of preputial separation, but vaginal opening was delayed by 1.7 days compared to controls. Because larger variations in pubertal development have been observed with known reproductive toxicants, the authors noted that this small delay in vaginal opening is probably not an adverse reproductive effect. Neurological testing was performed with tests conducted on specific days up to pnd 160, with some animals being exposed to multiple tests. In most tests, 1/pup/sex/litter was examined. Behavioral data were analyzed by one-way ANOVA with the litter as the unit of observation. The neurobehavioral battery failed to indicate any effect of methanol exposure on multiple

Table 33
Summary of developmental toxicity study in rats, Stanton et al. [100]

Experimental regimen	Number ^a	Dose in ppm ^b (mg/l blood level)	Maternal effects	Offspring effects
Prenatal exposure study with postnatal evaluation. Stanton et al. [100] exposed Crl:Long-Evans rats to methanol vapors on gd 7–19 for 7 h per day. Methanol concentrations were measured inside inhalation chambers. Maternal serum methanol levels were measured on gd 7, 10, 14, 18. Dams were weighed on gd 7–20 and pnd 1, 3, and 21. Dams were allowed to litter and nurse pups. Implantation sites were examined on pnd 23. Pups were examined externally and evaluated for postnatal growth, and pubertal landmarks. Neurobehavioral function was assessed at various time periods up to pnd 160. Generally 1 pup/sex/litter was assessed in each neurobehavioral test	5	0 (1.8–2.7)		
	6	15000 (3169–3826)	↓Bodyweight (gd 7–8)	↓Bodyweight (pnd 1, 21, and 35) ↑Age of vaginal opening (pnd 31.4 vs. 29.7) Anophthalmia and agenesis of optic nerve in two pups from one litter No effects on postimplantation loss, litter size, postnatal mortality, age of preputial separation, motor activity, and cognitive or sensory function

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a Number of dams delivering live litters.

^b The study authors estimated a dose of 6100 mg/kg bw per day.

Table 34
Summary of developmental toxicity study in rats [95] and [97,142]

Experimental regimen	Number ^a	Dose in ppm (mg/l blood level)	Maternal effects	Offspring effects ^b
Pre- and postnatal exposure study with postnatal evaluation of neurobehavioral toxicity. Weiss et al. [95] and Stern et al. [97,142] exposed 4 cohorts of pregnant Crl:Long-Evans Hooded rats to methanol vapors for 6 h per day from gd 6 to pnd 21. Pups were exposed together with the dams on pnd 1–21. Methanol concentrations in exposure chambers were monitored. Dams were weighed on pnd 7, 14, and 19. Pups were weighed on pnd 1, 4, 11, and 18. Neurobehavioral function was assessed in rats from two cohorts during the neonatal stage. Adult offspring from all cohorts were also tested for neurobehavioral function. Neurological testing was conducted on about one male and female rat/litter. Brain morphology was examined in select pups from cohort 2 and 3 on pnd 1 and 21. Neural cell adhesion molecules (NCAM) levels were measured in offspring at pnd 4 and at 15 months of age	46 (28)	0		
	46 (32)	4500 (555)	NE on bodyweight	↓Motor activity on pnd 18 ↑Motor activity on pnd 25 ↓Gender-related motor function and operant behavior in adult offspring ↓Cognitive function in adult offspring ↓NCAM 140 and NCAM 180 levels in brain on pnd 4 NE on brain morphology, nipple attachment, or olfactory response NE on postnatal bodyweight gain

NE: No effects.

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a Total number of pregnant dams in four cohorts (total number of litters with more than five pups).

^b Effects were subtle and it is not clear if statistical significance was obtained.

measures of sensory, motor and cognitive functioning when these animals were tested on pnd 13–63. The two animals with anophthalmia had aberrant visually evoked potentials.

Strengths/weaknesses: The strengths are that a number of different functions were assessed using a variety of measures. This would have been very important if effects had been found, to confirm their generality. The exposure dose and duration were reasonable choices, given the status of prior studies, and were well documented. Elevated maternal blood levels of methanol confirmed that actual exposure occurred and were in general agreement with an earlier study in rats (Nelson et al. [98]). In addition, dams were matched for bodyweight and then randomly assigned to treatment groups, the purity of methanol was reported, and methanol concentrations in chambers were measured and reported.

The overarching weakness of the study is that effects were not found and that the group size, ($n = 6-7$ with litter as the unit of measure) was too small for the tests employed to have statistical power to pick up deficits with known developmental neurotoxicants. The concentration of 15,000 ppm and/or duration (11 days) of exposure is in the range that produced evidence of prenatal developmental toxicity in rats.

Utility (adequacy) for CERHR evaluation process: This study revealed no effects on survival but decreased bodyweights in offspring from dams exposed prenatally to methanol vapors. The bodyweight effects were seen at birth and persisted through pnd 35. The utility of the absence

of neurobehavioral effect is limited due to the small group sample size.

Weiss et al. [95] and Stern et al. [97,142] sought to determine neurological effects in rat pups whose dams were exposed to methanol vapor for 6 h per day from gd 6 through pnd 21 (Table 34). [The Expert Panel noted that since litter and dam were exposed in the postnatal period, pup exposure during this time was direct and possibly through milk.] Four cohorts of Crl: Long-Evans hooded rats ($n = 10-12$ dams/treatment group/cohort) inhaled 0 (air only) or 4500 ppm methanol vapor (HPLC grade) for 6 h daily. The dose selection was based upon doses in other neurobehavioral studies. Three neonatal tests were selected to assess neurobehavior: (1) the suckling test, which measured the latency time to nipple attachment; (2) conditioned olfactory aversion test, which evaluated the sensory capabilities of neonates; and (3) a motor activity test. Two tests were performed on pups when they became adults; one assessed motor function and operant behavior while the second assessed cognitive function. A total of 13–26 rats per group were evaluated in neonatal tests and 8–13 rats per group were examined in adult tests of neurotoxicity. Data were analyzed by repeated measures ANOVA including both between and within animal factors.

Dam blood methanol concentrations were similar during gestation and lactation with a mean level of ~555 mg/l. Mean blood methanol levels, measured in pups on pnd 7 and

14, averaged 1260 mg/l, slightly more than twice the level of dams. Methanol levels in pups began a steady decline starting at pnd 11 and reached levels that were equivalent to maternal concentrations on pnd 48. There were no effects on dam weight gain during pregnancy, litter size, or postnatal pup weight gain to pnd 18. No effect on latency time to nipple attachment was observed when pups were tested on pnd 5. Methanol exposure had no effect on conditioned olfactory aversion response when pups were tested on pnd 10. Motor activity of treated pups was variable, being decreased on pnd 18 but increased on pnd 25. Neurological testing of pups was conducted prior to methanol exposure on pnd 18, but residual levels of methanol prior to testing were not measured. On pnd 25, 4 days had elapsed since the last methanol exposure. The authors opined that pnd 18 results were not likely due to residual methanol. In the test performed when pups were adults, small differences between control and treated adult offspring were noted in the fixed wheel running test only when results were analyzed separately by sex. The test measured motor function and operant behavior by assessing the ability of the rats to run in a wheel that had to be rotated a fixed number of times to receive a food pellet. Although there was no main effect of methanol, sex- and cohort-related interactions were noted. A stochastic spatial discrimination test assessed the ability to change patterns of sequential response requirements. Although methanol had no effect on the acquisition of the first pattern, methanol-treated rats failed to acquire the same level of responding on the reversal test. This indicated that methanol exposure may have produced subtle cognitive defects.

Morphological examination of brains revealed that methanol treatment did not delay neuronal migration, increase numbers of apoptotic cells in the cortex or germinal zones, or produce defective myelination on pnd 1 or 21. However, NCAM 140 and NCAM 180 expression were reduced in treated rats on pnd 4 but such differences were not apparent in rats killed 15 months after their last exposure. NCAMs are a family of glycoproteins that are needed for migration, axonal outgrowth, and establishment of the pattern for mature neuronal function.

A Health Effects Institute (HEI) Review Committee evaluated the study by Weiss et al. [95] and concluded that "... the investigators conducted many tests and found only isolated positive results that were small and variable. Because no compensation was made for multiple testing, care must be taken not to ascribe too much significance to these results."

[The Expert Panel noted the two-fold greater blood methanol concentration in neonatal pups compared to their dams when both were exposed to the methanol vapor. Several plausible factors may account for this difference: (1) pups' skin likely has a faster rate of absorption; (2) pups have a proportionally larger surface area per unit weight than do adults; (3) metabolism/excretion rates may be slower in neonates; (4) pups also are exposed to methanol through maternal milk.]

Strengths/weaknesses: A strength of this study is that it extended the dosing period into the postnatal period to more fully cover the extended period of brain development in the rat. The spectrum of neurobehavioral tests were also broader than those originally utilized by Infurna and Weiss [141]. In addition, a sufficient number of animals were randomly assigned to treatment groups, statistical analyses were appropriate, methanol purity was reported, and concentrations of methanol in chambers was measured and reported.

A weakness of this study was the lack of immunohistochemical studies to verify the NCAM expression findings.

Utility (adequacy) for CERHR evaluation process: The study indicated that there was no effect on viability and bodyweight of pups exposed prenatally and through pnd 21 to 4500 ppm methanol vapor. This study identified that blood methanol concentrations were approximately two-fold greater in nursing pups when compared to maternal levels. While some of this difference plausibly reflects innate age-related differences in toxicokinetics, exposure to methanol through mother's milk in addition to direct vapor exposure likely accounts for the majority of the difference. This study suggests that methanol exposure produced gender-related differences in methanol exposed pups in a test that assessed cognitive and motor function when the pups were tested as adults. Transient changes in NCAM isoforms were observed that could be suggestive of alterations in developmental processes (altered migration and differentiation). However, no gross neuropathological changes were found and immunohistochemical studies, which could have corroborated these findings, were not performed. An experimental design that does not permit evaluation of dose-response adds uncertainty to the utility of the findings.

Burbacher et al. [52,143] evaluated the reproductive and developmental effects associated with methanol exposure in *Macaca fascicularis* monkeys. In the study, two cohorts of monkeys (6/dose/group/cohort) were exposed to air only in chambers or 200, 600, or 1800 ppm methanol vapors (99.9% purity) for 2.5 h per day during a pre-mating and mating period (about 180 days), and during the entire pregnancy (about 168 days) (Table 35). Doses were selected to produce blood methanol concentrations from just above background to just below levels resulting in non-linear clearance kinetics. Monkeys in cohort 1 were all feral born and were 5.5–11 years old. Cohort 2 was made up of 15 feral born monkeys and nine colony-bred monkeys (Texas Primate Center, Charles River Primates, CV Primates, or Johns Hopkins University) ages 5–13 years. The two cohort design was selected to reduce the number of animals tested at the same time, but maintain an adequate sample size. Postnatal growth was monitored in the infants and neurological assessments were conducted to evaluate newborn health, reflexes, behavioral responses, and visual, sensorimotor, cognitive, and social behavioral development. A toxicokinetic study was also conducted and is described in detail in Section 2.1.3. Statistical analysis in this study included one-way ANOVA (to analyze growth, sensorimotor development, neonatal responses, and spatial and

Table 35
Summary of developmental toxicity study in monkeys [143]

Experimental regimen	Number ^a	Dose in ppm (mg/l blood level)	Maternal effects	Offspring effects
Burbacher et al. [143] exposed two cohorts of <i>Macaca fascicularis</i> monkeys to methanol vapors for 2.5 h per day, 7 per days per week during a premating and mating period (about 180 days) and throughout the entire gestation period (about 168 days). Methanol concentrations were monitored inside inhalation chambers. Parental monkeys were weighed weekly and menstrual cycles were evaluated prior to and during exposure. Infants were delivered naturally unless a Cesarean-section was required for complications. Infant size was measured weekly until 84 days of age and then monthly. Infant weight was measured daily for the first 147 days of life and weekly thereafter. Neurological assessments were conducted throughout the postnatal period	9 (8)	0 (2.4)		
	9 (9)	200 (5)	↓Gestation length (8 days) Vaginal bleeding in two monkeys	↓Sensorimotor development in males (goal achieved at 32 vs. 24 days of age) ^b ↓Visual recognition memory (time spent looking at unfamiliar monkey faces was 53% vs. 62% by controls) ^b
	9 (8)	600 (11)	↓Gestation length (6 days) Vaginal bleeding in two monkeys	One infant stillborn ↓Sensorimotor development in males (goal achieved at 43 vs. 24 days of age) ↓Visual recognition memory (time spent looking at unfamiliar monkey faces was 49% vs. 62% by controls) ^b
	10 (9)	1800 (35)	↓Gestation length (6 days) Unproductive labor in one monkey NE on menstrual cycles, conception rate, or live birth index NE on weight gain or overt signs of toxicity NE on formate accumulation	One premature infant Severe wasting in two females at 12–17 months of age ↓ Sensorimotor development in males (goal achieved at 41% vs. 24 days of age) ↓ Visual recognition memory (time spent looking at unfamiliar monkey faces was 57% vs. 62% by controls) ^b

NE: No effects.

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a Total number of pregnant monkeys in two cohorts (total number of live-born infants).

^b Statistical significance was not achieved.

recognition memory), repeated measures ANOVA (to analyze social behavior and secondary-outcome variables from the spatial memory test), and goodness-of-fit of all linear models through assessment of residuals.

Biweekly analysis of maternal methanol and formate blood concentrations revealed dose-related increases in methanol but not formate concentration throughout the exposure period, including pregnancy as described in Section 2.1.3. No information on fetal methanol or formate levels was collected. Maternal weight gain was not consistently affected and there were no clinical signs of toxicity. Methanol exposure had no effect on menstrual cycles prior to or during mating, conception rate, or live birth index. As discussed in greater detail in Section 4, the duration of pregnancy was reduced in all methanol treated groups but was not dose-related and was within the reported normal range for this species [144]. One infant in the high dose group was born after a 150-day gestation period and showed signs of prematurity including irregular breathing and body temperature, difficulty feeding, and a lower birth weight. Cesarean (C)-sections were conducted in two monkeys in the 200 ppm group and two in the 600 ppm group who experienced vaginal bleeding presumably due to placental

detachment. One C-section was performed in a monkey of the 1800 ppm group following three nights of unproductive labor.

Neurobehavioral testing was conducted during the first 9 months of life in a total of 8–9 infants per group, and revealed two effects that may have been due to methanol exposure. The Visually Directed Reaching Test evaluated the infant's sensorimotor development by determining their ability to reach for a brightly colored object containing a nipple dipped in applesauce. Performance of male infants in the Visually Directed Reaching Test was reduced in all treated groups. The mean ages for achieving the criteria of the test were 24, 32, 43, and 41 days for male and 34, 33, 28, and 40 days for females in the control- to high-dose groups, respectively. The results of the Visually Directed Reaching test were significant ($P = 0.04$) in the 1800 ppm group when males and females were evaluated together; when evaluated by sex, significance was obtained for males in the 600 ppm ($P = 0.007$) and 1800 ppm ($P = 0.03$) groups. The Fagan Test of Infant Intelligence assesses the time an infant spends looking at a familiar versus novel object and was conducted in the monkeys when they were 190–210-day-old. The Fagan test is thought to reflect information processing, attention, and

visual memory function in human and non-human primate infants and correlates well with IQ measures in children at later ages. In tests using monkey faces control infants spent more than 60% of the time looking at novel versus familiar faces. All three groups of prenatally methanol-exposed infants failed to show a significant preference for novel social stimuli (pictures of monkey faces), whereas the control group did show a significant novelty preference as expected. However, performance was not concentration-related, nor was there a significant overall methanol effect across the four groups (ANOVA $P = 0.38$). Methanol exposure had no effect on the remaining seven neurobehavioral tests that examined early reflex responses, gross motor development, spatial and concept learning and memory, and social behavior. Visual acuity, an important marker of methanol-induced toxicity, could not be evaluated due to a high test failure rate in control and treatment groups. Methanol exposure had no effects on infant growth or age of tooth eruption. However, at 12 and 17 months of age, two females in the 1800 ppm group (total of nine offspring in that group) experienced a wasting syndrome that occurred despite normal food intake. Tests for viral infection, hematology, blood chemistry, and liver, kidney, thyroid, and pancreas function revealed no cause for the symptoms. Both monkeys were euthanized and necropsies demonstrated severe malnutrition and gastroenteritis.

A committee assembled by HEI to review the Burbacher et al. [52,143] study expressed confidence in the data because the study was well designed and executed. The wasting syndrome observed in two females of the high dose group was identified as a concern by those reviewers. The committee noted the lack of dose response for the reduced gestation period in treated monkeys and also noted that there were no differences in body weight or other physical parameters of infants. They suggested that adrenocorticotropic hormone levels be measured in neonates in future studies to determine if premature labor was triggered by precocious development of fetal hypothalamus, anterior pituitary, or adrenal cortex. The committee urged caution in the interpretation of the two positive neurobehavioral effects since small numbers of animals were analyzed per group, especially per sex and cohort specific analysis where most significant effects were noted. In addition, the Committee noted that the results were not adjusted for multiple testing, there were usually no dose–response relationships, and results were inconsistent among the methanol exposure groups. Effects were small and often varied more between cohorts than treatment groups.

Strengths/weaknesses: The general strengths of this study are that it is detailed and well-designed with long dosing and follow-up periods and a thorough behavioral assessment. In addition, the animals were first separated into groups based on age, size, and parity and then randomly assigned to exposure groups. Purity of methanol was reported and concentrations in chambers were monitored and reported.

The number of animals used ($n = 9–10$) was large for a non-human primate study. However, the numbers of ani-

mals and singleton births, make this study, like many other primate studies, vulnerable to individual accidents that may or may not be treatment-related, thus reducing the power of the study. One weakness of the study is that small numbers of animals ($n = 2–4$ per group) were used during the analyses of subgroups such as sex and cohort. In addition, no correction for multiple comparisons was made.

Utility (adequacy) for CERHR evaluation process: Although most tests were negative, two critical findings were apparent on tests in the neurobehavioral battery used in this primate study. First a delay in sensorimotor development (assessed by the Visually Directed Reaching Test) was noted in male offspring during the first month of life. Delays in sensorimotor development were concentration-related in males as evidenced by delays of approximately 9 days for the 200 ppm (260 mg/m³) group to more than 2 weeks for the 600 and 1800 ppm (780 and 2300 mg/m³) groups. A concentration-related trend was also observed for both sexes combined, but not for the females alone. The basis for the gender-specific nature of this finding is unknown, but other developmental neurobehavioral phenomena, including the developmental toxicity of ethanol [145,146], are known to differ between sexes, and thus cannot be dismissed as necessarily chance occurrences. The second finding was that offspring prenatally exposed to methanol did not perform as well as controls on the Fagan Test of Infant Intelligence. Although there were not concentration-related trends observed in the Fagan test, this could well reflect the inherent constraint of the measured endpoint, which typically is an approximately 60% response preference for novel stimuli vis-a-vis a 50% chance response level. 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 necessarily quite limited, and thus the lack of a clear monotonic trend is not surprising.

The Expert Panel noted limitations such as small animal numbers, a lack of robust findings, and no control for multiple comparisons in the statistical analyses. However, the neurobehavioral findings are important from a qualitative perspective and warrant further investigation as to biological plausibility. More insight may be provided by an independent statistical analysis and further studies that are being conducted to evaluate the monkeys for latent and persistent functional deficits.

The HEI Review Committee noted that the maternal blood methanol level in the 200 ppm (260 mg/m³) group was only slightly higher than that of controls. But as the Committee also acknowledges, “These results may indicate sensitivity to even small increases in maternal blood methanol, or they may indicate random findings” [143]. Indeed, without a better understanding of the fetal pharmacokinetic and pharmacodynamic processes that could have been involved in these effects, it may be presumptuous to suppose that the measured maternal blood methanol levels are an adequate indicator of fetal exposure to the responsible toxic agent. In

sum, the HEI Committee's notes of caution do not warrant dismissal of the reproductive and developmental findings. This study does not address the issue of susceptibility due to folate deficiency and cannot address the issue of increased risk to the offspring.

