

Addendum to the March 2011 External Peer Review Draft IRIS Toxicological Review of Methanol (Non-Cancer) (EPA/635/R-11/001)

(June 16, 2011)

Note to peer reviewers: The text below highlighted in red underline includes revisions made to the Methanol (Noncancer) Toxicological Review subsequent to its release for public comment. The bracketed green text provides orientation as to the placement of the text within the toxicological review. These revisions were made to incorporate recently published literature.

3.1. OVERVIEW

[The following replaces page 3-6, line 10 to page 3-9, line 3 in March 2011 Tox Review]

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

rats or mice inhaling equivalent concentrations. If humans eliminate methanol at a comparable rate to rats and mice, then humans would also be expected to accumulate less methanol than those smaller species. However, if humans eliminate methanol more slowly than rats and mice, such that the ratio of absorption to elimination stays the same, then humans would be expected to accumulate methanol to the same internal concentration but to take longer to reach that concentration.

In all species, formaldehyde is rapidly converted to formate, with the half-life for formaldehyde being ~1 minute. Formaldehyde is oxidized to formate by two metabolic pathways ([Teng et al., 2001](#)). The first pathway (not shown in Figure 3-1) involves conversion of free formaldehyde to formate by the so-called low-affinity pathway (affinity = $1/K_m = 0.002/\mu\text{M}$) mitochondrial aldehyde dehydrogenase-2 (ALDH2). The second pathway (Figure 3-1) involves a two-enzyme system that converts glutathione-conjugated formaldehyde (*S*-hydroxymethylglutathione [HMGS]) to the intermediate *S*-formylglutathione, which is subsequently metabolized to formate and glutathione (GSH) by *S*-formylglutathione hydrolase.¹ The first enzyme in this pathway, formaldehyde dehydrogenase-3 (ADH3), is rate limiting, and the affinity of HMGS for ADH3 (affinity = $1/K_m = 0.15/\mu\text{M}$) is about a 100-fold higher than that of free formaldehyde for ALDH2. In addition to the requirement of GSH for ADH3 activity, oxidation by ADH3 is nicotinamide adenine dinucleotide- (NAD^+ -)dependent. Under normal physiological conditions NAD^+ levels are about two orders of magnitude higher than NADH, and intracellular GSH levels (mM range) are often high enough to rapidly scavenge formaldehyde ([Meister & Anderson, 1983](#); [Svensson et al., 1999](#)); thus, the oxidation of HMGS is favorable. In addition, genetic ablation of ADH3 results in increased formaldehyde toxicity ([Deltour et al., 1999](#)). These data indicate that ADH3 is likely to be the predominant enzyme responsible for formaldehyde oxidation at physiologically relevant concentrations, whereas ALDHs likely contribute to formaldehyde elimination at higher concentrations ([Dicker & Cedebbaum, 1986](#)).

¹ Other enzymatic pathways for the oxidation of formaldehyde have been identified in other organisms, but this is the pathway that is recognized as being present in humans ([Caspi et al., 2006](#); <http://metacyc.org>).

