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Developmental Toxicity of Methanol in Rodents

David C. Dorman and Frank Welsch

Methanol is a widely used commodity chemical that has also been proposed for large-scale use as an automotive fuel. Widespread use of methanol-based fuels would increase exposure of the general public to methanol vapors. Therefore additional data for an adequate human health risk assessment are needed, including information on pregnant women and potential health hazards to the unborn. In conventional bioassays with pregnant rats or mice, daily repeated inhalation exposure to high concentrations of methanol during major organogenesis causes developmental toxicity, including frank malformations at multiple sites. Mouse embryos display failure of the anterior neuropore to close (exencephaly) when dams inhale methanol concentrations of 10,000 ppm or greater on gestation day 8 (GD 8; day of mating and copulation plug = GD 0). This malformation is the most obvious lesion observed in mice and rats. Studies examining the pathogenesis of methanol-induced exencephaly (15,000 ppm for 6 hours on both GD 7 and GD 8) in pregnant mice suggest that cells in the region of the paraxial mesoderm are damaged by methanol. Methanol-induced structural anomalies in offspring may be explained by toxic effects of the chemical on mesodermal and neural crest cells. Methanol is metabolized (by oxidation) to formate, which is of special significance for the severe acute systemic toxicity encountered in adult humans. Studies at the Chemical Industry Institute of Toxicology (CIIT) examined the role of formate in the development of methanol-induced exencephaly in CD-1 mice, and evaluations of formate were conducted in parallel on cultured mouse embryos. Pharmacokinetic analyses in pregnant mice suggest that exencephaly is directly related to high concentrations of the parent compound methanol in maternal blood and embryos rather than to the accumulation of formate. These observations lend a new perspective to risk assessment for humans following very low-level exposure to methanol by inhalation.

Methanol is a natural constituent in humans, animals, and plants. Methanol arises in the human body from the metabolism of fresh fruits and vegetables. Aspartame, an artificial sweetener found in the diets of many people, is also a source of methanol. In the stomach, aspartame is broken down, and 10% becomes free methanol. Large volumes of methanol are used as a feed stock and as a solvent in the synthesis of other organic chemicals. Methanol is also an ingredient in paints and varnish removers and is used in the manufacture of plastics and coated fabrics.

Methanol has been considered for use as a major automotive fuel (Kavet and Nauss, 1990). The proposed heavier domestic reliance on methanol-based automotive fuels is likely to result in increased exposure of the general population to methanol vapors (Health Effects Institute, 1987; Kavet and Nauss, 1990). Methanol emission can arise from its release as uncombusted fuel in the exhaust or from its evaporation during refueling and after the engine is stopped. Methanol ingested by people has been recognized as a neurotoxicant since the early 1900s, and the role of formate as the cause of neuroocular toxicity has been well established. The possible use of methanol as an automotive fuel has spurred additional interest in the health risks posed by this alcohol, particularly by inhalation of methanol vapors.

Methanol Metabolism and Ocular Toxicity

Much of the information on the acute toxicity of methanol has accumulated since the early 1900s when exposure of people to relatively large acute doses of methanol (commonly known as wood alcohol) occurred through either accidental or intentional ingestion. The clinical toxicity experience shows that acute human exposure to methanol results in blindness and profound metabolic acidosis, which is apparently caused by the oxidation of methanol to formate. Severe metabolic acidosis can be fatal. The detoxification of formate in people is slow and determines toxicity. Blindness is a much-feared complication that develops in humans following a single moderate to large (greater than 0.4 to 1 g/kg) oral ingestion of methanol. Other common clinical manifestations of methanol poisoning in humans include central nervous system depression, weakness, headache, and vomiting (McMartin *et al.*, 1980).

While the enzymes responsible for oxi-

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Methanol in Rodents (from page 1)

dation of methanol to formate in humans and other primates are different from those in rodents (Figure 1), the rates of conversion are about the same. In primates, methanol is metabolized to formaldehyde predominantly by hepatic alcohol dehydrogenase (McMartin *et al.*, 1975). Formaldehyde is highly reactive and is not directly involved in the manifestations of methanol toxicosis (McMartin *et al.*, 1979). Formaldehyde is rapidly converted by formaldehyde dehydrogenase to formic acid. Under physiological conditions of the body, formic acid dissociates to formate and hydrogen ions, which cause metabolic acidosis (Tephly and McMartin, 1984) when too much formate is produced, as happens with methanol intoxication.

