HEALTH EFFECTS INSTITUTE

Reproductive and Offspring Developmental Effects Following Maternal Inhalation Exposure to Methanol in Nonhuman Primates

Part I: Methanol Disposition and Reproductive Toxicity in Adult Females

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Part II: Developmental Effects in Infants Exposed Prenatally to Methanol

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Includes the Commentary of the Institute's Health Review Committee

Research Report Number 89
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HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI supports research on all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate matter) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 200 projects at institutions in North America and Europe and published over 100 research reports.

Typically, HEI receives half its funds from the U.S. Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the United States. Occasionally, funds from other public or private organizations either support special projects or provide resources for a portion of an HEI study. The study described in the Research Report was part of a larger methanol program that received some initial support from the American Petroleum Institute in addition to the funds provided by core sponsors. The Institute gratefully acknowledges substantial funding to Dr. Burbacher from the National Institute of Environmental Health Sciences for studies conducted on the second cohort of animals. Regardless of funding sources, HEI exercises complete autonomy in setting its research priorities and in reaching its conclusions.

An independent Board of Directors governs HEI. The Institute's Research and Review Committees serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded studies are made available as Research Reports, which contain both the Investigators' Report and the Health Review Committee's evaluation of the work's scientific quality and regulatory relevance.

HEI Statement

Synopsis of Research Report 89

Effects of Prenatal Exposure to Inhaled Methanol on Nonhuman Primates and Their Infant Offspring

INTRODUCTION

In an effort to improve air quality and decrease dependence on petroleum, the federal government, industry, and other groups have encouraged development of alternative fuels such as methanol to substitute for gasoline or diesel fuel. Methanol is also a candidate to provide the hydrogen for fuel cells, which are being developed for a variety of power sources (including motor vehicle engines). Before people are exposed to increased concentrations of methanol, the potential health effects of such exposures require study.

Methanol, a simple alcohol containing one carbon atom, occurs naturally in plants and animals and participates in human metabolism. People regularly consume low doses of methanol in fruits, vegetables, and fermented beverages as well as soft drinks and foods sweetened with aspartame (which breaks down to methanol in the gastrointestinal tract). Despite its ubiquitous presence, methanol can be highly toxic if sufficient quantities are consumed. Ingestion of methanol (usually in the form of wood alcohol or tainted alcoholic beverages) can result in metabolic acidosis, blindness, and even death. Although the body has the capacity to metabolize the low doses of methanol to which people are regularly exposed, it cannot handle high doses because too much methanol overwhelms the body's ability to remove a toxic metabolite (formate). When formate accumulates, methanol poisoning occurs. One factor that regulates the rate at which formate is removed is the liver level of a derivative of the vitamin folic acid. People who are deficient in folic acid (including 15% to 30% of pregnant women) may be particularly susceptible to the toxic effects of methanol.

If methanol were to be widely adopted as a fuel, environmental exposures would increase through ingestion of contaminated drinking water, inhalation of vapors from evaporative and other emissions, and dermal contact. Current concentrations of methanol in ambient air are very low, 1 to 30 parts per billion (ppb). If all motor vehicles in the United States were converted to 100% methanol fuel, methanol levels in ambient air are estimated to increase approximately 1,000-fold (to 1 to 10 ppm in cities) and in a worst-case situation could occasionally reach concentrations as high as 200 ppm in enclosed spaces (HEI 1987). Inhaling these concentrations of methanol for short periods of time is not predicted to affect formate production and thus should not present a health risk. However, little is known about the consequences of long-term inhalation of methanol vapors, especially in susceptible populations of pregnant women and developing fetuses. HEI, therefore, developed a research program to address this information gap.

APPROACH

Dr. Thomas Burbacher and colleagues of the University of Washington studied the effects of long-term exposure to methanol vapors on metabolism and reproduction in adult female monkeys (*Macaca fascicularis*) and developmental effects in their offspring, who were exposed prenatally to methanol.

The investigators exposed adult female monkeys (11 to 12 animals/group) to one of four concentrations of methanol vapors (0, 200, 600, and 1,800 ppm) for 2.5 hours a day, seven days a week during the following periods: (1) before breeding, (2) during breeding, and (3) during pregnancy. They collected blood from the adults at regular intervals to monitor methanol levels (which served as a marker of internal dose) and formate concentrations. They also conducted pharmacokinetic studies to determine whether methanol disposition (which includes absorption, distribution, metabolism, and excretion) was altered as a result of

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of a research project sponsored by HEI. This study was conducted by Dr. Thomas Burbacher and colleagues of the University of Washington. The following Research Report contains both the detailed Investigators' Report (Parts I and II of *Reproductive and Offspring Developmental Effects Following Maternal Inhalation Exposure to Methanol in Nonhuman Primates*) as well as a Commentary on the study prepared by the Institute's Health Review Committee.

repeated methanol exposures and to assess pregnancy-related changes. Because high doses of methanol damage the central nervous system, the infants (8 to 9 animals/group) were examined at regular intervals during the first nine months of life to assess their growth and neurobehavioral development.

RESULTS

Exposure to methanol vapors did not affect the health of the adult monkeys prior to or during pregnancy. Single 2.5-hour exposures to methanol vapors caused short-term elevations in blood methanol concentrations of approximately 0- to 2-fold in the 200 ppm exposure group, 3- to 4-fold in the 600 ppm group, and 13- to 16-fold in the 1,800 ppm group. After long-term exposures, peak blood methanol concentrations declined slightly over the first month and remained constant thereafter. The concentrations of plasma formate (the toxic intermediate) remained at baseline levels during the entire course of the study in all exposure groups. Pregnancy had no effect on methanol disposition. Serum folate concentrations were not affected by pregnancy and methanol exposure.

Methanol exposure had no effect on most measures of reproductive performance, including menstrual cycles, conception rate, and live-birth delivery rate. However, all methanol-exposed animals had a decrease of about six to eight days in the duration of pregnancy compared to the control animals. It is not clear whether this decrease was related to methanol exposure as there was no dose response and no differences among offspring groups in body weight or other physical parameters. Prenatal exposure to methanol had no effect on infant growth and physical development for the first year of life. An unexplained wasting syndrome, characterized by growth retardation, malnutrition, and gastroenteritis, occurred after one year of age in two female offspring exposed in utero to 1,800 ppm methanol.

The investigators reported no systematic effects of prenatal methanol exposure on most of the measures used to test infant neurobehavioral development (neonatal behavior, early reflex responses, infant gross motor development, spatial memory, and social behavior). The investigators reported two possible methanol-related effects, one on visually directed reaching in male infants (a test of sensorimotor development), and one on novelty preference (a test of memory and cognitive function). Care must be taken in interpreting these results because a large number of neurobehavioral endpoints were analyzed and these results were based on a small number of subjects. Random fluctuations in the data may have appeared to be statistically significant. At the same time, however, both observations warrant further investigation as these central nervous system functions are complex perceptual processes that take time to develop and may be subject to latent neurotoxic effects.

CONCLUSIONS

This study adds substantially to our understanding of the effects of long-term exposure to inhaled methanol vapors. Because of the high quality of the study, the relevance of the animal model, the opportunities for dose-response analyses, and the availability of a marker of internal methanol dose, the results are appropriate for use in risk assessment. They can be readily used to predict the response of nutritionally competent people; they do not necessarily apply to women who are folate deficient.

The investigators' findings suggest that repeated inhalation exposure to concentrations of methanol vapors as high as 1,800 ppm would not result in accumulation of blood formate above baseline levels. With the exception of an unexplained shortening of gestation, methanol exposure had no effect on reproductive performance. The most significant result to emerge from this study was the wasting observed in two monkeys exposed in utero to 1,800 ppm methanol. Although this observation raises concern for prenatal exposures of this magnitude, pregnant women are unlikely to be exposed to such extremely high concentrations of methanol for prolonged periods of time.

Overall, the results provide no evidence of a robust effect of prenatal methanol exposure on the neurobehavioral development of nonhuman primate infants during the first nine months of life. However, improved understanding of methanol neurobehavioral toxicity will result from evaluation at later stages of development when more sophisticated tests of cognitive performance can be conducted and when latent effects may emerge. Such studies are now under way in the same monkeys at 4 to 5 years of age.

REFERENCE

Health Effects Institute. 1987. Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research. A Special Report of the Institute's Health Research Committee. Health Effects Institute, Cambridge, MA.

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Part II: Developmental Effects in Infants Exposed Prenatally to Methanol

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PREFACE

The use of methanol as an alternative fuel was proposed in the 1980s as a means to reduce petroleum imports, increase national security, and improve air quality (Gray and Alson 1989). Methanol appeared to be promising because it can easily and economically be produced from natural gas and a number of other feedstocks. Also, it has a high octane level and can reduce emission of hydrocarbons and other pollutants. More recently, methanol has emerged as a promising energy source for fuel cells, which are innovative electrochemical devices that convert fuel energy into hydrogen used to produce electric power for a variety of power sources (including motor vehicle engines). Although substituting methanol for gasoline and diesel fuels in motor vehicles would reduce the levels of some air pollutants, its expanded use raises concerns about the health implications of increased exposure of the general population to methanol vapors.

The prospect of introducing methanol as an alternative fuel prompted researchers and regulators to (1) resolve technical issues regarding fuel formulation and engine design, (2) characterize emissions from methanol-fueled vehicles and their effects on air quality, and (3) evaluate methanol's potential to cause health effects.

It is well known that methanol is toxic when ingested in sufficiently high amounts. In 1987, the Health Effects Institute (HEI) conducted an analysis of what emission-related health problems might emerge if methanol were to become more widely used as an automotive fuel. At that time, most information on the health risks of methanol exposure derived from clinical observations of humans who had accidentally or intentionally ingested methanol and from rodents exposed to very high concentrations of methanol. Human methanol poisoning is characterized by nausea, dizziness, metabolic acidosis, toxicity to the visual system (including blindness), and motor disturbances. If untreated, methanol poisoning can lead to coma and death (HEI 1987; Kavet and Nauss 1990; Kruse 1992; Marcus 1993; International Programme on Chemical Safety [IPCS] 1997). Visual toxicity is a hallmark of methanol intoxication. Clinical findings include visual disturbances, blood or edema in the optic disc, and an enlarged blind spot (Krause 1992). Autopsy reports have specified brain lesions, especially in the putamen and basal ganglia, in patients who died of a methanol overdose (Aquilonius et al. 1980; Koopmans et al. 1988; LeWitt and Martin 1988). The lethal methanol dose for humans is uncertain but appears to vary over a wide range (0.3 to 1 g/kg body weight) (IPCS 1997). Investigation of methanol toxicity in animals is difficult because normal rodents exposed to methanol do not display the metabolic acidosis and toxicity to the visual system that occur in humans (Roe 1982; Tephly and McMartin 1984; IPCS 1997).

The HEI Report (1987) found no evidence that short-term exposures to the concentrations of methanol vapors expected in ambient air as a result of methanol's use as a vehicular fuel would result in adverse health effects. The Report called for additional research, however, to reduce uncertainties regarding potential public health risks, especially the risks for susceptible subpopulations and the risks of prolonged low-level exposures. Because methanol was seriously being considered as a gasoline additive or replacement, HEI initiated a research program to address these issues. The study by Burbacher and colleagues, presented in this Research Report, was a key element in that program.

This Preface (1) provides background information on the sources of methanol exposures, (2) presents the regulatory context for the investigators' research, (3) briefly describes the known neurotoxic and developmental effects of methanol exposure, and (4) concludes with a description of the procedures that HEI used to develop its methanol research program and to review Dr. Burbacher's report.

METHANOL SOURCES AND USES

Methanol is a colorless, water-soluble liquid. Although methanol is a major chemical commodity, it also occurs naturally in humans, animals, and plants. In humans, methanol is derived both from the diet and from metabolic processes (Kavet and Nauss 1990; IPCS 1997). Dietary sources include fruits and vegetables, coffee, fruit juices, fermented beverages, and food containing the artificial sweetener aspartame, which hydrolyzes in the gut releasing 10% of its molecular weight as methanol (Stegink et al. 1981; IPCS 1997).

Methanol is widely used as a feedstock for chemical syntheses (for formaldehyde, acetic acid, and methyl tertiary-butyl ether [MTBE]) and as a solvent in a variety of consumer products (for example, paints and varnishes, antifreeze, windshield washers, cleansing solutions, and adhesives) (IPCS 1997; Malcolm Pirnie 1999). Methanol is also a component or byproduct in various commercial operations such as sewage treatment, fermentation, and the pulp and paper industry. In 1997, the U.S. Environmental Protection Agency (EPA) reported that methanol

ranked second for total release (221 million pounds annually) among chemicals listed on the agency's Toxic Release Inventory and first for air release (194 million pounds annually) (EPA 1999). However, it is the potential for expanded use of methanol as a vehicle fuel that has prompted research on the health effects of low-level exposures. Originally, proponents of methanol fuels envisioned their use as 100% (neat or straight) methanol, which is called M100. However, for technological, environmental, and safety reasons, most of today's methanol-based fuels are mixtures of 15% gasoline and 85% methanol, a blend known as M85.

Emissions testing of both light-duty and heavy-duty vehicles has shown that M85 significantly reduces emissions of carbon monoxide, hydrocarbons (including benzene and butadiene), and for heavy-duty vehicles, particulate matter (PM_{10}) (Gorse et al. 1992; Coburn et al. 1998). At the same time, methanol increases tailpipe emissions of methanol vapor as well as formaldehyde, which is toxic and has ozone-forming potential. Information on emissions from M100-fueled vehicles is more limited but generally shows the same trends (Auto/Oil Air Quality Improvement Research Program 1992). In addition to tailpipe emissions, inhalation of methanol vapor due to evaporative emissions from vehicles and fuel pumps, accidental ingestion from siphoning, and dermal exposure must be taken into account when considering possible human exposure to methanol as a consequence of its introduction into fuels.

REGULATORY AND POLICY ISSUES

In an effort to improve air quality and decrease dependence on petroleum, the U.S. government has encouraged the development of alternative fuels such as methanol by passing the Alternative Motor Fuels Act of 1988, the Clean Air Act Amendments of 1990, and the Energy Policy Act of 1992. As a result of these incentives, the petroleum industry has developed high-octane methanol fuels.

Under the provisions of the Clean Air Act, Congress authorized the EPA to establish national standards for air pollutants, set emission standards for motor vehicles, and regulate hazardous air pollutants. The original 1970 Act was amended in 1990, and those amendments deal with methanol in two ways—as a "hazardous air pollutant" (Section 112) and as a "clean alternative fuel" (Sections 241 and 242).

Section 112 of the Clean Air Act lists 189 hazardous air pollutants for which emission standards need to be set: methanol is one of those pollutants. In the 1990 amendments, Congress directed the EPA to review the list of hazardous air pollutants, to modify it as necessary, and to develop a national strategy to control their emissions from urban sources. Individuals and organizations have the right to petition the Agency to add to (or remove from) the list of hazardous air pollutants, substances for which the "emissions, ambient concentrations, bioaccumulation, or decomposition" are (or are not) reasonably anticipated to cause adverse health or environmental effects. The American Forest and Paper Association has submitted a petition requesting that methanol be delisted; this petition is now under consideration (Federal Register 1999).

The Clean Air Act Amendments of 1990 also established several programs for increasing the use of vehicles operated on "clean alternative fuels," which include fuels containing methanol. These amendments require the use of low-polluting fuels for buses in metropolitan areas if certain target levels are not met for ambient particles. In addition, the administrator of the EPA may extend the use of low-polluting fuels for other urban areas if "a significant benefit to public health could be expected to result from such an extension."

The Alternative Motor Fuels Act of 1988 is a federal statute that encourages widespread use of methanol, ethanol, and natural gas and the development of vehicles powered by these fuels. In 1992, Congress also passed the Comprehensive National Energy Policy Act to fulfill the low-polluting and clean-fuel mandates of the Clean Air Act Amendments of 1990 and to further stimulate alternative transportation fuels. The Act provides tax deductions for the purchase of alternative fuel vehicles and directs the U.S. Department of Energy to develop regulations to require the government and other organizations to acquire alternative-fuel vehicles. The final rule establishing the Alternative Fuel Transportation Program required "covered persons" to purchase increasing percentages of alternative fuel vehicles, beginning with 30% of the 1997 model-year light-duty vehicle acquisitions. To date, despite these efforts, methanol has not become an important component of the fuel supply in the United States except in its role as a feedstock for MTBE production.

Another important regulation would apply to methanol if it were proposed to be used widely as a fuel or fuel additive: Section 211 of the Clean Air Act mandates the EPA Administrator to require tests to determine the potential public health effects of fuels and fuel additives. Currently, no plans call for testing fuels containing methanol in conventional engines. Tailpipe emissions of many pollutants from fuel cell vehicles using methanol are much lower than those from conventional gasoline vehicles (Nowell 1999). Nevertheless, should methanol emerge as a fuel for

the generation of hydrogen in vehicles powered by fuel cells, this testing would likely need to commence in order to address the impact of evaporative emissions.

SCIENTIFIC BACKGROUND

The key to interpreting methanol toxicity is understanding its metabolism, including how species differ in the way they metabolize methanol and the impact of high-dose and low-dose exposures on these metabolic processes. Although methanol is metabolized through the same pathways in humans and animals, differences in the rate of formation of metabolic intermediates result in the marked variations in methanol-induced toxicity among species (Figure 1). After uptake (by either inhalation or ingestion) and distribution to body tissues, most methanol is metabolized in the liver to carbon dioxide and water; a small fraction is excreted directly through the lung and kidneys.

$$\underset{\text{methanol}}{CH_3OH} \xrightarrow{(1)} \underset{\text{formaldehyde}}{HCHO} \xrightarrow{(2)} \underset{\text{formate}}{HCOO^-} \xrightarrow{(3)} \underset{\text{water}}{H_2O} + \underset{\text{carbon dioxide}}{CO_2}$$

Figure 1. **Methanol metabolism.** (1) Reaction mediated by alcohol dehydrogenase in primates and by catalase in rodents. (2) Reaction mediated by formaldehyde dehydrogenase in both primates and rodents. (3) Reaction mediated by a number of enzymes that depend on liver tetrahydrofolate.

The first step in the metabolic sequence, the oxidation of methanol to formaldehyde, occurs at approximately the same rate in primates and rodents (Tephly and McMartin 1984). Formaldehyde is then rapidly oxidized to formate, the metabolite responsible for the toxic effects of methanol. The first two reactions are very fast, and formaldehyde has not been observed to accumulate in methanol-exposed animals or people. (However, some have suggested that given formaldehyde's high reactivity with cellular molecules, the failure to detect it does not preclude its possible involvement in methanol toxicity.) The key step in the metabolic pathway is the conversion of formate to carbon dioxide and water. This enzyme-mediated reaction requires the formation of a cofactor complex consisting of formate and tetrahydrofolate, which is derived from the vitamin folic acid (Makar and Tephly 1976). The concentration of tetrahydrofolate in the liver is a major determinant of the rate of formate removal (McMartin et al. 1977), and across species the rates of formate oxidation are directly related to liver levels of tetrahydrofolate. Removal of formate occurs about twice as fast in species that have high levels of liver tetrahydrofolate (rodents) as in those with lower levels (humans and other primates). In addition, levels of the enzyme responsible for oxidation of formate to carbon dioxide (10-formyl tetrahydrofolate dehydrogenase) are higher in rodents than primates (Johlin et al. 1987). The clinical effects of methanol depend on how much formate accumulates above the background level produced by normal metabolism (Tephly and McMartin 1984).

On the basis of limited emissions data, the concentrations of methanol vapors to which most people would be exposed if methanol were used as an alternative fuel are estimated to be too low to cause accumulation of formate and the onset of methanol toxicity. As reviewed by HEI (1987), ambient methanol concentrations resulting from vehicles operating on M100 are predicted to be 1 to 10 ppm in typical traffic situations but could be as high as 200 ppm in worst-case settings such as an enclosed garage with an engine idling or at hot ambient temperatures. Even these exposures are not expected to be high enough to cause an accumulation of formate above baseline levels in people with adequate folate levels (HEI 1987; Lee et al. 1992).

The above analysis suggests that inhaling low levels of methanol vapors should not present a health risk for most people because the body efficiently removes any excess formate. However, the clinical literature indicates that susceptibility to methanol toxicity varies widely (Kruse 1992; Marcus 1993). Because formate accumulation is a key factor in methanol toxicity, and its metabolism can be modified by liver folate concentrations or other factors that alter tetrahydrofolate regeneration, individuals who are folate-deficient may be at risk if exposed to methanol for prolonged periods of time. Pregnant and lactating women and patients with chronic alcoholism are among those with a high incidence of folate deficiency. It is well documented that folate deficiency during critical stages of pregnancy is associated with an increased risk of neural tube defects and that poor folate status may also be a risk factor for coronary disease (Selhub and Rosenberg 1990).[*] Animal studies have demonstrated that folate deficiency and treatments compromising the vitamin B_{12}–folate pathway decrease formate metabolism and exacerbate the toxic response to methanol (Eells 1992; Sahanashi et al. 1997). Thus, in some people who have mild folate deficiency, toxic symptoms could occur as a result of methanol exposures that are lower than those that cause toxicity in people who have adequate levels of folate.

[*] The Food and Drug Administration now requires U.S. Food manufacturers to fortify most enriched breads, flours, corn meal, pastries, rice and other grain products with folic acid to reduce the risk of neural tube birth defects in newborns.

The developing fetus may be particularly sensitive to the toxic effects of methanol exposure. The widely recognized teratogenic and neurotoxic effects of prenatal exposure to ethanol (which has similar physicochemical properties) raise concerns about the possible health effects of inhaled methanol vapors. Exposing the human fetus to high concentrations of ethanol (by maternal ingestion) can cause fetal alcohol syndrome, characterized by birth defects such as craniofacial malformations, growth retardation, and central nervous system disorders (Nulman et al. 1998; Mattson and Riley 1998). Consumption of moderate levels of ethanol can produce neurobehavioral deficits that are less severe but similar to those reported for fetal alcohol syndrome (Mattson and Riley 1998).

Evidence that methanol might be toxic for the developing fetus came initially from studies in laboratory rats indicating that fetal exposure to extremely high doses of methanol caused teratogenicity and neurotoxicity (Nelson et al. 1985; Infurna and Weiss 1986). Exposure of dams to 20,000 ppm methanol by inhalation for 7 hours daily throughout gestation increased the incidence of congenital malformations and urinary tract and cardiovascular defects in the offspring; lower exposure concentrations (10,000 ppm and 5,000 ppm) had no statistically significant effects (Nelson et al. 1985). Infurna and Weiss (1986) reported that early postnatal behavior (suckling behavior and time to locate nesting material) was delayed in rat pups after maternal ingestion of a high dose of methanol (2.5 g/kg/day) during gestational days 15 to 19.

More recent studies have not confirmed the teratogenicity of methanol in rats and point to differences in susceptibility between the mouse and the rat (Bolon et al. 1993; Rogers et al. 1993; Stanton et al. 1995; Weiss et al. 1996). Stanton and colleagues (1995) did not find significant changes in neurobehavioral and neurophysiological development in the offspring of rats exposed to 15,000 ppm methanol vapors (7 hours daily between gestational days 7 and 19). Weiss and coworkers (1996) also did not detect significant changes in a large number of behavioral endpoints (including suckling latency, motor activity, and cognitive function) in rats exposed to methanol (4,500 ppm/6 hours/day) in utero as well as after birth. In contrast, fetuses from pregnant mice exposed daily to very high concentrations of methanol vapors (5,000 to 15,000 ppm) from gestational days 6 to 15 showed an elevated incidence of exencephaly (failure of the skull to close over the brain) (Rogers et al. 1993). For this endpoint and under these exposure conditions, the parent compound methanol, rather than formate, may be responsible for the abnormality (Dorman et al. 1995). The difference in susceptibility of the two rodent species may be related to the higher blood methanol concentrations in mice than in rats exposed to the same concentrations of methanol vapors (Stanton et al. 1995; Pollack and Brouwer 1996).

Although toxicity resulting from single exposures to relatively high concentrations of methanol has been extensively studied, little information is recorded on the effects of prolonged, low-level exposures to methanol vapors. The most comprehensive study of the long-term effects of inhaled methanol vapors was conducted in rodents and monkeys by the New Energy Development Organization (NEDO) of Japan. The results have been presented in an abstract (Takeda and Katoh 1988) and in a report (NEDO 1987). Rats (F344/DuCrj) and mice (Crj:B6C3F_1) were exposed to methanol (0, 10, 100, or 1,000 ppm) for 12 or 24 months. No effects of exposure were reported for the two lower concentrations of methanol, but some increased body and organ weights were found in animals exposed to 1,000 ppm methanol. Pulmonary nodules increased in rats exposed to 1,000 ppm methanol; no methanol-related tumors were reported in mice. Two-generation reproductive studies in Crj:CD rats exposed continuously to methanol revealed no effects of methanol exposure on reproductive function (sexual cycle, days needed for insemination, insemination rate, pregnancy rate, litter size). The most notable finding was a decrease in brain weight in the offspring of rats exposed to methanol. Teratology studies were conducted on pregnant rats exposed to 0, 200, 1,000, or 5,000 ppm methanol for approximately 23 hours/day from day 7 to 17 of gestation. The highest exposure concentration (5,000 ppm) caused overt toxicity in the dams and fetal malformations in the first generation animals. Exposure concentrations of 1,000 ppm or less did not induce toxicity in the maternal animals, toxicity to the fetus, or effects on growth of the offspring.

NEDO also conducted long-term inhalation studies in monkeys *(Macaca fascicularis*) exposed to 10, 100, or 1,000 ppm methanol vapors for 21 hours/day for 7 months (2 animals/group), 1 year and 7 months (3 animals/group), or 2 years and 5 months (3 animals/group) (NEDO 1987). No changes were observed in physical growth or routine histologic parameters. The investigators reported degeneration of the basal ganglia of the cerebrum in animals exposed to 100 or 1,000 ppm methanol for 1 year and 7 months; this lesion was not found in animals exposed for 2 years and 5 months.

HEI METHANOL RESEARCH PROGRAM AND THE INVESTIGATORS' STUDY

As a result of concerns about the potential for health effects of methanol used as an alternative fuel, the HEI

Health Research Committee issued two Requests for Applications (RFAs) soliciting research proposals: RFA 87-1 "Behavioral and Neurotoxicological Effects of Methanol and Other Components of Automotive Emissions" and RFA 89-1 "Health Effects of Methanol Exposure: Metabolism and Pharmacokinetics; Fetal and Perinatal Neurotoxicity; Reproductive Toxicity."

RFA 89-1 requested proposals to study the metabolism of methanol following low-level exposures, especially in potentially susceptible populations, and methanol's effects on neurologic and reproductive function. Dr. Burbacher of the University of Washington submitted an application entitled "Primate Developmental Effects of Methanol," in which he proposed to evaluate the reproductive and developmental effects of methanol in nonhuman primates. After external peer review of all competing applications, the HEI Research Committee recommended Dr. Burbacher's proposal for one of the four studies funded under RFA 89-1. The Committee noted that the proposed study in macaque monkeys would provide critical information for risk assessments of methanol because of the similarities of humans and nonhuman primates in methanol metabolism, acute methanol-induced toxicity, placental structure, and neurobehavioral testing protocols. Because of the developmental and neurobehavioral effects reported in the offspring of pregnant rodents exposed to extremely high concentrations of methanol, it was important to confirm and extend these results using more sensitive endpoints, more suitable animal models, and more environmentally relevant methanol concentrations.