A discussion of the strengths/weaknesses and utility of this study for addressing reproductive toxicity is included in Section 4.2.

Reynolds et al. [147] conducted an aspartame feeding study in infant monkeys that pertains to methanol toxicity, since 10% of aspartame by weight is hydrolyzed to methanol in the gut of humans and animals [2]. Four 17–42-day-old *Macaca arctoides* monkeys per group (from Biologic Resources Laboratory) were fed formula with 0, 1000, 2000, or 2500–2700 mg aspartame/kg bw per day for 9 months. The doses would result in exposure to 0, 100, 200, or 250–270 mg methanol/kg bw per day according to Kavet and Nauss [2]. The solubility limit for aspartame was reached at the highest dose level and bottles had to be shaken in order to keep the aspartame in solution. Both formula only and phenylalanine in formula (1650 mg/kg bw per day) were used as controls and additional water was available at all times. Equal numbers of male and female infants were not included in each group because the monkeys were assigned to groups as they were born; the ratio of male to female monkeys was about 3:1. Water and formula intake rates were monitored and it was found that water intake was increased in the highest dose group during the 3rd, 5th, 8th, and 9th month of exposure. Exposure to aspartame had no effect on growth as measured by bodyweight gain and crown-heel length. Developmental milestones such as teething, vocalization, alertness, tractability, or general behavior were also unaffected by treatment. A limited number of hematological (hematocrit, hemoglobin, and white and red cell counts), serum chemistry (sodium, potassium, chloride, osmolality, and glucose) and urinalysis (pH, blood, protein, glucose, ketones, and bilirubin) parameters were measured at about every 2 months and were found to be unaffected in exposed groups. Electroencephalograms (EEGs) were obtained prior to exposure and at 4 and 9 months of treatment in all animals and at 4-month intervals after exposure in a total of eight animals. Treatment had no effect on EEGs. At about 1.5 years of age, the monkeys were tested for learning performance and hearing ability by Suomi [148]. Types of learning test included object discrimination, pattern discrimination, learning set discrimination, and oddity learning set discrimination as assessed in a Wisconsin General Test Apparatus. Orienting toward a sound was also tested. Data from learning tests was evaluated by ANOVA. Dietary aspartame exposure had no effect on learning performance or hearing ability. Learning performance in all groups was consistent with that reported by other laboratories for normal macaques at comparable ages in all groups.

Strengths/weaknesses: A strength of the studies by Reynolds et al. [147] and Suomi [148] (involving four monkeys in each of five groups) is the employment of

measures of known validity and sensitivity to neurotoxicant exposures. The data are clear, and the studies were accomplished in a rigorous manner. A clear strength of the studies was the inclusion of optional water so that diet was not a forced choice. In addition, animals were randomly assigned to groups as they were born. Several strengths were noted in the portion of the study conducted by Dr. Suomi. Although training monkeys to perform the tasks is difficult, Dr. Suomi's staff did an excellent job in all aspects of this research. The monkeys learned the tasks, indicating that appropriate behavioral change could be obtained under the current conditions. The number of animals was adequate to reach the conclusions that Dr. Suomi made, as much larger numbers would be required to determine if an aberrant monkey was truly affected.

A limitation in study design is that the statistical power of the hypothesis tests is unclear, as no calculations are presented. The studies did not find any effects at the doses used. To the extent that these were pre-subscribed dose parameters, one could not then say that this was a weakness of the studies. However, in a study design sense, the studies are flawed because the only useful information to come from them is that the highest dose appears to be tolerated. The study should have continued to higher doses, and in the view of this Panel member, if doing so required alternative routes of administration the effort would have been worth it.

Utility (adequacy) for CERHR evaluation process: This study, as well as the assessments subsequently carried out on the monkeys by Suomi [148], indicate that aspartame, at doses of up to 2500–2700 mg/kg bw per day or phenylalanine at 1650 mg/kg bw per day for 9 months early in life, do not result in significant effects on a variety of indices of growth, development, and learning in *Macaca arctoides*. The results reasonably rule out the possibility that the aspartame/phenylalanine doses employed have very large effects on the endpoints assessed, but what is unclear is the effect size with which the data are compatible. The NOAEL was the highest dose of aspartame tested which represented 250–270 mg/kg bw per day of methanol.

3.2.3. Mechanisms of toxicity

Bolon et al. [149] conducted a series of experiments in Crl: CD-1 ICR BR mice to determine the phase-specific developmental toxicity of methanol inhalation. In various experiments, mice were exposed to methanol vapors (HPLC grade) or HEPA-filtered air for 6 h per day during either the period of organogenesis (gd 6–15), neural tube development and closure (gd 7–9), or potential and abnormal neural tube reopening (gd 9–11). The methanol doses were based on doses producing teratogenicity in previous rodent studies such as Rogers et al. [96]. Methanol concentrations inside exposure chambers were verified. Dams were sacrificed on gd 17 and implantation and resorption sites were evaluated. In all studies fetuses were examined for external abnormalities, sexed, and weighed. Nonparametric tests were used for statistical analysis and the litter was considered the exper-

Table 36
Summary of developmental toxicity study in mice [149]

Experimental regimen	Exposure day	Number ^a	Dose (ppm)	Maternal effects	Fetal effects
Prenatal toxicity pilot study. Bolon et al. [149] exposed CrI:ICR BR CD-1 mice to methanol vapors for 6 h per day on gestation day specified in "exposure day" column. Methanol concentrations in exposure chambers were monitored. The dams were weighed and sacrificed on gd 17. Fetuses were weighed and examined for external malformations	gd 6–15	5 (5) 12 (11)	0 10000		↑Resorptions/litter (32% vs. 4%) ↑Litters with ≥1 resorptions (92% vs. 20%) ↓Fetal weight (13%) ↑Neural tube defects (12% fetuses in 46% litters), cleft palates (20% fetuses in 82% litters), and digit defects (8% fetuses in 36% litters) ^c
	gd 7–9	6 (6) 9 (9)	0 10000	NE	↑Resorptions/litter (13% vs. 1%) ↓Live fetuses/litter (10.4 vs. 12.8) ↑Neural tube defects (7% fetuses in 33% litters) ^b and cleft palate (13% fetuses in 33% litters) ^{b,c}
	gd 9–11	12 (12) 17 (17)	0 10000	NE	↑Cleft plate (4% fetuses in 24% litters) ^c ↑Digit defects (2% fetuses in 12% litters) ^{b,c}

NE: No effects.

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a Number of pregnant dams (litters examined).

^b Not statistically significant.

^c No malformations were noted in controls.

imental unit. In addition to the discussion of these studies below, Tables 36–38 list the incidence and statistical significance of developmental effects.

In the pilot study, groups of 5–17 dams were exposed to 0 or 10,000 ppm methanol on either gd 6–15, gd 7–9 or gd 9–11 (Table 36). Major developmental effects were seen on gd 6–15, and included reduced fetal body weight, resorptions, neural tube defects (NTDs), cleft palates, and digit defects. The same effects were noted on gd 7–9 with the exception of reduced fetal weight and digit defects. Cleft palate and digit defects were the only effects noted on gd 9–11.

Bolon et al. [149] next studied the dose–response relationship for NTDs by exposing 20–27 mice per group to 0, 5000, 10,000, or 15,000 ppm methanol on gd 7–9 (Table 37). In this study, fetuses fixed in Bouin's solution were examined for visceral malformations. Resorptions and dilated renal pelves were noted at all dose levels. Developmental effects in the gd 7–9 group were consistent with the pilot study with exposure to 10,000 ppm and higher resulting in NTDs, cleft palates, and eye and tail defects, and hydronephrosis. A reduction in fetal body weight and live fetuses/litter was observed in the 15,000 ppm group. In this study a group of 17 mice were also exposed to 15,000 ppm methanol on gd 9–11 to confirm the lack of neural tube effects observed in the pilot study. Maternal signs of intoxication (ataxia, circling, tilted heads, or depressed motor activity) were consistently noted following exposure to 15,000 ppm, but there were no effects on bodyweight when corrected for gravid uterus weight. Developmental effects were consistent with the pilot study with fetuses showing cleft palate,

limb and tail defects, renal pelvic dilation, and hydronephrosis.

Bolon et al. [149] conducted a third experiment to better define the window of susceptibility for neural tube effects (Table 38). Mice (8–22 per group) were exposed to 15,000 ppm methanol on gd 7, 8, 9, 7–8, 8–9, or 7–9. The key time period for NTDs was gd 7–8. NTDs were observed with all combinations of exposure days containing gd 7 and 8 and were not observed following exposure until gd 9 only. Resorptions were increased on any combination of exposure days that included gd 7. There were no resorptions observed following exposure on gd 8 or 9.

Following evaluation of all study results, the authors noted that methanol exposure during gd 7–9 causes neural tube (exencephaly most common) and eye defects and exposure on gd 10–12 results in limb defects. Hydronephrosis and cleft palate occurred following exposure during either time period. Malformations were sex specific with a greater incidence of NTDs and cleft palates in females and hydronephrosis in males.

Strengths/weaknesses: Strengths in study design included exposure throughout organogenesis as well as for shorter periods to determine phase specificity, adequate sample size for final study, good animal husbandry, carefully controlled methanol exposures, reporting of methanol purity, dose–response information, examination of embryotoxicity at different gestational days of exposure, and pathologic documentation of embryo defects.

Limitations in study design included no dose–response information for gd 6–15 exposure, no skeletal exams, and no information provided on plasma methanol concentrations.

Table 37
Summary of developmental toxicity study in mice [149]

Experimental regimen	Exposure day	Number ^a	Dose (ppm)	Maternal effects	Fetal effects	
Prenatal toxicity study that focuses on neural tube effects. Bolon et al. [149] exposed Crl:ICR BR CD-1 mice to methanol vapors for 6 h per day on gestation day specified in “exposure day” column. Methanol concentrations in exposure chambers were monitored. The dams were weighed during gestation and sacrificed on gd 17. Fetuses were weighed and examined for external and visceral malformations	Gd 7–9	22 (22)	0	NE	↑Litters with ≥1 resorption (56% vs. 27%) ↑Litters and fetuses with renal variations	
		27 (27)	5,000			
		20 (20)	10,000			NE
	20 (17)	15,000	Clinical neurological symptoms	↓Bodyweight (not corrected for gravid uterus weight) ↑Resorptions/litter (46% vs. 3%) ↑Litters with ≥1 resorptions (90% vs. 27%) ↓Live fetuses/litter (7.9 vs. 12) ↓Fetal weight (11%) ↑Neural tube defects (15% fetuses in 65% litters vs. 0) ↑Cleft plate (50% fetuses in 88% litters vs. 1% fetuses in 9% litters) ↑Litters and fetuses with renal variations ↑Litters and fetuses with eye and tail defects		
	Gd 9–11	17 (17)			15,000	

NE: No effects.

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a Number of pregnant dams (litters examined).

^b Not statistically significant, or statistical significance not specified.

Utility (adequacy) for CERHR evaluation process: The Panel has high confidence in these data for delineation of critical periods of exposure following high-dose inhalation of methanol. They noted that the pilot study and final study were in agreement. However, the relevance for humans is questionable because of the high exposure doses, especially the 10,000 and 15,000 ppm concentrations needed to cause embryotoxicity. The Panel expressed concern about the 15,000 ppm data (on which much of the paper is based) because of the maternal toxicity observed in about 20% of the animals at this exposure concentration. Lack of skeletal examination also weakens interpretation. In addition, a NOAEL was not identified for the gd 7–9 exposure.

A phase specificity study was also conducted in Crl:CD-1 mice by Rogers and Mole [150] in order to determine sensitive periods of developmental toxicity. Groups of 12–19 timed pregnant mice were exposed to filtered air or 10,000 ppm methanol vapors (Fisher Scientific Optima Grade) for 7 h per day on gd 6–7, 7–8, 8–9, 9–10, 10–11, 11–12, or 12–13. The doses were based on those producing malformations in previous studies by Rogers et al. [96]. Maternal blood methanol levels peaked at 4000 mg/l 1 h after the end of the gd 7 exposure and returned to baseline levels 19 h following exposure. Nine to 17 litters were examined per group with dams and litters considered the statistical unit. Statistical analysis included the General Lin-

Table 38
Summary of developmental toxicity study in mice [149]

Experimental regimen	Exposure day	Number ^a	Dose (ppm)	Maternal effects	Fetal effects
Prenatal phase specificity study. Bolon et al. [149] exposed CrI:ICR BR CD-1 mice to methanol vapors for 6 h per day on gestation days specified in "exposure days" column. Methanol concentrations in exposure chambers were monitored. The dams were weighed and sacrificed on gd 17. Fetuses were weighed and examined for external malformations	gd 7–9	22	0	Clinical neurological symptoms were observed for each exposure period	↑Resorptions/litter (39% vs. 3%) ↑Litters with ≥1 resorption (87% vs. 27%) ↓Live fetuses/litter (7.7 vs. 12) ↑Neural tube defects (1.4% fetuses in 8% litters) ^{b,c} ↑Neural tube defects (2.2% fetuses in 15% litters) ^{b,c} NE ↑Resorptions/litter (42% vs. 3%) ↑Litters with ≥1 resorption (100% vs. 27%) ↓Live fetuses/litter (8.4 vs. 12) ↓Fetal weight (12%) ↑Neural tube defects (16% fetuses in 67% litters) ^b ↑Neural tube defects (1.9% fetuses in 27% litters) ^{b,c} ↑Resorptions/litter (46% vs. 3%) ↑Litters with ≥1 resorptions (90% vs. 27%) ↓Live fetuses/litter (7.9 vs. 12) ↓Fetal weight (11%) ↑Neural tube defects (15% fetuses in 65% litters) ^b
	gd 7	15	15,000		
	gd 8	13	15,000		
	gd 9	8	15,000		
	gd 7–8	14	15,000		
	gd 8–9	11	15,000		
	gd 7–9	20	15,000		

NE: No effects.

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a Number of pregnant dams.

^b No malformations were noted in controls.

^c Not statistically significant.

ear Models procedure and multiple *t*-test of least squares method for continuous variables and the Fisher's exact test for dichotomous variables. Examination of fetuses was limited to bodyweight measurements and observations for external and skeletal malformations. The skeletal exam was conducted by placing fetuses in 70% ethanol, macerating in 1% KOH, and staining with Alizarin red S. An increase in prenatal mortality only occurred following exposure on gd 6–7 or 7–8. The incidences of fetal malformations/exposure day and their statistical significance are listed in Table 39. Exencephaly was observed with exposures on gd 6–9 with the highest incidences occurring with gd 6–7 exposure. The incidences of cleft palate peaked after exposure on gd 7–8. A significant percentage of cleft palates were also observed in the gd 6–7 group and low numbers of fetuses were affected after exposure up to gd 11–12. The greatest number of exoccipital bone and axis and atlas vertebrae defects occurred with exposure on gd 6–7. With the exception of atlas defects following gd 7–8 treatment, very few vertebral defects were noted when exposures were conducted after gd 7. Increased numbers of presacral vertebrae were also noted in the gd 7–8 group. Cervical ribs peaked with exposures on gd 6–7 but were also observed with gd 7–8 exposures. In contrast, the greatest incidence of lumbar ribs was noted

with exposure on gd 7–8 and significant increases were also observed on gd 6–7, 8–9, and 10–11.

As part of the same study, Rogers and Mole [150] examined the phase specificity in CD-1 mice exposed to 10,000 ppm methanol vapors for 7 h on gd 5, 6, 7, 8, or 9. A total of 12–17 litters/exposure day was evaluated. Fetal malformation results are listed in Table 40. Gd 7 was the most sensitive time period for the majority of fetal effects as observed by the highest incidence of resorptions, exencephaly, cleft palates, axis vertebrae defects, and cervical and lumbar ribs. Exoccipital malformations and reduced numbers of presacral vertebrae were noted at the highest frequency with exposure on gd 5. The highest occurrences of atlas vertebrae malformations were seen with gd 5 and 6 exposure.

The study authors noted that the occurrence of exencephaly coincided with treatment during the period of neurulation and neural tube closure. However the incidence of cleft palate peaked following exposure prior to the period of palatal development. Cleft palate and exencephaly appeared to be competing malformations because the two malformations rarely occurred in the same fetus. Some malformations (digit defects and hydronephrosis) observed in a study of mice by Bolon et al. [149] were not repeated in this study.

Table 39
Malformations in mice following 2-day exposure periods, Rogers and Mole [150]

Malformation	Percentage of fetuses/litter affected for each exposure period						
	Gd 6–7	Gd 7–8	Gd 8–9	Gd 9–10	Gd 10–11	Gd 11–12	Gd 12–13
Exencephaly	30 ^a	25 ^a	3	–	–	–	–
Cleft palate	20 ^a	22 ^a	12 ^a	3	3	1	–
Exoccipital defect	23 ^a	3	–	–	–	–	–
Atlas defect	72 ^a	18 ^a	1	1	2	5	2
Axis defect	22 ^a	5 ^a	–	1	–	–	–
Decreased vertebrae number	13	3	–	–	1	1	–
Increased vertebrae number	–	24 ^a	4	–	–	–	–
Cervical ribs	74 ^a	30 ^a	3	–	–	–	–
Lumbar ribs	10	68 ^a	43 ^a	52 ^a	45 ^a	17	18

^a Results achieved statistical significance.

Authors concluded that methanol exposure is most toxic during the gastrulation and early organogenesis stages. Skeletal defects suggest vulnerability to segmentation of the anterior region of the embryo.

A summary of the phase specification studies by Bolon et al. [149] and Rogers and Mole [150] is included in Table 41.

Strengths/weaknesses: Strengths of study design included exposures that were well-characterized, and characterization of plasma methanol levels over several time points during the course of the 7-day exposure. Although chamber concentrations were not reported, previous work with the same chambers demonstrated a highly stable atmosphere. Statistical analyses for the 2-day exposure periods were appropriate.

Weaknesses in study design included evaluation of small numbers of litters ($n = 12$ – 14) for most critical periods, measurement of plasma methanol levels only on gd 7, recording of only skeletal and external findings, no statistical comparisons reported for single-day exposures, and single-day exposures at only a single concentration (10,000 ppm). The single concentration was quite high, resulting in maternal toxicity at certain intervals and not providing information regarding interval-specific dose response patterns.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is moderate-to-high. They

provide valuable information regarding periods of sensitivity for critical developmental toxicity at a single high exposure level. The Panel noted that the number of resorbed/dead pups per litter was highly variable, possibly obscuring small effects on pup mortality. The usefulness of this study for human evaluation is questionable.

Bolon et al. [94] conducted additional studies in Cr1:CD-1 ICR BR (CD-1) mouse embryos and fetuses to identify the scope of methanol-induced cephalic malformations and to identify target sites in neurulating embryos. In an experiment to identify fetal pathology, 20–25 dams were exposed to 0 or 15,000 ppm methanol vapors for 6 h per day from gd 7–9 and were sacrificed on gd 17. As previously observed, methanol-treated dams were intoxicated. Fetal malformations were consistent with those previously observed by Bolon et al. [149] and Rogers et al. [96]. Cephalic NTDs affected about 15% of fetuses. Exencephaly was the most common NTD and was usually accompanied by malformed or missing cranial bones and eye anomalies (open eye, cataracts, and retinal folds). Malformations occurring at lower frequencies included anencephaly, encephaloceles, and holoprosencephaly. Bolon also measured the thickness of fetal frontal cortices, an endpoint that was not examined in previous studies. A total of 16–24 litters and 39–56 fetuses per group were examined. The data were analyzed by ANOVA with the individual animal as the unit. Significant reductions in

Table 40
Malformations in mice following one-day exposure periods [150]

Malformation	Percentage of fetuses/litter affected for each exposure period				
	Gd 5	Gd 6	Gd 7	Gd 8	Gd 9
Exencephaly	5	10	17	5	–
Cleft palate	8	20	47	16	3
Exoccipital defect	10	7	–	10 ^a	–
Atlas defect	56	56	31	37	6
Axis defect	19	24	29	17	4
Decreased vertebrae number	19	–	2	4	3
Increased vertebrae number	–	8	28	4	–
Cervical ribs	26	36	45	28	2
Lumbar ribs	2	31	39	27	15

^a This value contradicts the description in the text.