Although methanol may have direct toxic effects, formate is considered in clinical toxicology to be the toxic principle of methanol poisoning encountered in adult humans. The accumulation of formate causes metabolic acidosis and blindness, which is characteristic of methanol toxicity in sensitive species (e.g., humans and nonhuman primates) (McMartin *et al.*, 1980; McMartin *et al.*, 1977). Blood formate levels in excess of 10 mEq/liter are reported in humans with neuroocular toxicosis following methanol ingestion (McMartin *et al.*, 1980). Furthermore, administration of formic acid to monkeys causes optic disc edema with the loss of the pupillary light reflex, features that are also characteristic of methanol toxicity in humans (Martin-Amat *et al.*, 1977).

Formate in small amounts is a physiological product that is converted by a multistep pathway to carbon dioxide (Eells *et al.*, 1983). Formate metabolism is dependent on the activities of formyltetrahydrofolate synthetase as well as methenyltetrahydrofolate dehydrogenase and their rate-limiting cosubstrate tetrahydrofolate (Johlin *et al.*, 1987). Species susceptible to methanol toxicity appear to have lower liver tetrahydrofolate concentrations, slower formate metabolism, and thus increased sensitivity to methanol when compared with resistant species such as rats. The faster rate of formate removal in a nonsusceptible species is reflected by the fact that rodents do not accumulate formate above endogenous levels at any methanol dose. Therefore rodents are normally not susceptible to either methanol-induced metabolic acidosis or ocular toxicity.

Developmental Toxicity of Methanol

When pregnant CD-1 mice inhaled high concentrations of methanol on 10 consecutive days for 7 hours each day during major organ formation, prenatal development was severely disrupted (Rogers *et al.*, 1993a). The birth defects observed at multiple sites included exencephaly, cleft palate, and skeletal alterations. Neural tube defects also occurred when rats inhaled

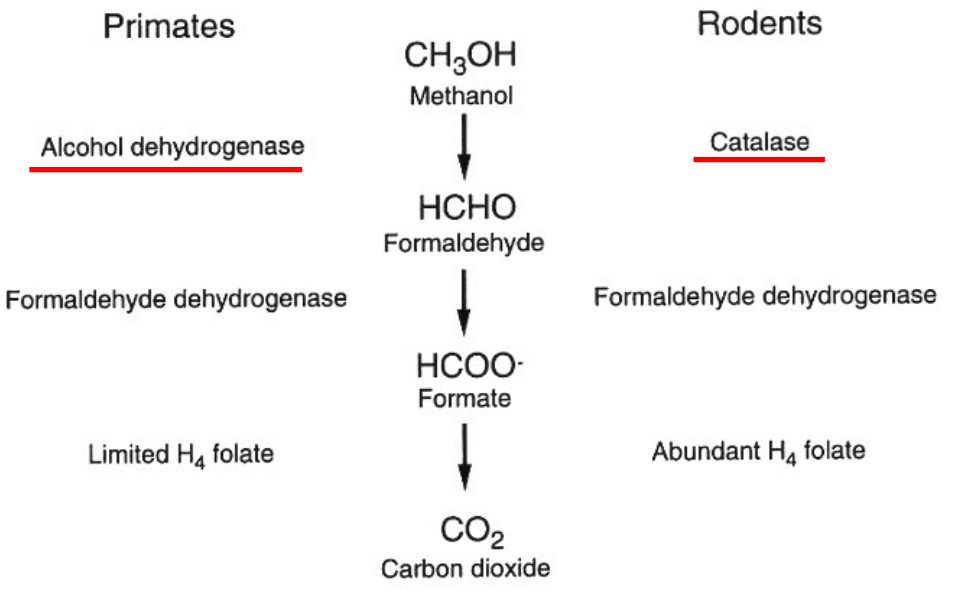

Fig. 1
Scheme for the metabolism of methanol. Major enzymes for primates (left) and rodents (right) are noted. Species differences in methanol toxicity are due primarily to the metabolic conversion of formate to carbon dioxide, which is rapid in rodents but slow in primates.