Dr. Burbacher's project was recognized as ambitious in that it involved exposure of adult female monkeys to methanol vapors by inhalation for a protracted period of time (before and during pregnancy) and behavioral testing of the offspring from birth to nine months of age. In order to achieve the desired sample size, the animals were divided into two cohorts, which were exposed and tested sequentially. Dr. Burbacher's study began in 1990 and lasted six years. HEI provided $2.6 million in funding for the project and the National Institute for Environmental Health Sciences provided $1 million to the investigator to support most of the costs associated with the second cohort of animals. The EPA supported maintenance of the infants after the 9-month testing period ended.

Three other studies funded under RFA 89-1 included a study on the uptake and disposition of methanol in rats and mice at different stages of gestation (Pollack and Brouwer 1996), a study on the effect of methanol on fetal development in rodents (Weiss et al. 1996), and a study on the uptake and disposition of methanol after a single exposure in nonhuman primates (Medinsky et al. 1997). This program provided comparative data on the metabolism and developmental effects of methanol across species.

All HEI-funded studies undergo an independent peer review of the methods, results, and data interpretation. Because of the complexity of his study, Dr. Burbacher reported the results in two parts. The investigators submitted a draft version of Part I, which addressed methanol disposition, adult toxicity, and reproductive toxicity, in March 1997. This report was evaluated by external peer reviewers and by members of the HEI Health Review Committee. Burbacher and colleagues submitted a revised draft of Part I in February 1998. In January 1998, they submitted a draft of Part II, which contained the developmental neurotoxicity results for the infant monkeys exposed to methanol in utero. HEI formed a Review Panel consisting of Review Committee members and consultants with expertise in pharmacokinetics, toxicology, and neurobehavioral toxicity to assist in the review of these reports. In March 1998 the panel met with the investigators and in executive session to discuss Parts I and II. Dr. Burbacher also presented his preliminary findings at the HEI Annual Conference in April 1998. On the basis of these discussions, the panel requested further revisions to Part I and additional analyses for Part II. The investigators submitted the revised final drafts, which form the basis of this Research Report, in July 1998 (Part I) and September 1998 (Part II). The panel reviewed these drafts and subsequent revisions that were made during the editorial process.

The Review Committee discussed the revised reports and the panel's evaluation and recommended Dr. Burbacher's reports for publication. The Committee, with the assistance of the panel and staff, then prepared its Commentary, which is included in this Research Report. The Review Committee's Commentary is intended to aid HEI's sponsors and the public by highlighting the strengths and limitations of the study and by placing the investigators' findings into scientific and regulatory perspective.

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HEI REVIEW COMMITTEE

The Review Committee gratefully acknowledges the cooperation of the investigators during the review process and the work of the members of the Review Panel who assisted the Committee in evaluating the Investigators' Report and developing its Commentary.

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INVESTIGATORS' REPORT

Reproductive and Offspring Developmental Effects Following Maternal Inhalation Exposure to Methanol in Nonhuman Primates

Part I: Methanol Disposition and Reproductive Toxicity in Adult Females

Thomas Burbacher, Danny Shen, Kimberly Grant, Lianne Sheppard, Doris Damian, Stephen Ellis, and Noelle Liberato

ABSTRACT

Increased use of methanol as an alternative motor fuel or fuel component could result in a more widespread exposure to the public, primarily from inhalation of methanol vapor. Although methanol has been shown to be both toxic and teratogenic at high levels, the health consequences of methanol exposure at lower, environmentally relevant levels have not been clearly established. Because the developing fetus is sensitive to many neurotoxic substances that pass readily through the placenta, fetal exposure to methanol via maternal inhalation may become an important public-health concern.

The aims of the present study were to determine 4 specific exposure–dose-response relations in nonhuman primates for exposure to methanol vapor:

1. changes in blood clearance and/or distribution kinetics of methanol related to repeated exposures to methanol vapor;
2. changes in blood clearance and/or distribution kinetics of methanol related to pregnancy;
3. toxic effects on maternal reproductive functions;
4. toxic effects on infant development.

The two-cohort study design utilized 48 adult female *Macaca fascicularis* monkeys (24/cohort), 4 adult male *M. fascicularis* (2/cohort), and their offspring. Adult females were exposed to 0 parts per million (ppm)*, 200 ppm, 600 ppm, or 1,800 ppm methanol vapor for approximately 2.5 hours/day, 7 days/week, both prior to and during pregnancy. Adult males were not exposed to methanol; offspring exposure ended at birth. Specific Aims 1 and 2 were addressed in the adult females by studying the time course of changes in blood methanol and plasma formate concentrations over the 6-hour period following exposure to methanol. Blood methanol toxicokinetics were determined after the first exposure, after approximately 3 months of daily exposures prior to breeding, and twice during pregnancy. Whole blood methanol and plasma formate concentrations were also monitored biweekly throughout the study. Specific Aim 3 was addressed by examining 5 factors in time-mated females: menstrual cycles; frequency of conception; frequency of complications during pregnancy, labor, and delivery; duration of pregnancy; and frequency of live births. Specific Aim 4 was addressed by examining the neurobehavioral development of *M. fascicularis* infants that had been exposed in utero to methanol compared with development in infants born to unexposed control females. Part I of this report presents the results related to Specific Aims 1 through 3. The results related to Specific Aim 4 are presented in Part II of this report.

The increase in blood methanol concentrations was proportionate to exposure level below 600 ppm, that is, between 200 ppm and 600 ppm. In contrast, a more than threefold increase in blood methanol concentrations occurred between the levels of 600 ppm and 1,800 ppm. The blood methanol–concentration time data from the 600 ppm– and 1,800 ppm–exposure groups were fitted with a one-compartment model featuring either first-order or Michaelis-Menten elimination kinetics. The limiting first-order kinetic parameters from the Michaelis-Menten model fits and the equivalent parameters from the linear

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

model fits (namely, elimination half-life, apparent blood clearance, and apparent distribution volume) were pooled to allow an assessment of the effects of dose, repeated exposure, and pregnancy on first-order blood methanol kinetics at environmentally relevant levels of exposure.

A consistent, but modest, increase in blood methanol clearance, as well as a shortening in elimination half-life, were noted in the chronically exposed monkeys. There was, however, no significant change in the apparent distribution volume of methanol. During pregnancy, the blood clearance of methanol remained stable for both the 600 ppm– and 1,800 ppm–exposure groups while a consistent reduction in distribution volumes per kilogram body weight was observed. In addition, there was a corresponding trend during pregnancy toward a shorter elimination half-life, particularly in the 600 ppm–exposure group. However, the change in elimination half-life was not statistically significant.

Plasma formate concentrations showed a slight but statistically detectable rise during the entire course of the study in both the control and methanol-exposure groups. There were no measurable differences in circulating plasma formate concentrations between control and methanol-exposed monkeys, which is consistent with earlier inhalation studies using radiolabeled methanol.

Chronic methanol exposure up to 1,800 ppm did not affect the health of *M. fascicularis* females prior to or during pregnancy. Overt signs of toxicity such as lethargy, uncoordinated motor movements (observed as staggering or clumsiness), and labored or irregular respiration were not observed following over 300 days of methanol exposure. The length of the menstrual cycle and the frequencies of conception and live births in the methanol-exposed and control females were very similar. Methanol exposure was, however, associated with a significant decrease in the duration of pregnancy, shortening the gestation period for offspring. The decrease in pregnancy duration was approximately 8 days, which did not result in an increase in the incidence of signs of prematurity in exposed offspring. The effect was observed at all methanol exposure levels tested. Because this is the first report of an association between low-level methanol exposure (200 ppm) and an effect on reproduction, further investigation is needed to confirm this finding. Investigations should focus on measuring possible changes in maternal and fetal endocrine parameters due to methanol exposure during pregnancy.

INTRODUCTION

Methanol (methyl alcohol, or wood alcohol) is a widely used industrial solvent that is necessary for production of consumer goods such as solid fuels (namely, Sterno®), antifreeze, and photocopying fluids. It is used in the pharmaceutical and agricultural industries and in the manufacture of chemicals such as ethylene glycol, methyl halides, formaldehyde, methacrylates, and methylamines (Von-Burg 1994). However, it is the use of methanol in fuel-related industries that bears most relevance to the public-health issues of this research. Methanol can be used as a motor fuel, as an intermediate in the production of methyl tertiary-butyl ether (MTBE), an effective octane enhancer, or as a direct octane booster for gasoline (Merck 1989, Hazardous Substance Data Bank 1992). Methanol has the potential to be an important motor fuel in the next century, replacing many of the existing uses for gasoline. The chief advantage of use of methanol over gasoline resides primarily in reduced hydrocarbon emissions. Hydrocarbon emissions contribute to atmospheric ozone formation, a significant health concern and the primary reason why many cities are unable to meet clean air standards. Fuels such as methanol provide the possibility of increasing air quality and decreasing the risk of adverse public-health effects from air pollutants.

If methanol is adopted as an alternative fuel, widespread exposure to the public, primarily from inhalation of methanol vapor, could result. Most exposures would probably take place on highways and urban streets, during refueling, and in private garages. For each scenario, calculations have been made to predict the extent of methanol exposure (Gold and Moulis 1988). Even in situations likely to result in the highest exposures, such as refueling (23 to 38 ppm) and hot-soak emissions in private garages (192 to 383 ppm), the length of exposures would be relatively brief and anticipated levels of methanol exposure would fall significantly below those associated with clinical neurotoxicity or morbidity. For reference, the current threshold limit value for methanol for an 8-hour workday, 40 hours per week, is a time-weighted average (TWA) of 230 $\mu g/m^3$ or 200 ppm (American Conference of Governmental Industrial Hygienists 1990).

METHANOL DISPOSITION AND METABOLISM (SPECIFIC AIMS 1 AND 2)

There are numerous reports on the metabolism and disposition of methanol in rodents and nonhuman primates (see reviews by Tephly and McMartin 1984; Tephly 1991). Limited data on human subjects have been obtained from clinical observations of patients with methanol poisoning

and from experimental exposure studies of healthy volunteers (HEI 1987).

The absorption of methanol is rapid following oral ingestion, inhalation of methanol vapor, or skin contact. Although absorption of inhaled methanol is often assumed to be complete, a study by Sedivec and colleagues (1981) in human volunteers and a series of studies by Perkins and colleagues (1995a, 1996a) and Pollack and Brouwer (1996) in rats and mice suggest that lung retention of methanol vapor is less than 100%. Sedivec and colleagues (1981) found that lung retention of methanol varied from 56.1% to 60.8% in five healthy men who were exposed to methanol vapor concentrations ranging from 103 to 284 mg/m^3 (78.6 ppm to 217 ppm). In the studies by Perkins and colleagues (1995a, 1996a), adult female Sprague-Dawley rats and CD-1 mice housed in an inhalation chamber absorbed 56% to 87% and 85% of the methanol vapor (> 1,000 ppm) from the airstream, respectively. After absorption, methanol distributes readily to all organs and tissues roughly in proportion to their water content (Yant and Schrenk 1937). Therefore, the volume of distribution of methanol is close to the volume of total body water.

In animals, metabolism is the predominant route of elimination at low or moderately high doses (< 500 mg/kg) of methanol (HEI 1987; Pollack and Brouwer 1996). Only at very high methanol doses when metabolism is saturated, do renal excretion and exhalation via the lungs become significant (> 10% of absorbed dose) (HEI 1987; Pollack and Brouwer 1996). Methanol is first converted to formaldehyde, which rapidly undergoes oxidation to formate. Formate enters the folate biochemical pathway and is eventually oxidized to carbon dioxide (CO_2), which is eliminated from the body. Although the overall metabolic sequence is the same for all mammalian species, there are important distinctions in the metabolism of methanol between rodents and primates.

In rodents, the initial oxidation of methanol to formaldehyde is accomplished primarily by a catalase perioxidative mechanism (Tephly and McMartin 1984; Liesivuori and Savolainen 1991). In contrast, in humans and nonhuman primates the initial oxidation of methanol is catalyzed by alcohol dehydrogenase, the same enzyme responsible for oxidation of ethanol. In the second metabolic step, formaldehyde is converted to formate by aldehyde dehydrogenase. Formaldehyde can also be oxidized to formate by combination with reduced glutathione to form *S*-formyl glutathione, which in the presence of glutathionine thiolase is hydrolyzed to the acid with subsequent release of reduced glutathione (Tephly and McMartin 1984). The quantitative importance of the latter pathway is unknown.

Another crucial difference between primates and rodents is in the metabolism of the toxic metabolite formate. Whereas formate undergoes oxidative degradation via the folate-dependent pathway in both primates and rodents, the level of total tetrahydrofolate is significantly lower in monkey livers compared with that in rat livers (Black et al. 1985). Because the rate of formate metabolism is related to the availability of tetrahydrofolate intermediates in the liver, this difference explains the well-recognized sensitivity of humans and nonhuman primates to methanol-induced toxicity (that is, metabolic acidosis and ocular toxicity).

The elimination kinetics of inhaled or intravenously administered methanol have been characterized in rodents, nonhuman primates, and human subjects. In humans, the elimination half-life of methanol from blood has been reported to be between 2 and 3 hours (HEI 1987). Slightly shorter blood half-lives, from slightly less than 1 hour to 1.5 hours, have been reported for monkeys and rats exposed to low doses of methanol (Horton et al. 1992; Dorman et al. 1994). As is well recognized in the case of ethanol, blood methanol elimination half-life becomes progressively longer as the methanol dose is elevated into the range that results in saturation of the primary metabolic enzymes (catalase or alcohol dehydrogenase in rodents and primates, respectively). Recent toxicokinetic modeling of blood methanol data from rodents, rhesus monkeys, and humans suggests that, at modest levels of exposure, the in vivo Michaelis constant or K_m (that is, the blood methanol concentration that results in a half-maximal rate of methanol clearance) is similar, in the range of 30 to 70 µg/mL or 1 to 2 mM (Horton et al. 1992; Perkins et al. 1995a; Pollack and Brouwer 1996). This means that a dose-dependent increase in elimination half-life is expected as blood methanol concentrations exceed 30 µg/mL or 1 mM. It should be noted that much higher estimates of in vivo K_m (~7 to 9 mM) were reported by Tephly and his associates from their very early studies in rats and rhesus monkeys (Tephly and McMartin 1984, Tephly 1991).

ALTERATIONS IN METHANOL METABOLISM

There is little information on methanol disposition kinetics and metabolism for chronic low-level exposure of pregnant subjects. The only available data come from recent HEI-sponsored studies in rodents after a single intravenous or oral dose of methanol was administered to pregnant Sprague-Dawley rats and CD-1 mice (Pollack and Brouwer 1996; Ward and Pollack 1996a). Maternal toxicokinetics of methanol were compared between nonpregnant rodents and rodents at mid- and near-term stages of

pregnancy. Overall, pregnancy had only a minor effect on maternal disposition of methanol. Total systemic exposure to methanol (measured by area under the blood concentration–time curve) increased slightly in pregnant rodents exposed to low methanol doses; this was explained by a progressive decrease during pregnancy in the V_{max} for methanol elimination. No methanol toxicokinetic data are currently available in chronically exposed nonhuman primates in either the pregnant or nonpregnant state. Because the metabolism of methanol in primates involves the same enzymes as the metabolism of ethanol, information on the effects of chronic ethanol consumption and pregnancy on ethanol disposition may be relevant to our consideration of methanol metabolism.

In humans, habitual alcohol consumption or alcohol abuse (in the absence of nutritional deficits) leads to two notable changes in ethanol toxicokinetics: an increase in the peak blood ethanol concentration and an accelerated rate of decline in blood ethanol concentrations after ingestion of a test dose of alcohol (Whitfield and Martin 1994). The increase in peak blood ethanol reflects a decrease in the first-pass metabolism of ethanol by alcohol dehydro-genase enzymes located in the gastric mucosa (Baraona et al. 1994). The more rapid elimination of ethanol is generally attributed to induction of the microsomal ethanol-oxidizing enzyme (namely, cytochrome P450 2E1) in the liver (Lieber 1994) although contributions from other ethanol-induced changes in hepatic function, such as induction of non-P450 enzymes (alcohol dehydrogenase and catalase), cannot be ruled out. An increase in the ethanol elimination rate has also been observed in animals following chronic ethanol feeding. A number of studies in rodents have examined the effects of chronic ethanol exposure on the activity of alcohol dehydrogenase enzymes in the liver. Inconsistent results in hepatic alcohol dehydrogenase activity were observed, varying from a decrease (Koivula and Lindros 1975), to no change (Kesaniemi 1974; Traves and Lopez-Tejero 1994; Kishimoto et al. 1995), to an increase (Dajani et al. 1963). The variable results are attributed in part to differences in the route of ethanol administration, experimental paradigm, or conditions employed for assay of alcohol dehydrogenase activity. Nonetheless, in the present context of methanol exposure, changes in alcohol dehydrogenase enzymes in the liver and/or other tissues due to chronic exposure remain a possibility.

The effects of pregnancy on alcohol-metabolizing enzymes and ethanol toxicokinetics have been studied extensively in mice (Petersen et al. 1977), rats (Kesaniemi 1974; Espinet and Argiles 1984; Traves and Lopez-Tejero 1994; Traves et al. 1995), guinea pigs (Clarke et al. 1986; Card and Brien 1988), and sheep (Brien et al. 1987; Clarke et al. 1987, 1989). In all these studies, maternal and fetal data were gathered in the third trimester or near the end of pregnancy. However, ethanol exposure schedules varied considerably, ranging from no exposure before pregnancy to short-term exposure before pregnancy to exposure throughout pregnancy. The results consistently showed that pregnancy alone (that is, pregnancy without preconception exposure to ethanol) had little or no effect on hepatic alcohol dehydrogenase activity in vitro or ethanol elimination rate in vivo. A recent study by Traves and Lopez-Tejero (1994) did show a modest reduction in the first-pass extraction of ethanol following intragastric administration in pregnant rats. These investigators also showed that pregnancy modified the chronic effects of ethanol on its own metabolism. Ethanol exposure before conception led to a significant reduction in hepatic alcohol dehydrogenase activity in pregnant rats, but not in control nonpregnant rats. Moreover, the inductive effect of chronic ethanol exposure in pregnant rats on the elimination rate of blood ethanol appeared to be attenuated. There was also a remarkable increase in blood–ethanol area under the curve (AUC) after intraperitoneal administration, a finding that is consistent with a decrease in first-pass metabolism due to down regulation of hepatic alcohol dehydrogenase enzyme. Overall, the reported studies with ethanol suggest that while pregnancy may not affect methanol toxicokinetics per se, it may modulate the effects of chronic exposure to methanol, that is, there may be an interaction between exposure and pregnancy.

There is an additional metabolic issue engendered by pregnancy, the potential effect of pregnancy on formate metabolism via the folate-dependent biochemical pathway. It is well recognized that pregnancy can induce folate deficiency. McNulty and colleagues (1993) investigated the rate of folate catabolism during pregnancy in three groups of female rats. These researchers monitored the amount of acetamidobenzoylglutamate, a catabolite of folate, in the urine of pregnant and nonpregnant rats. Excretion of this metabolite remained constant for the nonpregnant controls, whereas excretion rose continuously in the pregnant rats to a peak of approximately three times that of the controls. The authors concluded that this increased catabolism may be a major contributor to folate deficiency associated with pregnancy. McPartlin and colleagues (1993) studied pregnant women to determine if increased folate catabolism also occurs in humans. These researchers monitored the levels of acetamidobenzoylglutamate and aminobenzoylglutamate in the urine of 6 healthy pregnant volunteers throughout pregnancy and the postpartum period and in 6 nonpregnant volunteers. The results indicated that the pregnant

women had significantly higher levels of folate metabolites in their urine compared with the levels in the control women. These differences were observed in the second and third trimester of pregnancy, with the most significant increase occurring in the second trimester. A reduction in the hepatic reserve of tetrahydrofolate during pregnancy due to increased folate catabolism may retard the metabolism of methanol-derived formate. This reduction could also potentially lead to formate accumulation in fetal tissues. Therefore, the effect of pregnancy on the toxicokinetics of methanol-derived formate needs to be investigated.

METHANOL TOXICITY AND REPRODUCTIVE EFFECTS (SPECIFIC AIM 3)

The effects of acute, high-dose methanol exposure have been characterized in cases of human poisoning and in studies conducted in animals. The hallmarks of acute methanol toxicity in humans are well documented. In brief, the affected individual typically experiences a short period of intoxication followed by a period in which no symptoms of intoxication or toxicity are noted. This asymptomatic period is followed by physical symptoms of poisoning such as headache, nausea, vomiting, loss of equilibrium, severe abdominal pain, and difficulty in breathing. These symptoms can be followed by coma and death (Tephly and McMartin 1984, Jacobsen and McMartin 1986). Formate (formic acid) is considered to be the toxic metabolite of methanol, responsible for the disturbances of the visual system and metabolic acidosis that are the classic symptoms of acute poisoning (for extensive discussion on the toxicity of methanol, see Kavet and Nauss 1990). Humans and nonhuman primates display similar effects from high-dose methanol exposure due to their limited capability, compared with that of rodents, to metabolize formate to carbon dioxide (Black et al. 1985). Rodents typically do not exhibit the hallmark signs of methanol toxicity unless they are folate deficient (Makar and Tephly 1976) or are simultaneously exposed to nitrous oxide, a compound that interferes with formate metabolism (Black et al. 1985).

Individuals who recover from methanol intoxication can display permanent motor impairment, blindness, or both. For many years, the only permanent sequelae in survivors of methanol poisoning were thought to be optic atrophy and concomitant loss of visual acuity (Bennett et al. 1953). However, a German report published in the 1960s described the case of an individual who had experienced an episode of acute methanol toxicity and who was evaluated 20 years after exposure (Riegel and Wolf 1966). Clinical findings at follow-up revealed serious neurological abnormalities including focal cranial-nerve deficits, optic atrophy, and a Parkinsonian-like syndrome. This was one of the first reports of permanent motor impairment associated with methanol ingestion. Since that time, additional reports have linked methanol intoxication with Parkinsonian syndrome, usually involving symptoms such as rigidity, tremor, slowness of movement, and impaired balance (Guggenheim et al. 1971; Ley and Gali 1983). Overall, these reports show that persistent dysfunction of the central nervous system may result after recovery from an episode of methanol poisoning. Interestingly, an examination of the occupational and environmental causes of Parkinsonian syndrome reveals that methanol when used as an industrial solvent may have links to immediate or delayed onset of this clinical disorder (Tanner 1992).

In contrast to the effects of acute, high-level exposure, relatively little is known about the effects of chronic, low-level methanol exposure. In a review of clinical cases involving prolonged exposures to methanol, it was determined that the effects are similar in nature to those associated with acute episodes of high-level exposure (that is, both conditions seem to result in visual and central-nervous-system disturbances; see Kavet and Nauss 1990). The health effects of methanol exposure were evaluated in a study of teacher aides who operated spirit duplicating machines (Frederick et al. 1984). The measured levels of methanol vapor (365 to 3,080 ppm) were quite high relative to National Insitute for Occupational Safety and Health (NIOSH) standards (200 ppm TWA for an 8-hour workday), and the teacher aides reported significantly more episodes of headaches, blurred vision, nausea, or dizziness than did controls. Other occupational studies have found that methanol exposure is associated with changes in the rate of information processing and in muscle capacity; increases in headaches and mood swings are also frequently reported (for review, see Carson et al. 1981, 1987). In a report for the Health Effects Institute (Cook et al. 1991), the neurobehavioral effects following controlled low-level methanol exposure were described for adult male volunteers exposed to 192 ppm methanol vapor for 75 minutes. This exposure concentration was selected because it is slightly above the upper range of Environmental Protection Agency (EPA) estimates associated with traffic scenarios with methanol-based fuel (namely, 66 to 184 ppm). In addition to measuring both blood methanol and plasma formate concentrations, data from a battery of sensory-cognitive tasks were collected. The test battery included choice reaction time, a visual search and a visual tracking task, the Stroop color-word test, the symbol-digit substitution task, the Sternberg

memory scanning task, the speed addition task, and event-related potentials in the auditory and visual domains. Subjects were tested twice, once during methanol exposure and once with no exposure; they served as their own controls. The order of exposure versus sham conditions was counterbalanced across subjects. Exposure resulted in blood methanol concentrations approximately 3 times that of baseline; no rise in formate was observed. Results indicated methanol-induced effects on fatigue and concentration. Performance on the Sternberg memory-scanning task and latency of the P200 component of event-related auditory and visual potentials were also affected. The authors noted that most of the neurobehavioral measures were not adversely affected by methanol exposure and that many of the effects observed were not straightforward (that is, they were dependent on order of exposure) and were of relatively small magnitude. Although this study has many limitations, it does indicate that relatively low-level methanol exposure for a short duration may cause measurable changes in central nervous system function.

The reproductive effects of methanol exposure have been addressed in only a few studies to date. The earliest studies by Cameron and colleagues (1984, 1985) examined the neuroendocrine effects of methanol exposure in adult male rats. The initial study investigated the effects of inhalation exposure to 0, 200, 2,000, or 10,000 ppm methanol for 8 hours/day, 5 days/week for 1, 2, 4, and 6 weeks. Circulating free testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) levels were measured after the animals were killed. Hormone levels for the methanol-exposure groups were compared with levels for the controls. The results of the study indicated a significant decrease in circulating free testosterone after 200- and 2,000 ppm methanol exposure (but not 10,000 ppm methanol exposure) and a significant increase in circulating LH at 10,000 ppm methanol exposure. In a follow-up study, a decrease in circulating free testosterone was found after a single 6-hour, 200 ppm exposure to methanol. This decrease was not observed 18 hours after the exposure or immediately following 1 week of 200 ppm exposure for 6 hours/day. Lee and colleagues (1991) examined the effects of methanol exposure on testicular production of testosterone as well as morphology of the testes. Six weeks of 200 ppm methanol exposure for 8 hours/day, 5 days/week did not reduce serum testosterone levels or affect testicular morphology. There was, however, an indication of accelerated age-related degeneration of the testes in 18-month-old folate deficient rats following 13 weeks of 200 ppm methanol exposure. A later study by Cooper and colleagues (1992) failed to confirm an effect of 200 ppm methanol exposure on testosterone levels. A single exposure to 0, 200, 5,000, or 10,000 ppm methanol for 6 hours did not reduce serum testosterone or FSH levels. Hormone levels were significantly affected by the handling associated with placing the rats in the exposure chamber.

Studies of female reproductive effects following methanol exposure have concentrated on defining teratogenic effects in offspring following maternal exposure during pregnancy. The results of these studies do not indicate acute effects on offspring viability at methanol concentrations up to 10,000 ppm in rats (Nelson et al. 1985; Stanton et al. 1995) and 7,500 ppm in mice (Rogers et al. 1993). Studies aimed at examining neuroendocrine changes in nonpregnant or pregnant females following methanol exposure have not been reported.

The present study assesses whether repeated exposure to methanol changes methanol disposition kinetics in nonhuman primates; whether methanol disposition changes during pregnancy; whether chronic methanol exposure at levels from 200 ppm to 1,800 ppm is associated with overt adult toxicity, female reproductive toxicity, or both; and whether in utero exposure to methanol affects offspring development. The procedures used to determine changes in methanol kinetics included 6-hour toxicokinetic profiling of blood methanol and formate concentrations following inhalation exposures. Procedures to assess adult toxicity included observational techniques to detect labored respiration and deficits in visual and motor performance in exposed adult females. These procedures provided a rapid evaluation of whether or not overt maternal toxicity was associated with the methanol exposure levels used in this study. Procedures to evaluate subtle changes in adult central-nervous-system function were not included because the main focus of the study was on developmental effects on the central nervous systems of exposed offspring. The procedures to evaluate reproductive effects included (1) timed matings to evaluate conception rate, (2) daily and around-the-clock observations to detect pregnancy and labor delivery complications, and (3) determination of pregnancy duration and rate of live births in methanol-exposed and control female *M. fascicularis*. A battery of tests was included to evaluate offspring development throughout the first 9 months of life. The results of this battery are described in Part II of this Research Report.