Table 41
Comparison of phase specificity studies

Exposure day	Effects in [149] study with exposure of CD-1 mice to 10,000 ppm or 15,000 ppm methanol for 6 h per day ^a	Effects in [150] with exposure of CD-1 mice to 10,000 ppm methanol for 7 h per day ^b
5	Not examined	Exencephaly Cleft palate Exoccipital bone defect Atlas vertebra defect Axis vertebra defect Cervical ribs Resorptions
6	Not examined	Exencephaly Cleft palate Exoccipital bone defect Atlas vertebra defect Axis vertebra defect Cervical ribs Lumbar ribs Resorptions
6–7	Not examined	Exencephaly Cleft palate Exoccipital bone defect Atlas vertebra defect Axis vertebra defect Cervical ribs Resorptions
7	Neural tube defects Resorptions	Exencephaly Cleft palate Atlas vertebra defect Axis vertebra defect Cervical ribs Lumbar ribs Resorptions
7–8	Neural tube defects Resorptions	Exencephaly Cleft palate Atlas vertebra defect Axis vertebra defect Cervical ribs Lumbar ribs Resorptions
7–9	Neural tube defects Cleft palate Eye defects Tail defects Renal pelvis dilation Hydronephrosis Resorptions	Not examined
8	Neural tube defects	Exencephaly Cleft palate Exoccipital bone defect Atlas vertebra defect Axis vertebra defect Cervical ribs Lumbar ribs Resorptions
8–9	Neural tube defects	Exencephaly (not statistically significant) Cleft palate Lumbar ribs

Table 41 (Continued)

Exposure day	Effects in [149] study with exposure of CD-1 mice to 10,000 ppm or 15,000 ppm methanol for 6 h per day ^a	Effects in [150] with exposure of CD-1 mice to 10,000 ppm methanol for 7 h per day ^b
9	No significant effects reported (cleft palates not reported)	Cleft palate Lumbar ribs
9–10	Not examined	Lumbar ribs
9–11	Cleft palate Digit defects Limb defects Tail defects Renal pelvis dilation Hydronephrosis	Not examined
10–11	Not examined	Lumbar ribs
11–12	Not examined	No significant effects reported
12–13	Not examined	No significant effects reported

^a External malformations examined on all days and visceral exams were conducted on gd 7–9 and 9–11. Incidences are listed in Tables 39–41.

^b Skeletal and external malformations were examined. Incidences are listed in Tables 42 and 43.

frontal cortex thickness occurred in all methanol-treated litters, including litters with overtly normal fetuses. Individual layers of the cerebral cortex were affected as noted by reductions in intermediate cortex/subventricular plate and cortical layer one thickness, but an increased neuroepithelium thickness. An apparent increase in subventricular plate cellularity was also observed. Although the biological significance of changes in cortical thickness is not known, the observation led the authors to conclude that pathology may remain in older conceptuses in the absence of gross lesions and that looking at gross lesions alone may underestimate toxicity.

In the study of embryonic pathology, Bolon et al. [94] exposed the dams to air or 15,000 ppm methanol vapors for 6 h per day from gd 7–8 or gd 7–9. Dams exposed on gd 7–8 were sacrificed on gd 8.5 and 9.0 ($n = 3–5$ group per day) and dams exposed on gd 7–9 were sacrificed on gd 9.5 and 10.5 ($n = 4–9$ per group per day). Gross, histological, and morphometric evaluations were conducted on embryos. Data were evaluated by the Mann–Whitney *U*-test using the litter as the unit for dead and malformed fetuses and the embryo as the unit for cell density and mitotic index. At each sacrifice period, delays in growth and rotation and microcephaly were observed in treated embryos. The percentages of treated embryos with NTDs were 41 and 28% on gd 9.5 and 10.5, respectively, and the percentages were significant compared to controls. Study authors noted that the incidence of NTDs in gd 9.5 embryos was three times higher than the incidence in gd 17 fetuses in a previous study (Bolon et al. [149]) and postulated that less severe lesions may be repaired later in development. On gd 8.5 and

9, cephalic neural fold margins were swollen, blunted, and poorly elevated in the treated group. Consistent and severe reductions in the quantity, cell density, and mitotic index of cranial mesoderm were noted for each gestation day. Reduced proliferation and mitotic index were observed in the neuroepithelium. Decreased quantity and abnormal presence of neural crest cells in the folds dorsal to the foregut were also noted. These effects led authors to conclude that NTDs were apparently caused by permanent patency of the anterior neuropore due to an inability to raise the neural folds. Authors identified the neuroepithelium, neural crest, and mesoderm as the likely targets of methanol.

Strengths/weaknesses: The strengths of study design include a thorough pathological examination at term and pathogenesis after exposure, good animal husbandry, well controlled exposures with documentation of chamber concentrations of methanol, reporting of methanol purity, and excellent pathology and histopathology to document lesions.

A limitation of the study is that only a single, high exposure level, which caused maternal intoxication was studied. Although the number of litters examined at each timepoint was small (3–5 for control and 4–9 for treated groups), a large number of embryos was examined histopathologically at each timepoint. How embryos from a litter were divided for different analyses was not stated. Although appropriate statistical tests were done, the embryo, rather than the litter, was used as the experimental unit for examination of cortical thickness, cell density, and mitotic index.

Utility (adequacy) for CERHR evaluation process: The utility of this study for understanding the pathogenesis of fetal neural defects is moderate-to-high. In addition to confirming previous findings, it demonstrates effects on neuroepithelium at the histological level. The study indicates putative mode of action (reduced proliferation) and targets (neuroepithelium, mesoderm, neural crest). The relevance to humans may be very limited because of the high-dose exposure scenario.

Connelly and Rogers [151] conducted a study to determine if methanol-induced alterations in cervical vertebrae result from homeotic shifts in segment identity and/or patterning. A homeotic transformation is the development of one structure in the likeness of another. For example, a vertebra could assume the phenotype of a vertebra in front of (anteriorization) or behind it (posteriorization). A homeobox gene family controls developmental patterning and mutations in these genes can produce homeotic transformations. To study this mechanism, 6–7 Crl:CD-1 mice per group were gavaged with methanol (purity not specified) in distilled water twice daily on gd 7 at 0 (distilled water), 2000, or 2500 mg/kg bw for a total dosage of 0, 4000, or 5000 mg/kg bw. Doses were based on past studies by Rogers et al. [96]. On gd 18, the dams were sacrificed and fetuses were examined for vertebral alterations according to methods described above in the summary for the Rogers et al. [96] study. Data were evaluated with contrast *t*-tests of least square means within ANOVA with the dam and the litter as

Table 42
Cervical malformations in fetuses exposed to methanol [151]

Effect	Percent fetuses/litter affected at each dose (mg/kg bw)		
	0	4000	5000
Ribs on C7 ^a	0	10	28
Tuberculum anterior on C5 ^b	1	10	30
Split in C1	0	3	11
Split in C2	8	8	41

^a Normally found on thoracic rib 1 (T1).

^b Normally found on C6.

units of comparison. Observations that were consistent with homeotic transformations included ribs on cervical vertebra 7 (C7), tuberculum anterior on C5, and splits in C1 and C2; the effects were statistically significant at the high dose. The frequency of these vertebral effects is listed in Table 42. In an examination of disarticulated vertebrae, distinguishing characteristics were seen on vertebrae anterior to those normally displaying that characteristic. The authors concluded that methanol can alter segment patterning in mouse embryos, resulting in posteriorization of cervical vertebrae.

Strengths/weaknesses: A strength of this study is that skeletal malformations were more thoroughly examined than is generally done in developmental toxicity studies. In addition, the statistical analyses were adequate.

The limitation of this study is that small numbers of animals were used per group. Blood methanol levels were not measured.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is low-to-moderate. The authors have demonstrated skeletal malformations similar to those previously observed (Rogers et al. [96]), but it is not quite clear how these data fit into the overall picture of methanol-induced developmental toxicity. This is another study that provides information on mechanisms of high-dose toxicity in rodents. The Panel will need to discuss the relevance to the human situation.

Dorman et al. [66] conducted a series of experiments to examine the role of formate in methanol-induced exencephaly in Crl: CD-1 ICR BR (CD-1) mice. Their studies were a sequel to the studies of Bolon et al. [94,149] that delineated the critical period of methanol-induced exencephaly. The Dorman et al. studies routinely determined methanol and formate levels in maternal blood and decidual swellings. Dams treated with methanol were killed on gd 10 while formate-treated dams were killed on either gd 10 or 18. Controls were included as appropriate for the experimental design. HPLC grade methanol was used. Statistical significance for in vivo studies was conducted with one-way ANOVA and then Fisher's least significant differences test when F ratio indicated statistical significance. Dams ($n = 12$ – 14 per group) exposed to 10,000 ppm methanol for 6 h on gd 8 had litters with statistically significant increases in open neural tubes. Pretreatment of dams with

4-methylpyrazole (4-MP) prior to methanol exposure to inhibit metabolism by alcohol dehydrogenases produced a numerical, but not statistically significant, increase in the number of litters with open neural tubes. Treatment with 4-MP had no significant effect on end-of-exposure decidual swelling or maternal plasma methanol concentrations or peak blood or decidual swelling formate concentrations. Methanol levels in saline and 4-MP treated animals, respectively, were 65 and 75 mM (2080 and 2400 mg/l) in maternal plasma and 83 and 62 mmole/kg (2700 and 2000 mg/kg) in decidual swellings. Formate levels in decidual swellings were not altered and were in the range of 1.8–2.1 mmol/kg (83–97 mg/kg). However, treatment with 4-MP-modified methanol metabolism as evidenced by an increased 24-h-maternal-plasma methanol AUC of 1190 versus 990 mM/h (38,100 versus 31,700 mg/h/l) for controls and 4-MP groups, respectively. Decidual swelling AUC values were unaffected (1110 and 1005 mmol/h/kg = 35,500 and 32,200 mg/h/kg) for control and 4-MP, respectively. Six hours exposure to 15,000 ppm methanol on gd 8 increased end of exposure methanol concentrations to 223 mM (7140 mg/l) and 147 mmole/kg (4700 mg/kg) in maternal plasma and decidual swelling, respectively. AUC values for these samples were 2860 mM/h (91,520 mg/h/l) and 2130 mM/h/kg (68,160 mg/h/kg). As was observed at the 10,000 ppm study, there was no statistically significant increase in any formate levels after 15,000 ppm exposure.

In the same study, Dorman et al. [66] compared maternal blood and decidual levels of methanol and formate in mice that received a single 1500 mg/kg bw gavage dose of methanol in water on gd 8, with or without pre-treatment with 4-MP. As observed with the inhalation study, treatment with 4-MP increased the 24-h methanol AUC value in maternal plasma and decidua, but had no effect on peak maternal blood or decidual levels of methanol or formate. Maternal blood and decidual levels of methanol peaked at about 1 h following gavage. Methanol levels in saline and 4-MP treated animals, respectively, were 50.3 and 45.2 mM (1610 and 1450 mg/l) in maternal plasma and 33.3 and 20.4 mmol/kg (1070 and 653 mg/kg) in decidual swellings.

Dorman et al. [66] continued to study the role of formate in methanol-induced developmental toxicity by examining neural tube formation and embryo/fetal growth following gavage of dams with sodium formate in water at 0 or 750 mg/kg bw on gd 8. This formate dose mimics a maternal pharmacokinetic profile that is observed during a 6-h, 10,000 ppm methanol vapor exposure. The peak maternal plasma and decidual formate levels were 1.05 mM (48 mg/l) and 2.0 mmol/kg (92 mg/kg), respectively. Embryos or fetuses were examined following sacrifice at either gd 10 or 18. Exposure to formate did not increase the incidence of open neural tubes or adversely effect fetal growth at either time point.

Using different concentrations of either methanol or formate, Dorman et al. [66] investigated dysmorphogenesis in the in vitro culture of 7- and 8-day-old embryos. A

more detailed description of this study is included later in this section where the other in vitro studies are described. They observed a concentration-dependent increase in prosencephalic lesions and branchial arch hypoplasia with methanol at 250 mM (8000 mg/l) and prosencephalic lesions, cephalic dysraphism and branchial arch hypoplasia with methanol at 375 mM (12,000 mg/l) and formate at 40 mM (1840 mg/l); statistical significance was achieved from stage-matched controls. Noting the limited metabolic capacity of isolated embryos in culture, the authors assert that their findings provide strong evidence that methanol can act as a direct chemical teratogen.

Strengths/weaknesses: This is an important series of experiments designed to investigate the role of methanol metabolites in inducing exencephaly. The investigators had extensive experience with the mouse model of methanol-induced teratogenicity and thus were able to pinpoint critical periods to examine. In this case, the use of a high dose of methanol is not a defect because this is the dose that had previously been established to reproduce effects. These studies were innovative and well-designed. Strengths of study design included adequate numbers of animals/embryos per group, stable, well-controlled exposure, reporting of methanol grade, measurement of blood formate and methanol, and appropriate animal husbandry. The studies used in vivo and in vitro routes of exposure and compared metabolism inhibitor data with exposure to oral formate.

Appropriate statistical analyses were performed; however, it was not stated if the litter was used as the experimental unit for the in vivo studies.

Utility (adequacy) for CERHR evaluation process: These data are of high utility for defining the proximate developmental toxicant following methanol exposure in mice. The observation that the parent compound (administered at high concentrations) and not formate is responsible for methanol-induced exencephaly is noteworthy. The authors also noted that the in vivo and in vitro doses associated with these effects produce symptoms of clinical intoxication or delayed embryo growth. Given what is known about saturation of methanol metabolism under high exposure conditions, the relevance of the high-dose rodent developmental studies for human risk assessment is uncertain and needs careful consideration by the Expert Panel.

Sakanashi et al. [105] conducted a study to determine the effects of maternal folic acid intake on methanol-induced developmental toxicity in mice. Commencing 5 weeks prior to mating and throughout the entire study Crl: CD-1 mice were fed a purified, amino acid-based folic acid-free diet fortified with either 400, 600, or 1200 nmol/kg diet folic acid. The author described the three diets as containing low, marginal, and adequate folate levels, respectively. All diets contained 1% succinylsulfathiazole to prevent endogenous synthesis of folate by intestinal flora. On gd 6–15, mice were gavaged twice daily with water or methanol [**purity not specified**] in water at 2000 or 2500 mg/kg bw for a total daily dose of 0, 4000 or 5000 mg/kg bw. The original methanol dose of

Table 43
Developmental effects associated with methanol and dietary folic acid levels [105]

Effect	Methanol level (mg/kg bw per day)	Dietary folic acid level (nmol/kg)		
		1200 ^a	600 ^b	400 ^c
Resorptions (number/litter)	0	0.9	1.1	2.6
	4000	1.4	1.5	1.0
	5000	1.4	1.9	3.3
Live fetuses (number/litter)	0	10.4	11.4	7.9
	4000	9.7	9.5	11.0
	5000	10.3	9.3	7.7
Fetal weight (g)	0	1.19	1.17	1.11
	4000	1.11	1.04	0.82
	5000	1.12	0.94	0.88
Crown-rump length (cm)	0	2.3	2.3	2.3
	4000	2.2	2.2	2.1
	5000	2.2	2.2	2.1
Cleft palate (% fetuses/% litters)	0	0.71/7.4	0/0	3.7/18.5
	4000	4.8/30.8	0.7/6.7	57.6/100
	5000	7.0/34.5	24.3/66.7	43.9/86.2
Exencephaly (% fetuses/% litters)	0	0/0	0/0	1.4/3.7
	4000	1.6/7.7	0/0	0/0
	5000	0.67/3.4	1.4/13.3	6.7/34.5
Cleft palate or exencephaly (% fetuses/% litters)	0	0.71/7.4	0/0	5.1/18.5
	4000	6.3/30.8	0.7/6.7	57.6/100
	5000	7.7/37.9	25.7/66.7	50.7/89.7
C ₁ vertebrae defect (% fetuses/% litters)	0	0/0	2/6	7/20
	4000	2/8	2/7	7/33
	5000	8/27	12/25	28/50
C ₇ ribs (% fetuses/% litters)	0	0/0	4/11	3/10
	4000	5/15	5/13	0/0
	5000	13/27	30/56	24/25
C ₅ vertebrae defect (% fetuses/% litters)	0	0/0	3/11	0/0
	4000	22/54	7/13	0/0
	5000	29/46	35/62	33/46

^a Fetuses/litters examined in control; low; and high methanol groups = 282/27; 126/13; 300/29.

^b Fetuses/litters examined in control; low; and high methanol groups = 183/16; 143/15; 140/15.

^c Fetuses/litters examined in control; low; and high methanol groups = 214/27; 33/3; 223/29.

4000 mg/kg bw per day was based on the work of Rogers et al. [96] that observed significant developmental abnormalities. The dose of methanol was increased to 5000 mg/kg bw per day after results of a pilot study indicated that the frequency of malformations under their experimental regimen was less than that reported by Rogers et al. [96]. Dams were sacrificed on gd 18 and parameters standard in a Segment II developmental toxicity protocol were assessed as listed in Table 43. Three to 29 litters were examined per group. Skeletal data were analyzed with a general linear model using percent affected/litter. For continuous variables, the dam and litter were considered units of comparison and data were evaluated by two-way ANOVA and Fisher's protected least significant difference test. Incidence of abnormalities as percentage of affected litters was analyzed using binomial statistics.

The authors concluded that the level of induced folate deficiency in their study was not severe. After 5–7 weeks on

their respective diets, bodyweights of mice were similar and they presented no external evidence of deficiency. Maternal hematocrit and plasma folate levels were not affected by level of folic acid, but liver folate levels in the 400 nmol/kg group were decreased compared to the 600 or 1200 nmol/kg groups ($P = 0.06$). Pregnancy rate was similar across the folic acid groups. Gestational bodyweight gain, number of implantations, and number of live pups/litter were decreased in the dietary group that received 400 nmol folic acid/kg diet. An increase in the litter incidence of cleft palate in the 400 nmol/kg folic acid group was reported by the authors. **[However, the Expert Panel did not agree that reduced folic acid intake had an affect on cleft palate due to a lack of statistical significance.]**

Methanol treatment decreased gestational weight gain in groups fed diet containing 600 or 1200 nmol folic acid/kg diet; these effects were not seen in the 400 nmol/kg group. Methanol did not affect pregnancy or implantation

rate. There was no consistent effect of methanol exposure on hematocrit or liver folate level; plasma folate was increased in mice from the 1200 nmol/kg group that received 5000 mg/kg per day methanol. Methanol decreased fetal body weight in each of the folic acid dietary groups. An increase in the litter incidence of cleft palate was seen with methanol treatment in all dietary groups; the incidence was exacerbated in the 400 nmol/kg group. The litter incidence of exencephaly was increased by exposure to methanol in the 400 nmol folic acid/kg group. Methanol increased anomalies affecting the cervical region, although the incidence tended to decrease in dietary groups receiving larger amounts of folic acid.

The authors concluded the developmental toxicity of methanol was enhanced when maternal folic acid stores were low. They speculate that their data support a role for formate in the effects observed.

Strengths/weaknesses: This study had adequate numbers of animals in all groups except the group fed 400 nmol/kg folic acid and exposed to 4000 mg/kg bw per day methanol. Statistical analyses were adequate. Maternal liver folate levels were dramatically decreased in mice eating the 400 nmol folic acid/kg diet.