very high methanol concentrations on 20 consecutive days of gestation (Nelson *et al.*, 1985). Methanol exposure of pregnant women would most likely occur by inhalation of methanol vapors at concentrations that would be far below those examined in the rodent developmental toxicity studies. In spite of the known differences in metabolism among rodents and human beings, developmental toxicity induced by methanol in rodents is of potential relevance in assessing the impact of methanol-based fuels on human health. Rodent models for methanol developmental toxicity are of relevance because formate does not appear to play a direct role in the development of exencephaly. The well-established role of formate in human adult methanol toxicosis and the profound species differences in the metabolic fate of formate raise critical issues about the suitability of pregnant rodents as animal models of methanol developmental toxicity. As stated above, adults rodents are not susceptible to formate-mediated methanol toxicity.

Failure of the primitive brain (anterior neuropore) to close has been explored in some detail at the Chemical Industry Institute of Toxicology (CIIT) (Bolon *et al.*, 1993). Methanol inhalation was restricted to mouse gestation days (GD; copulation plug day is GD 0) 7, 8, and 9 by single-, two-, or three-day exposures to define the developmental phase specificity. Exposures lasted 6 hours each day at concentrations ranging from 5000 to 15,000 ppm. Those days include the differentiation processes during which the neural tube would normally close. The results revealed that exencephaly was not induced if methanol inhalation did not start before GD 9 (Bolon *et al.*, 1993) (Figure 2). Recent studies conducted in the same strain of pregnant mice have shown that a single methanol inhalation exposure as early as GD 5 can already induce exencephaly (Rogers *et al.*, 1993b). This outcome indicates that precursor cell populations in the mouse embryo are already vulnerable to methanol several days before cells destined to differentiate into the primitive brain have differentiated. That was the stage at which the CIIT experiments were conducted.

CIIT studies included experiments involving the development and progression of exencephaly. Histopathology of embryo heads on GD 8.5 (obtained from dams that had inhaled 15,000 ppm methanol for 6 hours on both GD 7 and GD 8) showed significant reductions in cell density and cell division (mitotic) index with occasional degenerating cells in the cranial mesoderm (Bolon *et al.*, 1994). The mitotic index in the neuroepithelium of embryos at this developmental stage was also reduced more than 50% by the two days of exposure to high methanol concentrations. Groups of neural crest cells were located in the neural folds dorsal to the foregut, compared to the more ventral location in the facial regions of control embryos (Figure 3). There is also decreased migration of neural crest cells to the branchial arches on GD 8.5. At this stage of development (GD 8.5), vital dye staining with Nile blue sulfate revealed minimal cell death in spite of an apparent loss of cranial

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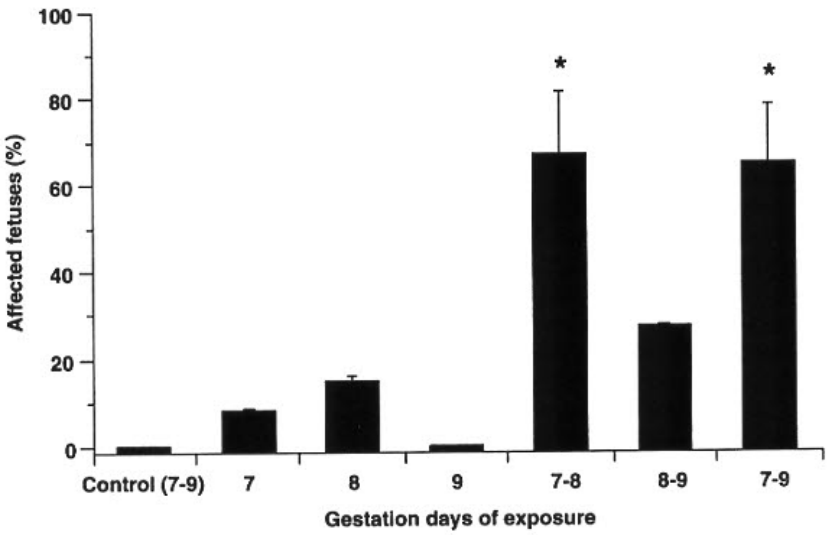

Fig. 2
Incidence of neural tube defects in CD-1 mouse embryos induced by maternal inhalation of methanol (15,000 ppm, 6 hours per day) during neurulation (data from Bolon *et al.*, 1993). * $P \leq 0.05$, significant difference from controls (Dunn's test).