SPECIFIC AIMS

Specific Aims 1 through 4 of the project were designed to determine the following exposure dose–response relations in nonhuman primates for exposure to methanol vapor:

1. changes in methanol disposition related to repeated exposures to methanol vapor, as defined by changes in the blood clearance, distribution volume, and/or elimination half-life of methanol in exposed nonhuman primate females;
2. changes in methanol disposition related to pregnancy, as defined by changes in the blood clearance, distribution volume, and/or elimination half-life of methanol in exposed, pregnant nonhuman primate females;
3. toxic effects on female reproductive function, as defined by decreased conceptions, increased frequency of complications of pregnancy and/or labored delivery, changes in pregnancy duration; and decreased frequency of live births among methanol-exposed nonhuman primate females; and
4. toxic effects on infant development, as defined by alterations in the physical and/or neurobehavioral development of nonhuman primate infants exposed in utero to methanol.

Specific Aims 1 and 2 were addressed by studies of the time course of blood methanol and plasma formate concentrations over a 6-hour period following exposure to methanol. Using appropriate kinetic models (Gibaldi and Perrier 1982), blood clearance, distribution volume, and the elimination half-life of methanol were estimated following initial exposure to methanol, after approximately 3 months of methanol exposure, and twice during pregnancy.

Specific Aim 3 was addressed by examining a number of factors—the menstrual cycles, frequency of conception, frequency of complications of pregnancy and labor and delivery, pregnancy duration, and frequency of live births for time-mated *M. fascicularis* females who were either exposed to methanol or not (controls) prior to conception and during pregnancy. The procedures used to address this specific aim were previously used to identify reproductive dysfunction due to chronic exposure to methylmercury (Burbacher et al. 1988). In that study, blood mercury levels significantly below those associated with overt maternal toxicity were associated with a reduced number of live births to mercury-exposed females.

Specific Aim 4 was addressed by examining the neurobehavioral development of *M. fascicularis* infants exposed in utero to methanol compared with the development of infants born to control females. Many of the neurobehavioral tests used to address this specific aim were adapted from studies of normal, high-risk, and teratogen-exposed human infants (Weisz and Zigler 1979; Streissguth et al. 1981; Fagan and Singer 1983; Rose 1983; Jacobson et al. 1985). Similar response patterns on these tests have been observed for both human and nonhuman primate infants (for review, see Burbacher et al. 1990). The results of these tests are presented in Part II of this report.

METHODS

SUBJECTS AND STUDY DESIGN

The two-cohort study design utilized 48 adult female *M. fascicularis* (24/cohort), 4 adult male *M. fascicularis* (2/cohort), and their offspring. This design, similar to that used in a previous investigation of the neurobehavioral effects of in utero methylmercury exposure (Burbacher et al. 1988), minimizes the number of subjects tested simultaneously, yet achieves a sufficient sample size to detect subtle changes in the neurobehavioral development of exposed offspring.

For each cohort, adult females were initially separated into six groups, with four animals per group based on known or estimated age, size, and colony parity. Females from each of the six groups were then randomly assigned to one of four methanol-exposure groups. The females in Cohort 1 were all feral born, imported to the University of Washington in October of 1990, and assigned to the project in May of 1991. The females were between 5.5 and 11 years of age and weighed between 2.3 and 3.7 kg when they were assigned to the project. Because all were recently imported, the actual parity of the females was unknown and their colony parity was thus considered 0. The females in Cohort 2 were a mixture of feral-born (n = 15) and colony-born (n = 9) monkeys (obtained from Texas Primate Center, Charles River Primates, CV Primates, or Johns Hopkins University) that arrived at the University of Washington between February and May of 1994 and were assigned to the project in August of 1994. These females were between 5 and 13 years of age and were, on average, larger than the Cohort 1 females, with weights between 2.2 and 5.7 kg. Five females had a colony parity of 0, equivalent to the Cohort 1 females, whereas the colony parity for the other nineteen females ranged between 1 and 4. The 4 adult males were feral born with age estimates between 10 and 12 years. The males weighed between 5 and 7.6 kg, and each had sired an offspring at the University of Washington's primate facility.

Age estimates for feral animals were obtained from a member of the veterinary staff at the University of Wash-

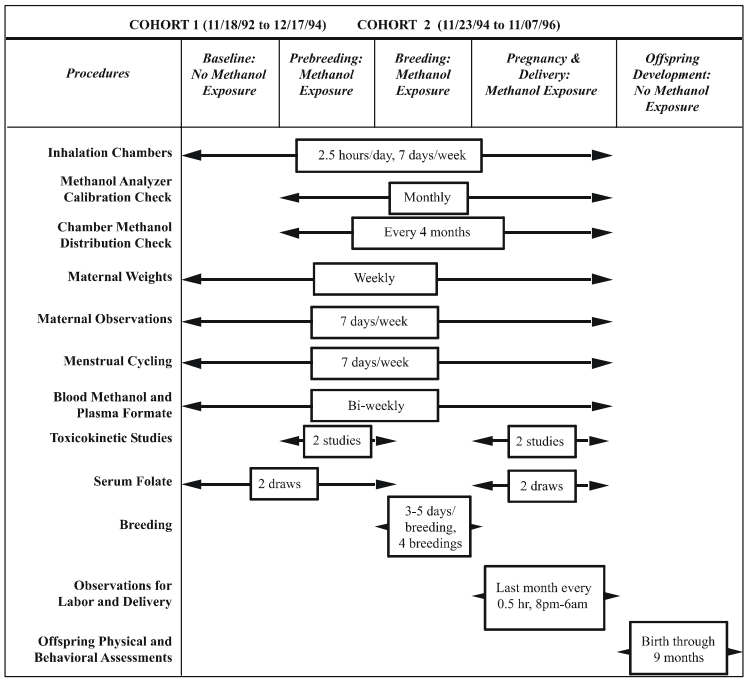

Figure 1. Experimental design and schedule of study.

ington who examined all of the animals. Ages were determined on the basis of dental records obtained during the examination. Measures of body weight, crown–rump length, head circumference, head length, and head width were obtained for each female. Two separate measurements were taken either by 1 tester (Cohort 1) or 2 independent testers (Cohort 2).

All adult females were housed at the Regional Primate Research Center at the University of Washington in a room located approximately 18 feet from the inhalation laboratory. The adult females were transferred to and from the laboratory in a transfer cage via a front corridor. Adult females were housed in individual cages with dimensions of 25 inches by 25 inches by 29.5 inches arranged in a four-pack design (2 cages on top and 2 on bottom) throughout the course of the study. A door with dimensions of 10 inches by 18.5 inches located in the common wall was opened every day prior to and after methanol exposures so adjacent females could have social contact through wire mesh. Females had free access to water in their home cages and were fed Purina Laboratory Fiber-Plus® Monkey Diet once per day in the afternoon, after all exposures had been completed. The adult males were housed individually in a different room, away from the females; they also had free access to water and were fed Purina Laboratory Fiber-Plus® Monkey Diet once per day in the afternoon. The facilities at the Regional Primate Research Center are certified by the American Association for the Accreditation of Laboratory Animal Care, and all procedures involving live animals had been approved by the University's Committee on the Use of Animals in Research.

The study design included a baseline period and 3 methanol-exposure periods (see Figure 1). During the entire study, females were transferred on a daily basis from their home cages to one of the inhalation chambers. Females were assigned to 1 of the 8 chambers at the beginning of the baseline period and were transferred to that chamber at the same time of day for the entire study. The schedule was arranged so that 2 females from each of the 4 exposure groups were always transferred in the morning, midday, and afternoon. Females remained in the chamber for approximately 2.5 hours before return to their home cages. During the baseline period prior to methanol exposure, all transfer, blood-draw, and observational procedures were performed according to the schedule in Figure 1. The baseline period was approximately 4 months long in order to characterize at least 3 menstrual cycles for each female, as well as to provide data regarding background (that is, endogenous) blood methanol and formate concentrations and behavioral responses. An initial methanol-exposure period of approximately 4 months was included prior to breeding females. During this latter period, all procedures initiated during baseline continued. In addition, 6-hour methanol clearance studies

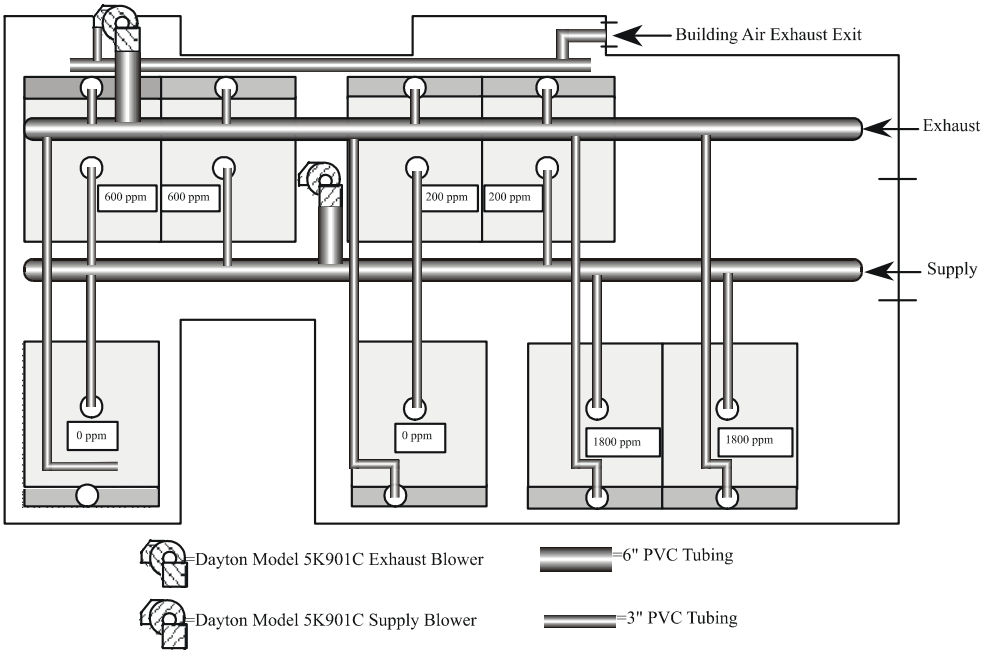

Figure 2. Inhalation laboratory with eight chambers for methanol exposures.

were performed after the initial exposure to methanol and after approximately 3 months of exposure. The breeding exposure period lasted until females were confirmed to be pregnant or until they had not conceived after 4 timed matings. For those females that did conceive, methanol exposure continued throughout pregnancy. All procedures continued during the periods of breeding and pregnancy, with 2 additional clearance studies scheduled during pregnancy.

Some changes in procedures did occur during the investigation. First, the females in Cohort 1 were not transferred to the inhalation laboratory during the 4-month baseline period. In addition, the exposure schedules of 2 females were changed during the end of the study to preclude long gaps in the transfer schedule. Last, 2 females were not exposed due to acute illness, one for 5 days and one for 11 days.

METHANOL EXPOSURES

The inhalation laboratory used in this study was approximately 10 feet by 18 feet in floor area with a ceiling height of 8 feet. The ventilation for the lab was controlled by a system that served the entire floor of the building (approximately 15 rooms). The system included several filters and warming and cooling devices for temperature control of incoming air. Two diffusers were located in the ceiling of the lab for air supply, and a duct located in the southeast wall provided an exhaust port for the air. The total air supply to the lab was 595 cubic feet/minute and the exhaust rate was 376 cubic feet/minute. The air exchange rate for the lab was 26 exchanges per hour, and the lab was positive to the front corridor at 0.011 inches H_2O. Eight inhalation chambers were arranged in the laboratory as shown in Figure 2. Each chamber housed one animal in a cage 47 cm wide by 61 cm high by 80 cm deep. The cage was supported in the chamber such that the animal was 0.5 m above the chamber floor. Each chamber had an accessible volume of 1.38 m^3 with a floor area of 0.70 m^2 (see Figure 3). Air was delivered to the chambers from the room via a Dayton Model 5K901C blower (Dayton Corporation, Moraine, OH). A second blower provided the air exhaust for the chambers. The pressures generated by the supply and exhaust blowers were measured with Magnehelic® pressure gauges (Dwyer Institute, Michigan City, IN), and flow adjustments were afforded by ball valves. The flow rate for each chamber was monitored with a linear flow element coupled to a Magnehelic® pressure gauge. The linear flow element for each chamber also served as the major pressure resistance in the air supply ductwork, thus achieving uniform distribution of air to each of the 8 chambers. On entering each chamber, air was forced through a perforated plastic plate that provided uniform air distribution within the chamber. Downward air velocity in the chamber below the level of the plenum was 1 cm/second. Air left the chamber via the outlet plenum through a fiberglass floor grate and was discharged into the room's exhaust system. The flow

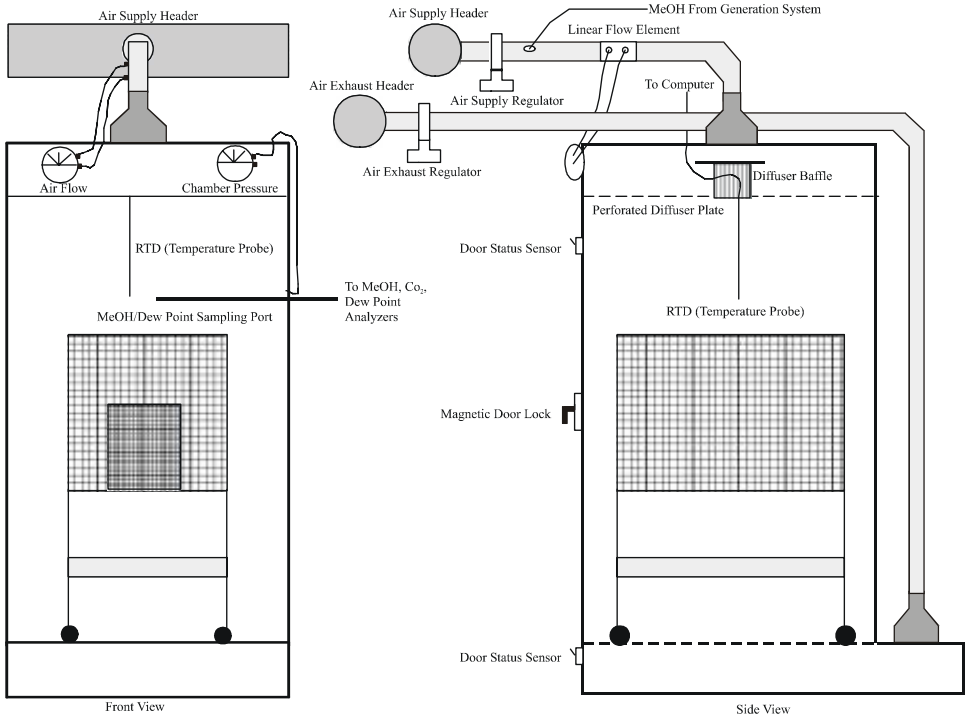

Figure 3. Schematic representation of inhalation chambers.

rate and air pressure for each chamber were set by adjusting individual ball valves at the end of the chamber containing the supply and exhaust ports. Chamber pressure was monitored with use of a second Magnehelic® pressure gauge. Flow to each chamber was set at a rate of 420 L/minute, and chamber pressure was adjusted to –0.10 inches H_2O relative to room pressure.

Methanol vapor was generated and delivered to each chamber using the system shown in Figure 4. Methanol vapor was generated by passing compressed air through gas dispersion bottles filled with high-performance liquid chromatography–grade (HPLC-grade) methanol (Fisher Scientific, Pittsburgh, PA; each bottle of methanol came with a "certificate of analysis" indicating at least 99.9% purity). The methanol was heated by placing the bottles in a water bath set at a temperature of approximately 36°C. The methanol vapor was delivered to the chamber via insulated polypropylene tubing that ran from the bottles to the vapor inlet port of each chamber. Delivery was provided just downstream from the inlet air-flow adjustment valve. Efficient mixing of vapor and supply air was produced by the turbulence induced by the partially closed adjustment valve. To achieve target air methanol concentrations, 8 independent generation and delivery systems permitted independent operation of the chambers through adjustment of the flow rates of compressed air to the individual dispersion bottles. The target air concentrations of methanol for the 4 exposure groups were as follows:

high methanol exposure = 1,800 ppm,

moderate methanol exposure = 600 ppm,

low methanol exposure = 200 ppm, and

controls = 0 ppm.

These target air concentrations were chosen to provide a range of blood methanol concentrations from just above background to just below that reported to cause nonlinear clearance kinetics in primates (Horton et al. 1992). To control the timing of the delivery of methanol to the chambers and the air sampling from each chamber, programs were developed using LABVIEW software, National Instruments interface equipment, and a Macintosh IIci computer. Methanol was delivered to any given chamber for 2 hours. At the end of each 2-hour exposure, the animals remained in the chamber for another 30 minutes while the methanol dissipated. Methanol, CO_2 , and dew point were measured by withdrawal of an air sample through a polypropylene tube located in the chamber at a level 5 cm above the monkey cage. The air sample was drawn at a rate

* Initially, carbon dioxide (CO_2) was going to be measured to provide data regarding ventilatory rates based on changes in CO_2 levels in the chamber. The final chamber design did not allow for this because the methanol and CO_2 levels were measured in the breathing zone of the animals. Thus, CO_2 levels are not reported.

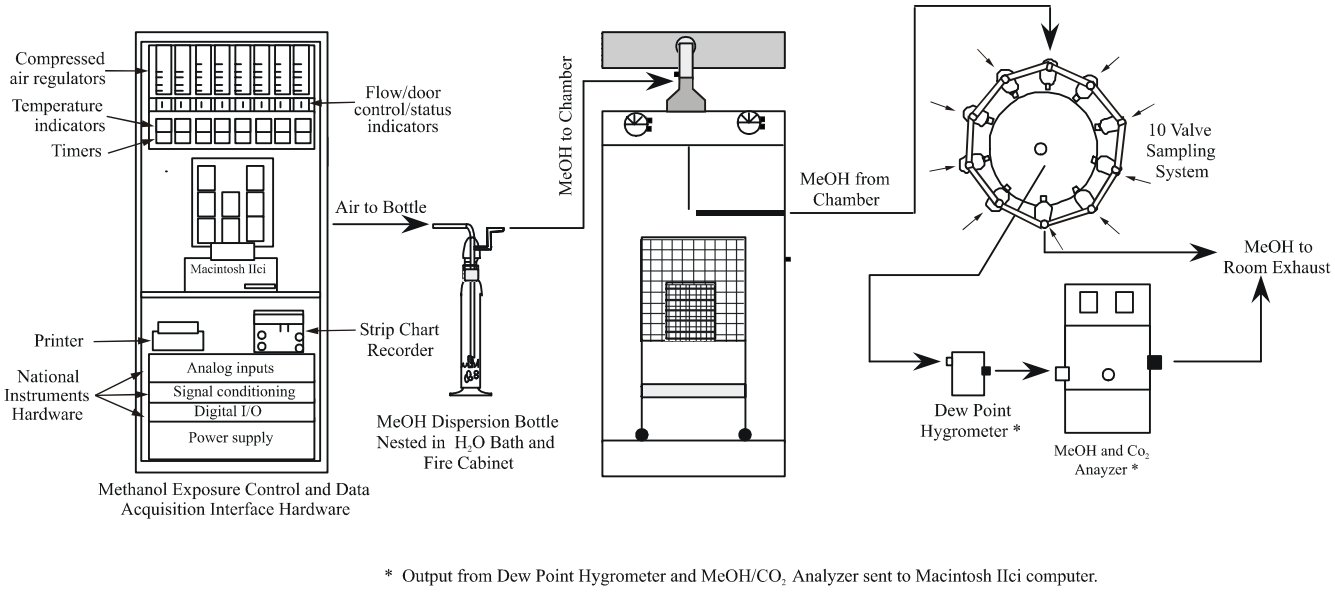

Figure 4. Methanol delivery and sampling system.

of approximately 1.5 L/minute. Methanol and CO_2 were measured by a General Analysis Corporation (Norwalk, CT) infrared analyzer.* Dew point was measured by a General Eastern Instruments (Woburn, MA) hygrometer, and chamber temperature was measured via resistance temperature detectors placed in each chamber. Dew point and temperature was used to calculate the relative humidity (RH) of each chamber according to the following equation:

$$\% \text{ RH} = 100 \times e^{\left(17.2694\left[\frac{\text{dew pt}}{\text{dew pt} + 238.3} - \frac{\text{temp}}{\text{temp} + 238.3}\right]\right)}$$

Because only one analyzer for methanol and CO_2 was available for all eight chambers, a 10-valve sampling system was constructed to allow flow from a particular chamber, control filter, or the room to the hygrometer and analyzer (see Figure 4). Sampling occurred repeatedly across the 10-valve system until the end of the day. Each chamber (plus the room and a control filter) was sampled for 1 minute with the initial sample from the chamber taken 4 minutes after the onset of methanol flow. With this schedule, the sample rate per chamber was 1 sample/10 minutes. Values for methanol, CO_2, temperature, dew point, and relative humidity were displayed on the computer screen after analysis of each sample. The values, along with appropriate identifiers, were entered into an Excel file and to an online printer after analysis of each sample. At the end of the day, the file containing complete results from the day's sampling of the chambers, usually 500 to 600 sample total, was copied to a 3.5-inch computer diskette for processing.

BLOOD METHANOL, PLASMA FORMATE, AND SERUM FOLATE ANALYSES

Sample Collection

Throughout the study, blood was collected from all females every other week for methanol and formate analyses. The blood draws occurred approximately 10 minutes after the females were removed from the inhalation chambers, that is, 30 minutes after the end of methanol flow to the chambers. Approximately 3 mL of blood were collected by venipunctures of the saphenous veins of the unanesthetized females. In addition to biweekly monitoring of blood methanol and formate, four full toxicokinetic profile studies were performed: after initial methanol exposure (Study 1), after approximately 3 months of exposure (Study 2), and twice during pregnancy (66 to 72 days [Study 3] and 126 to 132 days [Study 4]). For each toxicokinetic profile study, 2-mL blood samples were collected by venipunctures of the saphenous veins of the unanesthetized females at 7 time points: 30 minutes prior to exposure, and 30, 60, 120, 180, 240, and 360 minutes following termination of exposure.

Blood was also collected from all females for serum folate analyses. Blood was collected at five time points during the study: once prior to methanol exposure, twice after initial exposure but prior to breeding, and twice during pregnancy (51 to 57 days and 111 to 117 days). Samples were analyzed by the Hospital Clinical Laboratory at the University of Washington.

Blood Analysis

Upon arrival at the bioanalytical laboratory, each heparinized blood sample was split into two portions. One portion was immediately processed for the methanol assay. The other portion was centrifuged to yield plasma, which was stored at 4°C pending formate analysis.

Methanol in whole blood was analyzed in duplicate by a direct headspace capillary gas-chromatographic procedure developed in our laboratory. A 0.5-mL aliquot of 0.25 M NaOH was added to 0.5 mL of specimen to solubilize blood proteins. This step permits the use of high bath temperature (85°C) during headspace equilibration, which increases the sensitivity of the assay without creating the confounding problem of blood coagulation. Acetonitrile was added as an internal standard. Chromatographic resolution was achieved on a widebore carbowax column (J & W, DB-WAX, 30 m × 0.53 mm internal diameter, 1 µm thickness) with detection afforded by a flame ionization detector. Methanol analyses were typically run within 3 days after blood collection. A set of spiked methanol standards at 8 concentrations ranging from 0.5 to 60.0 µg/mL were prepared with use of monkey blood from nonexposed animals and processed along with each batch of blood samples. The data recorded for the methanol analyses included (1) calibration data consisting of blank subtracted peak area ratio of methanol and internal standard (acetonitrile) from the chromatogram of the injected standards, along with parameters from a regression analysis of the calibration data, and (2) sample data consisting of raw peak area ratios and the corresponding concentrations estimated by interpolation of the calibration plot. The assay had a limit of detection of 0.1 to 0.2 µg/mL and a limit of quantitation at 0.5 µg/mL. The intrabatch coefficient of variation of the assay was consistently < 6% at both low (3 µg/mL) and high (30 µg/mL) concentrations over the 4 years of analysis (see the Quality Assurance and Quality Control Procedures section and Appendix A, available on request).

Formate concentrations were measured using the enzymatic assay procedure of Buttery and Chamberlain (1988). This plasma assay relies on the colorimetric measurement of reduced nicotinamide adenine dinucleotide (NADH) produced by the action of formate dehydrogenase on formate. The color reagent consists of phenazine methosulfate and *p*-iodonitrotetrazolium, which reacts with NADH to form a stable red formazan. Formate analyses were typically performed within 1 week following the receipt of the blood samples. A set of spiked formate standards at 8 concentrations ranging from 0.125 to 2.5 mM were prepared using plasma from nonexposed females and processed along with each batch of blood samples. The data recorded for the formate analyses included (1) calibration data consisting of raw and blank subtracted absorbencies for the standards, as well as parameters from a regression analysis of the standard curve, and (2) sample data consisting of raw and enzyme blank-subtracted absorbencies, along with the corresponding calculated concentrations. The assay had a limit of quantitation at 0.01 mM. The intrabatch coefficient of variation of the enzymatic assay, which was determined at regular intervals over the project period, ranged from 5% to 10% (see the Quality Assurance and Quality Control Procedures section and Appendix A, available on request).

Serum folate concentrations were measured by the Hospital Clinical Laboratory at the University of Washington with use of a commercial radioimmunoassay kit (Quantaphase, Dawson et al. 1980). The limit of quantitation for the serum folate assay was 0.2 ng/mL and the within-run variability was < 6%. All blood analyses were run by technicians blind to the exposure status of the females.

MATERNAL HEALTH ASSESSMENTS

Maternal Weight

Throughout the study, all females were weighed on a weekly basis. Females were weighed every Tuesday while being transferred from the inhalation chamber to their homecages.

General Observations Test

The purpose of the General Observations Test was to provide a rapid assessment of females immediately following methanol exposure. Each female was observed for signs of lethargy, uncoordinated motor movements (staggering or clumsiness) and labored or irregular respiration approximately 5 minutes after return to the homecage. If the tester did not observe any of these signs, a code of "0" was entered on the data sheet. If signs of lethargy, motor incoordination, or irregular respiration were observed, a code of "1" was entered on the data sheet and the proper veterinary personnel were notified. The test was performed 5 days/week by the technicians who transferred the females to and from the inhalation lab. These technicians were not blind to the exposure history of the females. On the other 2 days, the test was conducted by the technicians who performed the clinical observation procedures (see the following section). These technicians were blind to the exposure history of the females.

Clinical Observations Test

The primary purpose of the Clinical Observations Test was to provide a quick evaluation of the visual function

and fine motor coordination of the females. Each female was assessed immediately after completion of the general observations procedures, or approximately 8 to 10 minutes after the end of the methanol exposure. Visual function was assessed by observing whether or not the female could visually orient to and/or follow a syringe filled with apple juice. Fine motor coordination was assessed by observing whether or not the female could reach for and pick up a small piece of fruit using only her thumb and index finger. If a female failed to visually orient or follow the syringe, or if she could not pick up a small piece of fruit using only her thumb and index finger, a code of "0" was entered on the data sheet. If the female successfully responded, a code of "1" was entered on the data sheet. The test was administered 2 days/week (Cohort 1) or 1 day/week (Cohort 2) by technicians who were "blind" to the methanol exposure history of the females.