Although the reproductive aspects of this study are well designed, there are limitations with the nutritional aspect of the study design. A common outcome of vitamin deprivation is loss of appetite and reduced food intake. Therefore, in studies of this type pair-fed animals are generally included. The pair-fed control animals are fed a normal diet but in amounts equivalent to their vitamin-deficient counterparts. This ensures equivalent consumption of calories and other nutrients. Without such controls there is a question whether the observed effects are due to folate deficiency, general malnutrition, or some other nutrient deficiency. As indicated in Fig. 1 of the study, the animals fed low-folate diets gained less weight during gestation; therefore, other nutrient deficiencies were probably present. For these reasons this study has limited value for evaluating the influence of maternal folate status on methanol developmental toxicity. In addition, folate determinations were done only one time and 3 days after the last methanol dose; if methanol had an effect on folate levels, there may have been time for recovery. Only maternal folate was determined; it is not clear if either the folate deficiency or methanol affected fetal folate levels. Since total folate was determined, it is not possible to determine if there may have been alterations in the folate subtypes present. Even at the lowest folic acid concentration, there was no difference in plasma folate level. It is also not clear if the diet was removed from the dams prior to sacrifice; plasma folate levels are sensitive to food consumption so if the chow was not removed, the animals may have eaten close to the time of sacrifice, which may account in part for the lack of effect on plasma-folate concentrations. The low folic acid group treated with 4000 mg/kg bw per day of methanol had only three litters analyzed and methanol purity was not reported.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in this study is moderate. The possibility of a contribution to methanol toxicity by a nutritional effect other than folate deficiency was not controlled for in the study. The lack of effect on plasma folate levels by the various folic acid deficient diets is somewhat troublesome, but this may have been due in part to the length of time between the animals' final meal and sacrifice. Plasma levels are very sensitive to food consumption, making them an insensitive indicator of tissue folate status which is more stable over time. Maternal hepatic folate levels were greatly reduced by the 400 nmol/kg folic acid diet, and hepatic levels may be the best measure of tissue folate status.

Fu et al. [80] performed studies in Crl: CD-1 mice to determine whether methanol influences maternal or fetal folate concentrations and whether maternal reticulocyte micronuclei formation is a marker for folate deficiency or methanol toxicity. The dietary and mating aspects and data analysis methods of this study are similar to those described above in Sakanashi et al. [105]. In the Fu et al. study the amino acid-based, folic acid-free diet was supplemented with either 400 or 1200 nmol folic acid/kg diet and 1% succinylsulfathiazole. The authors stated that the diets contained marginal and adequate folic acid supplementation, respectively. Methanol (HPLC grade) was administered on gd 6–10 in water at a dose of 0 or 5000 mg/kg bw per day given in two divided doses. Evaluations of dams and fetuses were conducted following sacrifice on gd 18; 21–24 litters per group were examined. Despite the shorter exposure period in this study, effects on fetal growth, survival, and external malformations were consistent with those reported by Sakanashi et al. [105]. Table 44 lists selected study results as a function of different dietary folate levels and methanol exposure. Folate levels in fetal liver, and in maternal plasma, liver, and erythrocytes were lower in mice on a 400 nmol folic acid/kg diet with and without exposure to methanol. Methanol treatment did not produce a further reduction of folate levels in maternal or fetal liver or maternal red blood cells. Neither folate intake nor methanol exposure affected the incidence of micronuclei formation in maternal reticulocytes, as described in Section 2. The study authors concluded that fetal folate stores were reduced despite a lack of overt signs of maternal folate deficiency and it appears that fetuses do not have preferred access to maternal folate stores. They also noted that folate levels were measured 8 days after methanol exposure ended and speculated that folate levels may have been lower during methanol exposure.

Strengths/weaknesses: The strengths of this study are that adequate numbers of animals were used, the grade of methanol was reported, statistics were adequate, and that folate reduction was achieved as determined by either hepatic, erythrocyte, or plasma levels in mice fed 400 nmol folic acid/kg diet. Measured fetal hepatic folate levels were also reduced.

The principal weakness in study design is that folate analyses were performed 8 days after the last methanol

Table 44
Effects of dietary folic acid intake and methanol exposure on selected maternal and fetal parameters [80]

Parameter evaluated	400 nmol folic acid/kg diet		1200 nmol folic acid/kg diet	
	Water control	Methanol	Water control	Methanol
Litters (<i>n</i>)	22	22	24	21
Maternal liver folate ^a	4.7 ± 0.4	4.6 ± 0.2	9.5 ± 0.5	9.3 ± 0.4
Maternal plasma folate ^a	14.1 ± 1.6	10.9 ± 1.4	20.1 ± 2.8	16.5 ± 1.8
Maternal erythrocyte folate ^b	610 ± 40	634 ± 30	902 ± 56	897 ± 74
Fetal liver folate (nmol/g)	1.9 ± 0.2	1.7 ± 0.1	5.0 ± 0.2	5.9 ± 0.4
Cleft palate (percentage litters affected)	13.6	72.7	0	19.0
Exencephaly (percentage litters affected)	13.6	22.7	4.2	19.0

^a Units not reported; units are most likely the same used in [105]: nmol/g for liver and nmol/l for plasma.

^b Units not reported.

dose. It is not clear why the plasma folate levels differ between this study and that of Sakanashi et al. [105]; the method of analysis appears to be different in the two studies which may account for some of the difference. Also, the time interval between the final methanol dose and the measurement of plasma folate was much longer in this study (8 versus 3 days). Food consumption was not monitored, but it is possible that the animals in the 400 nmol/kg folic acid group may have consumed more chow after methanol treatment leading to less of an effect on plasma methanol levels (55% decrease compared to 1200 nmol/g folate diet in the Sakanashi et al. study versus 30% decrease in the present study). The same criticisms (lack of pair-fed controls) that were discussed for the Sakanashi et al. [105] study apply to the Fu et al. study [80].

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is moderate. Length of exposure was altered from the earlier study (Sakanashi et al. [105]). The oral dose used in this study is similar to 10,000 ppm inhalation dose as determined by the severity of the defects observed. The level of hepatic folate deficiency achieved was very similar to that achieved with the 400 nmol dose of folate in the Sakanashi et al. study (62% decrease compared to adequate folate level in Sakanashi study versus 51% decrease in current study). However, there are quantitative, although not qualitative, differences in the results between this study and the earlier study of Sakanashi et al. Cleft palate and exencephaly were still the most common abnormalities observed. In the earlier study, folate deficiency produced neither cleft palate nor exencephaly in the absence of methanol; in the current study, cleft palate was significantly increased by folate deficiency. Exencephaly was also increased, but this difference was not statistically significant due to the presence of a fetus with exencephaly in the 1200 nmol folic acid/kg diet-water group. Although methanol increased the incidence of exencephaly in the 400 nmol folate/kg diet group, this difference was not statistically significant due to the high incidence of exencephaly in the 400 nmol folate/kg diet-water group; methanol had significantly increased the incidence of exencephaly in the Sakanashi et al. study. The lack of pair-fed controls severely lim-

its evaluation of the role of folate in methanol-induced toxicity.

De-Carvalho et al. [152] conducted a study to determine if methanol-induced fetotoxic effects in rats are altered by malnutrition. One group of pregnant Wistar rats (FIOCRUZ breeding stock) was fed ad libitum (well-nourished group) and a second group (protein-calorie malnourished group) received half the amount of diet consumed by the well nourished group. On gd 6–15, rats in each dietary group (*n* = 10–17 per group) were gavaged with distilled water or methanol (purity not specified) at 2500 mg/kg bw per day. Dams were sacrificed on gd 21. Dams in the malnourished groups gained less weight (corrected and uncorrected for gravid uterus) and liver weight was reduced. Methanol treatment further reduced weight gain in malnourished dams only during the treatment period but had no effect on liver weight. Evaluation of fetuses (*n* = 78–116 per group) was limited to mortality, bodyweight, external malformations, and skeletal malformations. After fetuses were preserved in 5% formalin, skeletal abnormalities were observed by clearing the fetuses with KOH and staining with Alizarin Red S. Resorption data were analyzed by chi-square test and all other fetal data by one-way ANOVA. Adverse fetal effects and their statistical significance are listed in Table 45. Malnourishment with or without methanol treatment resulted in reduced fetal weight and delayed ossification. Methanol treatment in malnourished dams potentiated delays in ossification but not reductions in fetal weight. Exposure to methanol resulted in decreased fetal weight and increased cervical ribs, regardless of nutritional status. Resorptions were increased in malnourished rats treated with methanol. Neither malnutrition nor methanol exposure caused an increase in external malformations. The authors concluded that malnutrition has no effect on methanol-induced structural malformations, but that delayed ossification in malnourished rats is aggravated by methanol treatment.

Strengths/weaknesses: The strengths of this study are that treatment occurred throughout gestation and generally an adequate number of animals were examined in each group.

Limitations in study design are that only one dose of methanol was used and this dose was administered once daily by gavage, methanol purity was not reported, the food

Table 45
Developmental effects associated with methanol and malnutrition [152]

Effect	Methanol dose (mg/kg bw per day)	Diet	
		Well-nourished	Malnourished
Resorptions/ implantation (%)	0	12.7	12.1
	2500	9.1	28.8 ^a
Fetal weight (g)	0	4.62	3.59 ^b
	2500	4.32 ^b	3.55
Delayed ossification (% fetuses)	0	18.6	39.7 ^b
	2500	25.4	78.4 ^a
Skeletal anomalies (% fetuses)	0	5.6	3.8
	2500	45.4 ^b	38.8 ^a
Cervical ribs	2500	35.4 ^b	31.0 ^a
	0	1.1	2.6

^a Significant compared to well-nourished and malnourished controls.

^b Significant compared to well-nourished controls.

deprivation was rather drastic, and visceral malformations were not examined.

Generally, appropriate statistical tests were done. However, the Fisher Exact Test should have been used rather than the chi-square test for numbers of 10–17. Additionally, it appears that the fetus, rather than the litter, was used as the experimental unit for the analysis of skeletal anomalies.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is low due to the study design (single oral dose administered once daily with no attempt to relate it to doses used in other studies as well as the rather drastic food deprivation). As indicated by the data in Table 1 of the study, these were severely malnourished animals. They had more than a 20 g loss in bodyweight compared with the 35 g bodyweight gain in the controls. The Panel could not see how these data would apply to any realistic human situation.

In vivo intrauterine microdialysis was used to measure methanol disposition in pregnant Sprague–Dawley rats (from Hilltop Laboratory Animals) on gd 20 after methanol administration by intravenous (IV) injection (100 or 500 mg/kg) or infusion (100 or 1000 mg/kg/h) in 3–4 rats/dose [65]. HPLC-grade methanol was used and saline was used as the vehicle in these studies. Statistical analyses included one-way ANOVA, linear least-squares regression, and two-tailed Student's *t*-test. Maternal blood and intrauterine dialysate were analyzed for methanol. Also, pregnant rats on gd 14 or 20 and pregnant Crl: CD-1 mice ($n = 4$ –6/dose/species) on gd 18 received methanol (0, 100, or 500 mg/kg) and tritiated water by IV injection, then maternal blood and intrauterine dialysate were analyzed for radioactivity. Methanol significantly reduced the rate of radioactivity uptake into the fetus in a dose-dependent manner, suggesting an inhibition of uteroplacental blood flow. For gd 20 rats, IV administration of 100 mg/kg caused a 31% decrease in initial radioactivity uptake, and 500 mg/kg caused a 45% decrease. For gd 14 rats, the decreases in ini-

tial uptake for the two doses were 30 and 57%, respectively. In gd 18 mice, the rate of radioactivity uptake was also decreased by methanol in a dose-dependent fashion. Initial uptake rate was decreased 26% by 100 mg/kg, and 47% by 500 mg/kg. The authors hypothesized that part of methanol's embryotoxic effects may be due to hypoxia resulting from decreased blood flow to the conceptus. Short-chain alcohols are known to affect the cardiovascular system, and the fetal effects of methanol are similar to those known to result from hypoxia (cleft palate, decreased survival, vertebral and rib formation, decreased birth weight). However, other mechanisms may be at work as well, because methanol frequently induces exencephaly in rodent embryos, while maternal hypoxia rarely does.

Strengths/weaknesses: This is technically a very sophisticated, well-done study that addresses an important issue—kinetics of methanol in the maternal-conceptual unit. The use of intrauterine dialysis to monitor blood flow is an impressive technique. In addition, the methanol grade was reported. Limitations are the high dose of methanol and the non-environmental exposure routes.

Utility (adequacy) for CERHR evaluation process: This study is useful for evaluating other rodent studies where high doses of methanol were employed. Under these exposure conditions, the reduction in maternal blood flow may contribute to the observed teratogenic effects in rodents. However, the results have not been reproduced under environmentally relevant exposure scenarios. Due to the doses used, administration as a bolus and route administered, the utility of this study to predict human health risks is limited.

Toxicokinetic studies by Perkins et al. [62] and Ward and Pollack [61] provide some insight into possible mechanisms of toxicity. Additional details of both studies are included in Sections 2.1.6 and 2.1.1.2, respectively. Perkins et al. [62] compared blood levels of methanol in female CD-1 mice and Sprague–Dawley rats following an 8-h exposure to methanol at 5000, 10,000, or 15,000 ppm. At equivalent doses, methanol blood levels in mice (3580, 6028, and 11,165 mg/l) were about 3.5 times higher than in rats (1047, 1656, and 2667 mg/l) despite the fact that the elimination rate of methanol in mice is about twice that of rats. Authors noted that higher blood methanol concentrations in mice versus rats may explain the increased sensitivity of mice to methanol-induced teratogenicity.

Ward and Pollack [61] compared the rate of methanol metabolism in liver homogenates from non-pregnant, pregnant (gd 20), and fetal CD-1 mice and Sprague–Dawley rats ($n = 4$ –5 per group). The homogenates were incubated with 0.005–1.0 mg/l methanol for 40 min and metabolism was measured by the production of formaldehyde. The metabolic rate of mouse homogenates was about twice that of rat homogenates. In both mice and rats, the metabolic rate was about 15% lower in homogenates from pregnant versus non-pregnant animals and about 95% lower in homogenates from fetal versus adult animals. According to the study authors, these data suggest that the fetus does not significantly con-

tribute to the elimination of methanol from the maternal-fetal unit. These results are consistent with an older study that found no or low (20% of maternal values) alcohol and aldehyde dehydrogenase activity in livers from 17- and 21-day-old Wistar rat fetuses. Essentially no activity was observed in placental tissue [153].

A series of in vitro studies were conducted to examine the embryotoxicity of methanol or its metabolites in the absence of confounding maternal factors.

Andrews et al. [154] conducted an in vitro methanol exposure study to compare methanol sensitivity in mouse versus rat embryos. CrI: Sprague–Dawley rat and CD-1 (CrI) mouse embryos were removed from pregnant dams during the stage of neural tube closure (gd 9 for rats and gd 8 for mice). Rat embryos (17–50 per group) were incubated in serum containing 0, 2, 4, 8, 12, or 16 mg/ml (0, 2000, 4000, 8000, 12,000 or 16,000 mg/l) methanol for 24 h. Mouse embryos (26–47 per group) were incubated for 24 h in serum containing 0, 2, 4, 6, or 8 mg/ml methanol (0, 2000, 4000, 6000, 8000 mg/l). Rats but not mice were incubated in serum without methanol for another 24 h. The dose level of 8000 mg/l is approximately equivalent to maternal methanol serum concentrations in mice that inhaled 15,000 ppm methanol or rats that inhaled 20,000 ppm methanol for 7 h [96,98]. At the end of the incubation period, the embryos were examined for viability and dysmorphogenesis. Growth and development were assessed by endpoints such as crown-rump length, head length, yolk sac diameter, somite number, developmental score, and protein content. In rat embryos, significant developmental effects were first noted at 8000 mg/l and included increased numbers of abnormal embryos and reduced growth. Increased embryo lethality was noted at 12,000 mg/l and abnormalities in surviving embryos included open neural tubes and abnormal brain and limb bud development. Nearly complete embryo lethality occurred at 16,000 mg/l. In mouse embryos, some significant signs of reduced growth and development were first noted at 2000 mg/l. Embryo lethality and an increased incidence of open neural tubes were noted at 6000 mg/l and higher. The study authors concluded that mouse embryos have a greater intrinsic sensitivity to methanol than rat embryos because developmental effects occurred at lower doses in mice. Authors suggested that the effects were due to methanol and not its metabolites because constant levels of methanol over the exposure period suggested a lack of significant metabolism.

Strengths/weaknesses: The strengths of this study are that doses used were similar to in vivo methanol levels after inhalation exposure and that length of exposure was the same for embryos of both species.

A weakness of this study is that different developmental stages were covered during the in vitro culture period for the two species. Although the exact developmental stages covered by the culture period were different for the two species, neural tube closure was completed in embryos of both species during the culture period.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is moderate-to-high. Outcomes of these in vitro studies were similar to those observed in vivo. This type of study insures that embryos of both species were exposed to the same concentrations of methanol for the same length of time, a situation that will probably not occur in vivo due to differences in pharmacokinetic and pharmacodynamic parameters in the two species. This is an important study because it suggests that the developmental effects associated with high-dose methanol exposures in rodents may be due to methanol, not formate. Unfortunately, the authors did not measure formate in the culture medium. They did, however, establish that there were no changes in methanol concentrations during the culture period. These results, together with those of Dorman et al. [66] point to methanol as being responsible for the dysmorphogenesis observed in rats and mice. This is most likely due to the accumulation of methanol under high-dose exposure scenarios.

Abbott et al. [155] conducted a study to further characterize methanol effects on rat and mouse embryos and determine if increased cell death occurs at sites with abnormal gross morphology. Gd 9.5 (0 somites) CrI: Sprague–Dawley rat embryos ($n = 4-5$ per group) were exposed to methanol at 0, 8, 12, or 16 mg/ml (0, 8000, 12,000, 16,000 mg/l) for 24 or 48 h. GD 8 (3–5 somites) CrI: CD-1 mouse embryos ($n = 17-18$ per group) were exposed to methanol at 0, 2, 4, or 8 mg/ml (0, 2000, 4000, 8000 mg/l) for 24 h. The embryos were examined for viability and dysmorphogenesis as described in Andrews et al. [154]. Results in rat and mouse embryos were similar to effects previously observed in this laboratory [154] and included reduced growth and development with increased numbers of abnormal embryos. Anomalies included erratic neural seam, open neural tube, and abnormal brain development. Again, mice were shown to be more sensitive than rats with significant adverse effects first noted at 8000 mg/l versus 12,000 mg/l, respectively. Effects on growth and development were generally similar but more pronounced in rats exposed for 48 versus 24 h. There was limited cell death noted in rat embryos exposed for 24 h. However, the 48-h exposure to 16,000 mg/l methanol resulted in increased cell death in the forebrain, optic vesicle, visceral arches, and otic vesicle. Increased cell deaths also occurred in the same regions of mouse embryos exposed for 24 h. Authors noted that cell death occurred in many regions that develop into structures (i.e. cranium, eye, ear, and cleft palate) displaying malformations following in vivo exposure. They also noted a lack of excess cell death in the neuroepithelium or neural folds, suggesting that NTDs occur through mechanisms other than cell death.

Strengths/weaknesses: A strength of this study is that cell death was examined in addition to the usual endpoints.

A limitation of this study is that different developmental stages were covered by treatment in the two species. Although the exact developmental stages covered by the culture period were different for the two species, neural tube closure was completed in embryos of both species during

the culture period. Small numbers of embryos were examined in some of the groups; it is not clear how many embryos were used for the analysis of cell death.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is moderate. The results observed in both species in this study are very similar to those reported by Andrews et al. [154] in both rats and mice increasing the confidence in the data. They observed cell death in control embryos that was enhanced by methanol treatment. The areas demonstrating cell death induced by methanol in this in vitro study were the same areas that were observed to be malformed following in vivo treatment with methanol [96]. That suggests that the mechanism for these malformations may be increased cell death. NTDs were observed in vitro, but increased cell death was not observed in the neuroepithelium or neural tube. The failure to find cell death in the neural tube region suggests that the failure of the neural tube to fuse may be occurring by a mechanism other than increased cell death.