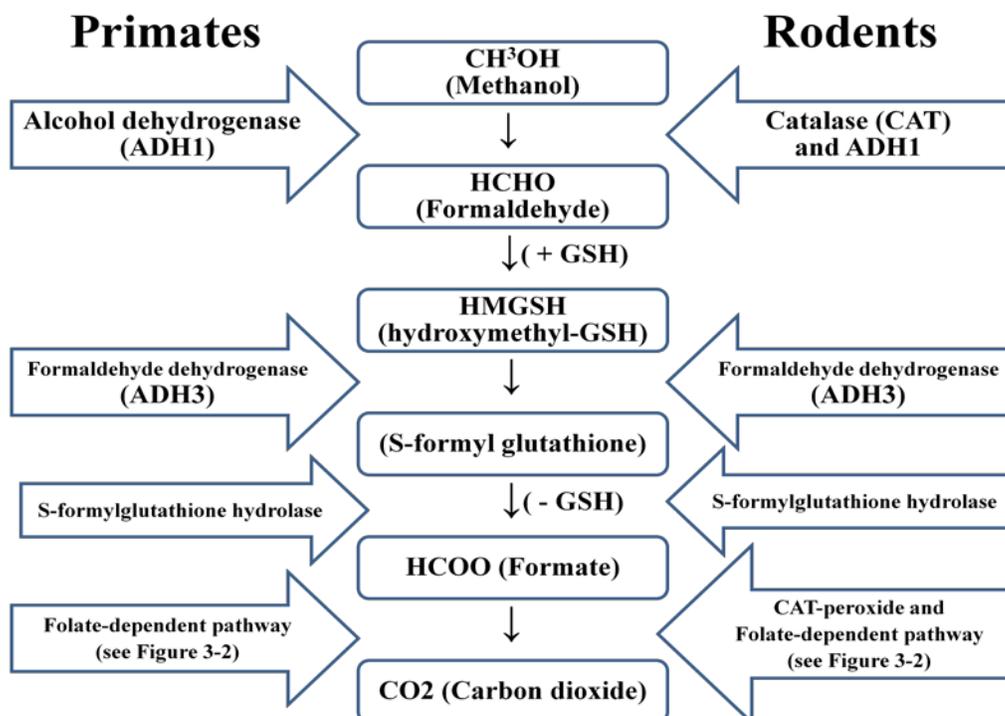


Figure 3-1. Methanol metabolism and key metabolic enzymes in primates and rodents.

Source: IPCS (1997).

Rodents convert formate to carbon dioxide (CO₂) through a folate-dependent enzyme system and a CAT-peroxide system ([Dikalova et al., 2001](#)). Formate can undergo adenosine triphosphate- (ATP-) dependent addition to tetrahydrofolate (THF), which can carry either one or two one-carbon groups. Formate can conjugate with THF to form *N*¹⁰-formyl-THF and its isomer *N*⁵-formyl-THF, both of which can be converted to *N*⁵, *N*¹⁰-methenyl-THF and subsequently to other derivatives that are ultimately incorporated into DNA and proteins via biosynthetic pathways (Figure 3-2). There is also evidence that formate generates CO₂⁻ radicals, and can be metabolized to CO₂ via CAT and via the oxidation of *N*¹⁰-formyl-THF ([Dikalova et al., 2001](#)).