Health Check Procedure

The primary purpose of the health check was to detect diarrhea and to administer and record all medications. Health checks were performed at the end of the day, 7 days/week, by the technicians who transferred the females to and from the inhalation laboratory.

MATERNAL REPRODUCTIVE ASSESSMENTS

Menstrual Cycles

The onset and duration of menstruation was assessed using a noninvasive observational method to detect menstrual bleeding: the females were trained to present their perinea to an observer for visual evaluation. A flashlight was used to aid in the inspection, and an apple juice reward was presented after evaluation was completed. The procedure was performed at the end of the day, 7 days/week, by the technicians who transferred the females to and from the inhalation laboratory.

Timed Matings

Females were bred to one of two nonexposed males for 4 hours each day on the 11th, 12th, and 13th day of the menstrual cycle after exhibiting a minimum of 7 menstrual cycles (3 cycles prior to methanol exposure and 4 cycles after initial exposure). Prior to separation at the end of the 4-hour breeding period, females and males were observed to detect the presence of semen as evidence of mating. Pregnancy was detected via blood progesterone analysis according to the method described by Gianessi and colleagues (1978). Typically, pregnancy was confirmed by 18 to 20 days of gestation. Females who did not conceive on the first breeding were bred again to the same male for 3 additional days. If necessary, a third and fourth breeding took place for 5 consecutive days (days 10 through 14 of cycle), again using the same male.*

Pregnancy Observations and Delivery Examinations

In addition to the observation procedures described above, all females were observed during the last month of pregnancy every half hour from 8 p.m. to 6 a.m. (that is, the lights out period) via infrared cameras. Females were observed for signs of labor so that the personnel who were scheduled to perform the delivery examinations could be contacted. Immediately after delivery of an infant, the female was sedated, and the infant was separated from the mother and placed in an isolette. Maternal weights were recorded, and the mother was returned to her home cage for observation while she remained sedated. The birth weight, crown–rump length, and head size of all infants were obtained immediately following delivery. Other infant assessment procedures were also performed at delivery. The results of these assessments on offspring are described in Part II of this Research Report.

QUALITY ASSURANCE AND QUALITY CONTROL PROCEDURES

Equipment Calibrations for Methanol Exposures

Two General Analysis Corporation (GAC) methanol and CO_2 infrared analyzers and two General Eastern dew point hygrometers were calibrated yearly within the specifications of each manufacturer's warranty. In addition, the calibration of the in-line GAC analyzer was checked locally on a monthly basis; the monthly checks were to ensure that methanol values were within 10% of known injected amounts.†

The resistance temperature detectors (RTDs) were also checked locally at intervals of approximately every 4 months with use of a DP100 ANALOGIC meter, calibrated within the specifications of the manufacturer's warranty (0.5°C). RTD checks were to ensure that temperature values were within 1°C.

* With Cohort 1, the male assigned to one female was changed due to fighting after two unsuccessful breedings. With Cohort 2, several females were bred to 2 different males after it became obvious that one of the males was an intermittent breeder (see Appendix C1 to Part II of this Research Report for list of female/male pairs).

† On 2 occasions, once with Cohort 1 and once with Cohort 2, the calibration checks indicated a problem with methanol values from the infrared analyzer. During these 2 periods, data from calibration checks were used to provide a correction factor for the daily methanol-exposure data.

Characterization of Exposure Chambers

Studies to investigate the distribution of methanol vapor within each of the chambers were conducted approximately every 4 months. During these studies, air samples were taken from 10 locations in a chamber with lines placed 2.5 inches from the 8 corners of the animal cage, from the sample line above the cage (the breathing zone site), and from a line located in the center of the animal cage. Methanol was delivered to the chamber for a little over 2 hours in order to provide 10 samples from each site while methanol concentrations inside the chamber were stable. The studies were conducted with the entire lab run according to the normal schedule. Chambers that were not being examined were sampled periodically for methanol using the backup analyzer; these studies were conducted to ensure that methanol concentrations at all sampled locations were within 10% of the samples from the breathing zone.

Blood Methanol Analysis

For internal quality control, intrabatch coefficients of variation (CV) for replicate measurements were determined at yearly intervals throughout the duration of the project (that is, a total of 4 runs between October 1990 and November 1994). For these studies, assays were performed on replicate samples of monkey blood (n = 5 to 20) that were spiked with either 3 or 30 µg/mL of methanol.

Two separate studies were conducted during the initial phase of the project to evaluate the stability of methanol in monkey blood stored at 4°C. In both studies, three batches of spiked samples were prepared from freshly collected, pooled blood from nonexposed monkeys. One batch was assayed immediately (Day 0). The other two batches of blood samples were stored at refrigerated temperature for 3 to 4 days and 6 to 7 days.

Last, the weekly regression curves for blood methanol standards were examined after each run to ensure acceptable linearity, goodness of fit ($r^2 > 0.998$), and week-to-week consistency in slope and intercept values. Also, the duplicate assays on each blood sample had to agree within 17.6% (that is, 3 × average intrabatch SD). If not, a third aliquot from the blood sample was analyzed when a sufficient volume of blood was available.

For external quality control, the laboratory participated in interlaboratory validation studies in May 1991, December 1991, and November 1993. These studies, coordinated by HEI, were conducted on samples of rat blood spiked with known amounts of methanol or on blood samples from rats exposed to a controlled air concentration of methanol vapor. Details of the procedure and results were presented in a summary report prepared by Sander Stern of the Department of Environmental Medicine, University of Rochester, on behalf of Maria Costantini of HEI in January 1994.

Plasma Formate Analysis

Intrabatch CV studies were also conducted on the plasma formate assay five times between October 1990 and September 1993. For these studies, plasma collected from several male nonexposed monkeys were pooled and spiked with sodium formate at concentrations between 0.3 and 0.6 mM. Two of the earlier studies included a higher concentration of spiked samples (~1 mM). Six to 12 replicate samples were analyzed.

The stability of formate in monkey plasma stored at 4°C was evaluated on two occasions. The first stability study covered storage intervals up to 19 days. A shorter-term storage internal (that is, 7 days) was examined in the second study. Aliquots of plasma samples containing about 0.4 to 0.5 mM of spiked sodium formate were prepared. One aliquot was assayed immediately to provide the Day 0 data. The remaining aliquots were divided into batches that were stored and assayed at designated times.

As in the case of the methanol assay, the standard curve for each formate run was examined for linearity, goodness of fit, and run-to-run consistency in slope and intercept values. No interlaboratory comparison studies were conducted for the formate assay because with the exception of Chemical Industry Institute of Toxicology (CIIT), none of the other laboratories included plasma formate measurements in their projects. The studies at CIIT were conducted with radiolabeled methanol. Plasma formate concentration was assayed by HPLC coupled with radioactive detection.

Quality Control Procedures for Maternal Assessments

The quality control program for maternal health assessments and for detection of menstruation included standard procedures for determining reliability across all testers, weekly checks of all completed data forms for detecting errors and omissions, a two-step data editing procedure, and frequent graphical data summaries for detecting outliers. The most important aspect of our quality control procedures were the reliability tests. The tests were performed every 3 to 4 months. For these tests, one of the project coinvestigators (KG), who was "blind" to the exposure history of the females, conducted an initial reliability test with a predesignated primary tester. This primary tester was typically also "blind" to the exposure history of the females. When the primary tester demonstrated reliability in testing, he/she then conducted the tests with the other individuals who routinely performed the procedures. Reliability criteria for all of the proce-

dures usually exceeded 90%. The results of the various quality control procedures are presented in Appendix A, available on request.

STATISTICAL ANALYSES

Raw data were transferred as ASCII files from the study database to computers in the Departments of Environmental Health and Biostatistics at the University of Washington. The statistical analyses were performed using Systat, SAS, or Splus. The individual statistical models were chosen based on the hypotheses and nature of the particular data sets. Four basic hypotheses were developed:

1. There will be no significant differences in methanol toxicokinetics
   (a) across the methanol-exposure groups following the initial methanol exposure,
   (b) between initial exposure and following repeated exposures (90 days) to methanol, and
   (c) between preconception and mid- and late-stage pregnancy.
2. There will be no significant differences across the methanol-exposure groups in plasma formate and serum folate concentrations during pregnancy.
3. There will be no significant differences across the methanol-exposure groups in overt signs of maternal toxicity.
4. There will be no significant differences across the methanol-exposure groups in signs of toxic effects on maternal reproduction.

In addition to Hypotheses 1 through 4, statistical analyses of maternal characteristics (age, weight, crown–rump length, gravidity, and parity) at the outset of the study were performed to examine the results of random assignment of adult females to the 4 exposure groups.

The general approach to testing the hypotheses was to first assess whether an exposure effect existed, both globally and specifically. A global *F* test (or equivalent) was used for assessing whether there were detectable differences among the 4 exposure groups. Because this test has less power than specific alternatives, a no exposure–effect hypothesis was also examined that compared the control group with the combination of all methanol-exposure groups. The control group was also compared with each methanol-exposure group using pairwise comparisons. Finally, the impact of controlling for cohort was assessed in mean models. These models are reported, however, only if there was a change in the results pertaining to methanol exposure. Type III *F* tests were used for all analysis of variance (ANOVA) analyses.

To test Hypotheses 1a through 1c, three separate measures of methanol disposition kinetics were used (elimination half-life, clearance, and distribution volume). Standard ANOVA models were used to examine Hypothesis 1a, whereas repeated measures ANOVA models were used to examine Hypotheses 1b and 1c.

To test Hypothesis 2, plasma formate concentrations obtained biweekly and serum folate concentrations obtained twice (prior to and during pregnancy) were used. Repeated measures ANOVA models were used to examine plasma formate and serum folate concentrations during pregnancy.

To test Hypothesis 3, four separate procedures were used to detect overt signs of maternal toxicity. Due to the low number of positive responses, no formal statistical analyses of the outcome measures were performed; instead, descriptive analyses are presented.

To test Hypothesis 4, the following measures of toxic effects on maternal reproduction were used:

(a) menstrual-cycle length,
(b) rate of conception,
(c) weight gain during pregnancy,
(d) frequency of pregnancy and delivery complications,
(e) pregnancy duration,
(f) frequency of live-birth deliveries, and
(g) offspring birth size (weight, crown–rump length, head circumference, length, and width).

Repeated measures ANOVA models were used to examine the menstrual-cycle length, whereas one-way ANOVA models were used to examine weight gain during pregnancy, pregnancy duration, and offspring birth size. Fisher's exact tests were used to examine the discrete outcome measures (conception rate, frequency of pregnancy and delivery complications, and frequency of live-birth deliveries). Logistic regression models could not be used for these measures because there was not enough differentiation among the various groups with respect to the outcomes.

Goodness-of-fit of all linear models was assessed through examination of residuals. Theoretically, residuals are random observations from a normal distribution curve with fixed variance. Whenever the empirical distribution of the residuals was too skewed to be considered normal, a log transformation was used. Individual observations for which a model did not seem to fit well, or that were considered potentially influential, were removed from the model and the model was refit without them. If our con-

Table 1. Maternal Characteristics at Assignment to Exposure Group[a]

	Maternal Characteristics			
Exposure Group	Age (years)	Weight (kg)	Crown–rump length (mm)	Head Circumference (mm)
Control (n = 11)	7.5 ± 0.4 (5.6–10.0)	3.10 ± 0.22 (2.35–4.91)	411 ± 8 (372–453)	241 ± 2 (229–255)
200 ppm (n = 12)	8.0 ± 0.6 (5.8–12.5)	3.09 ± 0.19 (2.25–4.37)	411 ± 8 (373–472)	242 ± 4 (225–264)
600 ppm (n = 11)	7.5 ± 0.3 (6.2–10.0)	3.22 ± 0.29 (2.37–5.73)	418 ± 9 (374–467)	243 ± 4 (225–264)
1,800 ppm (n = 12)	7.9 ± 0.6 (5.2–11.5)	3.10 ± 0.19 (2.35–4.70)	414 ± 4 (390–431)	240 ± 3 (231–258)

[a] Values are presented as means ± SE with range in parentheses on line below. No statistically significant differences were found in maternal age or maternal size across the four methanol-exposure groups (ANOVA; $p > 0.40$, all tests).

clusions were significantly affected by these removals, both the original fit (to the full data set) and the fit without these observations are reported. (For individual analysis tables, see Appendix C.)

RESULTS

SUBJECTS

For each cohort, 24 adult female *M. fascicularis* monkeys were randomly assigned to 1 of 4 methanol-exposure groups after being separated into subgroups according to maternal characteristics such as age, size, and parity. Of the 24 females, 2 had to be dropped from the study (1 in the control group and 1 in the 600 ppm group). The control female was dropped after testing positive for simian retrovirus after a prolonged illness. The female from the 600 ppm group was dropped after she was mistakenly exposed to methanol for 1 day during the baseline (nonexposure) period. The characteristics of the final exposure groups, which included the remaining 46 females, are summarized in Table 1 (see Appendix B, available on request, Figure B.1 for scatterplot figures of data from individual females). The age range for the females was roughly 5 to 13 years, with a mean age for females in the exposure groups of approximately 8 years. Although the majority of the females were under 10 years of age, 2 or 3 females in each exposure group were above 10 years of age. The weight range for the females was 2.2 kg to 5.7 kg, with a mean weight for the exposure groups of approximately 3 kg. Although the majority of the females were under 4 kg, 1 or 2 females in each exposure group were above this weight.

To test the assumption that the age and size of the females were not significantly different across the exposure groups, ANOVA models were fitted for age and maternal size (weight, crown–rump length, and head circumference). The results of the ANOVA models (see Tables C.1 through C.4) did not indicate a significant difference in maternal age or maternal size across the 4 methanol-exposure groups ($p > 0.40$, all tests).

To test the assumption that colony gravidity and parity were not significantly different across exposure groups (only females from Cohort 2 were considered because all females in Cohort 1 had a colony parity of 0), these variables were analyzed using Fisher's exact test. The Fisher's exact tests produced p values of 1 for both colony gravidity and colony parity, indicating there were no significant differences in either variable across the 4 methanol-exposure groups (see Tables C.5 and C.6).

We conclude that the counterbalancing procedure succeeded in providing 4 exposure groups similar in maternal age (~8 years), size (weight ~3 kg), and parity (~1).

METHANOL EXPOSURES

Results of the 10-minute sampling of chamber methanol concentration for the 2.5-hour exposure periods for each of the exposure groups are shown in Figure 5. The chamber methanol values shown were calculated by first averaging the chamber methanol concentrations at each sample time (4 minutes, 14 minutes, etc.) over all exposures for each female and then calculating the mean of these means for females within each exposure group. The exposure group data indicate that by 4 minutes of exposure, average concentrations were between 60% and 70% of the target chamber methanol concentrations. Average

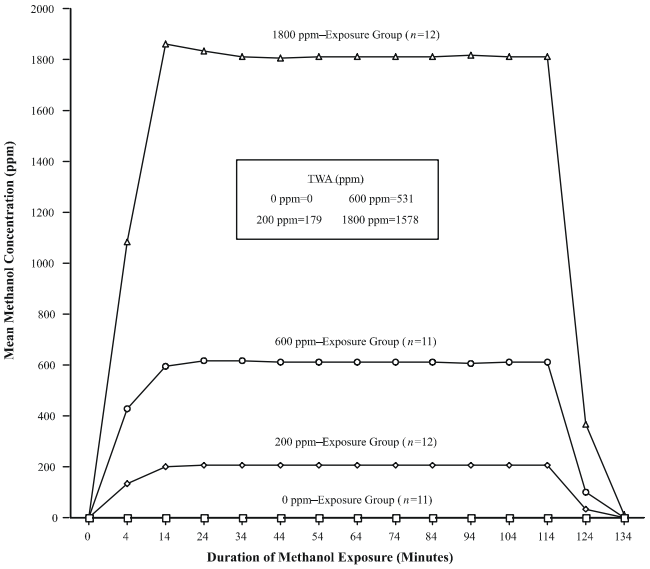

Figure 5. Results of 10-minute sampling of methanol concentration in the inhalation chambers for 2.5-hour exposure period. Symbols on graph represent average methanol concentration at specified time over all exposures for all females in the exposure group. TWA indicates time-weighted averages for methanol for the 2.5-hour period (methanol flow began at 0 minutes and stopped at 120 minutes).

chamber concentrations obtained for the 11 samples taken from 14 minutes after onset of methanol flow until 6 minutes prior to offset of methanol flow (total, 100 minutes) were all within 5% of the target concentrations. Average concentrations obtained 4 minutes following offset of methanol flow indicated a decline in chamber methanol concentrations of over 80%.

To examine the accuracy of each day's exposure, the 11 methanol samples taken from 14 minutes after onset of methanol flow until 6 minutes prior to offset of methanol flow were averaged for each female. The daily averages for chamber temperature and relative humidity were obtained from all 14 samples taken over the entire 2.5-hour exposure period. The results of the summaries for the daily methanol exposures are shown in Tables 2 through 5 and Figure 6. The chamber monitoring equipment was not available during the baseline period for females in Cohort 1; thus no data are available for comparisons. During the baseline period, the chamber methanol concentrations for the females in Cohort 2 were nearly always 0. The chamber temperatures were between 21°C and 27°C, and the mean temperatures across the exposure groups varied by less than 1°C. The relative humidity of the chambers varied greatly during this same period with values from 7% to 54%. Mean chamber relative humidity across the exposure groups, however, varied by less than 6%.

The duration of methanol exposure prior to breeding was similar for all females, with an average duration of approximately 120 days for all 4 exposure groups. The duration of exposure to methanol during breeding varied greatly across females (range, 3 days to 8 months) due to differences in the number of breedings before conception occurred. However, the average duration of methanol exposure during breeding was comparable for all of the exposure groups, with a range of 59 to 71 days (Table 5).

For the prebreeding and breeding periods, the average daily chamber methanol concentrations were near the target values of 0, 200, 600, or 1,800 ppm. During these periods, the mean chamber temperatures remained between 22°C and 24°C. The relative humidity of the chambers continued to vary greatly; values from Cohort 1 were higher, on average, than those from Cohort 2. The mean chamber relative humidity across the exposure

Table 2. Methanol Concentration in Inhalation Chambers During Baseline and Exposure Periods[a]

		Exposure Period		
Exposure Group	Baseline[b]	Prebreeding	Breeding	Pregnancy[c]
Control (n = 11)	0 ± 0 (0–2)	0 ± 0 (0–9)	0 ± 0 (0–0)	0 ± 0 (0–7)
200 ppm (n = 12)	0 ± 0 (0–0)	205 ± 0 (134–234)	208 ± 0 (172–232)	206 ± 0 (167–251)
600 ppm (n = 11)	0 ± 0 (0–4)	613 ± 1 (510–706)	610 ± 1 (540–706)	610 ± 1 (553–674)
1,800 ppm (n = 12)	0 ± 0 (0–0)	1,817 ± 1 (864–2,097)	1,824 ± 1 (1,572–1,957)	1,822 ± 1 (1,652–1,988)

[a] Values are presented as means ± SE in ppm with range in parentheses on line below.

[b] Cohort 2 monkeys only: n = 5 for control and 600 ppm–exposure groups; n = 6 for 200 ppm– and 1,800 ppm–exposure groups.

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

groups, however, continued to show little variation during these periods (see Tables 3 through 5).

For those females that conceived, the average daily chamber methanol concentrations during pregnancy were stable and within 10% of the target concentrations (see Figure 6 and Appendix B, available on request, Figure B.2). During pregnancy, the mean chamber temperatures and relative humidity were consistent with those observed during the breeding period.

In addition to the above, the TWA of the chamber methanol concentrations was calculated for each daily exposure. The average TWAs were very stable throughout the study for all of the females in both cohorts.

TOXICOKINETIC STUDIES

Time courses of mean blood methanol concentration data obtained from the four toxicokinetic studies (that is, Studies 1 through 4) are presented in Figure 7 (see Appendix B, available on request, Figure B.3 through Figure B.6 for scatterplots). Mean blood methanol concentrations in the control group remained reasonably stable over time within each toxicokinetic study and did not appear to differ among studies. Across the four studies in the 200 ppm–exposure group, mean blood methanol concentrations barely rose above pre-exposure concentrations (roughly two-fold, range of 4.3 to 5.5 μg/mL) at the first sampling time of 30 minutes following exposure, and they declined to near control con-

Table 3. Temperature in Inhalation Chambers During Baseline and Exposure Periods [a]

		Exposure Period		
Exposure Group	Baseline[b]	Prebreeding	Breeding	Pregnancy[c]
Control (n = 11)	23.2 ± 0.13 (21.6–25.0)	22.9 ± 0.13 (19.4–25.6)	23.0 ± 0.02 (20.2–27.4)	22.7 ± 0.01 (20.5–26.0)
200 ppm (n = 12)	23.9 ± 0.11 (22.2–26.5)	23.6 ± 0.02 (18.5–26.5)	23.8 ± 0.02 (20.4–26.9)	23.6 ± 0.02 (21.3–26.3)
600 ppm (n = 11)	23.8 ± 0.03 (22.0–26.0)	23.2 ± 0.02 (19.1–25.5)	23.4 ± 0.02 (21.0–26.4)	23.3 ± 0.01 (20.8–27.5)
1,800 ppm (n = 12)	23.7 ± 0.19 (21.7–26.3)	23.5 ± 0.02 (20.0–26.0)	23.8 ± 0.02 (21.2–26.7)	23.5 ± 0.02 (21.9–26.6)

[a] Values are presented as means ± SE in °C with range in parentheses on line below.

[b] Cohort 2 monkeys only: n = 5 for control and 600 ppm–exposure groups; n = 6 for 200 ppm– and 1,800 ppm–exposure groups.

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

Table 4. Relative Humidity in Inhalation Chambers During Baseline and Exposure Periods[a]

		Exposure Period		
Exposure Group	Baseline[b]	Prebreeding	Breeding	Pregnancy[c]
Control (n = 11)	33.7 ± 1.02 (7.5–53.8)	27.1 ± 0.26 (6.3–61.1)	34.0 ± 0.35 (7.5–53.8)	39.6 ± 0.26 (11.2–65.9)
200 ppm (n = 12)	32.5 ± 0.82. (10.1–48.3)	25.1 ± 0.23 (6.1–54.8)	34.1 ± 0.29 (7.5–53.8)	35.5 ± 0.27 (6.1–63.0)
600 ppm (n = 11)	29.1 ± 2.65 (7.6–49.4)	26.2 ± 0.26 (6.1–56.2)	35.2 ± 0.35 (7.5–53.8)	37.4 ± 0.24 (11.5–64.6)
1,800 ppm (n = 12)	30.6 ± 1.58 (10.1–49.4)	25.4 ± 0.23 (6.2–58.2)	32.7 ± 0.28 (7.5–53.8)	37.0 ± 0.25 (9.9–59.9)

[a] Values are presented as means ± SE in % with range in parentheses on line below.

[b] Cohort 2 monkeys only: n = 5 for control and 600 ppm–exposure groups; n = 6 for 200 ppm– and 1,800 ppm–exposure groups.

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

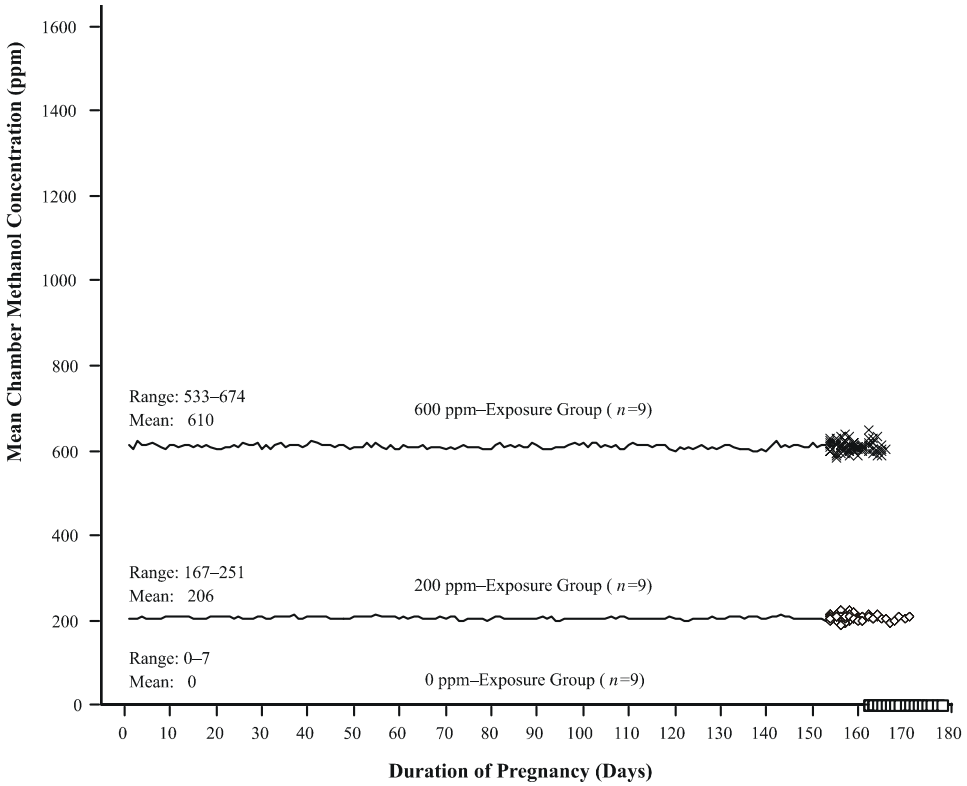

Figure 6. Methanol concentration (mean ± SE) in the inhalation chambers during pregnancy. Scatterplots shown when entire group is not represented due to delivery of offspring.

centrations by 120 minutes. In the 600 ppm–exposure group, mean blood methanol concentrations at 30 minutes after exposure ranged from 9.5 to 12.1 µg/mL across studies, which were three- to four-fold higher than pre-exposure concentrations or the corresponding levels in the control groups. Blood methanol concentrations declined to near control levels by 240 minutes. In the 1,800 ppm–exposure group, mean blood methanol at 30 minutes after exposure rose to concentrations that were about 13- to 16-fold higher than in the control group, that is, from 33.2 to 40.4 µg/mL across the four toxicokinetic studies. Mean blood methanol concentrations remained slightly (two times) above background or control concentrations at 360 minutes after exposure.

Table 5. Number of Days for Baseline and Exposure Periods[a]

		Exposure Period (days)		
Exposure Group	Baseline	Prebreeding	Breeding	Pregnancy[b]
Control (n = 11)	103 ± 4 (86–127)	123 ± 3 (104–138)	66 ± 25 (3–236)	167 ± 2 (162–178)
200 ppm (n = 12)	99 ± 3 (89–113)	121 ± 3 (108–146)	71 ± 21 (3–201)	160 ± 2 (152–171)
600 ppm (n = 11)	105 ± 3 (89–148)	118 ± 2 (107–130)	59 ± 19 (3–175)	161 ± 1 (153–166)
1,800 ppm (n = 12)	101 ± 2 (91–113)	120 ± 3 (105–141)	66 ± 21 (3–203)	163 ± 2 (150–178)

[a] Values are presented as means ± SE in days with range in parentheses on line below.

[b] n = 9 for control, 200 ppm–, and 600 ppm–exposure groups; n = 10 for 1,800 ppm–exposure group.