Abbott et al. [156] extended the in vitro analysis of methanol-induced developmental toxicity by examining cleft palates in cultures of Crl:CD-1 mouse embryo mid-craniofacial regions. Twelve-day-old embryos ($n = 20$ –44 per group) were dissected and cultured in serum-free media containing 0, 6, 8, 10, 12, 15, 18, or 20 mg/ml (0, 6000, 8000, 10,000, 12,000, 15,000, 18,000, or 20,000 mg/l) methanol. Methanol exposures lasted for 6, 12 h, 1 day, or 4 days, and all explants were incubated for a total of 4 days. At the end of the incubation period, the cultures were examined for morphology, fusion, proliferation, and growth. Examination by electron and light microscopy revealed that exposure to methanol for 1 day or more reduced the incidence and completeness of fusion. The posterior epithelium was degenerated in unfused palates that were exposed for 1 day, but was intact in unfused palates exposed for 4 days. A dose-related reduction in DNA content after 6 h of exposure indicated that cellular proliferation was a specific and sensitive target. The dose that produced a significant reduction in DNA content was not specified by the authors, but it appears that reductions were first noted at 10,000 mg/l after 6 h of exposure and at the lowest dose, 6000 mg/l, after 12 h of exposure. Reductions in total protein content were first noted after 12 h of exposure, but occurred to a lesser degree than DNA reductions. Measurement of ^3H -thymidine uptake by scintillation counting demonstrated increased uptake in cultures exposed for 12 h and decreased uptake in cultures exposed for 4 days. Examination of ^3H -thymidine intake by autoradiography revealed a selective dose and duration-dependent decrease in labeled palatal mesenchymal cells in cultures exposed to $\geq 15,000$ mg/l methanol for 1 day and ≥ 8000 mg/l for 4 days. Uptake of ^3H -thymidine was reduced in epithelial cells after 4 days of exposure to the highest dose, $\geq 20,000$ mg/l. The authors also examined ethanol and found that it was more potent than methanol but did not produce toxicity through inhibition of cell proliferation.

Strengths/weaknesses: The strength of this study is that a large range of doses was tested for various periods of time.

Some limitations were noted for this study. A single time-point for DNA, protein and cell proliferation (as measured by tritiated thymidine uptake) was used. All cultures were treated with methanol at the beginning of culture and terminated at the same time, leading to differences in the length of time between methanol treatment and analysis; the differences in this recovery time could account for some of the observations. The lowest concentration used in vitro corresponds to the highest dose used in vivo [96]; in vivo that dose produced over 48% incidence of cleft palate, but there was no effect on palatal fusion in vitro.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is low due in part to the very high concentrations of methanol used. Additionally, previous in vivo work had shown that the sensitive period for cleft palate formation was much earlier than the timeframe used in the current study. Although effects were demonstrated on palatal fusion in the present study, the differences in the sensitive period between in vivo and in vitro exposure may indicate that different mechanisms are responsible for the defect. Despite these weaknesses, the study does point out the embryotoxic effects of methanol per se.

Andrews et al. [157] assessed the in vitro toxicity of formate in rat and mouse embryos using the same procedure they employed for the assessment of methanol toxicity [154] as described above. The purpose of this study was to compare the intrinsic toxicity of formate to that of methanol, to compare sensitivity in rats versus mice, and to assess the toxicity of formate when administered as the acid versus sodium salt. Crl:CD (Sprague–Dawley) BR rat (16–30 per group) and Crl:CD-1 (ICR) mouse (17–29 per group) embryos (9- and 8 days old, respectively) were incubated for 24 h in media containing sodium formate or formic acid at levels resulting in equimolar concentrations of formate (2.95–44 mM = 136–2020 mg/l). Exposures were also conducted for 48 h in rat embryos treated with formic acid. As expected, addition of formic acid reduced the pH of media. In all treated embryos, dose-related trends were noted for reduced growth and development. Anomalies were observed in rat and mouse embryos treated with ≥ 11.8 mM (543 mg/l) formate through addition of either the salt or acid to the media. The most frequently observed anomalies involved the central nervous system (CNS) and included open anterior and posterior neuropore and erratic neural seam. Other anomalies observed with sodium formate treatment included rotational and tail defects in rats. In addition to the CNS defects described above, enlarged maxillary processes were observed in rat embryos treated with formic acid. A significant increase in embryo lethality was observed only in rat and mouse embryos treated with formic acid (17.6–44 mM = 810–2020 mg/l formate). The study authors concluded that formate exposure in rat and mouse embryos result in quantitatively and qualitatively similar results. Exposure to sodium formate for 48 h in rats resulted in the same

types of effects that occurred at a higher frequency compared to the 24-h exposure. Formate (as either the sodium salt or the acid form) exposure produced embryo lethality and dysmorphogenesis at molar concentrations that were 4–10-fold lower than those observed with methanol. Acidosis may be partially responsible for embryotoxicity since treatment of cultures with formic acid appeared to lower the amount of formate ion needed to induce lethality compared to adding Na-formate. In closing, the authors stated that developmentally toxic levels of formate are not likely to occur in humans as a result of environmental exposures. If this belief is true, rodents would be a good model for extrapolation to lower doses according to the study authors.

Strengths/weaknesses: A strength of this study is that both formic acid and sodium formate were examined, as were effects of these compounds on pH. Potencies of the two compounds were compared in embryos of the two species.

A weakness of this study is that slightly different developmental stages were evaluated in the two species. Although the exact developmental stages covered by the culture period were different for the two species, neural tube closure was completed in embryos of both species during the culture period.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is moderate-to-high for the same reasons as stated above for Andrews et al. [154]. The study is useful because it allows for an examination of direct effects of compounds on embryonic growth and development in the absence of maternal confounds. This study, in combination with results from Dorman et al. [66] and Brown-Woodman et al. [158], increases confidence in the observation that high concentrations of formate produce embryotoxicity. However, as stated by the authors, the concentrations of formate required to produce adverse effects are unlikely to be achieved by the expected increased exposure to methanol that would result from its addition to gasoline.

An *in vitro* study by Brown-Woodman et al. [158] further examined the toxicity of methanol versus formate and evaluated the role of acidosis in developmental toxicity. Sprague-Dawley rat embryos (8–17 per group; source not specified) were removed from dams on gd 10 and incubated for 40 h in serum containing 0 or 51.3–411.7 mM (1640–13,170 mg/l) methanol; a second group (9–19 per group) was incubated in 0 or 3.74–27.96 mM (172–1290 mg/l) formic acid. At the end of the exposure period, the embryos were assessed for viability and growth by examination of endpoints such as yolk sac vasculature, embryonic rotation, heartbeat, crown-rump length, somite number and protein content. No effect levels of 211.7 and 3.74 mM (6774 and 172 mg/l) were identified by study authors for methanol and formic acid, respectively. Growth and developmental retardations were noted at higher concentrations for both methanol and formic acid. The lowest concentrations to produce embryotoxicity were 286.5 mM (9168 mg/l) methanol and 18.66 mM (858 mg/l) formic acid. A series of experiments were next conducted to determine if toxicity associ-

ated with formic acid exposure resulted from a reduction in pH. To assess the effects of formate in the absence of a pH reduction, embryos were incubated in sodium formate or mixtures of sodium formate and formic acid at levels resulting in formate concentrations associated with embryotoxicity in the formic acid study. Embryos were also incubated in serum that was adjusted with hydrochloric acid to pH levels obtained with formic acid exposure. The results of these experiments indicated that both low pH and formate contribute to toxicity. The authors noted that embryotoxicity in this study occurred with serum methanol levels that were equivalent to those producing developmental toxicity in rats exposed through inhalation by Nelson et al. [98]. In closing, the authors stated that occupational exposure to methanol at a TWA of 200 ppm would not result in blood levels of methanol or formate associated with developmental toxicity. However, the authors did note that pregnant women are at an increased risk of folate deficiency, a condition that may lead to a greater extent of formic acid accumulation.

Strengths/weaknesses: The strengths of this study are that serum methanol concentrations were measured at several time points, pH effects were examined from two different approaches, and pH was measured at several time points during culture.

The weakness of this study is that only a small number of embryos were treated in each group.

Utility (adequacy) for CERHR evaluation process: The Panel notes that the developmental stage of exposure was different from that used by Andrews et al. [154,157] in rats. However, the concentrations used are similar and results were very comparable. The Panel's confidence in these data is moderate to high. In spite of the differences in study design and the low numbers of embryos used per group, the similarity of results to those reported by Andrews et al. [154,157] in rats increases the Panel's confidence in the reported results. Brown-Woodman et al. [158] found that at sufficiently high concentrations, both methanol and formate were embryotoxic and that low pH contributed to the toxicity of formic acid in culture. These are important observations for the CERHR process. The study also provides useful dose-response data.

In vitro experiments to examine the role of formate in developmental toxicity were also conducted by Dorman et al. [66] as part of a series that also included *in vivo* studies that are addressed earlier in this section. On Gd 7, CrI: CD-1 ICR BR (CD-1) mouse embryos were explanted and cultured in media with 0, 62, 125, or 187 mM (0, 2000, 4000, or 6000 mg/l) methanol for 12 h. Gd 8 embryos were cultured with 0, 62, 125, 187, 250, or 375 mM (0, 2000, 4000, 6000, 8000, or 12,000 mg/l) methanol or 4, 8, 12, 20, or 40 mM (180, 370, 550, 920, or 1840 mg/l) formate for 12 h. Embryos were examined on gd 9 for the size and shape of head, neuropore patency, somite numbers, and growth. At least nine embryos were exposed per group and experiments were replicated a minimum of two times. In embryos explanted on gd 8, significant increases in pros-

encephalon lesions and branchial arch hypoplasia were observed with methanol treatment at ≥ 250 mM (8000 mg/l) and increased numbers of cephalic dysraphisms were noted with exposure to 375 mM (12,000 mg/l) methanol. A dose of 250 mM (8000 mg/l) methanol is approximately equal to plasma methanol levels in mice inhaling 15,000 ppm methanol for 6 h (223 mM = 7140 mg/l). Treatment with 40 mM (1840 mg/l) formate also resulted in increased numbers of cephalic dysraphisms and prosencephalon lesions. A plasma formate level of 40 mM (1840 mg/l) greatly exceeds the level observed mice inhaling 15,000 ppm methanol (0.75 mM = 35 mg/l) but according to authors, can occur in humans with acute methanol toxicosis. Exposure to a 187 mM glycerol osmolality control resulted in prosencephalon and branchial arch defects, but no neural tube effects. Dose-related reductions in embryo growth and rotation were also reported for methanol and formate exposure. The findings of this study, in addition to those obtained in *in vivo* experiments described above, led Dorman et al. [66] authors to conclude that "... methanol and not formate is the proximate teratogen in pregnant CD-1 mice exposed to high concentrations of methanol vapor."

Strengths/weaknesses: This study design is good for comparing effects of methanol and formate. Strengths of the study design include the selection of methanol and formate concentrations that were calculated to approximate blood levels that occur under typical rodent exposure scenarios. Exposure concentrations approximate peak maternal blood methanol levels following 15,000 ppm exposure *in vivo*.

A weakness is the lack of information on formate and methanol levels during the culture period and the limited information on pH. The Panel also noted that embryos cultured on gd 7 did not grow well and stated that those results were questionable.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these studies is high. Results strongly suggest that formate is not involved in the teratogenicity of methanol in mice. The exposures in the Dorman study (12 h) were shorter than those used by Andrews et al. [157] (24 and 48 h). Also, the investigators examined different endpoints, so it is difficult to compare the studies directly. However, results of the *in vitro* studies are similar to Andrews et al. [157] and Brown-Woodman et al. [158].

Andrews et al. [159] conducted *in vitro* studies with rat embryos to compare toxicities of methanol and formate alone and in combination. The studies were based on a developmental scoring system that takes into account embryonic growth and stages of development. Doses from previous *in vitro* experiments [155,157] were used in a dose-addition predictive model to estimate doses of methanol, formate, and methanol/formate mixtures that would reduce developmental scores by 13.5% (Simplex 1) and 27% (Simplex 2). Methanol/formate doses were 0/0, 6.11/0, 2.25/0.56, and 0/0.89 mg/ml (0/0, 610/0, 2250/560, 0/890 mg/l) in Simplex 1. In Simplex 2 doses were 0/0, 8.75/0, 5.90/0.49, 2.25/1.12, and 0/1.51 mg/ml (0/0, 8750/0, 5900/490, 2250/1120, and

0/1510 mg/l). Gd 9 Sprague-Dawley (CrI:CD [SD] BR) rat embryos were treated for 48 h and examined for signs of toxicity. Fifteen to 26 embryos were examined in each group. Treatment with individual compounds produced significant decreases in development score, somite number, crown-rump length, and head length in Simplex 1 and Simplex 2. In Simplex 2, the methanol/formate mixtures also produced significant decreases in those parameters. However, in all cases, the reductions following exposure to either methanol or formate alone were greater than reductions observed with methanol/formate mixtures. The observation led authors to conclude that methanol and formate have an infra-additive (less than additive) interaction and produce effects through different mechanisms of toxicity.

Strengths/weaknesses: A strength of this study was that a sufficient number of embryos per group were examined. In addition, the combined effect of methanol and formate was investigated; such a mixture of the two compounds is the most likely occurrence *in vivo*. The embryotoxicity of the mixtures was predicted based on the results of previous studies; the results of either compound alone were almost exactly as predicted.

Some limitations were noted for this study. Previous work had demonstrated that the rat was less sensitive than the mouse to the effects of methanol or formate; however, rather than choosing the most sensitive species for this study, the authors chose to study the effect of mixtures in the rat. Little information is presented in the Methods section regarding the actual concentrations of methanol and formate used in these studies. In Fig. 6 of the study, data are presented in the figure that are not found (or discussed) elsewhere in the manuscript.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is moderate-to-high. The methanol and formate concentrations used gave almost exactly the results predicted by previous work showing the reproducibility of the results and increasing confidence in them.

3.3. Utility of data

The human data are inadequate to assess the developmental toxicity of methanol.

Data from animal prenatal exposure studies are sufficient to demonstrate that methanol is a developmental toxicant following inhalation exposures resulting in blood methanol levels of 537 mg/l in the mouse and 1840 mg/l in the rat. Studies in mice sufficiently demonstrated the same developmental pattern of response following oral or inhalation exposures resulting in equivalent blood levels of methanol.

Studies that evaluated neurobehavioral effects in Long-Evans rats exposed prenatally and/or during the neonatal stage are sufficient to demonstrate that methanol blood levels of 555 mg/l in dams and 1260 mg/l in offspring are associated with adverse neurological effects.

Neurobehavioral studies in primates suggested minor alterations in cognitive function following prenatal exposure to methanol but due to study limitations, were judged to be insufficient for assessing human hazard.

The mechanistic studies sufficiently define the period(s) of embryonic development that are most sensitive to exposure to methanol or its metabolites. There are *in vitro* data that suggest that methanol or formate are developmental toxicants and that acidosis contributes to effects seen with formate. Other studies suggest that simultaneous exposure to methanol and formate are not additive. *In vivo* studies clearly indicate that methanol per se is the likely developmental toxicant in mice.

The results of the animal developmental toxicity studies are assumed to have biological relevance to human. They have clear value to risk assessment by identifying methanol blood level as a useful biomarker of exposure and effect.

3.4. Summary of developmental toxicity

A single study [104] was reviewed in which a variety of occupations and consequent exposure to complex mixtures were determined in women who gave birth to infants with and without cleft lip or palate. The study did not find an association between methanol exposure and oral clefts, but several limitations in the study were noted including: small number of subjects exposed to methanol, lack of individual exposure data, and confounding by other chemical exposures. Because of these limitations the Panel judged the study results to be uncertain and concluded there are insufficient human data upon which to evaluate the developmental toxicity of methanol.

Since methanol is metabolized by a folate-dependent pathway, the Expert Panel reviewed a number of epidemiological studies that examined folate supplementation and various birth defects [108–112]. In general these studies suggest that periconceptional supplementation with multivitamins containing folic acid decreases the incidences of birth defects including NTDs and orofacial clefts. These studies suggest that it will be important to consider possible interactions between methanol exposure and folate status in animal studies in view of various interspecies differences such as differences in folate levels, methanol metabolism, and toxicokinetics.

3.4.1. Experimental animal data

The Panel reviewed developmental toxicity studies that were performed in rats, mice, and non-human primates. Results of these reviews are summarized below.

3.4.1.1. Prenatal rat studies. The results of Nelson et al. [98] are sufficient to conclude that inhalation exposure of Crl:Sprague–Dawley rats to 20,000 ppm methanol vapor for 7 h per day on gd 7–15 causes prenatal developmental toxicity as evidenced by reduced fetal weight, increased litter incidence of exencephaly and encephalocele, and skeletal

malformations. This dose caused clinical signs of maternal intoxication in early days of exposure but no other maternal effects. Developmental toxicity was also observed following exposure to 10,000 ppm for 7 h per day on gd 1–19 as evidenced by statistically significant reductions in fetal body weight. The Expert Panel designated 10,000 ppm inhaled methanol as a maternal NOAEL and 5000 ppm as a fetal NOAEL. Blood methanol levels were determined in non-pregnant rats with exposures similar to the pregnant dams and were reported at 1840–2240 mg/l and 5250–8650 mg/l in rats exposed to 10,000 and 20,000 ppm methanol, respectively. In the study by NEDO [99], maternal toxicity and adverse developmental effects were observed in Sprague–Dawley rats after inhalation of 5000 ppm methanol on gd 7–17 for an average of 22.7 h per day. Increased numbers of late resorptions, reduced numbers of live fetuses, decreased fetal weight, and increased numbers of malformed fetuses were observed. The observed malformations were similar to those observed by Nelson et al. [98]. No adverse effects were observed at 1000 ppm. Deficiencies in design or completeness of data presentation led the Expert Panel to conclude that the studies of Cummings [138] and Youssef et al. [140] were of limited utility in this evaluation.

3.4.1.2. Prenatal mouse studies. The studies of Rogers et al. [96] are sufficient to conclude that prenatal exposure of Crl:CD-1 mice to methanol vapor at doses of 2000 ppm or greater for 7 h per day on gd 6–15 causes developmental toxicity as evidenced by cleft palate, exencephaly, and skeletal malformations. The initial appearance of malformations was dose-associated with cervical ribs seen at 2000 ppm and cleft palate and exencephaly at 5000 ppm. Effects on the number of live pups per litter and fetal weight were seen at 7500 and 10,000 ppm, respectively. Methanol blood levels in the 2000, 5000, 7500, 10,000, and 15,000 ppm groups were measured at 537, 1650, 3178, 4204, and 7330 mg/l, respectively. The developmental toxicity NOAEL was 1000 ppm. The maternal NOAEL was judged to be 15,000 ppm by the Expert Panel. Rogers et al. [96] also established dose comparability across inhalation and oral gavage exposure by demonstrating that twice daily gavage with 2000 mg/kg bw per day methanol on gd 6–15 results in a methanol blood level (3856 mg/l) and developmental pattern of response similar to that in mice exposed to 10,000 ppm methanol vapor. No postnatal studies were performed in the mouse.

3.4.1.3. *In vivo* rodent mechanisms studies. A considerable literature (10 reports or publications) was reviewed relevant to characterizing mode of action of methanol's effects on developmental toxicity in the rodent. Two laboratories conducted phase-specificity studies in CD-1 mice exposed to teratogenic concentrations of methanol through inhalation [149,150]. The majority of findings were consistent between laboratories. As expected, methanol exposure during the period of neural tube development and closure (gd 7–9) resulted in exencephaly. The incidence of cleft palate was

also increased following exposure during gd 7–9, despite the fact that cleft palate closure occurs later in gestation. Gd 7 was found to be the most sensitive day for developmental effects, since treatment on that day resulted in the greatest incidence of resorption, exencephaly, cleft palate, and vertebral and rib defects. Bolon et al. [94] subsequently identified a putative mode of action (reduced proliferation) and targets of toxicity (neuroepithelium, mesoderm, neural crest) for methanol-induced NTDs in embryos of CD-1 mice exposed to 15,000 ppm methanol vapors for 6 h per day from gd 7–9. Connelly and Rogers [151] studied whether cervical vertebrae were associated with homeotic shifts and concluded that methanol can alter segment patterning in CD-1 mouse embryos, resulting in posteriorization of cervical vertebrae.