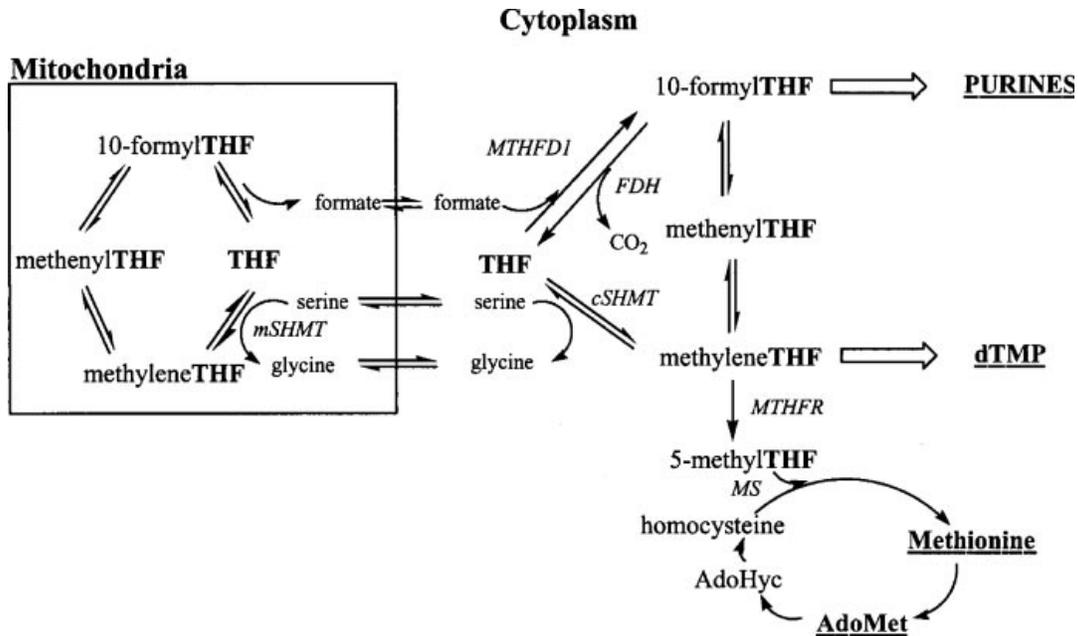


Figure 3-2. Folate-dependent formate metabolism. Tetrahydrofolate (THF)-mediated one carbon metabolism is required for the synthesis of purines, thymidylate, and methionine.

Source: Montserrat et al. (2006).

Unlike rodents, formate metabolism in primates occurs solely through a folate-dependent pathway. Black et al. (1985) reported that hepatic THF levels in monkeys are 60% of that in rats, and that primates are far less efficient in clearing formate than are rats and dogs. Studies of [human subjects](#) involving [¹⁴C]formate suggest that ~80% is exhaled as ¹⁴CO₂, 2-7% is excreted in the urine, and ~10% undergoes metabolic incorporation ([Hanzlik et al., 2005, and references therein](#)). [Sweeting et al. \(2010\)](#) have reported that [formic acid accumulation in primates within 6 hours of a 2 g/kg ip exposure to methanol was 5-fold and 43-fold higher than in rabbits and mice, respectively](#). Mice deficient in formyl-THF dehydrogenase exhibit no change in LD₅₀ (via intraperitoneal [i.p.]) for methanol or in oxidation of high doses of formate. Thus it has been suggested that rodents efficiently clear formate via [high capacity](#) folate-dependent pathways, peroxidation by CAT, and by an unknown third pathway; conversely, primates do not appear to exhibit such capacity and are more sensitive to metabolic acidosis following methanol poisoning ([R. J. Cook et al., 2001](#)).

3.2. KEY STUDIES

[The following replaces page 3-9, line 27 to page 3-9, line 31 in [March 2011 Tox Review](#)]

Some recent toxicokinetic and metabolism studies ([Burbacher, Grant, et al., 1999](#); [Burbacher, Grant, et al., 2004](#); [Dorman et al., 1994](#); [Medinsky et al., 1997](#); [Pollack & Brouwer, 1996](#); [Sweeting et al., 2010](#); [2011](#)) provide key information on interspecies differences, methanol metabolism during gestation, metabolism in the nonhuman primate, and the impact of folate deficiency on the accumulation of formate.

[The following replaces page 3-12, line 10 to page 3-12, line 23 in [March 2011 Tox Review](#)]