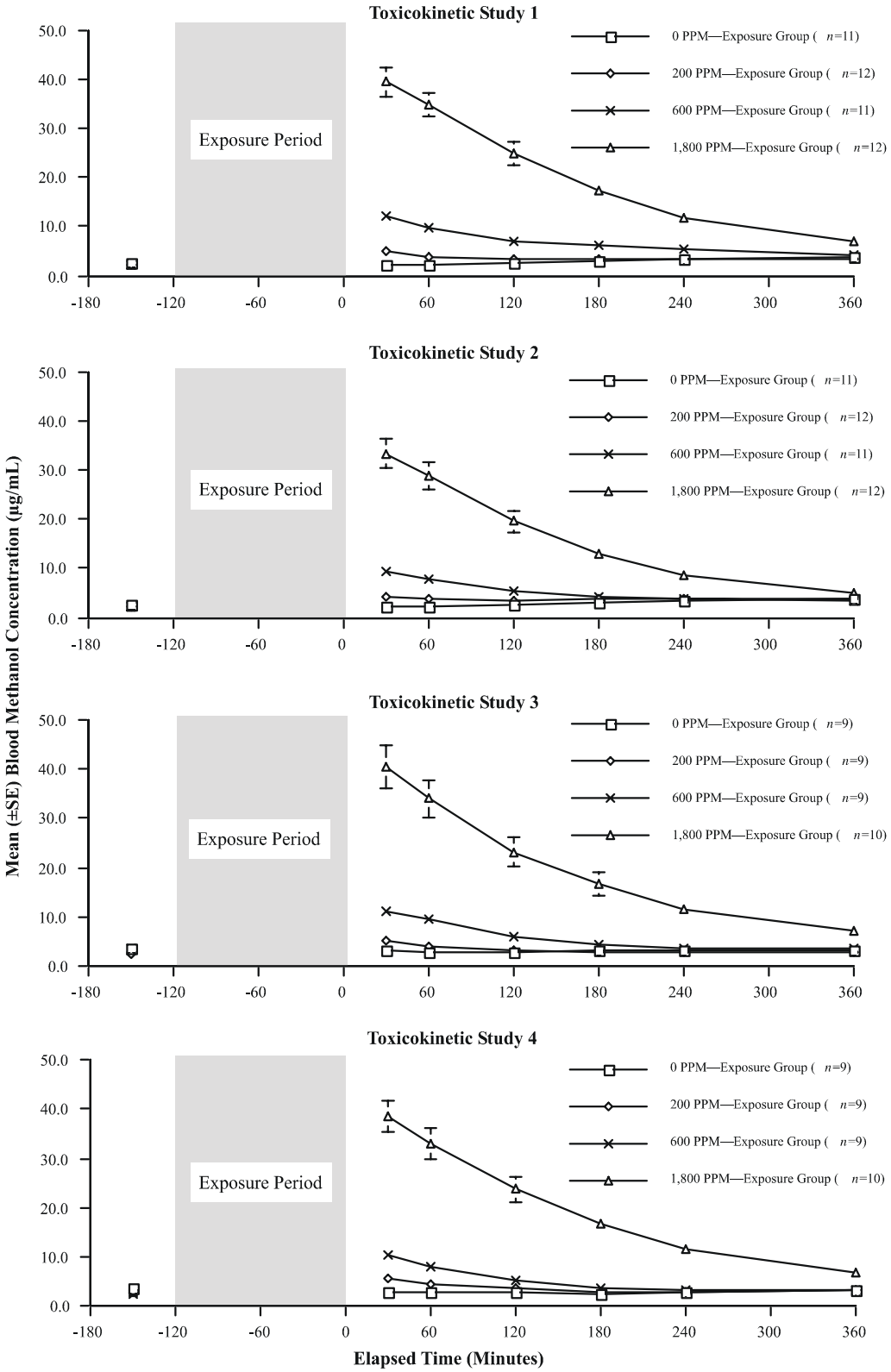

Figure 7. Blood methanol concentration data from toxicokinetic studies. Study 1 was performed after initial methanol exposure; Study 2 was performed after 87 days of methanol exposure; Studies 3 and 4 were performed during pregnancy. Samples were taken at 30 minutes prior to exposure and at 30, 60, 120, 180, 240, and 360 minutes after exposure.

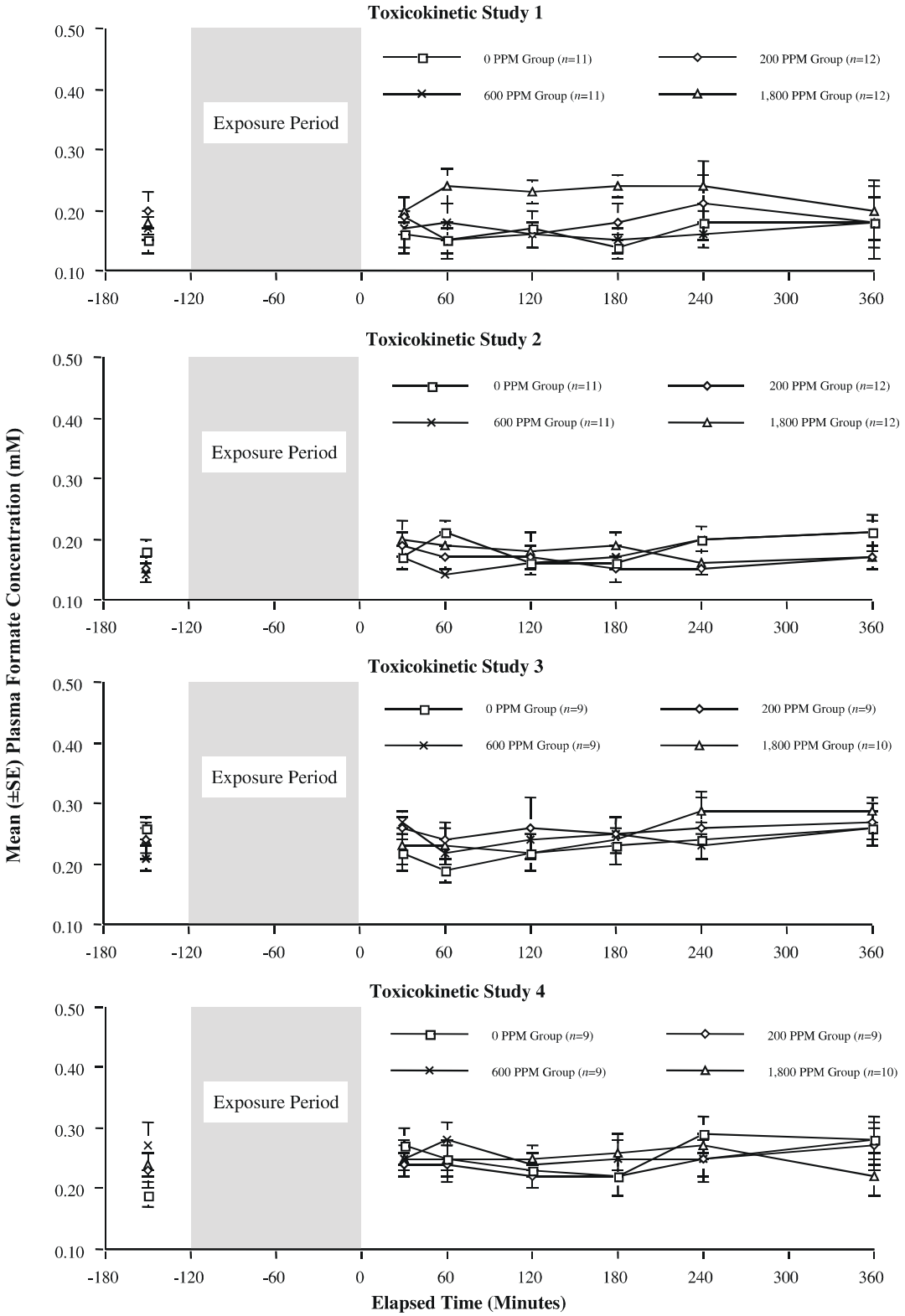

Figure 8. Plasma formate concentration data from toxicokinetic studies. Study 1 was performed after initial methanol exposure; Study 2 was performed after 87 days of methanol exposure; Studies 3 and 4 were performed during pregnancy. Samples were taken at 30 minutes prior to exposure and at 30, 60, 120, 180, 240, and 360 minutes after exposure.

Time courses of mean plasma-formate-concentration data from Toxicokinetic Studies 1 through 4 are shown in Figure 8 (see Appendix B, available on request, Figure B.7 through Figure B.10 for scatterplots). For all methanol-exposure groups, mean plasma formate concentrations did not show consistent rises following methanol exposure; the range of mean formate concentrations across time in the methanol-exposure groups was comparable with those observed in the control group (< 0.1 to 0.3 mM). Given the absence of a measurable elevation in plasma formate concentration following methanol exposure, we focused our toxicokinetic analysis on the blood methanol data.

The net rise in blood methanol concentration following exposure to methanol vapor (C_{net}) was estimated by subtracting the pre-exposure blood methanol concentration from each observed blood methanol concentration. A full kinetic analysis of blood methanol data from the 200 ppm–exposure group was not feasible because, for many females, there were not enough time points for which a net concentration could be meaningfully estimated. Therefore, only the data from the 600 ppm– and 1,800 ppm–exposure groups were used for toxicokinetic modeling.

Figure 9 shows semilogarithmic plots of the mean net blood methanol concentration data for the 600 ppm– and the 1,800 ppm–exposure groups for Toxicokinetic Studies 1 through 4. Although the mean net blood methanol concentrations appear to decline in a log linear fashion, the rate of decline for the 1,800 ppm–exposure group tended to be slower than for the 600 ppm–exposure group, especially for studies performed during chronic methanol exposure (Studies 2 through 4). Further inspection of individual data sets revealed subtle curvature in the semilogarithmic plots of net blood methanol concentration data in some females in the 1,800 ppm–exposure group (see Figure 10). In addition, at corresponding times during each of the toxicokinetic studies, the mean net blood methanol concentrations for the 1,800 ppm–exposure group were consistently more than threefold higher than those for the 600 ppm–exposure group. This disproportionate relation between net blood methanol concentration and exposure dose is illustrated by comparing time plots of mean net blood methanol concentration data between the 600 ppm– and 1,800 ppm–exposure groups that have been normalized by the exposure chamber methanol concentration (C_{net}/C_{ch}). As seen in Figure 11, the mean exposure, dose-normalized, net blood methanol concentrations are significantly higher in the 1,800 ppm–exposure group than in the 600 ppm–exposure group. These observations collectively point to the presence of nonlinear elimination kinetics in the 1,800 ppm–exposure group.

One-Compartment Model

Based on the observations previously noted, net blood methanol concentration–time data were fitted to a one-compartment model featuring either first-order or saturable (Michaelis-Menten) metabolic kinetics. The linear one-compartment model is described by the following rate equations for net blood methanol concentration (C_{net}) in micrograms per milliliter during and after the 2.5-hour methanol vapor exposure. Lung uptake of methanol was assumed to occur at a constant rate throughout the exposure period.

$$\text{During exposure,} \quad \frac{dC_{net}}{dt} = \frac{C_{ch} \times 1.31 \times R_v}{1000 \times (V/F)} - K \times C_{net} \qquad (1)$$

$$\text{After exposure,} \quad \frac{dC_{net}}{dt} = -K \times C_{net} \qquad (2)$$

C_{ch} is methanol concentration in the inhalation chamber in ppm, and R_v is the ventilatory rate in mL/minute. The constant 1.31 allows for conversion of chamber methanol concentration from ppm to mg/m^3. The ventilatory rate of each monkey in mL/min was estimated by the allometric equation of Adolph (1949), that is, $R_v = 2 \times B^{0.74}$, where B is body weight in grams. The apparent distribution volume (V/F) of methanol in liters is a hybrid parameter of the true distribution volume (V) and fractional absorption of methanol vapor via the lungs (F). Both the ventilatory rate and fractional absorption were assumed to be constant across the range of exposure concentrations. The first-order elimination rate constant (K) is expressed in units of $minutes^{-1}$. The corresponding elimination half-life is computed by ln 2/K. Apparent blood clearance (adjusted for lung-uptake fraction), that is, Cl/F, was computed from the product of K and V/F. Estimates of both the apparent blood clearance and apparent distribution volume were normalized by kilogram body weight. In subsequent text, the terms blood clearance and distribution volume refer to the respective apparent estimates (that is, the terms are not corrected for the fraction of methanol dose absorbed via the lungs).

For the Michaelis-Menten model, the one-compartment model equations were modified to reflect saturation in methanol metabolism as follows:

$$\text{During exposure,} \quad \frac{dC_{net}}{dt} = \frac{C_{ch} \times 1.31 \times R_v}{1000 \times (V/F)} - \frac{\frac{V_{max}}{V} \times C_{net}}{K_m + C_{net}} \qquad (3)$$

$$\text{After exposure,} \quad \frac{dC_{net}}{dt} = -\frac{\frac{V_{max}}{V} \times C_{net}}{K_m + C_{net}} \qquad (4)$$

V_{max} is defined as the maximum rate of methanol metabolism in units of µg/min, and K_m is the Michaelis constant

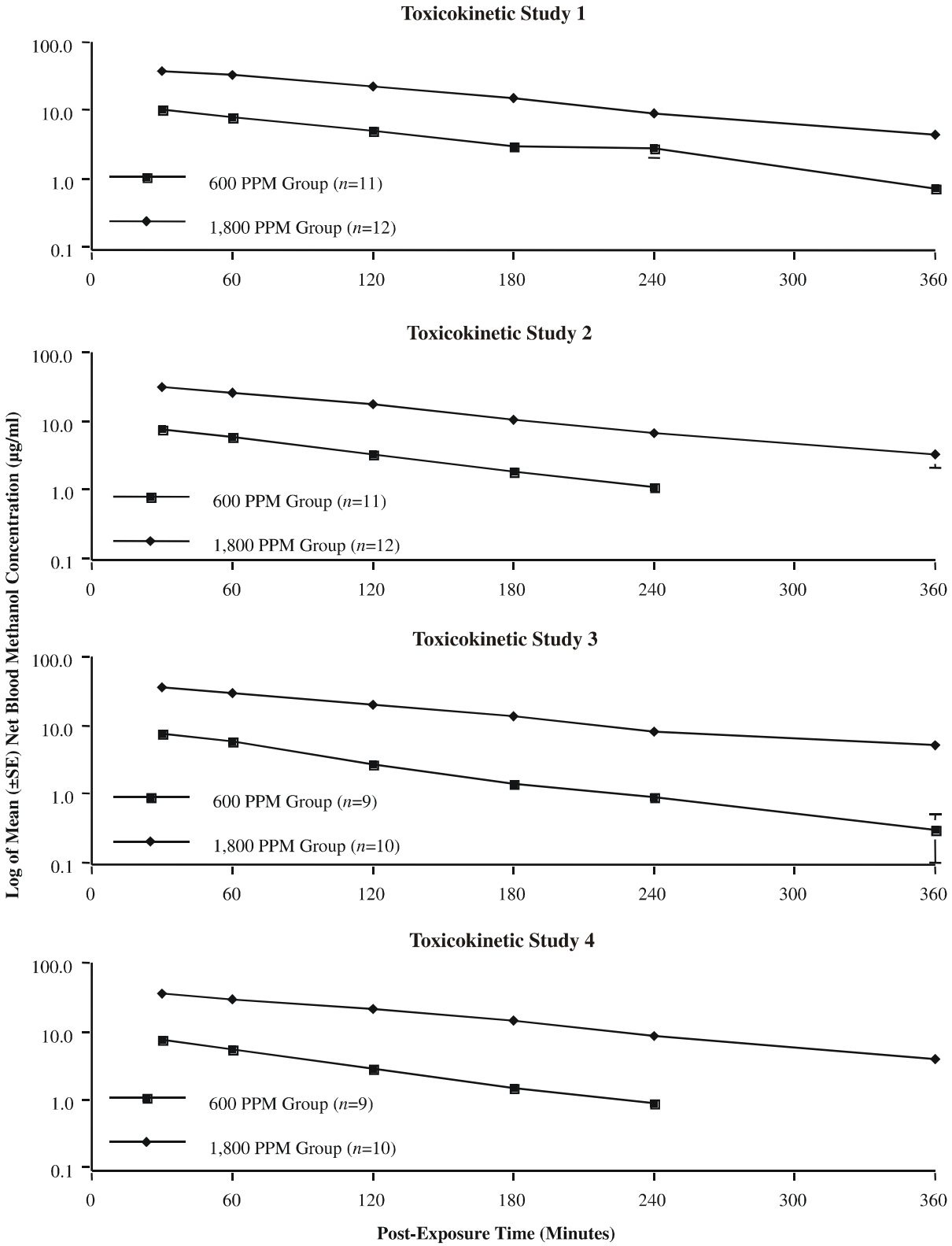

Figure 9. Net blood methanol concentrations (mean ± SE) from toxicokinetic studies for 600 ppm– and 1,800 ppm–methanol exposure groups. Study 1 was performed after initial methanol exposure; Study 2 was performed after 87 days of methanol exposure; Studies 3 and 4 were performed during pregnancy.

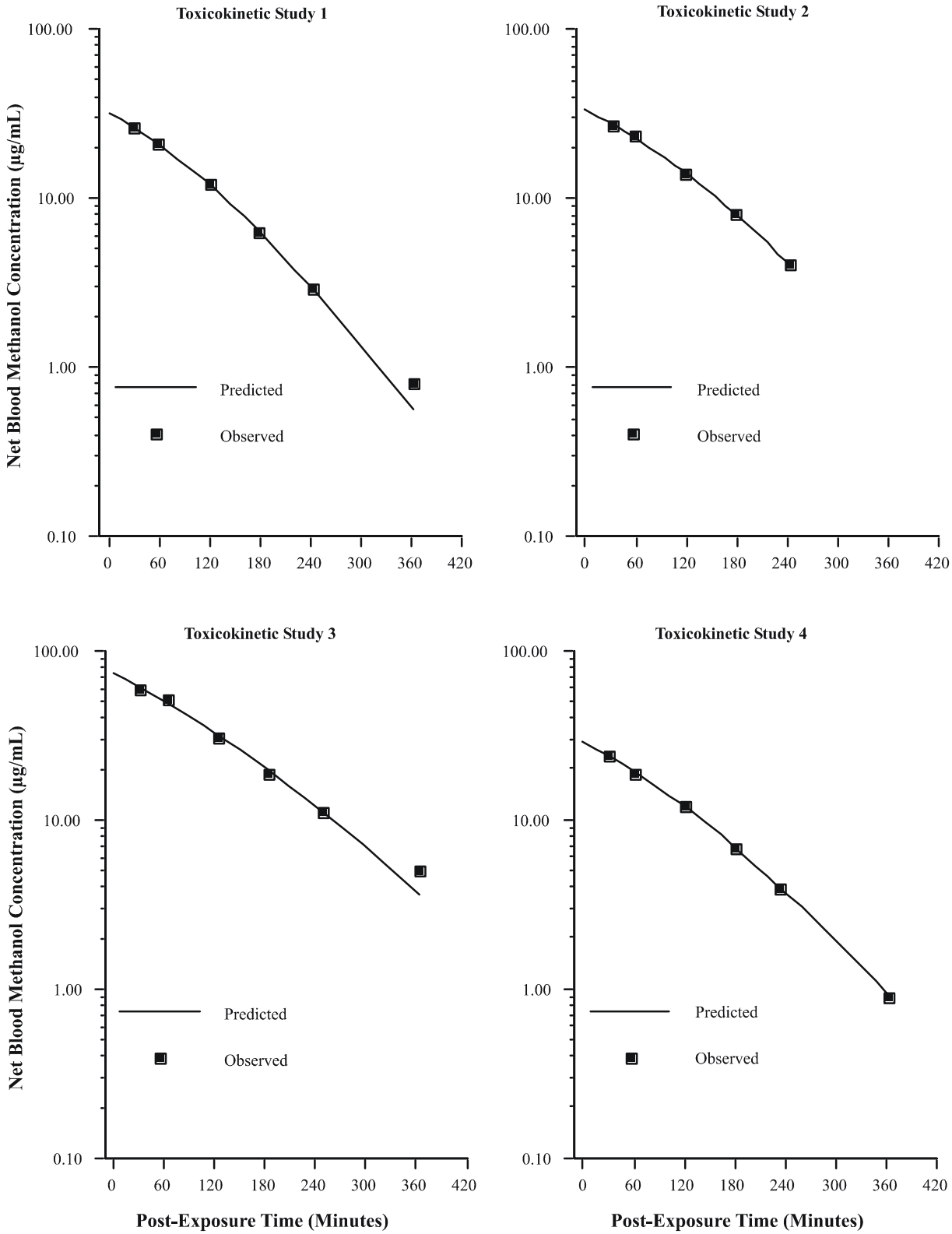

Figure 10. Example of comparison of Michaelis-Menten model predictions to observed net blood methanol concentration data after 1,800 ppm–methanol exposure in monkey #A94040 across toxicokinetic studies. Study 1 was performed after initial methanol exposure; Study 2 was performed after 87 days of methanol exposure; Studies 3 and 4 were performed during pregnancy.

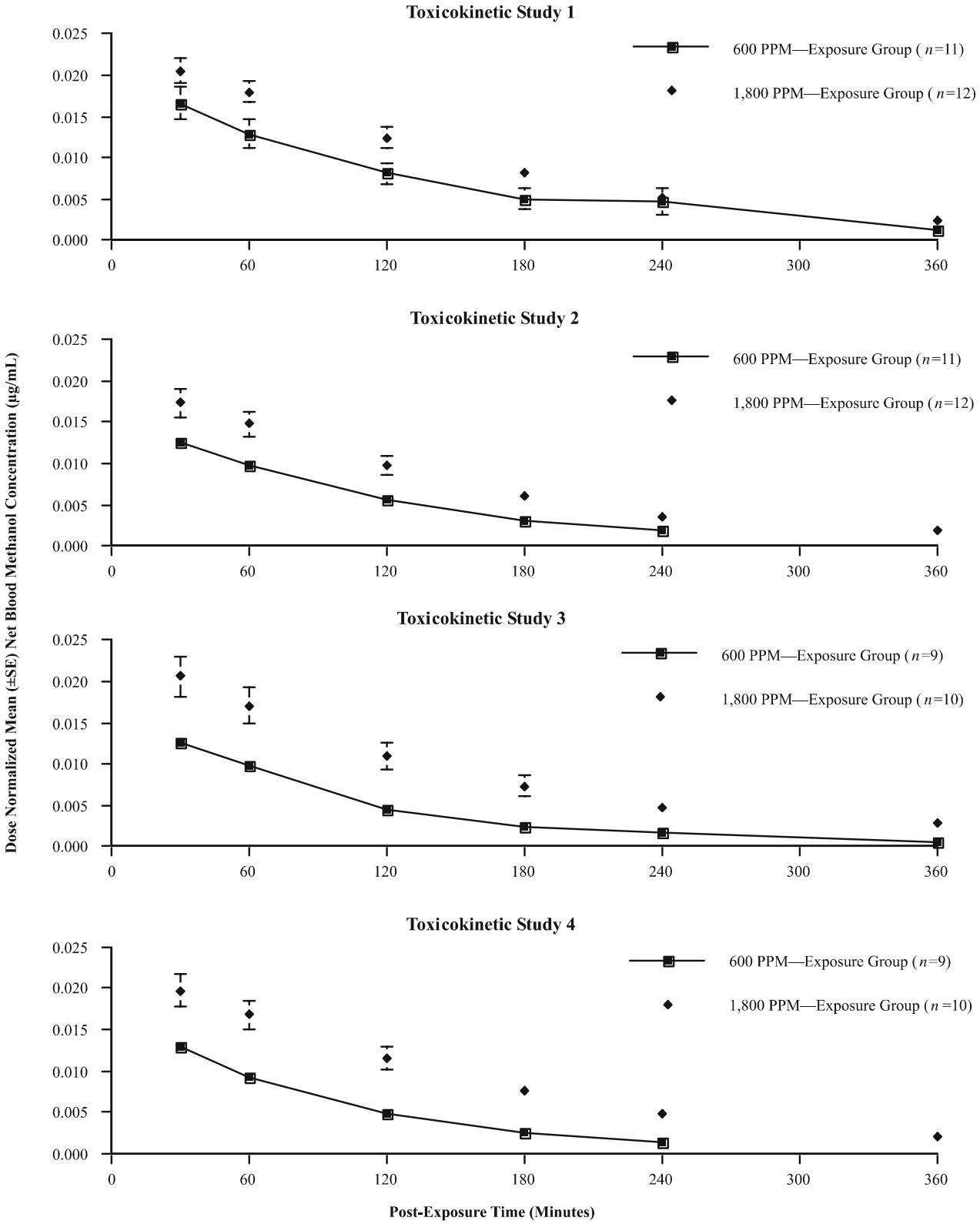

Figure 11. Dose-normalized net blood methanol concentrations (mean ± SE) from toxicokinetic studies for 600 ppm– and 1,800 ppm–methanol exposure groups. Study 1 was performed after initial methanol exposure; Study 2 was performed after 87 days of methanol exposure; Studies 3 and 4 were performed during pregnancy.

in units of μg/mL. Since the fraction of methanol dose absorbed is not known, the apparent maximum rate of metabolism (that is, V_{max}/F) is estimated. It should be noted that the ratio of apparent V_{max}/K_m is the apparent first-order clearance (that is, Cl/F in the linear model) when the blood methanol concentration falls below K_m. In this Michaelis-Menten model (as well as in the equivalent linear model), we assumed the existence of a single saturable metabolic pathway or several parallel metabolic pathways with a similar K_m; in other words, there is no parallel, nonmetabolic pathway of elimination for methanol in humans. The apparent first-order elimination rate constant (that is, K in the linear model) is computed by dividing Cl/F by V/F. The corresponding terminal or first-order elimination half-life is calculated by ln 2/K.

The postexposure blood methanol concentration–time data from each toxicokinetic study in individual monkeys were fitted with either the linear or the Michaelis-Menten model. In an effort to optimize model fit, different weighting schemes were used for the two models. A fractional standard deviation of 0.1 was used to compute weights for each datum in the linear model fits, whereas an absolute

Table 6. Estimated Half-Life of Blood Methanol from Toxicokinetic Studies[a]

Exposure Group	Study 1 Initial Exposure (Prebreeding)	Study 2 85 Days of Exposure (Breeding)	Study 3 70 Days Pregnant[b]	Study 4 129 Days Pregnant[b]
600 ppm (n = 11)	90.7 ± 8.0 (48.4–143.5)	72.2 ± 10.6 (29.4–141.2)	55.4 ± 4.3 (38.2–78.9)	59.8 ± 2.9 (44.8–70.0)
1,800 ppm (n = 12)	81.1 ± 10.7 (47.8–146.2)	75.7 ± 10.9 (39.9–158.6)	78.0 ± 7.8 (48.2–122.5)	84.8 ± 7.0 (56.9–117.5)

[a] Values are presented as means ± SE in minutes with range in parentheses on line below.

[b] n = 9 for 600 ppm–exposure group; n = 10 for 1,800 ppm–exposure group.