Dorman et al. [66] reported an important series of experiments designed to investigate the role of methanol and its metabolite, formate, on development using CD-1 mice. Using a dose of sodium formate (750 mg/kg bw) that is equivalent to the formate concentration following inhalation of 15,000 ppm methanol, no exencephaly was observed. However, 15,000 ppm methanol exposure is a dose that produces exencephaly in mouse fetuses, thus suggesting exencephaly in mice requires direct exposure to methanol as opposed to only accumulation of formate. Two studies examined the impact of folate pools on methanol-induced developmental toxicity in CD-1 mice fed diets with adequate or reduced folate levels [80,105]. In dams fed folate-deficient diets, maternal and fetal hepatic folate levels were reduced. Folate deficiency enhanced the toxicity of methanol as noted by increased incidences of cleft palate and exencephaly in mice treated with methanol at 4000–5000 mg/kg bw per day. The Expert Panel noted that the folate deficiency studies were limited due to a lack of pair-fed controls. Using *in vivo* intrauterine microdialysis, Ward and Pollack [65] collected data in mice and rats to indicate that at doses that are developmentally toxic (100 or 500 mg/kg or 1000 mg/kg/h, IV) there is also a reduced uteroplacental blood flow. They postulated that, under these conditions, hypoxia may have a role in the etiology of embryotoxic effects of methanol. Ward and Pollack [61] compared the rate of methanol metabolism in pregnant and non-pregnant mice and rats and fetal mice. Pregnancy appeared to reduce metabolic rate by ~15%; metabolic rate in mouse liver homogenate was about two-fold greater than rat liver homogenates. Metabolic rates in fetal homogenates were only 5% of those seen in adults. These fetal data are consistent with earlier observations on alcohol and aldehyde dehydrogenases in rat fetuses [153].

3.4.1.4. Postnatal rat studies. Stanton et al. [100] exposed Long-Evans rats to 15,000 ppm methanol vapor for 7 h per day on gd 7–19 and observed modest but statistically significant reduced bodyweight in pups at birth, weaning, and pnd 35. Effects were not observed postnatally in the pups that were subjected to a range of tests for neurobehavioral function; however, small sample size limits confidence in these negative results.

Offspring from Long-Evans dams that drank water containing 2% methanol on either gd 15–17 or 17–19 were observed to have an increased latency to effect nipple attachment or to reach their home nesting site [141]. In a later study, Weiss et al. [95] determined neurological function in Long-Evans pups following 6 h per day exposure to 4500 ppm methanol vapor to dams from gd 6 through pnd 21 and to pups from pnd 1–21. No effects were observed on dam weight during gestation, litter size or postnatal pup weight gain to pnd 18. No effects were observed in latency to nipple attachment or olfactory sensory capabilities. Changes in motor activity were variable or inconsistent, but performance on an operant tests suggested subtle cognitive effects.

In the aggregate, the data from postnatal assessments of Long-Evans rats give no indication of maternal toxicity or effects on pup viability following prenatal doses of up to 15,000 ppm methanol vapor [100], or pre and postnatal exposure to 4500 ppm [95]. Modest reduction in bodyweight was observed postnatally in pups whose dams had been exposed to 15,000 ppm methanol vapor on gd 7–19 [100]. Suckling behavior was affected in a drinking water study [141], but not replicated in an inhalation study [95]. While numerous behavioral outcomes were assessed and found to be negative, one significant effect, the failure of methanol-exposed rats to adjust to a change in response requirements in an operant task, suggested subtle cognitive effects following exposure to 4500 ppm with peak maternal blood levels reported at 555 mg/l [95].

There is sufficient evidence in Long-Evans rats that extended exposure via methanol inhalation at 4500 ppm with peak maternal blood levels reported at 555 mg/l, and blood methanol levels in rat offspring at pnd 21 reported at 1260 mg/l is associated with adverse neurological outcomes.

3.4.1.5. *In vitro* rodent studies. To gain a better understanding of mechanisms of toxicity, seven *in vitro* studies were conducted with methanol or formate. Exposure of rat and mouse embryos to methanol demonstrated effects consistent with those observed *in vivo* with a greater intrinsic sensitivity of mouse versus rat embryos; developmental toxicity in rats and mice was noted with methanol concentrations of ≥ 8000 mg/l and 2000 mg/l, respectively [154]. Increased cell death was noted in mouse (dose not clear) and rat (16,000 mg/l) embryo structures associated with malformations following *in vivo* exposures; however, increased cell death was not noted in neural tube regions, suggesting a mechanism other than cell death for NTDs [155]. Treatment of mouse and rat embryos with formate demonstrated effects similar to those of methanol, but the formate concentrations that caused effect (543–1840 mg/l) were 4–10-fold lower [66,157,158]. Toxicity appeared to be induced by both the formate ion and resulting acidosis. In a study testing mixtures of methanol and formate in rat embryos, it was found that the effects of the two compounds were less than additive [159]. According to Andrews et al. [157] and

Table 46
Nominal exposure levels to methanol vapor and corresponding blood methanol levels in rats [98] and mice [96]

Species	Maternal NOAEL	Fetal NOAEL	Maternal LOAEL	Fetal LOAEL
Sprague–Dawley rat	10,000 ppm (1840–2240 mg/l) ^a	5000 ppm (1000–2170 mg/l) ^a	20,000 ppm (5250–8650 mg/l) ^a	10,000 ppm (1840–2240 mg/l) ^a
CD-1 Mouse	15,000 ppm (7330 mg/l) ^a	1000 ppm (97 mg/l) ^a	Unknown	2000 ppm (537 mg/l) ^a

^a Maternal blood methanol level.

Brown-Woodman et al. [158], the formate levels that produced toxicity in *in vitro* studies are not likely to occur in humans following environmental or occupational exposures.

3.4.1.6. Rat/mouse comparison. In comparing similar studies in rodents, the data are sufficient to demonstrate that exposure to high concentrations of methanol vapor can cause similar prenatal developmental toxicity and frank malformations. There are good, but limited, data to indicate that the nature and incidence of fetal effects correlates with blood methanol concentration when methanol exposure is by inhalation or the gavage route. Mice are judged to be the more sensitive species since effects were noted at lower chamber concentration doses than rats. However, at equivalent chamber concentrations, mice had higher maternal blood methanol levels. Table 46 compares NOAELs from the definitive prenatal developmental toxicity studies in rat and mouse.

Cross species comparisons as to postnatal effects are not possible as there are only data in rats.

3.4.1.7. Postnatal nonhuman primate studies. Burbacher et al. [143] studied the effects of methanol on general and neurobehavioral development of *M. fascicularis* infants whose mothers were exposed to methanol vapors (200–1800 ppm for 2.5 h per day leading to blood methanol levels of 5–35 mg/l) throughout gestation. It was reported that duration of pregnancy was reduced in primates exposed to methanol vapors, and that C-sections were performed in some treated animals but not in controls (see Section 4 for discussion). Adult monkeys experienced no effects on weight gain or overt toxicity as a result of methanol exposure. Normal weight gain and physical development was observed through the first year of infant life. Neurobehavioral performance was similar in control and methanol groups in seven of nine tests. A subtle, statistically significant, dose-related delay in sensorimotor function was seen in males of the 600 and 1800 ppm groups and in both sexes at 1800 ppm when data were combined for both cohorts. Prenatal methanol exposure decreased preference for novel social stimuli; however, there was no evidence of a dose response relationship. An additional study looking at postnatal exposure to aspartame demonstrated no effects on general health, development, or learning in *M. arctoides* monkeys fed with up to 2700 mg/kg bw per day aspartame (equivalent to 270 mg/kg bw per day methanol) during the first 9 months of life [147,148]. The differences between effects observed in these nonhuman primate studies may be

explained by exposures occurring during different critical windows of nervous system development (i.e. prenatal versus postnatal exposures). These nonhuman primate studies taken together suggest that despite presumed higher levels of blood methanol achieved in the postnatal exposure study, prenatal exposure may be the more sensitive period leading to altered neurological function in nonhuman primates.

The Expert Panel agreed that these neurobehavioral findings in monkeys were not robust and recognized issues regarding the failure to control for multiple comparisons in the statistical analysis. The findings, however, are important from a qualitative perspective and the biological plausibility for effects on these two early tests of cognitive performance in the Visually Directed Reaching task and novelty preference in the Fagan test warrants further investigation. The Panel recommended that an independent statistical analysis of the Burbacher et al. [143] study might provide additional insights. In addition, the Panel recognizes that monkeys from this methanol study are still being evaluated for latency and persistence in functional deficits.

While the primate data examining the postnatal neurological outcomes raise some concerns it has identified insufficiencies that prevent making a clear determination about human risk.

Both the rodent and primate neurobehavioral outcomes do suggest that alterations in cognitive function are consistent and subtle.

3.4.2. Role of methanol as the proximate teratogen

The Expert Panel considered several possible metabolites as being responsible for methanol-induced developmental toxicity. The first was that, as with acute methanol toxicity, formate would be the proximate teratogen. *In vitro* embryo culture studies suggest that formate can induce structural abnormalities in rats or mice [157,158]. Data from Dorman et al. [66], however, provide direct evidence that formate is unlikely to play a significant role in methanol-induced teratogenesis in mice *in vivo*. The Panel concluded that methanol is the most likely proximate teratogen; however, the biological basis by which it induces defects remains unknown. Gastrulating and early organogenesis-stage rodent embryos were particularly sensitive to adverse developmental effects of methanol. The Panel concluded that the available rodent data are relevant for humans despite known differences between species with respect to methanol metabolism. The Expert Panel concluded that rodents are a good model for human exposures to methanol at levels where formate is not accumulated, since rodents do not accumulate formate

even at very high doses of methanol. Therefore, the developmental toxicity of methanol alone (without formate) can be analyzed in rodents at dosages high enough to determine LOAELs and NOAELs. In conclusion, there is sufficient evidence in rodents that inhalation of methanol at doses of 2000 ppm or greater in mice (blood methanol level of 537 mg/l) or 10,000 ppm or greater in rats (blood methanol level of 1840 mg/l) for 7 h per day throughout organogenesis does cause developmental toxicity. These data are assumed relevant to consideration of human risk.

The Panel concluded that there is sufficient evidence to assume that methanol could be a developmental toxicant in humans. The Panel also noted that the blood methanol concentrations that have been associated with developmental toxicity in rodents are in the range associated with formate accumulation, metabolic acidosis, and other signs of acute toxicity in humans.

4. Reproductive toxicity

This section contains evaluations of original studies.

4.1. Human data

There were no human data located.

4.2. Experimental animal toxicity

Because methanol is so commonly used in industry, Cameron et al. [160] studied the effects of methanol exposure on the male reproductive system. Groups of five mature male Sprague–Dawley rats per group [source and age not specified] were exposed to methanol vapors (99.5% purity) at 0, 200, 2000, or 10,000 ppm for 8 h per day, 5 days per week, for 1, 2, 4, or 6 weeks (Table 47). [There was no discussion of rationale for dose selection.] Five control animals were exposed to air only. Animals were sacrificed 16 h following the last exposure and serum levels of testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were measured by radioimmunoassay (RIA).

(The number of animals examined at each time period was not specified.) Statistical significance was evaluated by Student's *t*-test. Significantly reduced levels of testosterone were noted at week 2 for the 200 ppm group and at week 6 for the 200 and 2000 ppm group. The greatest reduction in testosterone level occurred in the 200 ppm group at week 6. A significant increase in LH levels was noted in the 10,000 ppm group at week 6, the only time point of measurement. To determine the cause of reduced testosterone levels, the authors exposed five mature male rats per group to 200 ppm methanol vapors for 6 weeks, intravenously injected the rats with [¹⁴C]testosterone 16 h following the last exposure, and measured levels of plasma [¹⁴C]testosterone. The experiment demonstrated that methanol does not increase the rate of testosterone removal from blood; the authors therefore concluded that methanol exposure affects the rate of testicular testosterone synthesis.

Strengths/weaknesses: The strengths of this study are that the exposures appear to have been well-controlled as the variations around the target concentrations appear relatively small and the purity of methanol was reported.

Some weaknesses were noted for this study. The lack of detail regarding measurement of chamber concentrations reduces confidence in the chamber concentrations reported. It was not stated if rats were randomly assigned to treatment groups and the ages of rats were not reported. The number of rats used is quite small for hormone studies (*n* = 5), thus increasing the chances of finding spurious effects. Because of inter-animal variations, at least 15 animals are required and 20 per group are measurably better when doing single-point hormone evaluations [161,162]. The time between the end of exposure and death is long. The animals were killed ~16 h after the end of the last exposure, by which time circulating methanol levels would have declined, and any compensatory change in hormone levels would have had time to occur. Thus, the reader is not sure whether the effects seen are due to methanol exposure, or if they are “rebound effects” resulting from the absence of the main methanol effect. The data in the Cameron et al. [163] paper (discussed below) are consistent with a possible rebound effect, although how a rebound would result in depressed values and in an inverse dose–response is not immediately clear. Methodologic de-

Table 47
Summary of reproductive toxicity study in rats [160]

Experimental regimen	Number ^a	Dose (ppm)	Effects
Cameron et al. [160] exposed mature male rats (source not specified) to methanol vapors for 8 h per day, 5 days per week, for 1, 2, 4, or 6 weeks. Methanol concentrations in inhalation chambers were verified. The animals were sacrificed 16 h following the last exposure period to determine serum levels of testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH)	5	0	
	5	200	↓Testosterone on week 2 (55% of control level)
	5	2,000	↓Testosterone on week 6 (32% of control level)
	5	10,000	↓Testosterone on week 6 (59% of control level)
	5		↑LH on week 6 (311% of control level)

↑: Statistically significant increase.

↓: Statistically significant decrease.

^a The number of rats exposed/each sacrifice period.

tails are sparse at best. For example, no details of the RIA assay are provided, so the Panel has little idea of the confidence in the assay that generated the numbers. Of greater concern is the fact that the statistics are inappropriate (hormone data are almost never normally distributed, and repetitive *t*-tests assures too many false-positive comparisons). Minimal data or methods were provided for the radiolabeled clearance study, which prevents significant weight being placed on these data. Lastly, the Panel noted the lack of LH measurements for most time periods. The inverted dose–response for testosterone is intellectually challenging to interpret, as no known mechanism can be invoked. The fact that the pattern of changes in LH are mirrored by change in testosterone suggests that the primary effect is on the CNS, which drive changes in testosterone production, but the fact that normal LH levels are coupled with testosterone values that are 60% of control suggest that there are peripheral effects as well.

Utility (adequacy) for CERHR evaluation process: The Panel's confidence in these data is low because of the weaknesses of this study and limited reporting of data. The Panel is not confident in the link between exposures and effects reported by this study. The data might be useful in confirming data from another study without these limitations.

In a second study, Cameron et al. [163] assessed four alcohols (methanol, ethanol, *n*-propanol, and *n*-butanol) to determine the effects on male hormonal levels. Groups of five male mature Sprague–Dawley rats (source and age not specified) were exposed to methanol vapors (99% purity) at 0 or 200 ppm for 6 h per day for 1 day or 1 week (Table 48). [The basis for dose selection was not discussed.] Five control animals were exposed to air only. Animals were sacrificed either immediately or 18 h after the last exposure period. Serum levels of testosterone, LH, and corticosterone were measured by RIA. [The number of rats examined was not specified.] Statistical significance was determined by Student's *t*-test. A significant reduction in testosterone level was noted immediately following the first 6-h exposure for each of the four alcohols. Levels returned to control values after 18 h in all but the *n*-butanol group. No other changes in hormone levels were observed. [According to the Expert Panel, these data seem to suggest that methanol affects both peripheral testosterone production and central LH secretion, as LH was not elevated when testosterone was reduced.]

Strengths/weaknesses: Many of the strengths and weaknesses for this study are similar to those in the previous study [160]. A strength of this study is that some animals were killed immediately after the end of exposure, thus addressing one of the concerns noted for the Cameron et al. [160] study. There does seem to be some recovery of testosterone levels that occurs within 18 h after ceasing exposure. A second strength is that there are both LH and testosterone data for these timepoints, allowing a sense of site(s) of action.

The weaknesses of this study include no reporting of chamber concentrations or methods used to measure the concentrations, insufficient reporting of methods, use of a small number of animals, and no information about assay performance (a relatively minor point). See previous study [160] for an explanation about these limitations.

Utility (adequacy) for CERHR evaluation process: Some of the data from the Cameron et al. [163] study were apparently similar to those reported in the Cameron et al. [160] paper, which slightly increases the Panel's confidence in the 1984 paper. Collectively, the Panel believes that the data from the Cameron et al. [163] study have more value for the Evaluative Process, and places moderate confidence in these data. However, the study is still limited by the small numbers of animals per group. These two papers [160,163] are viewed as best used to corroborate other data.

Lee et al. [164] noted the lack of dose- and time-related responses of testosterone levels in rats exposed to methanol in the Cameron et al. [160,163] studies. Therefore, they conducted a series of studies to further investigate the testicular effects following methanol exposure. In the first study, 8-week-old male Sprague–Dawley [CrI: CD(SD)BR VAF/Plus] rats ($n = 9–10$ per group) were exposed to 200 ppm methanol (purity not specified) by inhalation for 8 h per day, 5 days per week, for 1, 2, 4, or 6 weeks (Table 49). (It is assumed the dose level was selected because it was the dose evaluated by Cameron et al. [160,163].) Nine control rats were exposed in chambers to clean filtered air. Serum testosterone levels were measured by RIA at the end of exposure in 9–10 rats/exposure period between 9:00 and 11:00 a.m. in order to avoid diurnal fluctuations in testosterone levels. Statistical significance was determined by one factor analysis of variance followed by Student's *t*-test. Methanol treatment had no effect on serum testosterone concentration, the gross appearance of reproductive tissues, or testes or seminal vesicle weight. These testes were next

Table 48
Summary of reproductive toxicity study in rats, Cameron et al. [163]

Experimental regimen	Number ^a	Dose (ppm)	Effects
Cameron et al. [163] exposed mature Sprague–Dawley male rats (Source not specified) to methanol vapors for 6 h per day for 1 day or 1 week. Methanol concentrations in inhalation chambers were verified. One group of animals was sacrificed immediately after each exposure period and a second group was sacrificed 18 h following the last exposure period. Serum levels of testosterone, luteinizing hormone (LH), and corticosterone were measured	5	0	
	5	200	↓ Testosterone immediately after one day of exposure (41% of control level)

↓: Statistically significant decrease.

^a The number of rats exposed/each sacrifice period.

Table 49
Summary of reproductive toxicity study in rats [164]

Experimental regimen	Number ^a	Dose (ppm)	Effects
Lee et al. [164] exposed mature Sprague–Dawley male Crl:CD(SD) BR VAF/Plus rats (8-weeks old) to air or methanol vapors for 8 h per day, 5 days per week, for 1, 2, 4, or 6 weeks. Methanol concentrations in inhalation chambers were verified. The animals were sacrificed on the last day of exposure between 9:00 and 11:00 a.m. Testes and seminal vesicles were weighed and serum levels of testosterone were measured. Testes were examined for in vitro production of testosterone with and without human chronic gonadotropin	9 9–10	0 200	No effects on testosterone levels, gross appearance of reproductive tissues, testes seminal vesicles or body weight, or in vitro testosterone production

^a Number of rats exposed/each sacrifice period.

incubated in vitro and it was determined that methanol treatment had no effect on testosterone production, with or without the addition of human chronic gonadotropin hormone.