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

[Sweeting et al. \(2010; 2011\)](#) studied methanol and formic acid pharmacokinetics in male C57BL/6 mice, male C3H mice, male CD-1 mice, male NZW rabbits and male cynomolgous monkeys (*Macaca fascicularis*) following a 0.5 or 2 g/kg ip exposure to methanol. Blood samples were taken over the entire methanol elimination period for rabbits (48 hours) and CD-1 mice (12 hours for 0.5 g/kg exposure; 24 hours for 2 g/kg exposure), over a 12-hour exposure window for the C57BL/6 and C3H mice and a 6-hour post exposure window for monkeys. Following the 2g/kg dose, methanol blood levels exhibited saturated elimination kinetics in all three species, and peak methanol concentrations were similar (68, 87 and 79±10 mmol/L in C57BL/6, C3H and CD-1 mice, respectively; 114±7 mmol/L in rabbits and 94±14 mmol/L in monkeys), though the peak concentrations in C57BL/6 (p<0.01) and CD-1 (p<0.05) mice were significantly lower than rabbits. Methanol clearance rates were 2.5-fold higher in CD-1 mice than in rabbits after the 2 g/kg exposure, and 2-fold higher after the 0.5 g/kg exposure. When measured over the entire elimination period, plasma methanol AUCs in the rabbits were 175±27 after a 0.5 g/kg dose and 1893±345 mmol/L x hr after a 2 g/kg dose. Comparable plasma methanol AUCs in CD-1 mice were more than 2-fold lower, 71±9 after a 0.5 g/kg dose and 697±50 mmol/L x hr after a 2 g/kg dose. At 12-hours, the plasma methanol AUC values for C57BL/6, C3H and CD-1 mice were 465±14, 550±30 and 640±33 mmol/L x hr, respectively, and rabbits had an AUC value of 969±77 mmol/L x hr. The elimination period plasma formic acid AUCs in the rabbits were 3.02±1.3 after a 0.5 g/kg dose and 10.6±1.4 mmol/L x hr after a 2 g/kg dose. In comparison, plasma formic acid AUCs in CD-1 mice were nearly 6-fold lower at 0.5 g/kg (71±9 mmol/L x hr) and more than 3-fold lower at 2 g/kg (697±50 mmol/L x hr). At 12-hours, the plasma formic acid AUC values for C57BL/6, C3H and CD-1 mice were 2.1±0.3, 1.6±0.2 and 1.9±0.15 mmol/L x hr, respectively, and rabbits had a formic acid AUC value of 3.0±0.3 mmol/L x hr. All of the 12-hour formic acid AUCs for the mice were significantly lower (p<0.05) than the rabbit, but none of the mouse strains differed from each other (p<0.05). Formic acid accumulation at 6-hours post-exposure in monkeys (7.75±3.5 mmol/L x hr) was 5-fold and 43-fold higher than in rabbits (1.5±0.2 mmol/L x hr) and CD-1 mice (0.15±0.04 mmol/L x hr), respectively.

Burbacher, Shen et al. (1999; 2004) examined toxicokinetics in *Macaca fascicularis* monkeys prior to and during pregnancy. The study objectives were to assess the effects of repeated methanol exposure on disposition kinetics, determine whether repeated methanol exposures result in formate accumulation, and examine the effects of pregnancy on methanol disposition and metabolism. Reproductive, developmental and neurological toxicity associated with this study were also examined and are discussed in Sections 4.3.2 and 4.4.2. In a 2-cohort design, 48 adult females (6 animals/dose/group/cohort) were exposed to 0, 200, 600, or 1,800 ppm methanol vapors (99.9% purity) for 2.5 hours/day, 7 days/week for 4 months prior to breeding and during the entire breeding and gestation periods. Six-hour methanol clearance studies were conducted prior to and during pregnancy. Burbacher, Shen et al. (1999; 2004) reported that:

4.3.3. Other Reproductive and Developmental Toxicity Studies

[The following replaces page 4-41, line 14 to page 4-41, line 25 in [March 2011 Tox Review](#)]

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

[Sweeting et al. \(2011\) performed a series of experiments in NZW rabbits, C57BL/6J mice and C3H mice to compare plasma pharmacokinetics of methanol and formic acid and embryotoxicity. For the pharmacokinetic portion of the study, male mice and rabbits were given a single i.p. dose of 2 g methanol/kg body weight or its saline vehicle control. For the teratology portion of the study, pregnant female mice and rabbits were given two i.p. doses of 2 g methanol/kg body weight on GD 7 or 8, for a total daily dose of 4 g methanol/kg body weight, or two i.p. doses of a saline vehicle control. Methanol exposure did not significantly impact fetal body weights for any of the species and strains tested. No statistically significant effects were reported on rabbit growth parameters and mortality. However, postpartum lethality was nearly 2-fold higher in the methanol exposed \(11%\) versus control \(5%\) fetuses, and stillbirths were also increased \(4% versus 0%\). Though these increased incidences were not statistically significant, they may be biologically significant given that postpartum lethality \(“wasting syndrome”\) and a shortened gestational period were possible adverse outcomes observed in methanol exposed monkeys \(see discussion of Burbacher, Shen et al., 1999; 2004 in Section 4.3.2\). A 4.4-fold increase in tail abnormalities per litter, including shortening and absence, was reported in rabbit fetuses. However, due to the variability of this endpoint among litters, this](#)