Table 7. Estimated Clearance of Blood Methanol from Toxicokinetic Studies[a]

Exposure Group	Study 1 Initial Exposure	Study 2 85 Days of Exposure	Study 3 70 Days Pregnant[b]	Study 4 129 Days Pregnant[b]
600 ppm (n = 11)	10.3 ± 1.2 (4.4–19.4)	13.5 ± 1.5 (8.7–24.0)	12.8 ± 1.2 (9.0–18.8)	11.9 ± 1.0 (8.5–16.9)
1,800 ppm (n = 12)	11.9 ± 1.5 (4.9–19.9)	15.5 ± 2.5 (4.0–37.3)	10.9 ± 1.7 (5.0–22.5)	10.9 ± 1.4 (6.5–18.9)

[a] Values are presented as means ± SE in mL/min/kg with range in parentheses on line below.

[b] n = 9 for 600 ppm–exposure group; n = 10 for 1,800 ppm–exposure group.

Table 8. Estimated Distribution Volume Methanol from Toxicokinetic Studies[a]

Exposure Group	Study 1 Initial Exposure	Study 2 85 Days of Exposure	Study 3 70 Days Pregnant[b]	Study 4 129 Days Pregnant[b]
600 ppm (n = 11)	125 ± 0.10 (0.64–1.68)	1.30 ± 0.15 (0.58–2.10)	1.00 ± 0.12 (0.64–1.85)	1.02 ± 0.08 (0.74–1.43)
1,800 ppm (n = 12)	1.19 ± 0.08 (0.8–1.60)	1.41 ± 0.15 (0.82–2.80)	1.10 ± 0.11 (0.61–1.57)	1.23 ± 0.08 (0.85–1.55)

[a] Values are presented as means ± SE in L/kg with range in parentheses on line below.

[b] n = 9 for 600 ppm–exposure group; n = 10 for 1,800 ppm–exposure group.

standard deviation of 0.1 was used in the Michaelis-Menten model fits.

The data from the 600 ppm–exposure group, which consistently showed log-linear decline, were well described by the linear model. The mean ± SE of the estimates for elimination half-life, blood clearance, and distribution for Toxicokinetic Studies 1 through 4 in the 600 ppm–exposure group are presented in Tables 6 through 8 (see Appendix B, available on request, Figures B.11 through B.13 for scatterplots).

For the 1,800 ppm–exposure group, better fits were observed for 32 of the 44 data sets with the Michaelis-Menten model than with the linear model (see Figure 10). The visual fit, standard error of the mean parameter estimates, and the Akaike's Information Criterion guided our model selection. Failure to converge was the principal cause of failure in fits with data from the remaining 12 studies. The nonconvergence problem was not consistently encountered across Studies 1 through 4 in individual monkeys. Visual inspection indicated that the problem was mainly due to the lack of apparent curvature in the semilogarithmic decline of blood methanol concentration over time for these 12 studies; that is, the data do not exhibit saturable metabolic kinetics. Therefore, data from these 12 studies were fitted with the linear model.

The parameter estimates obtained from the Michaelis-Menten model fits of the data from the 1,800 ppm–exposure group are presented in Tables 9 and 10. Summarizing over all 4 toxicokinetic studies, the K_m estimates ranged from 32.7 µg/mL to 107.7 µg/mL (mean ± SE = 63.0 ± 11.3 µg/mL). The apparent V_{max} estimates ranged from 1,502 µg/min to 4,672 µg/min (mean ± SE = 2,855 ± 403 µg/min). For the 1,800 ppm–exposure group, the observed blood methanol concentrations at the earliest sampling time of 30 minutes were generally around 30 to 40 µg/mL. Hence, peak blood methanol concentrations in the monkeys of the 1,800 ppm–exposure group were just below or within range of the apparent K_m of methanol. There was a large variation in K_m and V_{max} estimates between monkeys as well as within a monkey over multiple toxicokinetic studies. Moreover, there was no discernible trend in the variation of Michaelis-Menten estimates across the 4 studies within individual animals. The mean apparent volume of distribution ranged from 1.12 L/kg to 1.44 L/kg (mean ± SE = 1.25 ± 0.07 L/kg). Elimination half-life for the terminal first-order decline in blood methanol concentration ranged from 56.6 minutes to 77.6 minutes (mean ± SE = 64.9 ± 2.8 minutes). The first-order clearance estimate ranged from 11.7 mL/min/kg to 18.2 mL/min/kg (mean ± SE = 14.3 ± 1.1 mL/min/kg).

The limiting first-order elimination half-life and clearance estimates as well as the volume estimates from the Michaelis-Menten model fits are comparable with the linear model estimates for the 600 ppm–exposure group (see Tables 6 through 8). Hence, for the 1,800 ppm–exposure group, we pooled the estimates from the linear model fits (12 of 44 studies) with the corresponding first-order parameters from the Michaelis-Menten model fits (32 of the 44 studies). The pooled data are presented in Tables 6 through 8 (see Appendix B, available on request, Figures B.11 through B.13 for scatterplots). The following statistical comparisons of blood methanol kinetics in the first-order regime among exposure groups and among toxicokinetic studies were performed.

Hypothesis 1a: Dose Effects of Initial Methanol Exposure

The estimates for 3 parameters, elimination half-life, blood clearance, and distribution volume of methanol, obtained from Toxicokinetic Study 1 were analyzed by ANOVA. These initial analyses examined whether the 3 toxicokinetic parameters differed between the 600 ppm– and the 1,800 ppm–exposure groups after the first methanol exposure. The results from the ANOVA models (see Tables C.7 through C.9) did not indicate a significant dose effect for any of the 3 toxicokinetic parameters ($p > 0.32$). Outliers were detected through residual analysis for all 3

Table 9. Estimates of Methanol Toxicokinetic Parameters for the 1,800 ppm–Exposure Group[a]

Toxicokinetic Study	Distribution Volume (L/kg)	K_m (µg/mL)	V_{max} (µg/min)	Elimination Constant (min^{-1})	Half-Life (min)	Clearance (mL/min/kg)
1	1.17 ± 0.10	32.7 ± 6.1	1502 ± 214	0.0122 ± 0.0006	57.7 ± 2.6	14.4 ± 1.5
2	1.44 ± 0.18	38.5 ± 10.0	2084 ± 481	0.0126 ± 0.0008	56.6 ± 3.4	18.2 ± 2.7
3	1.12 ± 0.14	78.5 ± 15.1	3313 ± 668	0.0105 ± 0.0009	69.1 ± 6.5	12.1 ± 2.2
4	1.23 ± 0.09	107.7 ± 37.1	4672 ± 1158	0.0094 ± 0.0008	77.6 ± 6.4	11.7 ± 1.5
Overall	1.25 ± 0.07	63.0 ± 11.3	2855 ± 403	0.0112 ± 0.0004	64.9 ± 2.8	14.3 ± 1.1

[a] Derived from SAAM II fits to a one-compartment model with Michaelis-Menten elimination using SD = 0.1 for weighting. Values are presented as means ± SE.

Table 10. Estimates of Methanol Toxicokinetic Parameters for the 1,800 ppm–Exposure Group[a]

Animal Number	Distribution Volume (L/kg)	K_m (µg/mL)	V_{max} (µg/min)	Elimination Constant (min^{-1})	Half-Life (min)	Clearance (mL/min/kg)
Study 1						
90066	NC[b]	NC	NC	NC	NC	NC
90067	0.80	46.8	2556	0.0112	61.8	9.0
90083	0.91	47.1	1234	0.0115	60.3	10.5
90093	NC	NC	NC	NC	NC	NC
90118	0.99	21.8	710	0.0129	53.9	12.8
90122	NC	NC	NC	NC	NC	NC
T88189	1.60	28.9	1904	0.0121	57.1	19.4
T89226	1.03	25.5	912	0.0110	62.9	11.4
90095	1.32	13.7	1200	0.0142	48.8	18.7
94011	1.34	29.5	1651	0.0101	68.9	13.5
A94033	NC	NC	NC	NC	NC	NC
A94040	1.37	26.7	1851	0.0145	47.8	19.9
Study 2						
90066	NC	NC	NC	NC	NC	NC
90067	1.34	19.43	1914	0.0174	39.9	23.2
90083	1.17	34.88	1270	0.0125	55.3	14.7
90093	NC	NC	NC	NC	NC	NC
90118	2.80	7.10	693	0.0133	52.1	37.3
90122	NC	NC	NC	NC	NC	NC
T88189	1.54	112.0	5650	0.0094	73.8	14.4
T89226	1.11	41.3	1955	0.0119	58.4	13.1
90095	1.07	49.3	2617	0.0102	67.8	11.0
94011	1.03	24.9	1508	0.0144	48.2	14.8
A94033	1.62	27.0	1263	0.0116	59.7	18.8
A94040	1.33	30.8	1889	0.0127	54.4	16.9
Study 3						
90066	0.91	50.8	2290	0.0127	54.6	11.6
90067	NC	NC	NC	NC	NC	NC
90083	NC	NC	NC	NC	NC	NC
90093	0.83	87.1	1696	0.0068	101.2	5.7
90118	NC	NC	NC	NC	NC	NC
90122	1.04	151.5	6721	0.0093	74.3	9.7
T88189	NA[c]	NA	NA	NA	NA	NA
T89226	NA	NA	NA	NA	NA	NA
90095	1.36	61.9	4181	0.0092	75.3	12.5
94011	1.57	24.9	2009	0.0144	48.2	22.5
A94033	1.49	78.9	3832	0.0111	62.7	16.5
A94040	0.61	94.5	2461	0.0103	67.3	6.2
Study 4						
90066	1.02	338.5	10771	0.0065	106.0	6.7
90067	1.02	35.4	1975	0.0117	59.4	11.9
90083	NC	NC	NC	NC	NC	NC
90093	0.85	57.2	1691	0.0086	80.8	7.3
90118	1.43	127.9	5858	0.0084	82.5	12.0
90122	1.20	140.6	7474	0.0081	85.4	9.7
T88189	NA	NA	NA	NA	NA	NA
T89226	NA	NA	NA	NA	NA	NA
90095	1.55	15.5	1808	0.0122	56.9	18.9
94011	1.31	112.5	5131	0.0075	92.4	9.8
A94033	NC	NC	NC	NC	NC	NC
A94040	1.42	28.9	2671	0.0121	57.1	17.3

[a] Derived from SAAM II fits to a one-compartment model with Michaelis-Menten elimination using SD = 0.1 for weighting.

[b] NC = Nonconvergence in regression fit.

[c] NA = Not applicable.

toxicokinetic parameters. The results did not change after deleting the outliers and refitting the models. The mean elimination half-life, blood clearance, and distribution volume of methanol for the 600 ppm–exposure group were comparable with those of the 1,800 ppm–exposure group (90.7 ± 8.0 minutes vs 81.1 ± 10.7 minutes, 10.3 ± 1.2 mL/min/kg vs 11.9 ± 1.5 mL/min/kg, and 1.25 ± 0.10 L/kg vs 1.19 ± 0.08 L/kg) (Figure 12).

Hypothesis 1b: Effects of Repeated Methanol Exposures

ANOVA models were also fitted to parameter estimates for the toxicokinetic study following the initial methanol exposure and for the study following approximately 3 months of exposure (that is, Study 1 versus Study 2, see Tables 6 through 8). These analyses examined whether significant changes in the elimination half-life, blood clearance, and distribution volume of methanol occurred among the various exposure groups following repeated methanol exposures. The results from the ANOVA models (see Tables C.10 through C.12) indicated a significant change in elimination half-life ($p = 0.03$), a significant change in blood clearance of methanol ($p = 0.02$), and no change for the distribution volume of methanol ($p = 0.46$) following repeated methanol exposures (see Figure 12). These effects were not dose dependent; that is, there was no significant interaction effect between study and dose ($p > 0.18$). The mean elimination half-life of methanol decreased by 20% and 7% after 90 days of exposure to methanol vapor at levels of 600 ppm and 1,800 ppm, respectively. The percentage increase in mean blood clearance was the same for the two exposure groups (~30%) following daily exposure for 90 days.

Hypothesis 1c: Pregnancy Effects

For monkeys in the 600 ppm– and 1,800 ppm–exposure groups that conceived, repeated measures ANOVA models were used to compare the values of the 3 toxicokinetic parameters from studies conducted prior to conception with values from the studies conducted during early and late pregnancy (see Tables 6 through 8). There were 9 such monkeys in the 600 ppm–exposure group and 10 in the 1,800 ppm–exposure group. The ANOVA examined whether significant changes in elimination half-life, blood clearance, and distribution volume of methanol occurred due to pregnancy among the various groups and whether any pregnancy effect was dose dependent. The results of the ANOVA models (see Tables C.13 through C.15) indicated a significant change due to pregnancy in the mean distribution volume of methanol ($p = 0.01$); however, no changes in the mean elimination half-life and blood clearance of methanol were indicated ($p > 0.17$). These effects were not dose dependent (that is, no study-by-dose interaction was found). The respective mean distribution volume of methanol per kilogram body weight decreased by approximately 22% and 17% in the 600 ppm–exposure and 1,800 ppm–exposure groups during pregnancy (see Figure 13). The results also indicated a significant main effect of dose on the elimination half-life of methanol, reflecting a consistently shorter mean elimination half-life over all 3 studies for the 600 ppm–exposure group (that is, overall average of 63 minutes versus 79 minutes for the 600 ppm– and 1,800 ppm–exposure groups, respectively). Outliers were detected through residual analysis for all 3 toxicokinetic parameters. The results did not change after deleting the outliers and refitting the models.

In summary, postexposure blood methanol concentrations were elevated by more than threefold as exposure

Table 11. Results of Biweekly Monitoring of Blood Methanol Concentrations for Baseline and Exposure Periods[a]

		Exposure Period		
Exposure Group	Baseline	Prebreeding	Breeding	Pregnancy[b]
Control ($n = 11$)	2.3 ± 0.1 (0.9–8.7)	2.3 ± 0.1 (0.9–5.3)	2.3 ± 0.1 (0.7–5.7)	2.7 ± 0.1 (0.5–8.4)
200 ppm ($n = 12$)	2.2 ± 0.1 (1.0–11.0)	4.7 ± 0.1 (2.0–8.3)	4.8 ± 0.1 (1.9–9.4)	5.5 ± 0.2 (2.9–12.1)
600 ppm ($n = 11$)	2.4 ± 0.1 (0.9–10.2)	10.5 ± 0.3 (5.5–22.4)	10.9 ± 0.2 (6.4–15.5)	11.0 ± 0.2 (5.8–17.7)
1,800 ppm ($n = 12$)	2.4 ± 0.1 (0.9–5.2)	35.6 ± 1.0 (18.6–77.5)	35.7 ± 0.9 (23.1–54.9)	35.5 ± 0.9 (19.9–62.2)

[a] Values are presented as means ± SE in µg/mL with range in parentheses on line below.

[b] $n = 9$ for control, 200 ppm–, and 600 ppm–exposure groups; $n = 10$ for 1,800 ppm–exposure group.

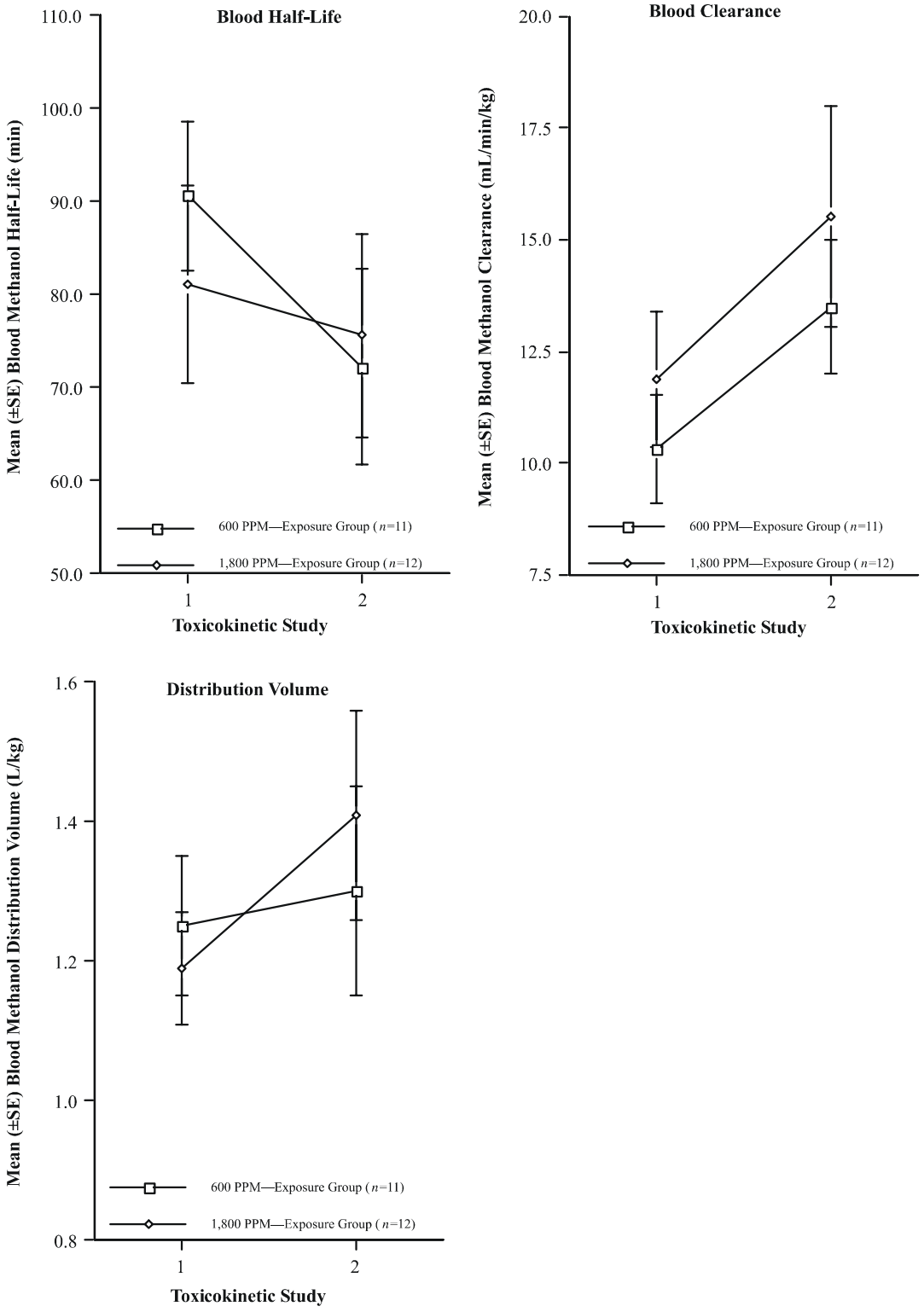

Figure 12. Estimated blood half-life, clearance, and distribution volume of methanol from toxicokinetic studies after initial exposure (Study 1) and after approximately 90 days of exposure (Study 2). After the initial methanol exposure (Study 1), half-life, clearance, and distribution volume were not significantly different for the 600 ppm versus the 1,800 ppm–exposure groups (ANOVA; $p > 0.32$, all tests). There was a statistically significant decrease in the blood half-life of methanol (ANOVA; $p = 0.03$) and a significant increase in clearance (ANOVA; $p = 0.02$) from Study 1 to Study 2. No significant change was observed for the distribution volume of methanol (ANOVA; $p = 0.46$).

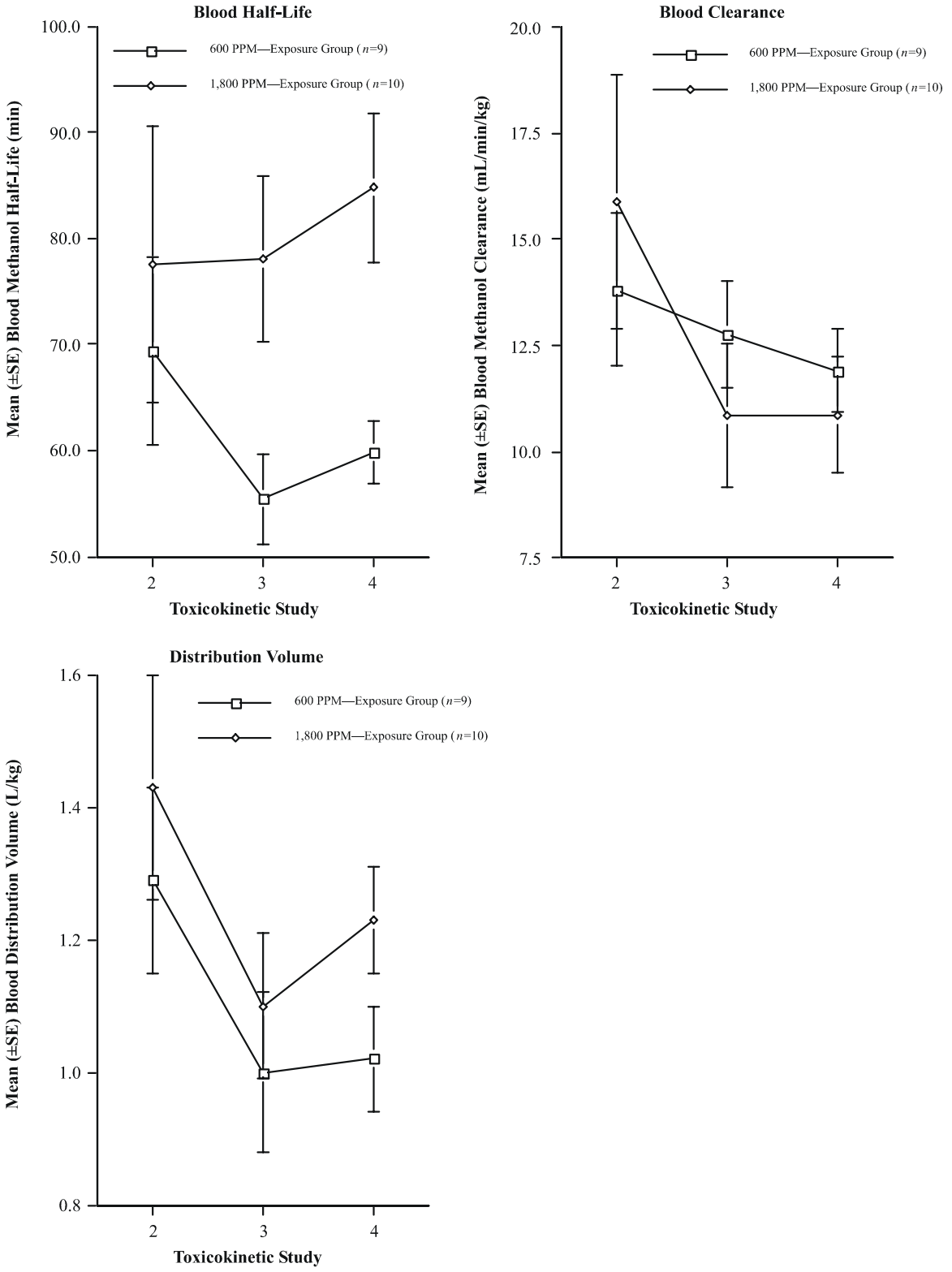

Figure 13. Estimated blood half-life, clearance, and distribution volume of methanol from toxicokinetic studies prior to pregnancy (Study 2) and during mid (Study 3) and late (Study 4) pregnancy. There was a significantly longer blood half-life for the 1800 ppm–exposure group when compared with the 600 ppm–exposure group across all studies (ANOVA; $p = 0.02$). There were no significant changes in half-life and clearance across the toxicokinetic studies (ANOVA; $p > 0.17$, both tests). Distribution volume decreased significantly across the toxicokinetic studies (ANOVA; $p = 0.01$).

level increased from 600 ppm to 1,800 ppm. Moreover, saturable metabolic kinetics were observed in the highest methanol exposure group, at 1,800 ppm. A significant decrease in the elimination half-life and blood clearance of methanol was observed in the *M. fascicularis* females following repeated daily exposures to methanol vapor. There was a significant decrease in distribution volume of during pregnancy, but no consistent changes in elimination half-life or clearance of methanol were observed. The mean elimination half-lives were consistently shorter in the 600 ppm–exposure group than in the 1,800 ppm–exposure group across the 2 toxicokinetic studies performed before pregnancy and those performed during mid and late stages of pregnancy.

MONITORING BLOOD METHANOL, PLASMA FORMATE, AND SERUM FOLATE

The results of the biweekly blood methanol concentration tests at 30 minutes post-exposure are summarized in Table 11 (see Appendix B, available on request, Figure B.14 for scatterplots). During the baseline period, the mean endogenous methanol concentrations in blood were at or below 3 µg/mL for all of the exposure groups, with individual values ranging from 0.9 µg/mL to as high as 11.0 µg/mL. During the prebreeding period, mean blood methanol concentrations of approximately 5 µg/mL were reached during methanol exposure in the 200 ppm–exposure group, with individual values ranging from 2.0 µg/mL to 8.3 µg/mL. For the 600 ppm–group, the mean postexposure blood methanol concentrations were approximately 10 µg/mL, with individual values ranging from 5.5 µg/mL to 22.4 µg/mL. The mean blood methanol concentrations were approximately 35 µg/mL for the 1,800 ppm–exposure group, with individual values ranging from 18.6 µg/mL to 77.5 µg/mL.

For the females that conceived, there was little change in blood methanol concentrations during pregnancy (see Figure 14). The mean blood methanol concentrations during pregnancy were approximately 3 µg/mL for the controls, 5 µg/mL for the 200 ppm–exposure group, 11 µg/mL for the 600 ppm–exposure group, and 35 µg/mL for the 1,800 ppm–exposure group.

Hypothesis 2: Methanol-Exposure Effects on Plasma Formate and Serum Folate Concentrations

Plasma formate concentrations remained low during the entire study (see Appendix B, available on request, Figure B.15 for scatterplots), with mean values ranging between 0.14 mM and 0.24 mM (see Table 12 and Figure 15). Initially, ANOVA models were used to compare plasma formate concentrations across the 4 methanol-exposure groups during the baseline period. Repeated measures ANOVA models were then used to compare formate concentrations during baseline with those obtained during the initial 3 months of methanol exposure (prebreeding period). Repeated measures ANOVA models were

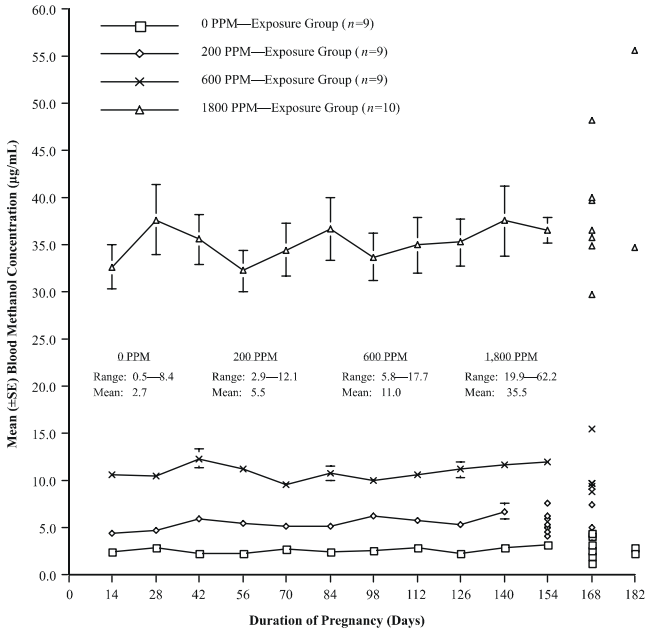

Figure 14. Blood methanol concentrations (mean ± SE) 30 minutes after exposure during pregnancy. Scatterplots shown when entire group is not represented due to delivery of offspring.