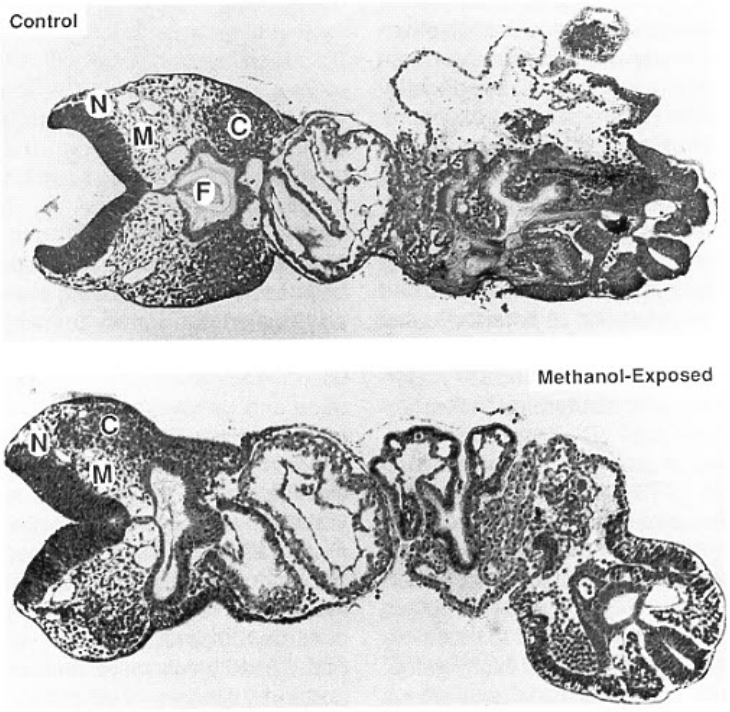

Fig. 3
Longitudinal sections of gestation day 8.5 mouse embryos showing neural tube pathology induced by maternal inhalation of methanol (15,000 ppm, 6 hours per day) on gestation days 7–8. Major structures of interest include cephalic neural folds (N), mesoderm (M), foregut (F), and neural crest cells (C). When compared to a control embryo, consistent lesions included reduction and rounding of the cephalic neural folds, insufficiency of the mesoderm, collapse of the foregut, and displacement of neural crest cells. (Adapted from Bolon *et al.*, 1994.)

mesenchyme cells in the neural folds. Structural anomalies caused by methanol can be partially explained by alterations in the number of progenitor (mesodermal and neural crest) cells. The overall micromorphological appearance of mesodermal and neuroepithelial cells in methanol-exposed embryos was quite different from that resulting from high doses of the structurally related teratogen ethanol (Sulik *et al.*, 1988). Ethanol altered cell death patterns in the neuroepithelium prior to neural tube closure.

Methanol Toxicokinetics in Pregnant Mice

Knowledge of the pharmacokinetics of a chemical or its embryotoxic metabolites during pregnancy can be used to determine whether developmental toxicity will occur following maternal exposure (Clarke, 1993). The relative roles of methanol and formate in the pathogenesis of methanol-induced neural tube development were the subjects of a recent CIIT investigation (Dorman *et al.*, 1995). Formaldehyde, an intermediary oxidation product with a very short half-life in blood, is not a likely teratogen candidate and has been carefully evaluated for its developmental hazard potential by other investigators.