difference was not statistically significant. Non-significant increases were reported in exposed rabbit litters for several other effects that were not observed in controls, including two fetuses with open posterior neuropores, one with an abdominal wall defect (prune belly), and three with frontal nasal hyperplasia. In C3H mice, methanol in utero exposure caused a 2-fold increase in fetal resorptions, but this increase was not statistically significant over saline treated controls (p<0.01). In C57BL/6, methanol caused a 66% incidence of fetal ophthalmic abnormalities (P<0.001) compared to a non-significant 3% incidence in C3H mice. Ophthalmic anomalies were not observed in saline-exposed controls of either strain. Methanol also caused a 17% increase in fetal cleft palates in C57BL/6 mice (p<0.05) compared to 0% in saline controls, and 0% in C3H mice treated with either methanol or saline. No increase in cephalic NTDs, an endpoint commonly observed in CD-1 mice, was observed in C57BL/6 or C3H mice. The different teratological results across these mouse strains could not be explained by differences in methanol or formic acid disposition (the pharmacokinetic results of this study are described in Section 3.2). The authors hypothesize that these differences in embryotoxicity could be due to strain differences in ADH activity and the amount of catalase available for ROS detoxification, or differences in other pathways that involve ROS formation.

Pregnant female C57BL/6J mice received 2 i.p. injections of methanol on GD7 ([J. M. Rogers et al., 2004](#)). The injections were given 4 hours apart to provide a total dosage of 0, 3.4, and 4.9 g/kg. Animals were sacrificed on GD17 and the litters were examined for live, dead, and resorbed fetuses. Rogers et al. ([2004](#)) monitored fetal weight and examined the fetuses for external abnormalities and skeletal malformations. Methanol-related deficits in maternal and litter parameters observed by Rogers et al. ([2004](#)) are summarized in Table 4-8.

[The following replaces page 4-46, line 10 to page 4-46, line 17 in [March 2011 Tox Review](#)]

In contrast to the in vitro and in vivo findings of Dorman et al. (1995), Andrews et al. (1995) demonstrated that formate can induce ~~similar~~ developmental lesions in whole rat and mouse ~~conceptuses was demonstrated by Andrews et al. (1995), who evaluated the developmental effects of sodium formate and formic acid in rodent whole~~ embryo cultures in vitro. Day 9 rat (Sprague-Dawley) embryos were cultured for 24 or 48 hours and day 8 mouse (CD-1) cultures were incubated for 24 hours. As tabulated by the authors, embryos of either species showed trends towards increasing lethality and incidence of abnormalities with exposure concentration. Among the anomalies observed were open anterior and posterior neuropores, plus rotational defects, tail anomalies, enlarged pericardium, and delayed heart development.

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

[The following replaces page 4-72, line 21 to page 4-73, line 6 in [March 2011 Tox Review](#)]

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

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

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

4.8. NONCANCER MOA INFORMATION

[The following replaces page 4-84, line 26 to page 4-85, line 8 in [March 2011 Tox Review](#)]

While it is well established that the toxic consequences of acute methanol poisoning arise from the action of formate, there is less certainty on how the toxicological impacts of longer-term exposure to lower levels of methanol are brought about. For example, since developmental effects in experimental animals appear to be significant adverse effects associated with in utero methanol exposure, it is important to determine potential MOAs for how these specific effects are brought about.