In an additional experiment Lee et al. [164] determined if testicular lesions indicating changes in testosterone levels were present in rats exposed to methanol. These experiments also examined the effects of both dietary folate intake and age. Four-week-old male Long-Evans (Crl: [LE] BR VAF/Plus) rats were fed diets with sufficient or reduced folic acid (3–4 mg/kg or <0.05 mg/kg with 1% succinylsulfathiazole, respectively). At 7 months of age, rats (≥ 9 per group) were exposed to methanol vapors [**purity not specified**] at 0, 50, 200, or 800 ppm for 20 h per day continuously for 13 weeks (Table 50). A group of 15-month-old rats (8–12 per group) were exposed to 0 or 800 ppm methanol vapors for 20 h per day for 13 weeks. [**Treatment of controls was not discussed, no rationale was provided for dose selection.**] The authors stated that acidosis and visual impairment occurred in the rats fed low folic acid diets and exposed to methanol. At the end of exposure, testes were removed, weighed, and preserved in 10% neutral buffered formalin, embedded in glycol methacrylate,

and stained with PAS or methylene blue. The testes from 8–12 rats per group were examined. [**There was no statistical evaluation of histological effects.**] At the end of exposure, there were no methanol-induced, dose-related increases in testicular lesions or changes in testes or body weights in 10-month-old rats fed diets with sufficient or reduced amounts of folate. The rats that received sufficient folic acid and were 18 months old at the end of exposure also experienced no dose-related increases in testicular lesions. However, in the 18-month-old rats fed reduced folic acid diets, methanol exposure increased the incidence but not severity of age-related testicular lesions. Specifically, mild, age-related testicular degeneration, consisting of subcapsular vacuoles in the germinal epithelium of seminiferous tubules, was noted in 3/12 control rats and 8/13 rats exposed to 800 ppm methanol. [**This lesion appeared to the Panel to be more properly a fixation-induced shrinkage artifact. The Panel could not interpret an increased likelihood to shrink upon fixation as an adverse treatment-related health effect.**] Additional lesions included atrophy of seminiferous tubules in 1 rat and Leydig cell hyperplasia in another rat of the 800 ppm methanol group.

Table 50
Summary of reproductive toxicity study in rats, Lee et al. [164]

Experimental Regimen	Number ^a	Dose (ppm)	Effects in folate-sufficient group	Effects in folate-reduced group
Lee et al. [164] fed 4-week-old Crl: Long-Evans (LE) BR VAF/Plus rats folate-sufficient (3–4 mg folic acid/kg) or folate-reduced (<0.05 mg folic acid/kg with 1% succinylsulfathiazole) diets. At ~7 months of age, the rats were exposed to air or methanol vapors for 20 h per day for 13 weeks. Methanol concentrations were monitored inside inhalation chambers. At the end of the exposure period (10 months of age), body and testes weight were measured and testes (preserved in 10% neutral buffered formalin) were examined histologically	11–13 12 12 9–12	0 50 200 800	1/11 With testicular lesions 0 Testicular lesions 0 Testicular lesions 0 Testicular lesions	0 Testicular lesions 2/12 With testicular lesions 1/12 With testicular lesions 0 Testicular lesions
The same experiment was conducted in rats that were ~15 and 18 months old at the beginning and end of methanol exposure, respectively	10–12 8–13	0 800	4/10 With testicular lesions 3/8 With testicular lesions No effects on body or testes weights	3/12 With testicular lesions 8/13 With testicular lesions 1/13 with Leydig cell hyperplasia No effects on body or testes weights

^a Number of rats in folate-sufficient and folate-reduced groups.

Strengths/weaknesses: The ages of animals were appropriate. Strengths include evaluation of hormone status by several means: (1) direct RIA measure of testosterone (complete with assay performance data), (2) weight of androgen-sensitive organs, (3) an assessment of the capability of in vivo exposed testes to produce testosterone in vitro, and (4) histologic assessment of the seminiferous epithelium, which would show a specific low-androgen lesion if a biologically-meaningful reduction in testosterone had occurred. The concordance among all these endpoints confers great credibility to the conclusion of no methanol effect on testosterone. The methods of fixing and preserving the testis were sufficient, although not entirely without some artifact. It appears as though some of the vacuoles are shrinkage-induced artifacts that may occur during fixation. However, the fixation methods are better than those used by many investigators. In addition, the authors used sufficient animals to allow confidence in the data, randomly divided animals into treatment groups, provided some details on the analytic methods for verifying chamber methanol concentrations, and used appropriate statistics for comparing testosterone levels.

A limitation of this study was the number of animals in which testosterone levels were measured. The variances in Table 2 of the study are all large (in some cases, almost the same value as the mean), indicating large inter-animal variability. However, this is compensated by the other testosterone-dependent measures (seminal vesicle weight, in vitro testosterone production, and testis histology), all of which are concordant with no change in testosterone production. A second limitation was that testosterone was not measured in folate-reduced rats, but only in folate-sufficient rats. If reduced-folate rats are a physiologically-relevant surrogate for methanol-exposed humans, it would have been useful to have measured serum testosterone in folate-reduced rats. Lastly the purity of methanol was not reported.

Utility (adequacy) for CERHR evaluation process: The Panel has high confidence in the methods and data resulting from these studies. The Panel considers that these data collectively show little or no ability of methanol, at 200 ppm in SD rats, or up to 800 ppm in Long Evans rats, to reduce testosterone signaling in vivo. The apparent increase in aging changes in the 800 ppm-exposed Long-Evans rats is of uncertain significance.

Cooper et al. [101] studied the effects of methanol exposure on rat serum pituitary hormone levels in an attempt to replicate the findings of Cameron et al. [160,163] and to determine if hormone levels were affected by handling of animals during inhalation exposure. The experiments used male Long-Evans hooded rats (Harlan Sprague–Dawley) that were or were not acclimated to exposure and handling conditions. Rats were acclimated by removing them from their home cages and transferring them to inhalation chambers for 2 weeks. In the first experiment 10 rats per group (90 days old) were exposed to methanol vapors (Optima Grade from Fisher Scientific [136], >99.9% purity) at levels of 0, 200, 5000, or 10,000 ppm for 6 h. The doses were

based on those used in studies conducted by Cameron et al. [160,163], Nelson et al. [98], and Infurna and Weiss [141]. A control group consisted of sham-exposed rats. One group of rats was sacrificed immediately following exposure and a second group was sacrificed 18 h later (24 h after the start of exposure). Statistical significance was evaluated by analysis of variance; when significant interactions were observed further comparisons were made by Student's *t*-test. Serum methanol levels in acclimated rats immediately after exposure were measured at 7.4, 680, and 1468 mg/l in the 200, 5000, and 10,000 ppm methanol treatment groups, respectively. At 24 h following exposure, serum methanol levels exceeded the detection limit only in the high-dose group and were measured at 235 mg/l. Analyses were conducted to measure serum levels of testosterone, LH, and FSH and testicular interstitial fluid testosterone ($n = 10$) by RIA in 10 rats per group. Results of hormone analyses are illustrated in Fig. 4. The following discussion on serum hormonal levels includes only effects that were statistically significant. Immediately after exposure, change in LH level was the only effect noted. The non-acclimated rats exposed to methanol at 5000 ppm showed an apparent ~40% reduction in LH. An increased LH level in non-acclimated versus acclimated controls indicated that higher LH levels were associated with handling of the rats, but this was not seen at 24 h after the start of the last exposure. Methanol treatment resulted in an increased LH level in acclimated rats exposed to 10,000 ppm when killed immediately after exposure, but reduced LH at 5000 ppm in non-acclimated rats. At 24 h, a methanol-induced increase in LH was noted in acclimated rats of the 10,000 ppm group. At 24 h, the serum testosterone level was reduced in acclimated rats exposed to 10,000 ppm methanol, but increased in non-acclimated rats exposed to 5000 ppm methanol. Changes in testosterone levels occurred in opposite directions in acclimated versus non-acclimated rats of all methanol treatment groups. Results were similar for testicular interstitial fluid testosterone levels. The authors noted that the experiment did not reproduce the results of Cameron et al. [160,163] because exposure to 200 ppm methanol did not reduce serum testosterone levels.

In the second experiment, Cooper et al. [101] measured serum methanol, testosterone, LH, and prolactin levels in ten, 90-day-old male Long-Evans rats per group exposed to 5000 ppm methanol vapors for 1, 3, or 6 h. Unless otherwise specified, the details were the same as the previous experiment by Cooper. Measurements were conducted immediately after exposure. Serum methanol concentrations in acclimated rats were 242, 397, and 752 mg/l after exposure for 1, 3, and 6 h, respectively. In non-acclimated rats, serum methanol concentrations after 1, 3, and 6 h of exposure were 299, 683, and 873 mg/l, respectively. The increased concentrations of serum methanol in non-acclimated rats after 3 or 6 h of exposure were statistically significant. Methanol treatment had no effect on serum testosterone and LH levels when compared to unexposed controls in the same acclimation group. However, both testosterone and LH levels were

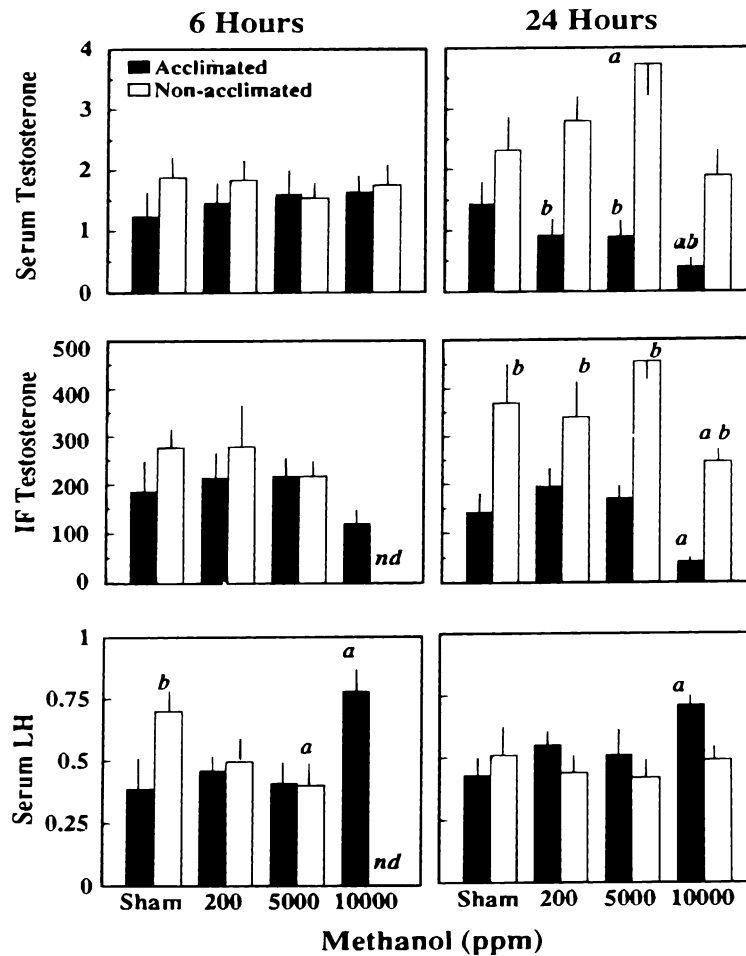


Fig. 4. Hormonal levels in rats exposed to methanol. Reprinted with permission from Elsevier Science [101].

significantly higher in non-acclimated versus acclimated rats with or without methanol exposure. Methanol treatment significantly increased serum prolactin levels in comparison to non-exposed controls of the same acclimation group and prolactin levels were highest in the non-acclimated rats at 1 and 6 h of exposure. The authors concluded that methanol exposures can affect serum hormonal levels, but the magnitude and direction of change depends upon the handling of the animal.

Strengths/weaknesses: The strengths of these studies are that age of the animals were appropriate, significant methodological detail was provided, appropriate statistics were used, methanol purity was reported, methanol concentrations in chambers were monitored and reported, internal evaluations (method-checks on methanol analyses and RIA assay performance) were conducted, serum methanol levels were measured, and the animals were randomly divided into exposure groups.

A limitation of the studies is that numbers of animals ($n = 10$) are barely sufficient for most hormone measures. These studies are limited primarily by the complexity of the study design. The authors themselves note that han-

dling appears to change both the direction and magnitude of any hormone changes, which makes the interpretation of any methanol effect (in the words of the authors) “most difficult.”

Utility (adequacy) for CERHR evaluation process: While the Panel had high confidence in the methods of the investigators and the resulting quality of these data, it is difficult to put these data into perspective with other data in the literature. It appears that methanol inhalation is a stressor (based on serum prolactin levels), and any effects of methanol exposure on testosterone require high levels of exposure (5000 ppm or greater), and may be modified by how well-acclimated the rats are to the exposure apparatus and process. Taken at face value, these studies appear to support the lack-of-effect noted by Lee et al. [164].

The Japanese New Energy Development Organization [99] sponsored a two-generation study in CrI:CD Sprague-Dawley rats. At 8 weeks of age, male and female rats ($n = 30$ /sex per group) were randomly assigned to groups that were exposed to 10, 100, or 1000 ppm methanol vapors (reagent grade, stated to have <1 ppm vinyl chloride monomer and <3 ppm formaldehyde). Dose selection was

based upon the ACGIH TLV and observations in other studies sponsored by this group. Chamber concentrations of methanol were monitored and reported. A group of 30 control rats/sex per group was exposed to air in chambers, while a second group of 30 control rats/sex per group was not handled. Exposures were conducted for approximately 20 h per day. Males and females were exposed for 8 weeks prior to mating and throughout the mating period which lasted up to 21 days. Females continued to be exposed throughout gestation and lactation. F₁ pups continued to receive exposures throughout the study duration. Methanol blood levels were measured in 5–8 offspring/sex per group at 9 weeks of age and the respective mean levels from the control to high dose group were 2.00–2.97, 2.94–3.48, 1.02–4.20, and 53.16–99.48 mg/l. Development landmarks (eyelid opening, auricle development, incisor eruption, testes descent, vaginal opening) were monitored in F₁ pups. Two F₁ pups/sex/litter were selected for a breeding study similar to that conducted in the F₀ parental rats. Authors stated that new rats would be added to the study if there were not enough F₁ rats to obtain 20 litters per group. Parameters evaluated in both generations of rats included “sexual cycle” (2 weeks prior to mating), days to insemination, insemination rate, and fertility. Data were analyzed by *t*-test, Mann–Whitney *U*-test, Fisher’s exact test and/or Armitage’s χ^2 -test. Data from the experiment were incompletely reported, but some explanation of findings was provided. Treatment with methanol had no effect on fertility, pup delivery, or lactation behavior in either generation. Testicular descent occurred earlier in F₁ rats of the 1000 ppm group and in the F₂ rats of the 100 and 1000 ppm groups. Systemic effects included significantly reduced bodyweight gain in F₀ males from the 1000 ppm dose group following 7 weeks of treatment; a similar trend was observed in female rats but did not reach statistical significance. Food intake was significantly reduced in F₀ rats from the 1000 ppm dose group. Several other non-reproductive parameters were evaluated, but findings are not being evaluated by CERHR due to the incomplete reporting of data.

Strengths/weaknesses: This appears to have been a well-conducted study that followed the accepted protocol for the conduct of a multigeneration reproduction study. The number of animals was sufficient to detect a treatment-related effect and the conditions of exposure appear to be adequate. The study is enhanced by the measurement of blood methanol concentrations in F₁ animals at 9 weeks of age.

The primary weakness of this study is that very few data are actually presented to support the authors’ conclusions regarding the presence or absence of effects on reproductive and most other parameters. Without data actually being presented, it is not possible for a reader to independently reach the same conclusion as the authors. Other weaknesses include the apparent substitution of animals during the course of the study. It is not clear how many animals were

substituted and the exposure histories of the substituted animals.

Utility (adequacy) for CERHR evaluation process: This study is of limited utility for a CERHR evaluation due to the absence of actual data and uncertainty around the issue of the degree of independent scientific review this document has received.

Ward et al. [165] examined sperm morphology in 4-month-old CrI: B6C3F1 mice that were gavaged with 0 ($n = 5$) or 1000 mg/kg bw per day methanol [**purity not specified**] in water ($n = 10$) for 5 days. The dose resulted in 10 times the methanol level found in formalin, the main interest of the study. Non-parametric tests were used to determine significance of differences among all treatment groups (Kruskal–Wallis test) and between groups (Mann–Whitney *U*-test). Treatment with methanol significantly increased the number of mice with “banana-type” sperm morphology, an effect of unknown biological significance.

There were no histopathological effects observed in the reproductive organs of 15 male and female CrI: Sprague–Dawley rats/sex per group (4–5 weeks old) that were exposed to 2500 ppm methanol vapors for 6 h per day, 5 days per week for 4 weeks [79]. A detailed summary of the study and a discussion of strengths/weaknesses and utility is included in Section 2.2.2.

In two cohorts of *Macaca fascicularis* monkeys (six per group/cohort) that were exposed to methanol vapors at up to 1800 ppm, there were no effects on menstrual cycles or conception rate [52,143]. A non-dose-related reduction in pregnancy duration and increased complications during birth were noted in monkeys treated with 200–1800 ppm methanol and are discussed in greater detail under Section 3.2.2.

The Panel noted that Dr. Alice Tarantal, a primate reproduction expert from the California Regional Primate Research Center, reviewed the reproductive findings of the Burbacher et al. [52,143] study for the American Forrester and Paper Association [166]. Dr. Tarantal noted that there may be an association between methanol exposure and early deliveries. However, she concluded that findings are more likely coincidental and of limited biological significance, since: (1) all deliveries were within the range of historically observed gestational ages for *Macaca fascicularis*, and (2) the birth weight and size of all infants were within normal ranges. Dr. Tarantal stated that there does not appear to be sufficient evidence to support the claim of increased pregnancy complications following methanol exposure. She stated that vaginal bleeding sometimes occurs in macaques 1–4 days prior to delivery of a healthy infant and that it does not necessarily imply a risk to the fetus. An ultrasound examination would have been required to diagnose fetal or placental problems. Lastly, Dr. Tarantal stated that, “It would be useful to review the findings discussed above within the context of normative colony data.”

Strengths/weaknesses: The strengths of these data in terms of a reproductive evaluation are the use of a relevant sub-

human primate model in sufficient numbers to make initial evaluations meaningful, a carefully-designed and executed exposure situation, and evaluation of functional endpoints that comprise female reproduction and are sensitive to toxicant perturbations. General strengths and weaknesses of this study are discussed in Section 3.2.2.

Utility (adequacy) for CERHR evaluation process: The Panel had confidence in the reproductive data, and found them relevant to the consideration of human reproductive risk. No significant reproductive effect distinguished the methanol-exposed groups from the control group, except for a statistically significant ($P = 0.03$) decrease in the duration of pregnancy. Pregnancies resulting in live births were about 6–8 days (5%) shorter in the methanol-exposed groups. Although no other adverse reproductive outcomes (e.g. reduced fertility, spontaneous abortion, reduced neonatal size or weight) were statistically significant, it is noteworthy that C-sections were performed only on methanol-exposed females. Five C-sections were performed in methanol treated groups (two in both the 200 and 600 ppm group and one in the 1800 ppm group) versus no C-sections in the controls. These operations were performed in response to signs of possible difficulty in the maintenance of the pregnancy (e.g. vaginal bleeding) and thus suggest late reproductive dysfunction in the methanol-exposed females. There were no reports of ultrasound confirmation of placental separation in this study. Though concerning, these findings have uncertain utility in demonstrating methanol-induced reproductive toxicity because of the: (1) lack of dose–response over a wide range of blood methanol concentrations, (2) lack of clinical findings indicative of prematurity in the newborns, (3) the small numbers of animals used, and (4) the unavailability of historical control data from the laboratory. The utility of this study for addressing developmental toxicity is included in Section 3.2.2.