CIIT scientists used several research strategies to study methanol and formate toxicokinetics in pregnant mice and conceptuses during and after methanol exposure. Limitations exist in using a rodent model to study the role of methanol-derived formate in methanol-induced exencephaly. To overcome these limitations, three research strategies were employed. First, methanol metabolism was pharmacologically modified with 4-methyl-pyrazole, a potent alcohol dehydrogenase inhibitor. 4-Methyl-pyrazole has been applied in mice to modify maternal metabolism and exacerbate the teratogenicity of ethanol (Blakley and Scott, 1984a, b) or attenuate profoundly that of 2-methoxyethanol (Sleet *et al.*, 1988). Second, formate was also administered directly to pregnant mice with the objective of assessing direct developmental toxic effects from formate by achieving maternal blood formate concentrations similar to those observed following inhalation of high methanol concentrations. Finally, the toxic effects of methanol and formate on development of whole embryos cultured under exclusion of confounding maternal factors were evaluated.

Methanol inhalation exposures were conducted in a Cannon nose-only system modified to produce a whole-body exposure of mice held within a rat nose-only tube (Dorman *et al.*, 1996). This apparatus allowed exposure of unrestrained pregnant

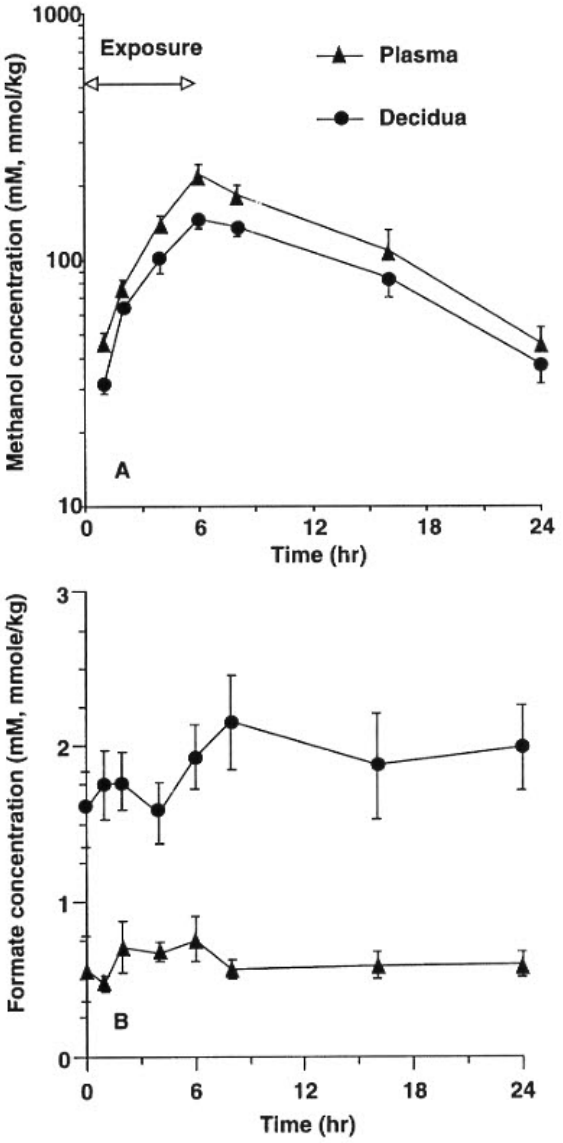

Fig. 4
Mean (± SEM) maternal plasma and decidual swelling methanol (A) and formate (B) concentrations following maternal inhalation of methanol (15,000 ppm, 6 hours per day) on gestation day 8. (Adapted from Dorman *et al.*, 1995.)

mice and removal of animals from the exposure system for pharmacokinetic analyses without disrupting the ongoing inhalation exposure. Uterine tissue that included the embryo (i.e., decidual swellings) and maternal plasma were analyzed for methanol and formate (Figure 4). End-of-exposure maternal plasma methanol concentrations after 15,000 ppm for 6 hours were 223 ± 56 mM. These levels were similar to those reported by Rogers *et al.* (1993b) in a conventional developmental toxicity study using the same mouse strain and a methanol exposure of 15,000 ppm for 7 hours per day. Comparable methanol levels were also achieved in those animals by oral bolus dosing with two doses (Rogers *et al.*, 1993b). The end-of-exposure maternal plasma methanol concentrations following a 10,000 ppm exposure in the modified rat nose-only tubes were 65 ± 25 mM. The end-of-exposure plasma methanol concentrations of individual mouse dams approached those reported for people with methanol toxicity caused by acute oral intake. Human blood methanol levels exceeding 100 mM have been measured during methanol poisoning following acute methanol ingestion, and they occurred in conjunction with blood formate levels ranging from 10 to 26 mM (for review, see Brown-Woodman *et al.*, 1995). In contrast, there were no changes in either blood or decidua formate concentrations in pregnant mice and their conceptuses under any of the methanol inhalation exposure conditions.