Sweeting et al. (2011) have postulated that mouse embryo tissue may have a high sensitivity to oxidative damage relative to other species due to a strong reliance on catalase over ADH to metabolize embryonic methanol. The authors suggest that the low ADH activity in mouse embryo relative to rats (Harris et al., 2003; Section 4.3.3), combined with the preference of catalase to metabolize methanol over hydrogen peroxide, could lead to a greater depletion of catalase and a higher level of ROS in mouse versus rat embryos, partially explaining the higher sensitivity of mice to the embryotoxic effects of methanol. If ROS accumulation due to this catalase consumption makes a significant contribution to methanol teratogenicity in sensitive mouse strains, then sensitive mouse strains may not adequately reflect risk to humans, assuming human fetuses do not rely on catalase for methanol metabolism. However, there is reason to believe that human infants can metabolize methanol via a mechanism other than ADH, and that this alternative mechanism could involve catalase (Tran et al., 2007). As discussed in Section 3.3, ADH activity in human fetuses and infants is as low as 10% and 20% of adult activity, respectively (Pikkarainen and Raiha, 1967; Smith et al., 1971). Yet, some human infants are still able to efficiently eliminate methanol at high exposure levels (Tran et al., 2007).

As described in Section 4.6.1, data from experiments carried out by Dorman et al. (1995), formate is not the probable proximate teratogen in pregnant CD-1 mice exposed to high concentrations of methanol vapor. This conclusion is based on the fact that there appeared to be little, if any, accumulation of formate in the blood of methanol-exposed mice, and exencephaly did not occur until formate levels were grossly elevated. Another line of argument is based on the observation that treatment of pregnant mice with a high oral dose of formate did not induce neural tube closure defects at media concentrations comparable to those observed in uterine decidual swelling after maternal exposure to methanol. Lastly, methanol- but not formate-induced neural tube closure defects in mouse embryos in vitro at media concentrations comparable to the levels of methanol detected in blood after a teratogenic exposure.

[The following replaces page 4-86, line 6 in March 2011 Tox Review]

In adult humans, metabolism of methanol occurs primarily through ADH1, whereas in rodents

5.3.8. Choice of Species/Gender

[The following replaces page 5-32, line 1 to line 5 in March 2011 Tox Review]

The RfC and RfD were based on decreased brain weight at 6 weeks postbirth in male rats (the gender most sensitive to this effect) (NEDO, 1987). This decrease in brain weight also occurs in female rats; however, if the decreased brain weight in female rats had been used, higher RfC and RfD values would have been derived (approximately 66% higher than the male derived values). As shown in Table 5-4, the HEC POD of 182 mg/m³ derived from the NEDO (1987) rat study was lower than the HEC POD derived from the Rogers et al. (1993) mouse study, but

slightly higher than the HEC POD derived from the Burbacher et al. (1999) monkey study. As discussed in Section 5.3.1, while existing developmental and chronic studies suggest that monkeys may be the more sensitive and relevant species, the substantial deficits and uncertainties in the reported data preclude their use for derivation of an RfC. The Rogers et al. (1993) mouse study was not chosen as the basis for the RfC because it results in a higher HEC POD than the chosen rat study. Sweeting et al. (2011) have suggested that mouse embryos may not be a suitable endpoint for assessing human risk because they postulate that mouse embryos have a relatively high sensitivity to oxidative damage due to a relatively high reliance on catalase over ADH to metabolize embryonic methanol. If ROS accumulation due to this catalase consumption makes a significant contribution to methanol teratogenicity in sensitive mouse strains, then sensitive mouse strains may not adequately reflect risk to humans, assuming human fetuses do not rely on catalase for methanol metabolism. However, there is reason to believe that human infants can metabolize methanol via a mechanism other than ADH, and that this alternative mechanism could involve catalase (Tran et al., 2007). As discussed in Section 3.3, ADH activity in human fetuses and infants is as low as 10% and 20% of adult activity, respectively (Pikkarainen and Raiha, 1967; Smith et al., 1971). Yet, some human infants are still able to efficiently eliminate methanol at high exposure levels (Tran et al., 2007).

References

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