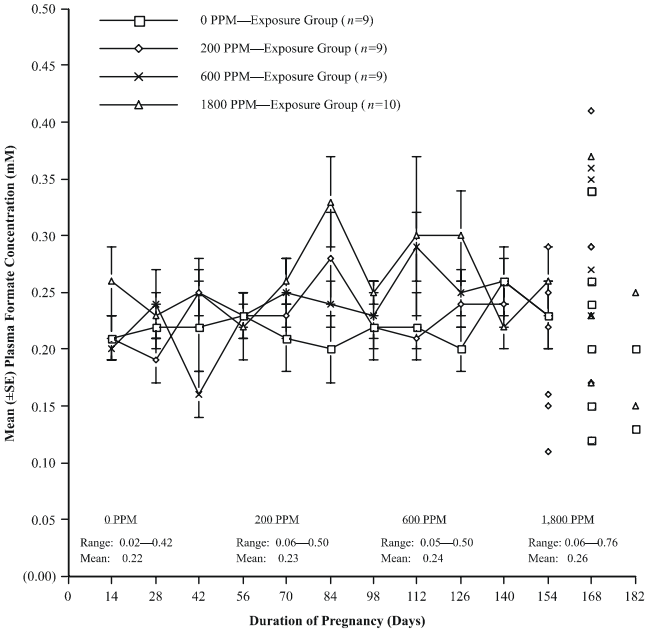

Figure 15. Plasma formate concentrations (mean ± SE) 30 minutes after exposure during pregnancy. Scatterplots shown when entire group is not represented due to delivery of offspring.

also used to compare formate concentrations obtained during the initial 3 months of methanol exposure with those obtained during pregnancy. The plasma formate concentrations obtained during breeding were not used in the analyses due to large differences in the duration of this period (from 3 days to 236 days). The number of formate concentrations obtained during this period ranged from 0 (for females that conceived after the initial breeding) to 16 (for females that required repeated timed-matings). The results of the ANOVA models (see Tables C.16 through C.18) did not indicate a significant difference in the plasma formate concentrations across the 4 methanol-exposure groups during the baseline period ($p = 0.60$). Significant changes in plasma formate concentrations were observed, however, when the baseline formate concentrations were compared with those obtained after approximately 3 months of methanol exposure ($p = 0.005$) and when formate concentrations obtained after 3 months of

Table 12. Results of Biweekly Monitoring of Plasma Formate Concentrations for Baseline and Exposure Periods[a]

		Exposure Period		
Exposure Group	Baseline	Prebreeding	Breeding	Pregnancy[b]
Control ($n = 11$)	0.18 ± 0.2 (0.01–0.64)	0.17 ± 0.01 (0.02–0.44)	0.22 ± 0.02 (0.05–0.54)	0.18 ± 0.03 (0.02–0.42)
200 ppm ($n = 12$)	0.16 ± 0.02 (0.00–0.51)	0.18 ± 0.01 (0.03–0.46)	0.21 ± 0.01 (0.06–0.36)	0.17 ± 0.01 (0.06–0.50)
600 ppm ($n = 11$)	0.15 ± 0.01 (0.00–0.69)	0.17 ± 0.01 (0.05–0.43)	0.20 ± 0.01 (0.10–0.35)	0.19 ± 0.03 (0.05–0.50)
1,800 ppm ($n = 12$)	0.14 ± 0.02 (0.00–0.79)	0.19 ± 0.01 (0.04–0.44)	0.24 ± 0.01 (0.05–0.51)	0.22 ± 0.03 (0.06–0.76)

[a] Values are presented as means ± SE in mM with range in parentheses on line below. No statistically significant differences were found in plasma formate concentrations across the four methanol-exposure groups during the baseline period (ANOVA; $p = 0.60$). Statistically significant changes were noted in plasma formate concentrations from baseline to prebreeding (ANOVA; $p = 0.005$) and from prebreeding to pregnancy (ANOVA; $p = 0.0001$). These changes were not dependent on dose.

[b] $n = 9$ for control, 200 ppm–, and 600 ppm–exposure groups; $n = 10$ for 1,800 ppm–exposure group.

Table 13. Serum Folate Concentrations for Baseline and Exposure Periods[a]

		Exposure Period			
		Prepregnancy[b]		Pregnancy[b,c]	
Exposure Group	Baseline	70 Days	98 Days	55 Days	113 Days
Control ($n = 11$)	14.4 ± 1.0 (11.0–20.0)	14.0 ± 1.2 (8.2–20.0)	13.4 ± 1.2 (7.3–20.0)	16.0 ± 1.1 (8.8–20.0)	15.6 ± 1.1 (9.4–20.0)
200 ppm ($n = 12$)	11.9 ± 1.3 (3.9–19.4)	13.2 ± 1.6 (5.8–20.0)	12.9 ± 1.3 (6.8–20.0)	15.5 ± 1.5 (8.3–20.0)	13.4 ± 1.3 (7.2–20.0)
600 ppm ($n = 11$)	12.5 ± 1.4 (5.8–20.0)	15.4 ± 1.2) (9.2–20.0)	13.4 ± 1.0 (9.6–20.0)	14.8 ± 1.1 (10.3–20.0)	16.4 ± 1.0 (9.8–20.0)
1,800 ppm ($n = 12$)	12.6 ± 0.7 (7.6–16.1)	14.8 ± 1.2 (8.9–20.0)	15.3 ± 1.1 (8.0–20.0)	15.9 ± 1.2 (8.4–20.0)	15.7 ± 1.0 (9.9–20.0)

[a] Values are presented as means ± SE in ng/mL with range in parentheses on line below. No statistically significant differences were found in plasma folate concentrations across the four methanol-exposure groups during the baseline period (ANOVA; $p = 0.47$). Statistically significant changes were noted in plasma folate concentrations from baseline to prebreeding (ANOVA; $p = 0.02$) and from prebreeding to pregnancy (ANOVA; $p = 0.007$). These changes were not dependent on dose.

[b] Number of days exposed to methanol.

[c] $n = 9$ for control and 600 ppm–exposure groups; $n = 10$ for 200 ppm– and 1,800 ppm–exposure groups.

methanol exposure were compared with those obtained during pregnancy ($p = 0.0001$). The changes observed following initial methanol exposure were nearly dose dependent (dose-by-period interaction, $p = 0.06$), reflecting a slightly greater rise in plasma formate concentrations for the methanol-exposed monkeys than for the controls. The changes observed during pregnancy were not dose dependent, reflecting a small overall increase in plasma formate concentrations for all exposure groups.

The results of the folate assays are summarized in Table 13. Serum folate concentrations were typically within the normal range of values for macaques. The mean serum folate concentrations during baseline and prepregnancy were 12 to 15 ng/mL for the 4 methanol-exposure groups, with individual values ranging from 5.5 to 20 ng/mL. For the females that conceived, mean folate concentrations during pregnancy remained between 13 and 17 ng/mL. ANOVA models similar to those described above were used to examine whether there were differences in serum folate concentrations prior to methanol exposure, after methanol exposure but prior to pregnancy, and during pregnancy. The results of the ANOVA models (see Tables C.19 through C.21) did not indicate a significant difference in the serum folate concentrations across the methanol-exposure groups during the baseline period ($p = 0.47$). Significant changes in serum folate concentrations were observed, however, when the baseline folate concentrations were compared with those obtained after methanol exposure but prior to pregnancy ($p = 0.02$) and when folate concentrations obtained prior to pregnancy were compared with those obtained during pregnancy ($p = 0.007$). The observed changes in serum folate concentrations were not dose dependent.

MATERNAL HEALTH ASSESSMENTS

Hypothesis 3: Methanol Exposure and Maternal Toxicity

Several indicators were utilized to detect overt maternal toxicity due to methanol exposure. These indicators included the following paragraphs.

Maternal Weight The weights of all females were quite stable during the study. The mean weight for each of the 4 methanol-exposure groups during the baseline period and through breeding was approximately 3.5 kg. The mean weight at conception for the females in the 4 exposure groups was between 3.2 kg and 3.7 kg. Mean weight gain during pregnancy varied from 1.3 kg to 1.8 kg across all exposure groups (see Table 14 and Appendix B, available on request, Figure B.16 for scatterplots). Weight gain during pregnancy was calculated for each female and used in ANOVA models to test for differences across the methanol-exposure groups. The results of the ANOVA models did not indicate a significant methanol-exposure effect on maternal weight gain during pregnancy ($p > 0.12$, all tests, see Table C.22). ANOVA models controlling for cohort differences, however, did indicate a significant methanol-exposure-group-by-cohort interaction for weight gain during pregnancy ($p = 0.05$). The results for offspring in Cohort 1 indicated a slight decrease in maternal weight gain for the methanol-exposed females compared with the weight gains of the controls, whereas the opposite pattern (a slight increase) was observed for the 600 ppm– and 1,800 ppm–methanol exposure groups in Cohort 2. One monkey (a female in the 600 ppm–exposure group) was detected through residual analysis as an outlier; she was deleted, and the models were refitted. The results did not change after removing her data.

Table 14. Maternal Weight Gain During Pregnancy and Duration of Pregnancy[a]

Exposure Group	Weight Gain[b] (kg)	Duration of Pregnancy[c] (days)
Control $n = 9$	1.67 ± 0.07 (1.33–2.05)	168 ± 2[d] (162–178)
200 ppm $n = 9$	1.27 ± 0.14 (0.51–1.76)	160 ± 2 (153–172)
600 ppm $n = 9$	1.78 ± 0.25 (1.09–3.45)	162 ± 2[d] (153–166)
1,800 ppm $n = 9$	1.54 ± 0.20 (0.52–2.31)	162 ± 2 (150–169)

[a] Values are presented as means ± SE with range in parentheses on line below.

[b] No statistically significant differences were found in maternal weight gain during pregnancy across the four methanol-exposure groups (ANOVA; $p < 0.12$, all tests).

[c] Pregnancy durations for the methanol-exposure groups were significantly shorter than that for the control group (ANOVA post hoc tests; $p = 0.04$, all tests).

[d] Live-born offspring only, $n = 8$.

General Observations Test Observations made during the daily General Observations Tests did not indicate the presence of overt toxicity in adult females from any of the 4 methanol-exposure groups. That is, lethargy, uncoordinated motor movements, and labored respiration were not observed during the study.

Clinical Observations Test The Clinical Observations Tests were performed in order to detect visual problems and fine-motor incoordination. Consistent with the findings of the General Observations Tests, the results of 50

(Cohort 2) to 100 (Cohort 1) clinical observations did not indicate the presence of overt toxicity in the adult females. During the entire study, only 6 females failed to respond to the visual task (3 control females and 3 methanol-exposed females). The 3 control females failed the task once, as did a 200 ppm–exposed female. One female in the 600 ppm–exposure group failed the visual task twice, once during the baseline period and once after approximately 3 months of methanol exposure. The remaining female, from the 1,800 ppm–exposure group, failed the visual task 8 times, twice during the baseline period, 4 times during the first 2 months of exposure, and twice after approximately 8 months of exposure. None of the above females exhibited a pattern of responses indicative of visual deficits due to methanol exposure. Of the 46 females observed, 23 failed the motor coordination task during the study. Of these 23 females, 15 failed it 3 times or fewer. Of the remaining 8 females, who failed the task 5 to 13 times during the study, 4 were from the control group, 1 was from the 200 ppm–exposure group, and 3 were from the 600 ppm–exposure group. All of these females failed the task during the baseline period as well as during methanol exposure, and none exhibited a pattern of responses indicative of fine-motor incoordination due to methanol exposure.

Health Check Procedures The number of females who became ill and required medication during the study was quite low. Only 1 female required intensive medication and intervention (long-term antibiotics, intravenous fluids) to treat severe diarrhea with dehydration. This female, part of the 200 ppm–exposure group, could not be transferred to the inhalation laboratory for 11 days during her illness and was not exposed to methanol until she had fully recovered. The remaining treatments were typically Pepto-Bismol® tablets for signs of diarrhea, which lasted for only a few days.

MATERNAL REPRODUCTIVE ASSESSMENTS

Hypothesis 4: Methanol Exposure and Reproductive Toxicity

Several indicators were also used to detect maternal reproductive toxicity due to methanol exposure. These indicators included the following:

Menstrual Cycles All females exhibited 3 menstrual cycles before methanol exposure, 1 during the period in which exposure was started, and 3 after exposure was started (see Table 15). One female exhibited an abnormal cycle length of 88 days prior to methanol exposure. This cycle was not included in the analysis. All females exhibited at least 4 normal cycles (> 20 days and < 50 days) prior to breeding.

In analyzing cycle lengths, two issues were addressed: first, whether, in addition to the natural differences among individuals, there were significant differences among the methanol-exposure groups during the baseline period (prior to methanol exposure); second, whether the cycle lengths were affected by exposure to methanol. To address the first issue, repeated measures ANOVA models were fitted for the 3 cycle lengths observed during the baseline period. The results of the ANOVA models (see Table C.23) did not indicate significant differences in the lengths of menstrual cycle of females across the 4 methanol exposure-groups during the baseline period ($p > 0.12$, all tests).

In order to test whether cycle lengths were affected by exposure to methanol, further repeated measures ANOVA models were fitted to the data regarding the 7 observed menstrual cycles. These models allowed for a test of

Table 15. Lengths of Menstrual Cycles for Baseline and Prebreeding Exposure Periods[a]

Exposure Group	Baseline[b]			Exposure[c]			
	Cycle 1	Cycle 2	Cycle 3	Cycle 1	Cycle 2	Cycle 3	Cycle 4
Control (n = 11)	31 ± 1	30 ± 1	30 ± 1	31 ± 1	30 ± 1	30 ± 1	30 ± 1
200 ppm (n = 12)	29 ± 1	29 ± 1	28 ± 1	29 ± 1	29 ± 1	30 ± 1	29 ± 1
600 ppm (n = 11)	29 ± 1	29 ± 1[d]	30 ± 1	30 ± 1	29 ± 1	30 ± 1	29 ± 1
1,800 ppm (n = 12)	31 ± 1	29 ± 1	29 ± 1	29 ± 0	29 ± 1	29 ± 1	31 ± 1

[a] Values are presented as means ± SE in days.

[b] No statistically significant differences were found in the length of the menstrual cycle across the four methanol-exposure groups during the baseline period (ANOVA; $p < 0.12$, all tests).

[c] No statistically significant differences were found in the length of the menstrual cycle due to methanol exposure (ANOVA; $p = 0.45$).

[d] n = 10 due to abnormal cycle length (88 days) for one cycle in one animal.

Table 16. Results of Timed-Mating Procedures[a,b]

Exposure Group	Conception Rate	Pregnancy Complication Rate	Live-Born Delivery Rate
Control	9/11 (82%)	2/9 (22%)	8/9 (89%)
200 ppm	9/12 (75%)	2/9 (22%)	9/9 (100%)
600 ppm	9/11 (82%)	3/9 (33%)	8/9 (89%)
1,800 ppm	10/12 (83%)	3/10 (30%)	9/10 (90%)

[a] No statistically significant differences were found in conception rates, complication rates or live-birth delivery rates (Fisher's exact test; $p = 1.0$, all tests).

[b] Conception Rate = number of conceptions/number of animals mated. Pregnancy Complication Rate = number of complications during pregnancy and delivery/number of animals pregnant. Live-Birth Delivery Rate = number of live-birth deliveries/number of animals pregnant.

whether there is a change in the line fitted to the cycle lengths from the time point at which exposure begins. The results of the ANOVA models did not indicate a significant methanol-exposure effect on cycle lengths in the females ($p = 0.45$, see Table C.24). The duration of menstrual cycle remained stable at approximately 30 days (see Table 15).

Timed Matings The results of the timed-mating procedures are summarized in Table 16. The frequency of conception was approximately the same across the 4 exposure groups (82% in the 0 ppm–exposure group, 75% in the 200 ppm–exposure group, 82% in the 600 ppm–exposure group, and 83% in the 1,800 ppm–exposure group). The conception frequencies were analyzed through contingency tables with use of Fisher's exact test. No significant differences across methanol-exposure groups were found ($p = 1.0$, see Table C.25).

Pregnancy Observations and Delivery Examinations

A total of 37 infants were delivered from the 46 females. Two females delivered stillborn infants, 1 in the control group and 1 in the 600 ppm–exposure group. One female in the 1,800 ppm–exposure group required a Cesarean section (C-section) to deliver a dead fetus. Five methanol-exposed females received C-sections to deliver live-born infants. The criteria established by the veterinary staff for C-section delivery included observations of vaginal bleeding with no signs of labor and unproductive labor for at least 3 consecutive nights. Four females, 2 in the 200 ppm–exposure group and 2 in the 600 ppm–exposure group, showed signs of bleeding during the day with no signs of labor. These females were evaluated by the veterinary staff, who decided that placental detachment had occurred and C-section delivery was required to save the fetus. In addition, 1 female in the 1,800 ppm–exposure group received a C-section after evaluation by the veterinary staff following 3 nights of long, unproductive labor. One female in the 1,800 ppm–exposure group delivered a 150 day–gestation infant that exhibited signs of prematurity (irregularities in respiration and temperature, feeding difficulties). The birth weight of this infant (260 grams) was also the lowest of all of the live-born offspring. One female in the control group delivered a 178 day–gestation infant that exhibited signs of postmaturity (meconium staining, hyperemia). The birth weight of this infant was normal.

The rate of complications during pregnancy or labor and delivery and the rate of live-birth deliveries (see Table 16) were also analyzed through contingency tables using Fisher's exact test. The rate of complications during pregnancy or labor and delivery were 22% for the control females and the 200 ppm females, 33% for the 600 ppm females, and 30% for the 1,800 ppm females. No significant differences across methanol-exposure groups were found ($p = 1.0$, see Table C.26). The live-birth delivery rate (numbers of live-birth deliveries/number of conceptions) were between 90% and 100% for all 4 exposure groups (see Table C.27).

ANOVA techniques were employed to examine whether or not methanol exposure affected the duration of pregnancy for the live-born deliveries (see Table 14 and Appendix B, available on request, Figure B.17 for scatterplots). Because only live-born deliveries were used, this analysis also provides a comparison of the durations of gestation for the offspring of the 4 methanol-exposure groups (see Table 17). A preliminary ANOVA was performed to test for possible effects on pregnancy length due to the sire, rather than the mother. The results of this analysis indicated that the null hypothesis, that distributions of pregnancy lengths from the 4 sires were homogeneous, could not be rejected (at $\alpha = 0.05$). Thus, sire-related effects were not considered further.

In order to test for differences in pregnancy length, an ANOVA model was fitted to the data (see Table C.28). The behavior of the residuals of the model was good, indicating that the underlying assumptions were reasonable. The results of the ANOVA model indicated a significant effect on pregnancy length due to methanol exposure ($p = 0.03$). Post hoc testing indicated that each of the 3 methanol-exposed groups had significantly shorter durations of pregnancy than did the control group ($p < 0.04$, all tests).

A summary of the birth characteristics of the 34 live-birth deliveries is shown in Table 17 (see Appendix B, available on request, Figure B.18 for scatterplots). ANOVA procedures were used to examine whether or not meth-

Table 17. Birth Characterisitcs of Live-Born Offspring[a]

Exposure Group	Gestation Length (days)	Infant Characteristics[b]				
		Birthweight (g)	Crown–Rump Length (mm)	Head Circumference (mm)	Head Length (mm)	Head Width (mm)
Control (n = 8)	168 ± 2 (168–178)	369 ± 14 (295–425)	180 ± 3 (167–192)	182 ± 1 (177–186)	63 ± 1 (60–64)	49 ± 0 (47–50)
200 ppm (n = 9)	160 ± 2 (153–172)	344 ± 14 (290–420)	175 ± 3 (165–188)	179 ± 1 (194–183)	62 ± 1 (59–65)	48 ± 1 (45–50)
600 ppm (n = 8)	162 ± 2 (153–166)	368 ± 25 (280–475)	176 ± 4 (165–196)	180 ± 3 (170–191)	62 ± 1 (58–67)	48 ± 1 (59–65)
1,800 ppm (n = 9)	162 ± 2 (150–169)	369 ± 21 (260–465)	177 ± 3 (158–189)	181 ± 2 (170–188)	63 ± 1 (59–65)	48 ± 1 (45–51)

[a] Values are presented as means ± SE with range in parentheses on line below.

[b] Gestation lengths for the methanol-exposed groups were significantly shorter than that for the control group (ANOVA post hoc tests; $p < 0.04$, all tests). No statistically significant differences were found in the measurements of birth size across the four methanol-exposure groups (ANOVA; $p < 0.24$, all tests).

anol exposure affected the size of newborn offspring (see Tables C.30 through C.34). These analyses included all of the 34 live-born infants because the infants delivered via C-section were not considered outliers. The offspring characteristics analyzed were birth weight, crown–rump length, head circumference, head length, and head width. The results of the ANOVA models did not indicate a significant effect of methanol exposure on the size of the offspring at birth ($p > 0.24$, all tests). ANOVA models controlling for cohort differences, however, did indicate a significant methanol-exposure-group-by-cohort interaction for birth weight ($p < 0.002$) and head circumference ($p < 0.03$). Similar to the results for maternal weight gain, results from offspring in Cohort 1 indicated a slight decrease in these parameters for the methanol-exposure groups compared with the controls, whereas the opposite pattern (a slight increase) was observed for these parameters for the 600 ppm– and 1,800 ppm–methanol exposure groups in Cohort 2.

DISCUSSION AND CONCLUSIONS

Adult female *M. fascicularis* were exposed to 0 ppm, 200 ppm, 600 ppm, or 1,800 ppm methanol vapor for approximately 2.5-hours/day, 7 days/week, prior to and during pregnancy. Two cohorts of subjects were studied, with each cohort consisting of 24 adult females, 2 adult males, and their offspring. Changes in the disposition kinetics of methanol due to repeated methanol exposure and due to pregnancy were evaluated. Observational and timed-mating procedures assessed the effects of methanol exposure on maternal health and reproductive function. Assessments of the physical and behavioral development of methanol-exposed and control offspring were used to evaluate the effects of in utero methanol exposure on offspring. The results of these assessments are presented in Part II of this Research Report.

METHANOL EXPOSURES AND BLOOD METHANOL CONCENTRATIONS

The protocol selected for exposing adult female *M. fascicularis* monkeys to methanol via inhalation succeeded in providing stable daily exposures over the entire study near the expected target concentrations of 0, 200, 600, and 1,800 ppm. Although the temperature and relative humidity inside the inhalation chambers fluctuated somewhat during the study, the fluctuations were observed in all of the chambers and did not vary with chamber methanol concentrations.

The daily blood methanol concentrations obtained following methanol exposure were quite consistent across the entire course of the study, including during pregnancy. Exposures produced peak blood methanol concentrations from a little less than twice background to over 10 times background. Differences in blood methanol concentrations within exposure groups did not seem to be dependent upon the age or weight of the females.

METHANOL DISPOSITION AND METABOLISM

The postexposure time courses of blood methanol and derived formate in the female adult *M. fascicularis* monkeys

were studied after the first exposure and after chronic exposures to methanol vapor, with the latter obtained both prior to breeding and twice during pregnancy. Very limited data are available in the literature on the toxicokinetics of methanol following low-level inhalation exposure. Investigators at the CIIT have recently reported two toxicokinetic studies in monkeys following a single controlled exposure to low-to-moderate levels of methanol vapor. The present study provides the first assessment of the effects of chronic, low-level inhalation exposure in combination with pregnancy on methanol disposition kinetics in nonhuman primates. In accordance with the study design, the following discussion is divided into three segments: single exposure, continuous exposure in nonpregnant monkeys, and continuous exposure in pregnant monkeys.

Single-Exposure Methanol Toxicokinetics

We initially compared our toxicokinetic data from the first methanol exposure with reported single-exposure data in rhesus and *M. fascicularis* monkeys. Horton and colleagues (1992) studied the washout kinetics of blood methanol in awake, adult male rhesus monkeys following a 6-hour exposure to 50, 200, 1,200, or 2,000 ppm of methanol vapor. Blood methanol concentrations did not show a measurable rise above endogenous (background) levels in the 50 ppm–exposure group. A mean (± SE) rise of 3.9 ± 1.0 µg/mL was observed at the end of a 6-hour exposure to 200 ppm methanol. This compares well to the mean estimated end-of-exposure concentrations in our female *M. fascicularis* subjects, that is, 3.7 ± 0.4 µg/mL. Horton and colleagues (1992) reported a mean blood methanol elimination half-life of 3.2 ± 1.2 hours and 2.9 ± 0.6 hours for their 1200 ppm– and 2,000 ppm–exposure groups, respectively. These mean half-lives are longer than the estimates obtained in our monkeys, which ranged from 81 minutes (1.4 hour) in the 600 ppm–exposure group to 91 minutes (1.5 hour) in the 1,800 ppm–exposure group (see Figure 12 and Table 6). At their highest exposure level of 2,000 ppm, Horton and colleagues (1992) observed a mean (± SE) end-of-exposure blood methanol concentration of 64.4 ± 10.7 µg/mL, which is consistent with model predictions of concentrations of 49.3 ± 3 µg/mL. Overall, this means that results for lung absorption and systemic distribution and clearance of methanol are comparable between the two studies.

The single-exposure toxicokinetic data from a more recent study by Dorman and colleagues (1994) in 4 adult female *M. fascicularis* appear to differ significantly from our study and from the earlier study by Horton and colleagues (1992). It is important to note that Dorman and colleagues (1994) exposed anesthetized monkeys to ^{14}C-methanol vapor via an inhalation system, and then they collected exhaled $^{14}CO_2$ over a 48-hour period in an effort to account for quantitative metabolism of methanol. A more rapid elimination of methanol was observed in these monkeys when the data were compared with the data collected by Horton and colleagues (1992) and data from the present study. At exposure levels ranging from 10 to 900 ppm, the mean half-lives of blood ^{14}C-methanol varied between 0.56 and 0.95 hr. More notably, Dorman and colleagues (1994) observed very low peak or end-of-exposure blood concentrations. The mean blood ^{14}C-methanol concentration at the end of a 2-hour exposure in the highest exposure group (900 ppm) was 106 ± 84 µM or 3.4 ± 2.7 µg/mL, which is only one third the concentration that we observed in our female *M. fascicularis* at a lower exposure level (600 ppm). The very low circulating methanol concentration could reflect low lung retention, more extensive extravascular distribution, or a higher clearance rate of methanol (or a combination thereof) in anesthetized monkeys compared with awake, freely moving animals.

A complicating feature of methanol disposition that is evident in our study was the presence of nonlinear elimination kinetics at the 1,800 ppm level of exposure. Nonlinear kinetics are immediately apparent when one examines the relation of blood methanol concentration to exposure level of methanol vapor. Blood methanol concentrations for the 1,800 ppm–exposure group increased more than proportionately when compared with concentration data for the lower methanol-exposure groups. This was evident in the data from all four toxicokinetic studies and from biweekly blood monitoring. The disproportionate blood concentration to exposure-level dose relation is well illustrated in Figure 11 by the distinct separation in the mean, dose-normalized, net blood methanol concentration–time profiles for the 600 ppm– and 1,800 ppm–exposure groups. Likewise, area under the curve (AUC) for net blood methanol concentration from end-of-exposure to infinity following a single exposure also increased more than proportionately with elevation in exposure level (data not shown).

A somewhat similar relation of dose dependence in blood concentration to acute exposure level was observed in the earlier study of Horton and colleagues (1992), except that a slight nonlinearity extended across all three exposure levels (200, 1,200, and 2,000 ppm) for both the mean end-of-exposure concentrations and AUCs. In contrast, Dorman and colleagues (1994) observed excellent linear correlations between end-of-exposure concentration or AUC and inhaled dose of ^{14}C-methanol, which

may be related to poor lung uptake and resulting low circulating concentrations of methanol (that is, peak concentrations < 10 μg/mL).