In the CIIT studies, abnormal development of the neural tube was also observed in mouse whole-embryo cultures exposed to 250 mM methanol or 40 mM formate (Dorman *et al.*, 1995). That formate concentration bears no relationship to the formate

levels detected in maternal plasma and conceptus tissues after methanol inhalation at a high concentration or administration of formate by bolus dosing. However, the 250-mM methanol concentration is similar to that found in plasma after inhalation exposure of pregnant mice to 15,000 ppm methanol for 6–7 hours. The formate concentrations used in these *in vitro* studies greatly exceeded those observed in mouse decidual tissues following maternal methanol exposure. These results indicate that exposure of mouse embryos in culture to formate concentrations close to those encountered during acute methanol neurotoxicosis in humans (i.e., greater than 10 mM) can result in dysmorphogenic effects. Our *in vitro* results are in general agreement with those reporting dysmorphogenic responses in CD-1 mouse embryos cultured for 24 hours in the presence of 182 mM methanol (Andrews *et al.*, 1993). These investigators also reported a significant effect on the overall developmental score of mouse embryos (GD 9.5) when cultured for 24 hours in the presence of 17 mM sodium formate in culture medium with a pH of 7.75. Considering the very limited metabolic capacity of cultured embryos and the high formate concentrations needed to induce adverse effects, these data provide strong additional evidence that methanol at high concentrations is a direct-acting chemical teratogen in rodents.

Finally, CIIT investigators also examined the developmental neurotoxicity of formate (Dorman *et al.*, 1995). An oral formate dose that produces blood formate levels corresponding to those observed following a high inhalation concentration methanol exposure (10,000 ppm, 6 hours per day) was defined. Pregnant mice given a single oral dose of sodium formate (750 mg/kg) on GD 8 have peak formate levels in both maternal plasma (1.05 ± 0.4 mM) and decidua (2.0 ± 0.2 mM) similar to those observed following methanol inhalation of very high concentrations (Figure 4). No significant increase in the incidence of open neural tubes was observed in formate-exposed mice on either GD 10 (2.0 ± 1.1%) or near term (GD 18, 0% open neural tubes). These findings also indicate that metabolic acidosis resulting from formate production is not involved in the development of neural tube defects.

Role of Folate in Methanol Developmental Toxicity

As mentioned earlier, formate metabolism is dependent on the activity of formyltetrahydrofolate synthetase, methenyltetrahydrofolate dehydrogenase, and the tissue cosubstrate tetrahydrofolate (Black *et al.*, 1985; Johlin *et al.*, 1987). Thus endogenous folate levels in the body are a critical determinant as to whether sufficient tissue formate concentrations are reached that would induce toxicity following exposure to methanol. Along with its critical function in formate metabolism, folate also plays an important role in normal fetal development and nervous system function.

A relationship between the dietary status of the dam (folate-normal vs. folate-deficient) and developmental neurotoxicity has been recognized since at least the 1950s. Folate deficiency in humans is associated with an increased incidence of nervous tissue damage (Reynolds *et al.*, 1973). Many pregnant women are borderline folate-deficient. Dietary folate supplementation of women during pregnancy has been associated with a decreased incidence of posterior neural tube defects (i.e., spina bifida) (Milunsky *et al.*, 1989; MRC Vitamin Study Research Group, 1991). This insight is the foundation for the recommendation that women contemplating pregnancy should take folate supplements before conception and the recent effort to add folate to flour used for making bread.