4.3. Utility of data

Insufficient data were available in humans to evaluate the reproductive toxicity of methanol. The animal data set included studies conducted in male and female rats and a study conducted in female non-human primates. For male rats, the data were sufficient to evaluate hormonal changes and structural effects on the reproductive system. However, insufficient data were available for the evaluation of structural effects on the female reproductive system and functional reproductive toxicity in male and female rats. In female non-human primates, the data were sufficient to evaluate estrous cyclicity and fertility but were insufficient to evaluate effects on parturition. The data in these species are assumed to be biologically relevant to judging potential hazard in humans.

4.4. Summary of reproductive toxicity

4.4.1. Human data

No human data were located.

4.4.2. Experimental animal data

The Panel reviewed various studies relevant to reproductive toxicity, including hormonal assays in rats. The Panel also reviewed a reproductive function study in female primates.

4.4.2.1. Rat. Four studies examined serum hormone levels in male rats exposed to methanol through inhalation and two studies included a histological evaluation of reproductive organs. The definitive work was a study by Lee et al. [164]. The Panel had high confidence in the results of their study that exposed 8-week-old Sprague–Dawley rats to 200 ppm for 8 h per day for 1–6 weeks and observed no effect on testosterone, weight of androgen sensitive organs, capability of in vivo-exposed testes to produce testosterone in vitro, and lack of gross morphological effect. In the second part of the Lee et al. study, normal and folate-deficient, methanol-sensitive Long-Evans rats exposed to 800-ppm methanol for 20 h per day, 7 days per week for 13 weeks had no adverse testicular histology at 10 months of age. A higher incidence, but not severity, of age-related testicular degeneration was observed in the folate-deficient, 18-month-old rats exposed to 800 ppm methanol for 13 weeks; but the incidence of age-related testicular lesions in the 18-month-old folate-sufficient rats was equal in treated and control rats. The results of Poon et al. [79] who found no lesions in the reproductive organs of 4–5 week-old male and female Sprague–Dawley rats that inhaled 2500-ppm methanol vapors for 6 h per day for 4 weeks were consistent with findings of Lee et al. [164] in 10-month old rats. Their methodology was adequate to detect major testicular effects and of modest utility to detect more subtle effects. The Cameron et al. [160,163] studies examining serum hormone levels were found to be of limited utility because of deficiencies in experimental design and incomplete reporting of data. Their results were not confirmed by Lee et al. [164] or by Cooper et al. [101]. Cooper et al. found that treatment with ≥ 5000 ppm methanol for 6 h could affect serum levels of LH, testosterone, and prolactin. However, the magnitude of the response, and in the case of LH and testosterone, the direction of the response depended on whether or not the animal was acclimated prior to treatment. These data underscore the need to consider the impact that experimental conditions may exert upon hormonal results. Applying such considerations to the reviewed studies may limit the utility of these data.

The NEDO [99] developmental toxicity study that included a postnatal phase demonstrated a significant prolongation of gestation length and reductions in litter size and pup viability following exposure to 5000 ppm by inhalation (see Section 3.2.1). Blood methanol levels were not reported by NEDO but based on other studies where rats were exposed to 5000 ppm methanol it is speculated that blood levels in rats ranged from 700–1000 mg/l [62,98,101]. No effects were observed at 1000 ppm. Because exposure began on gd 7 (i.e. after conception), this study is more

indicative of developmental toxicity than of reproductive function.

The database on methanol's effects on reproduction is fragmented and uneven. The data are sufficient to conclude that 800 ppm by inhalation (20 h/day \times 7 days/week \times 13 weeks) represents a probable NOAEL in rats for male reproductive system structure [164]; blood methanol levels were not measured in this study. Although somewhat contradictory, the weight of the evidence on male reproductive hormones is sufficient to conclude that exposures resulting in blood methanol levels up to approximately 1500 mg/l [101] have no consistent effect on male hormones. The data in rodents are currently insufficient to allow a conclusion regarding methanol's effects on female or male reproductive function. However, the submission of more detailed results from an existing two generation reproduction study in rats [99] could address this data deficiency. Effects on parturition, litter size and pup survival were only observed in a developmental toxicity study at inhalation levels of 5000 ppm, corresponding to a speculated blood methanol level of ≥ 700 mg/l [99].

The reproductive physiology in rodents is assumed to be relevant to humans.

The blood levels of methanol associated with reproductive toxicity in rodents are 700 mg/l and greater. Blood methanol levels of this magnitude in humans would be associated with frank methanol (formate) toxicity.

4.4.2.2. Primate. One study examined reproductive function in female *Macaca fascicularis* monkeys exposed to 200–1800 ppm methanol vapors for approximately 2.5 h per day during a pre-mating and mating period (about 180 days) and the entire pregnancy (about 168 days), producing blood methanol levels of about 35 mg/l at the highest dose [52,143]. There were no effects found on menstrual cycles or conception rates. Variations within the normal range of gestation length [144] were noted in treated animals along with a non-dose-related increase in Cesarean sections performed only in treated animals. While the Panel noted and was concerned with this as a possible sequela of exposure, the lack of a dose–response over a wide range of blood methanol concentrations, the lack of clinical findings indicative of prematurity in the newborns, the small number of animals, and the unavailability of historical control data from this laboratory all prevent the Panel from concluding whether these effects were methanol-related. These data were considered sufficient to demonstrate the lack of a treatment-related effect on menstrual cycles or conception rates, but were considered insufficient to assess effects of methanol on parturition in primates. Nevertheless, the effects on gestation length cannot be discounted, and this left the Panel with some concern about the effects of methanol on primate parturition.

The reproductive physiology and the pharmacokinetics/metabolism of methanol in this study are considered to be relevant to humans.

5. Summaries, conclusions, and critical data needs

5.1. Summary and conclusions of reproductive and developmental hazards

5.1.1. Developmental toxicity

The Expert Panel judged that there are insufficient human data upon which to evaluate the developmental toxicity of methanol. The Panel reviewed developmental toxicity studies that were performed in rats, mice, and non-human primates. The data in mice and rats were consistent and deemed to be sufficient to determine that inhalation or oral exposure to methanol is a developmental hazard. Mice were judged to be more sensitive than rats to inhaled methanol, since effects were noted at lower chamber concentrations. The Panel also concluded that there was sufficient evidence that methanol is a developmental neurotoxicant in rodents; however, the data from inhalation studies in primates were insufficient to draw the same conclusion. In the primate study [143], neurobehavioral performance was similar in control and methanol offspring in seven of nine areas tested. However, two early tests of sensorimotor and cognitive performance provided evidence of subtle, but not definitive, adverse effects. The study of Rogers et al. [96] was determined to be a critical study for the assessment of developmental toxicity. This study is sufficient to conclude that prenatal exposure of mice to methanol vapor at concentrations of 2000 ppm or greater for 7 h per day on gd 6–15 can cause developmental toxicity as evidenced by cleft palate, exencephaly and skeletal malformations (mean maternal blood methanol concentrations were 537 mg/l at the end of exposure to 2000 ppm). The developmental toxicity NOAEL was 1000 ppm (corresponding to mean maternal blood methanol concentrations of 97 mg/l). Maternal toxicity was not observed in this study following exposure to concentrations up to 15,000 ppm, the highest concentration tested. There are good, but limited, data to indicate that the nature and incidence of fetal effects correlate with blood methanol concentration when methanol exposure is by inhalation or the gavage route. Studies by Bolon and coworkers [149] and Rogers and Mole [150] demonstrated that the gastrulating and early organogenesis-stage embryo is particularly sensitive to the adverse developmental effects of methanol. Results from Dorman et al. [66] led the Panel to conclude that methanol rather than formate is the most likely proximate teratogen. However, the biological events by which methanol induced defects remain unknown. The Panel concluded that the available rodent data are assumed to be relevant for humans because of the known similarity among species in early embryonic development and that the experimental models used to evaluate methanol teratogenesis (i.e., in vivo and in vitro studies with rodents) have been shown to be useful for known human teratogens.

5.1.2. Reproductive toxicity

The Expert Panel judged that there are insufficient human data upon which to evaluate the reproductive toxicity

of methanol. The Panel noted that the methanol database on reproduction in rodents is fragmented and uneven. The Panel also reviewed a study on reproductive function in female primates. The data are sufficient to conclude that 800 ppm by inhalation (20 h/day \times 7 days/week \times 13 weeks) in rats did not affect the structure of the male reproductive system [164]; blood methanol levels were not measured in this study. No consistent effect on male hormones resulted from exposures that led to blood methanol levels of \sim 1500 mg/l [101]. In a single rat developmental toxicity study [99] effects on parturition, litter size, and pup survival were observed at inhalation levels of 5000 ppm (blood methanol level not reported but speculated by Panel to be \sim 700–1000 mg/l based on other studies); effects were not observed at levels of 1000 ppm and lower. The existence of a two-generation study in rats was noted but results were incompletely reported in English. Therefore, the Expert Panel concluded that the data in rodents are currently insufficient to allow a conclusion regarding methanol's effects on female or male reproductive function. The reproductive physiology in rodents is assumed to be relevant to humans. Noting that decrements in male reproductive performance typically occur at doses higher than those causing histological or hormonal change, the aggregate data available to the Panel was judged to be sufficient to indicate that adverse reproductive effects would not occur in male rats following inhalation exposure to \leq 800 ppm.

One study examined reproductive function in female *Macaca fascicularis* monkeys exposed to methanol vapors (up to 1800 ppm) for approximately 2.5 h per day during a pre-mating and mating period (about 180 days) and the entire pregnancy (about 168 days), producing blood methanol levels of \sim 35 mg/l. Burbacher et al. [52,143] found no effects on menstrual cycles or conception rates. Burbacher and coworkers [143] also reported a decrease in pregnancy duration in treated animals with no effect on the weight or other physical or behavioral parameters of offspring at birth. The Burbacher et al. data were considered sufficient to demonstrate the lack of a treatment-related effect on menstrual cycles or conceptions rates but were considered insufficient to assess effects of methanol on parturition in primates. The Panel could not determine whether or not the possible effects observed in late gestation were treatment-related, thus leaving the Panel with uncertainty about the effects of methanol on primate parturition.

5.2. Summary of human exposure

Methanol is produced naturally in the human body and is found in expired air and body fluids. Humans are also exposed to methanol through contact with anthropogenic and natural sources. Natural sources of methanol include fruits and vegetables and fermented spirits. Methanol is also released during the metabolism of food additives such as the artificial sweetener, aspartame, and DMDC, a yeast inhibitor added to a variety of beverages. Methanol is one of the

highest ranking US chemicals in terms of production volume as well as environmental releases. The use of methanol in US gasoline is currently limited, but increased use of alternative fuels and developments in fuel cell technology could result in much greater use of methanol in the future.

Humans can be exposed to and absorb methanol by inhalation, oral intake, and dermal contact. The Panel determined that blood methanol concentration is a useful biomarker of exposure and that the metabolism and toxicity of methanol are independent of the route of exposure. The Panel focused on three aspects of potential methanol exposure: dietary, occupational, and accidental conditions. Dietary exposure is pervasive in the general population and has been characterized through survey studies. It is generally believed that dietary sources contribute to the observed background blood methanol concentrations ($<$ 5–10 mg/l). These levels of methanol will not result in formate accumulation or adverse health effects. The second exposure scenario considered by the Panel was anticipated occupational exposures to inhaled methanol that occur at or below the current TWATLV (200 ppm). Human chamber studies have shown that short-term inhalation exposure to 200 ppm methanol results in blood methanol concentrations of $<$ 10 mg/l with no observed increase in blood formate concentration. The third scenario examined by the Panel was accidental exposure to high doses of methanol. The clinical literature reports that 2474 people were accidentally exposed to high (poisoning) doses of methanol in the year 2000 [9]. The magnitude of these exposures is often poorly documented and blood methanol concentrations may approach or exceed levels observed in the cited high-dose rodent and monkey studies. Exposure to high levels of methanol will result in elevated blood formate concentrations and the development of ocular toxicity and other hallmark features of methanol poisoning. The Panel noted that 5859 children under six years of age with gasoline ingestion were reported to poison control centers in 2000 [9]. It can be plausibly speculated that greater use of methanol in automotive fuels and fuel cells could increase the incidence of methanol poisoning in children.

The Expert Panel review of data germane to methanol exposure from dietary sources was limited. Although information was available on the distribution of populations exposed to methanol from common dietary sources, e.g. fruits, vegetables, fermented spirits and the food additive aspartame, data on the potential contribution of the food additive DMDC or other sources (drinking water) were scant. *Federal Register* notices on final rules permitting specific uses of DMDC did specifically cite that consideration of methanol exposure was a factor in assessing safety of the permitted use [16–18,20]. The Expert Panel did not review the scientific data available to the FDA that underpin these conclusions of safety.

The distribution of the total daily population exposure to methanol from all sources has not been characterized. Aggregate exposure information is needed for common or typical conditions and for higher, but not necessarily accidental,

exposure conditions that may apply to small but significant portions of the population. While blood methanol levels are a useful biomarker of exposure, population data on blood methanol levels are limited.

Finally, the Panel is aware that subpopulations of undefined size may exist that have diminished capacity to clear methanol from the bodies. This diminished capacity may reflect polymorphisms in dehydrogenase enzymes that metabolize methanol or disease states, dietary factors, or medications that reduce folate levels, which that, in turn, may compromise later stages of methanol metabolism. Studies were not located that considered the degree, if any, to which these subpopulations may be more prone to adverse consequences from methanol exposure levels than the general population.

5.3. Overall conclusions

The Expert Panel recognized the need to consider species differences in methanol metabolism and toxicity in its evaluation of the risk to reproduction posed by methanol exposure in humans. The Expert Panel agreed that blood methanol concentrations provide a useful dose metric for the comparison of results among various studies. There are sufficient pharmacokinetic data to determine blood methanol concentrations in rodents associated with adverse reproductive and developmental effects. Mean maternal blood methanol concentration observed in mice following inhalation exposure to 1000 ppm methanol for 7 h per day on gd 6–15 (i.e. the fetal NOAEL for teratogenicity) was 97 mg/l. Mean maternal blood methanol concentration observed in mice following inhalation exposure to 2000 ppm methanol for 7 h per day on gd 6–15 (i.e. the fetal LOAEL for teratogenicity) was 537 mg/l. In humans, achievement of such a blood methanol concentration has resulted in formate accumulation, metabolic acidosis, ocular toxicity, and other signs of methanol toxicity. These observations suggest that there may be overlap between exposures resulting in clinical signs of acute toxicity and those that might result in developmental toxicity in humans. The toxicity data available to the Panel that was collected in monkeys provide suggestive but insufficient evidence that adverse developmental effects may occur in primates exposed by inhalation to methanol at maternally nontoxic doses. The Panel's confidence in these data may have been strengthened had statistical analyses that adjust for multiple testing been applied to the data. The Expert Panel concludes that there is insufficient evidence to determine if the human fetus is more or less sensitive than the most sensitive rodent species (i.e. mouse) to methanol teratogenesis. Moreover, other factors (e.g. genetic polymorphisms in key metabolizing enzymes, maternal folate status) that alter methanol metabolism may predispose some humans to developmental toxicity at lower blood methanol concentrations (<100 mg/l). This caveat is especially important since the Expert Panel recognized that there are limited human exposure data

for pregnant women and other potentially susceptible subpopulations.

The Expert Panel concluded that developmental toxicity was the most sensitive endpoint of concern with respect to evaluating the risk to reproduction posed by methanol exposure in humans. In particular, the data obtained from rodent studies indicate that the gastrulating and early organogenesis-stage embryo is particularly sensitive to the adverse developmental effects of methanol. The Panel concluded that methanol is the most likely proximate teratogen; however, the biological basis by which it induces such effects remains unknown. The Panel assumed the available rodent data were relevant for humans.

The Panel has minimal concern that methanol exposures resulting in low (<10 mg/l) blood methanol concentrations may result in developmental toxicity in humans. These methanol concentrations have been associated with consumption of a common American diet and with work exposures that are below U.S. occupational exposure limits.

- The Panel has concern that methanol may be a developmental toxicant in pregnant women following exposure to high levels of methanol.
- The Panel has negligible concern that methanol may be a male reproductive toxicant in humans under dietary conditions or occupational exposure that result in blood methanol concentrations <10 mg/l. However, there were not sufficient data to rule out the possibility that high, acutely toxic doses of methanol might affect male reproduction.
- The Panel determined that the data are insufficient to assess whether or not methanol is a reproductive hazard in females.

5.4. Critical data needs

Critical data needs are defined as tests or experiments that could provide information to substantially improve an assessment of human reproductive risks. The items listed in Sections 5.4.1 and 5.4.2 are considered by the Panel as critical data needs.

5.4.1. Exposure

- Studies are needed to assess total exposure to methanol from all sources, including foods, food additives, occupational and environmental exposures. Such studies would allow better quantification of human blood methanol concentrations that, in turn, would improve estimations of human risk. Including methanol as one of the chemicals assessed in a NHANES survey could be a means for characterizing the range of methanol blood levels in the US population.

5.4.2. Effects

- A summary of a two-generation rat reproductive toxicity study done by the Japanese NEDO was received, but data

were not available in sufficient detail for Expert Panel review. The complete document is understood to be available in Japanese, and a translation of the two-generation study to English is a critical data need. Translated data may allow an expert review to substantiate the information available in the NEDO summary and provide a basis for more definitive judgment about methanol effects on reproductive function.

- The Panel also noted that the NEDO developmental toxicity study protocol included several tests of neurobehavioral function in offspring from treated dams. Translation of these studies could also contribute to a more robust assessment of developmental neurotoxicity of methanol. Translation of these data was also identified as a critical data need.
- Certain aspects of the statistical analyses done in the studies by Burbacher et al. [52,143] were discussed. Most Panel members recommended that data from these studies be reanalyzed, particularly in regard to reported effects on duration of gestation and neurobehavioral effects in offspring. A more rigorous statistical evaluation that adjusts for multiple comparisons may permit consensus as to whether there is evidence that methanol is a developmental neurotoxicant in monkeys.
- The Panel was generally aware that Dr. Burbacher continues to evaluate neurobehavior in the offspring from the original studies [52,143]. The Panel believes that periodic reports or publications of these follow-on studies would be of value to a reassessment of methanol effects on human reproduction and development. The Panel also expressed the view that terminal histopathological examination of brain could materially contribute to the scientific database.

Although not considered critical data needs, the following studies would provide information that would contribute to our understanding of the toxicity of methanol.

- *Basis for Toxicity.* Studies are needed to elucidate the basis for the developmental toxicity of methanol, both in terms of its teratogenic effect on early embryos and potential neurobehavioral effects of fetal exposures. Pathogenesis studies of the potential for methanol to perturb essential developmental processes including, but not limited to, cell proliferation, cell migration, cell death, and morphogenesis are needed, as are studies at the biochemical and molecular levels to elucidate the target sites for methanol developmental toxicity. Such data may have allowed the Panel to determine whether methanol and ethanol share common mechanisms of toxicity, thus allowing the Panel to draw additional conclusions based in part on the more extensive literature reporting on the toxicity of ethanol.
- *Susceptibility.* Little information is available concerning factors that may increase susceptibility to the reproductive and/or developmental toxicity of methanol. Genetic polymorphisms of methanol metabolizing enzymes, including CYP2E1 and alcohol dehydrogenases, may be important. Because of potential interactions between folate status

and methanol toxicity, polymorphisms in folate transport or metabolizing proteins, as well as folate nutritional status, may impact susceptibility to methanol. The Panel identifies as research needs the elucidation of the role of genetic polymorphisms in methanol or folate metabolism, and folate status, in determining susceptibility to the reproductive or developmental toxicity of methanol. Such data would be useful in identifying individuals within the population who are potentially at increased risk of reproductive or developmental toxicity of methanol.

- *Cumulative Risk.* Considering the effects of methanol on reproduction, developmental toxicity is the most sensitive endpoint of methanol toxicity in rodents. Also, methanol has chemical and metabolic properties that are similar to ethanol. Therefore, it would be helpful to have data from developmental toxicity studies using concurrent exposures to methanol and ethanol.

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