Interactions between folate deficiency and chemical teratogens may occur that potentiate the developmental toxicity of the teratogen. For example, Sakanashi and coworkers (1994) found an increase in methanol-induced exencephaly and other terata in CD-1 mice on a folate-deficient diet and also reported that folate supplementation ameliorated those adverse developmental effects. In studies conducted at CIIT, exposure to a high concentration of methanol resulted in transient, albeit statistically insignificant, decreases in total maternal red blood cell and decidua folate levels (Dorman *et al.*, 1995). Whether a significant trend might have been observed if larger numbers of animals had been examined or if a more direct measure of the tetrahydrofolate had been used is unknown. Taken together, these observations raise the question as to whether folate-deficient individuals exposed to methanol are at special risk.

In earlier studies at CIIT, the clearance of methanol and formate was examined in cynomolgus monkeys with low folate levels in the red blood cells arising from dietary manipulation (Dorman *et al.*, 1994). Even with a compromised folate status, monkeys inhaling 900 ppm of methanol for 2 hours still had peak concentrations of methanol-derived formate that were well below the endogenous formate levels and orders of magnitude lower than levels that produce acute methanol toxicity (Dorman *et al.*, 1994). These results were obtained after a single exposure and therefore preclude broad generalizations. However, the data suggest that even folate-deficient primates have sufficient endogenous tissue folate stores to effectively detoxify small amounts of methanol-derived formate produced from the inhalation of methanol vapors at concentrations relevant to human exposures arising from the use of methanol-based automotive fuels. Estimates of such exposures are in the range of 1 ppm or less under normal anticipated use conditions (Health Effects Institute, 1987; Kavet and Nauss, 1990).

Relevance to Risk Assessment

The CIIT studies provide strong evidence that methanol, not formate, is the proximate developmental toxicant and teratogen in pregnant CD-1 mice exposed to high concentrations of methanol vapor. This conclusion is based on the following evidence. (1) No significant accumulation of formate occurred in any of the methanol-treated mice, regardless of high concentrations inhaled. (2) Exencephaly was induced without elevated formate levels in maternal plasma or decidual tissue. (3) Treatment of pregnant mice with a high oral formate dose did not cause exencephaly, even at formate tissue levels comparable to those measured after a high concentration methanol inhalation. Furthermore, methanol, but not formate, induced neural tube defects in mouse embryos cultured in media containing methanol concentrations comparable to those observed in the decidua after maternal exposure to methanol. Further research is needed to determine whether significant methanol-formate-folate interactions occur that might affect the development of methanol-induced exencephaly.

When assessing the possible adverse effects on human health resulting from exposure to low concentrations of methanol, other important sources of methanol and formate arising from the diet and natural metabolic processes must also be considered. The amounts of methanol and formate from these sources might be equal to or higher than those from exposure to methanol vapors from automotive fuel during normal use. Diet soft drinks sweetened with aspartame constitute an important source of methanol for many Americans since such products contain about 555 mg of aspartame per liter. Thus drinking a 350-ml (12-ounce) diet beverage is roughly equivalent to a methanol intake of 20 mg. Additionally, methanol is generated during intermediary metabolism by normal enzyme processes. Endogenous levels of formate, which is also the toxic metabolite of methanol, range from 0.07 to 0.4 mM. Formate is an essential building block for many molecules such as nucleic acids, which are the components of DNA.

The methanol concentrations used in the CIIT studies in mice greatly exceed estimates of likely exposure scenarios to methanol vapors related to its use as an

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automotive fuel (Health Effects Institute, 1987). For example, the estimated upper human exposure limit occurs with hot-soak emissions from a malfunctioning vehicle housed in a poorly ventilated, closed garage. Under this worst-case scenario, ambient air methanol concentrations may reach 240 mg/m³ (185 ppm). Only short-term human exposures (less than 15 minutes) are anticipated to occur under these extreme conditions. For reference purposes, the threshold limit value (TLV) of the American Conference of Governmental Industrial Hygienists (ACGIH) for exposure to methanol over an 8-hour work day is 260 mg/m³ (200 ppm). This value is for worker populations and not the general public. Exposure of healthy humans to methanol at the current 8-hour, time-weighted average (TWA), permissible exposure limit (200 ppm) set by the Occupational Safety and Health Administration (OSHA) is unlikely to result in a toxicologically significant increase in either total blood methanol or formate concentrations. Inhalation of 200 ppm methanol for 6 hours by human beings did not result in an increase in background level of formate (Lee *et al.*, 1992). Even with a compromised folate status, monkeys inhaling 900 ppm of methanol for 2 hours still had peak concentrations of methanol-derived formate that were approximately 10 times below the endogenous formate levels (Dorman *et al.*, 1994). The blood level of methanol in human beings after inhalation of methanol at this TLV for 6 hours was 0.25 mM after light exercise (Lee *et al.*, 1992). This value is approximately 250 times less than the concentrations found to be embryotoxic in the CIIT studies. For example, the end-of-exposure maternal mouse plasma methanol concentrations following a teratogenic methanol exposure (6 hours, 10,000 ppm) were 65 ± 25 mM. These data suggest that the current industrial safety limits should provide an adequate margin of safety for pregnancy for inhalation exposures at the current TLV.

An additional component of the CIIT research regarding prenatal methanol toxicity is the application of an existing physiologically based pharmacokinetic (PBPK) model of gestation (Clarke *et al.*, 1993; Terry *et al.*, 1995). Such PBPK models can account for the dynamics of physiological changes associated with pregnancy and represent a significant advantage in quantitatively assessing potential exposure of the conceptus. In collaboration with scientists at the University of North Carolina at Chapel Hill, CIIT investigators recently published a PBPK model for methanol developmental toxicity studies (Ward *et al.*, 1996). At the developmental stages examined, the existing PBPK model provided a good description of methanol disposition in dam and conceptus of both species. In analogy to the simulated 2-methoxyethanol exposures of pregnant women (Welsch *et al.*, 1995), the ability to extrapolate to humans is presently being examined by coupling a model of the physiology of human pregnancy to the PBPK model of methanol disposition. These simulation efforts at CIIT are devoted to the development of generic, rather than toxicant-specific, PBPK gestation models. Accurate descriptions of the disposition of toxicants with applicability across species, classes of toxicants, and extrapolations from high to low dose are important components of risk characterization. In the case of methanol, the PBPK model may provide a better scientific foundation for risk assessment of human developmental toxicity.

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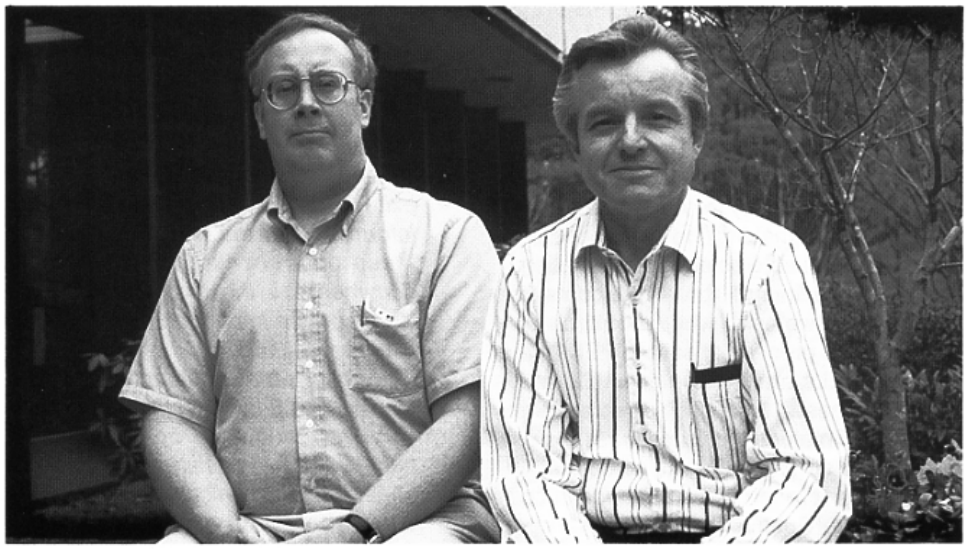

Drs. Dorman (left) and Welsch

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