The nonlinear elimination kinetics at the 1,800 ppm level of exposure most likely reflected saturation of methanol metabolism, presumably by hepatic alcohol dehydrogenase. Saturable metabolism of methanol has been amply demonstrated by previous studies in monkeys and rodents at moderate-to-high doses of methanol (Tephly 1991; Pollack et al. 1993; Ward et al. 1995). The occurrence of saturable metabolic kinetics in our monkeys was evidenced by the characteristic convex semilogarithmic plot of blood methanol concentration–time course for the majority of monkeys in the 1,800 ppm–exposure groups (see Figure 10).

To account for nonlinear kinetics of methanol in the 1,800 ppm–exposure group, we conducted a separate toxicokinetic analysis of the 1,800 ppm–blood methanol concentration–time data using a Michaelis-Menten model to describe metabolic elimination of methanol. Perkins and colleagues (1995a) have recently summarized in vivo K_m and V_{max} estimates for nonhuman primates and human volunteers based on modeling of blood methanol data from the literature. The same investigative team has also reported in vivo K_m and V_{max} values for female mice and rats (Pollack et al. 1993; Ward et al. 1995; Pollack and Brouwer 1996). These reported values, along with the estimates from our two cohorts of female *M. fascicularis*, are compared in Table 18. The present estimates of K_m are significantly lower than the previous estimates (~1/10th) for monkeys based on the analysis of literature data by Perkins and colleagues (1995a), although the V_{max} estimates are reasonably similar across studies. The apparent discrepancy could be related to (1) the biased nature of our estimates because they were obtained from a subset of the 1,800 ppm–exposure group that had sufficiently low K_m values relative to their end-of-exposure blood methanol concentrations and (2) possible differences between toxic and nontoxic dose exposure. It should be noted that our present in vivo estimates of K_m are quite similar to the reported values from in vitro studies with monkey livers (~35 μg/mL) as compiled previously by Horton and colleagues (1992) in their development of a physiologically based toxicokinetic model for methanol. Finally, the apparent K_m and V_{max} are quite similar to the values found in rodents, even though they represent saturation of different enzyme systems (that is, alcohol dehydrogenase in monkeys versus catalase in rodents).

In the current research, the inconsistent appearance of nonlinear disposition kinetics in some, but not all, studies in individual monkeys within the 1,800 ppm–exposure group posed a problem for statistical comparison of kinetic parameters across the four toxicokinetic studies (that is, missing data). The problem was solved in part by merging the estimates of parameters representing limiting first-order kinetics (namely, distribution volume, elimination rate constant, and blood clearance) from the Michaelis-Menten model fits with the estimates from the linear model fits. Hence, our statistical analysis only examines the effects of dose and pregnancy on parameters pertaining to the linear range of methanol kinetics and does not address possible changes in methanol disposition kinetics at blood methanol concentrations approaching or exceeding K_m. For example, we are not able to resolve effects of pregnancy on the K_m and apparent V_{max} of methanol metabolism, even though analysis is performed on the ratio of apparent V_{max}/K_m, or the first-order clearance estimate. Nonetheless, the results are clearly applicable to environmentally relevant levels of exposure to methanol vapor.

Statistical analysis of the acute methanol-exposure data from the female *M. fascicularis* revealed no remarkable differences in the first-order elimination kinetics of blood methanol across the 600 ppm– and 1,800 ppm–exposure

Table 18. Comparison of Present In Vivo Michaelis-Menten Parameter Estimates for Female *M. fascicularis* to Estimates in Literature for Humans and Other Animals Following Acute Exposure to Methanol

Sex, Species, or Strain	Dose and Route of Administration	K_m (μg/mL)	V_{max} (μg/min)	Source
Human subjects	Intoxication via ingestion	320	1,550	Perkins et al. 1995
Cynomolgus monkeys	2 g/kg i.p.	716	2,200	Perkins et al. 1995
Cynomolgus monkeys	1 g/kg i.p.	278	800	Perkins et al. 1995
Rhesus monkeys	0.05–1.0 mg/kg i.p.	252	983	Perkins et al. 1995
Female, nonpregnant and pregnant *M. fascicularis*	1,800 ppm methanol vapor for 2 hours	32.7	1,502	Present study
Female Sprague-Dawley rats	100 or 2,500 mg/kg i.v.; and 100 or 2,500 mg/kg p.o.	18.0–48.7	387–1,053	Ward and Pollack 1995
Female CD-1 mice	2,500 mg/kg i.v.	48.7	1,955–2,233	Ward and Pollack 1995

groups. We surmise that the low-level toxicokinetic characteristics of methanol, specifically lung retention, distribution volume, and intrinsic metabolic clearance of methanol, do not differ between the two exposure groups. Moreover, the apparent nonlinear kinetics of methanol at the 1,800 ppm level of exposure can be fully explained by saturation in oxidative metabolism as opposed to a toxic effect from exposure to methanol vapor. Our conclusion of a consistent bioavailability of inhaled methanol at the two higher levels of exposure is interesting in view of recent reports from Perkins and colleagues (1995b, 1996a,b), which showed in rats that lung retention of methanol vapor is dependent upon the inhaled methanol concentration and that high blood concentrations of methanol can exert a centrally mediated suppressive effect on ventilation rate. Our data suggest that, at low-to-modest air concentrations of methanol vapor, methanol retention in the lungs of unanesthetized, unrestrained monkeys is independent of exposure concentration.

Some further comments on our estimates of methanol distribution volume and clearance rate are in order. As stated in the Methods section, the volume and clearance estimates were not corrected for the retained fraction of inhaled dose of methanol vapor (that is, inhalation bioavailability). Inhalation studies in human volunteers (Sedivec et al. 1981) and in rats (Pollack and Brouwer 1996) indicate that lung retention of methanol vapor is about 60 to 70%. No lung retention estimates are available for inhalation exposure in monkeys. If we assumed a 60% lung retention, the true distribution volume of methanol would be in the range of 0.6 L/kg for an average apparent volume of about 1.0 L/kg across all exposure groups. The corrected volume is close to the volume of body water, which is consistent with the hypothesis that methanol distributes freely in all body fluid spaces. The same consideration applies to the clearance estimates. The true systemic clearance would be in the range of 6 mL/min/kg, given that the average apparent clearance for the first exposure was in the range of 10 mL/min/kg (see Table 7 and Figure 12), and both are moderately low clearance values relative to the known renal and liver blood flows (> 20 mL/min/kg).

In Toxicokinetic Study 1, there was no evidence of an acute rise in plasma formate in response to an initial methanol exposure. Plasma concentrations of derived formate were measured in both of the earlier acute-exposure monkey studies from CIIT. Similar to our findings, Horton and colleagues (1992) were not able to see a measurable rise in plasma formate concentration with use of a relatively sensitive enzymatic assay. By using radiolabeled methanol, Dorman and colleagues (1994) were able to measure the very low concentrations of plasma formate derived from methanol. A peak blood ^{14}C-formate concentration of 2.8 ± 1.7 µM was observed in their highest exposure group (900 ppm) after a 2-hour exposure, a concentration that is around 1% of the normal level of endogenous formate (~0.1 to 0.2 mM).

Chronic-Exposure Methanol Toxicokinetics in Nonpregnant Monkeys

Toxicokinetic Study 2 was performed after approximately 3 months of daily exposure to methanol vapor. There was little or no accumulation of methanol as evident by the near basal concentrations of methanol in blood samples drawn before the regular 2-hour exposure session in all of our monkeys. This is reasonable given the short elimination half-life of methanol relative to the time interval between exposures (that is, 22 hours or more than 10 half-lives). Near complete washout of methanol occurred before the next exposure.

Statistical modeling of the first-order kinetic parameter from Toxicokinetic Studies 1 and 2 (that is, comparing acute exposure with chronic exposure) revealed significant variations in elimination half-life and clearance across the two studies, but not between the two exposure levels of 600 ppm and 1,800 ppm. Chronic exposure to methanol vapor did not alter the mean distribution volume of methanol. The mean apparent volume also did not differ between the 600 ppm– and 1,800 ppm–exposure groups. Because V/F is dependent on F, lung retention of methanol vapor must not have been affected by chronic exposure. Furthermore, the change in apparent blood clearance should then reflect a true acceleration in methanol metabolism following chronic exposure to methanol vapor. It should be noted that the apparent inductive effects of chronic exposure were rather modest (30% or less) compared to the interanimal (~300%) variations.

The biweekly blood monitoring data support our conclusion of an accelerated metabolic clearance of methanol following chronic exposure. The average, biweekly blood methanol concentration (sampled at 30 minutes postexposure) showed a small decline over the first month of repeated exposures. This was particularly evident at the highest exposure group (1,800 ppm). It would appear that the change in kinetics occurred quickly upon regular exposure of the monkeys to methanol vapor. The average, biweekly blood methanol concentrations remained quite constant for at least 3 months of exposure following this early change.

Our study is the first examination of the effects of chronic methanol exposure on its own disposition kinetics. As noted in the introduction, chronic alcohol

(ethanol) consumption or alcohol abuse in humans induces the microsomal ethanol-oxidizing enzyme (namely, cytochrome P450 2E1) in the liver. The effect of chronic alcohol consumption on alcohol dehydrogenase, the principal metabolizing enzyme of methanol in nonhuman primates, has not been elucidated. Both in vitro and in vivo studies in rodents have produced conflicting results. Some earlier studies have suggested induction of alcohol dehydrogenases (Dajani et al. 1963), whereas other studies have yielded negative results (Kesaniemi 1974; Traves and Lopez-Tejero 1994; Kishimoto et al. 1995) or even an inhibitory effect (Koivula and Lindros 1975). Our results in the female *M. fascicularis* may be the first evidence of an in vivo induction of alcohol dehydrogenases with a substrate other than ethanol.

In all exposure groups, including the control females, a small but statistically significant rise in the biweekly plasma formate concentration was observed. No significant elevation in plasma formate could be observed from the toxicokinetic studies. Therefore, the consistent rise in the biweekly plasma formate must represent a slight elevation in the circulating pool of endogenous formate. There is no evidence of a measurable contribution of methanol-derived formate to the endogenous pool of formate found during chronic methanol exposure.

Chronic-Exposure Methanol Toxicokinetics in Pregnant Monkeys

Pregnancy in the female *M. fascicularis* did not appear to have any effect on the metabolic clearance of methanol (expressed per kilogram body weight) (see Table 7 and Figure 13). There was a modest but statistically significant reduction in the apparent distribution volume of methanol per kilogram of body weight between the prebreeding and the two pregnancy studies (see Table 8 and Figure 13). As a result, there was a slight but statistically insignificant decrease in the elimination half-life, particularly in the females in the 600 ppm–exposure group (see Table 6 and Figure 13).

Given the complex changes in maternal physiology (for example, in body fluid spaces, cardiac output, and regional blood flow) and the presence of the fetal compartment, changes in the apparent distribution volume of methanol are not unexpected. However, the underlying physiologic mechanism(s) of the observed reduction in apparent volume is difficult to explain. Because this reduction occurred in the absence of a corresponding decrease in blood clearance, we can conclude that the change does not represent an increase in the pulmonary absorption of methanol. There does appear to be a shift in extravascular distribution of methanol during pregnancy in the monkey.

The pattern of toxicokinetic changes observed in our monkeys appears to differ from the recent data obtained by Ward and colleagues (1996a,b) in pregnant female Sprague-Dawley rats and in CD-1 mice. These investigators gathered an extensive set of blood concentration–time data after single intravenous or intragastric doses of methanol at early- and mid-stage pregnancy and near term. The blood concentration–time data were fitted with a two-compartment model featuring Michaelis-Menten elimination kinetics. A modest decrease in the in vivo V_{max} of methanol was observed at term in both rodent species. The decreased rate of liver metabolism in the near-term rodents was confirmed by in vitro measurements of the oxidation rate of methanol to formaldehyde. These investigators also reported gestation-related changes in the rat in the intercompartmental transfer rate constant and a trend toward an increase in the central and steady-state volumes for methanol. Therefore, the effects of pregnancy on the disposition of methanol appear to be species specific.

Finally, plasma formate concentrations from the biweekly monitoring did show a slight, statistically significant upward trend when the results following 30 days of exposure were compared with those obtained during pregnancy. Because there was a wide range in the duration of the breeding period (3 days to 236 days), plasma formate concentrations obtained immediately prior to conception were not used for many of the females. The mean values displayed in Table 12 indicate that the slight increase in plasma formate concentrations took place prior to pregnancy, that is, during the prebreeding period. Therefore, physiologic changes associated with pregnancy are not the likely cause of this small increase. However, because the increase was observed in all 4 exposure groups, including the controls, the additional generation of formate was most likely not due to methanol vapor exposure. This lack of significant contribution of methanol metabolism to plasma formate during chronic exposure is consistent with earlier conclusions reached by Dorman and colleagues (1994) in their study of acute methanol exposure in female *M. fascicularis*. In that study, folate deficiency stemming from poor dietary intake did not contribute to a significant elevation in formate burden at low environmental levels of methanol exposure.

The observed lack of a measurable addition to circulating formate from methanol exposure does not preclude the possibility of localized accumulation of formate in critical tissues such as the maternal retina and in the fetus. Tissue-specific accumulation of formate could occur as a result of metabolic generation of formate at those sites

and/or of intracellular sequestration of formate. For example, Eells and colleagues (1996) reported that the concentration of formate present in the vitreous humor and retina in methanol intoxication frequently exceed those measured in the blood. Also, Garner and colleagues (1995) provided evidence suggesting that intraretinal metabolism of methanol to formate rather than distribution of formate into vitreous humor may be responsible for the ocular toxicity. Very little information is available on fetal metabolic formation or maternal-fetal distribution of formate; only data in rodent species are available. Although metabolism of methanol does occur in the fetus, recent studies in pregnant rats and mice showed that the in vitro metabolic rate of methanol in fetal liver is very low, less than 5% of that in the maternal liver (Pollack and Brouwer 1996; Ward and Pollack 1996a). Also, formate in the maternal circulation can gain access to fetal tissues (Infurna and Berg 1982). However, the distribution kinetics of formate between fetal and maternal circulation during continuous, low-level exposure have not been investigated. Therefore, further studies are needed to evaluate fetal toxicokinetics of formate during chronic, low-level exposure of pregnant females to methanol vapor and the relevance of kinetics to possible effects on fetal development.

METHANOL EFFECTS ON MATERNAL HEALTH AND REPRODUCTION

Exposure to methanol at concentrations of up to 1,800 ppm for over 1 year did not produce overt signs of toxicity in adult female *M. fascicularis*. The observational procedures included in this study to assess maternal toxicity would not have detected subtle changes in visual function or behavior, and no postmortem examinations were performed on adult females to assess possible pathologic changes due to methanol exposure. Procedures did, however, evaluate whether motor incoordination, blindness, and/or respiratory effects were present. These are the typical signs of toxicity associated with acute, high-dose methanol exposure. Because no positive findings were found on these assessments, further studies did not seem warranted. In a previous study of subchronic inhalation exposure to methanol (6 hours/day, 5 days/week for 4 weeks) in nonhuman primates, no overt signs of toxicity were reported at exposure concentrations up to 5,000 ppm (Andrews et al. 1987). The only other study of chronic methanol exposure in nonhuman primates is that conducted by the New Energy Development Organization in Japan (1987). Unfortunately, the results from this study have not been reported in enough detail to make meaningful comparisons. It should be noted that prior to initiating the current study, a pilot study using 2,700 ppm methanol as our highest concentration was conducted. Reduced food intake was observed for females after the initial exposures (Burbacher, personal observations). Whether this was a transient or chronic effect was not evaluated because our goal was to examine concentrations well below those associated with even transient observed effects in adults. Thus, our highest concentration was reduced to 1,800 ppm.

Chronic methanol exposure did not interfere with the menstrual cycle or the ability of females to conceive. The timed-mating procedures used (3 matings/day between days 11 and 13 of the menstrual cycle) typically produce close to 100% conception rates in normal groups of *M. fascicularis* females (Mahoney 1975). The overall conception rate for this study was lower than expected, at 80%. This was due to a sire effect: 1 of the males in Cohort 2 successfully impregnated only 4 females. There are no reports of reproductive studies following chronic methanol exposure in nonhuman (or human) primates. Studies using rodent models have reported reproductive effects in males due to methanol exposure (Cameron et al. 1984, 1985). A significant decrease in circulating free testosterone was reported in rats after a single 6-hour exposure to 200 and 2,000 ppm, but not 10,000 ppm, methanol vapor, and a significant increase in circulating LH was reported with 10,000 ppm methanol exposure. A study by Cooper and colleagues (1992) failed to confirm an effect of 200 ppm methanol exposure on testosterone levels in rats. A single exposure to 0, 200, 5,000, and 10,000 ppm methanol for 6 hours did not reduce serum testosterone or FSH levels. Rodent studies exploring female reproductive effects following methanol exposure have concentrated on defining teratogenic effects in offspring following exposure during pregnancy. These reproductive and developmental studies typically do not report conception rates. Thus, comparative data for females are lacking.

Serum folate concentrations were obtained to examine whether possible methanol-exposure effects were associated with pregnancy-induced folate deficiency in these animals (Hall et al. 1976). Serum folate concentrations increased slightly over the course of the study for all of the methanol-exposure groups. Thus, there was no sign of decreased serum folate concentrations during pregnancy for either the methanol-exposed or control females. Although serum folate concentrations did not decrease, folate concentrations in red blood cells have been shown to more accurately reflect tissue folate stores (Scott et al. 1994). Whether changes in red blood cell folate concentrations occurred due to methanol exposure is not known.

Fetal and newborn mortality frequencies were low for all of the exposure groups. One female (in the 1,800 ppm–

exposure group) had to be C-sectioned to deliver a dead fetus, and two females (1 control and 1 in the 600 ppm–exposure group) vaginally delivered full-term stillborn infants. The autopsy on the fetus delivered by C-section indicated the presence of hydrocephalus with significant autolysis in all of the major organs. Autopsies on the 2 stillborn infants delivered vaginally indicated that the lungs were not inflated and that they had died close to or during delivery. No malformations were observed, and the cause of death for both infants was asphyxiation. In general, studies using rats have not reported increases in fetal mortality or malformations following exposure to methanol at concentrations up to 10,000 ppm (Nelson et al. 1985; Infurna and Weiss 1986; Stanton et al. 1995). Increases in malformations were reported, however, in full-term mouse fetuses at exposure concentrations of 2,000 ppm methanol and above (Rogers et al. 1993).

Four methanol-exposed females, 2 in the 200 ppm– and 2 in the 600 ppm–exposure group, were C-sectioned following observations of uterine bleeding without productive labor, presumably due to placental detachment. All 4 infants were delivered alive and without complications. In humans, ingestion of ethanol during pregnancy has been associated with an increased incidence of uterine bleeding and abruptio placentae (Kaminski et al. 1978; Conner 1984). In the study by Conner (1984), abruptio placentae was associated with alcohol intake of 14 or more drinks per week. However, given the small number of *M. fascicularis* females exhibiting this condition in the present study and the lack of a response at the highest exposure concentration, conclusions concerning methanol exposure as a causative factor in uterine bleeding are not warranted at this time.

Methanol exposures were associated with a reduction in the length of pregnancy, thus shortening the gestation period of the offspring. Exposures from 200 ppm to 1,800 ppm methanol vapor were associated with an average 6-to-8-day (approximately 5%) reduction in the length of pregnancy. One female in the 1,800 ppm–exposure group delivered an infant at 150 days gestation that exhibited signs of prematurity (irregular respiration, temperature instability, and feeding difficulties). Five of the methanol-exposed females delivered infants at or before 155 days gestation, whereas the shortest gestation length for the controls was 162 days. Previous reports have indicated that the normal average length of pregnancy for large-scale breeding colonies of *M. fascicularis* females ranges from 160 to 168 days (Fujiwara and Imamichi 1966; Valerio et al. 1969). Thus the averages for both the methanol-exposure groups and the control groups are within the norms expected for this species. Premature delivery has not been reported in the few studies of rats exposed to methanol during pregnancy (Infurna and Weiss 1986; Stanton et al. 1995; Weiss et al. 1996). Changes in the duration of pregnancy may be related to alterations in the onset of parturition. Previous studies have indicated that a sudden increase in cortisol secretion by the fetal adrenal glands may play a pivotal role in leading to parturition (for a review, see Thorburn 1991). The decrease in the duration of pregnancy, therefore, may be of fetal origin, possibly related to accelerated development of the fetal hypothalamus, pituitary gland, or adrenal cortices (Thorburn 1991; Thorburn et al. 1991). Further studies will be required to confirm the association between low-level methanol exposure and effects on pregnancy duration. These studies should focus on measuring possible changes in maternal and fetal endocrine parameters as a result of methanol exposure during pregnancy.

Although the average gestation period of the methanol-exposed offspring was significantly shorter than that of the controls, methanol exposure did not affect the size of the offspring at birth. The average birth weight, crown–rump length, and head size of infants in the methanol-exposure groups were comparable to those of the control infants. Reduced fetal weights have been reported in studies of rats and mice following maternal exposure to methanol at 10,000 ppm and above (Nelson et al. 1985; Rogers et al. 1993). Reduced size in human newborns following alcohol ingestion during pregnancy has been reported in numerous studies (Virji 1991; Sampson et al. 1994; Windham et al. 1995). The results of the present study do not indicate that reduced offspring size at birth is associated with methanol exposure concentrations insufficient to cause overt maternal toxicity.

SUMMARY

The results of the present study indicate that, for this nonhuman primate model, 2.5-hour daily exposures to methanol vapor at levels from 200 ppm to 1,800 ppm produce end-of-exposure blood concentration of methanol from 2 times to 10 times background endogenous levels. Although blood methanol concentrations increased as expected (threefold) as the exposure level rose from 200 ppm to 600 ppm, a more-than-proportionate increase in blood methanol concentrations was observed in the 1,800 ppm–exposure group. This increase was accompanied by the appearance of nonlinear elimination kinetics at the 1,800 ppm level of exposure, a finding that most likely reflects saturation of methanol metabolism (presumably by hepatic alcohol dehydrogenase). There was a modest but consistent increase in the mean blood clearance and

elimination half-life of methanol following 90 days of repeated methanol exposure, which may represent autoinduction of methanol metabolism. Minimal changes in methanol toxicokinetics were observed in pregnant females, with the only significant change a reduction in the distribution volume of methanol per kilogram of maternal body weight. Blood clearance of methanol did not appear to be affected by pregnancy.

Chronic methanol exposures for up to 1 year did not cause overt maternal toxicity in *M. fascicularis* females. The menstrual cycles and the ability of females to conceive and give birth to healthy live-born infants were also unaffected. Methanol exposures, however, were associated with a reduction in the length of pregnancy in this animal model, thus shortening the gestation length of the offspring. The reduction in the length of pregnancy was not dose dependent. Exposures from 200 ppm to 1,800 ppm methanol vapor were associated with an average 6-to-8-day (approximately 5%) reduction in the length of pregnancy. No concomitant decrease in offspring birth size was observed. Future studies focusing on maternal and fetal endocrine parameters are needed to confirm this finding.

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APPENDIX A. Results of University of Washington Quality Assurance and Quality Control Procedures

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APPENDIX B. Scatter Plots

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APPENDIX C. Statistical Analysis Tables

Table C.1. Maternal Age (years) at Assignment

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	1.97	3	0.66	0.21	0.89
Error	134.27	42	3.20		
Linear Contrasts[a]					
Controls (n = 11) vs. all exposed (n = 35)	0.47	1	0.47	0.15	0.70
Controls vs. 200 ppm (n = 12)	1.06	1	1.06	0.33	0.57
Controls vs. 600 ppm (n = 11)	0.01	1	0.01	0.004	0.95
Controls vs. 1,800 ppm (n = 12)	0.64	1	0.64	0.20	0.66

[a] The n values given in the left column are applicable throughout the tables in this appendix unless otherwise noted.

Table C.2. Maternal Weight (g) at Assignment

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	131800.13	3	43933.38	0.08	0.97
Error	23745930.30	42	565379.29		
Linear Contrasts					
Controls vs. all exposed	13623.58	1	13623.58	0.02	0.88
Controls vs. 200 ppm	627.27	1	627.27	0.001	0.97
Controls vs. 600 ppm	84072.73	1	84072.73	0.15	0.70
Controls vs. 1,800 ppm	356.26	1	356.26	0.0006	0.98

Table C.3. Maternal Crown–Rump Length (mm) at Assignment

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	410.91	3	136.97	0.22	0.89
Error	26688.24	42	635.43		
Linear Contrasts					
Controls vs. all exposed	140.71	1	140.71	0.22	0.64
Controls vs. 200 ppm	3.56	1	3.56	0.006	0.94
Controls vs. 600 ppm	328.41	1	328.41	0.52	0.48
Controls vs. 1,800 ppm	82.35	1	82.35	0.13	0.72

Table C.4. Maternal Head Circumference (mm) at Assignment (mm)

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	55.27	3	18.42	0.14	0.94
Error	5651.10	42	134.55		
Linear Contrasts					
Controls vs. all exposed	2.61	1	2.61	0.02	0.89
Controls vs. 200 ppm	5.40	1	5.40	0.04	0.84
Controls vs. 600 ppm	18.18	1	18.18	0.14	0.72
Controls vs. 1,800 ppm	7.12	1	7.12	0.05	0.82

Table C.5. Frequency of Maternal Gravidity at Assignment

	Maternal Gravidity[a]						
Exposure Group	0	1	2	3	4	6	Total
Control	2	1	2	0	0	1	6
200 ppm	1	2	2	1	0	0	6
600 ppm	1	2	2	0	1	0	6
1,800 ppm	1	2	2	0	1	0	6
Total	5	7	8	1	2	1	24

[a] The *p* value of the Fisher's exact test is 1, indicating that the distributions of maternal gravidity are not significantly different across the exposure groups.

Table C.6. Frequency of Maternal Parity at Assignment

	Maternal Parity[a]				
Exposure Group	0	1	2	4	Total
Control	2	1	2	1	6
200 ppm	1	2	3	0	6
600 ppm	1	2	2	1	6
1,800 ppm	1	2	2	1	6
Total	5	7	9	3	24

[a] The *p* value of the Fisher's exact test is 1, indicating that the distributions of maternal parity are not significantly different across the exposure groups.

Table C.7. Methanol Half-Life (log of minutes) Following Initial Exposure

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.14	1	0.14	1.01	0.32
Error	2.82	21	0.13		

Table C.8. Methanol Clearance (log of mL/min/kg) Following Initial Exposure

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.07	1	0.07	0.39	0.54
Error	3.93	21	0.19		

Table C.9. Methanol Distribution Volume (log of L/kg) Following Initial Exposure

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.01	1	0.01	0.16	0.69
Error	1.38	21	0.06		

Table C.10. ANOVA for Methanol Half-Life (log of minutes) from Toxicokinetic Study 1 vs. Study 2

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	1	21	0.11	0.74
Within-Subject Factors				
Study	1	21	5.76	0.03
Study × exposure group	1	21	1.87	0.18

Table C.11. ANOVA for Methanol Clearance (log of mL/min/kg) from Toxicokinetic Study 1 vs. Study 2

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	1	21	0.27	0.61
Within-Subject Factors				
Study	1	21	6.41	0.02
Study × exposure group	1	21	0.08	0.78

Table C.12. ANOVA for Methanol Distribution Volume (log of L/kg) from Toxicokinetic Study 1 vs. Study 2

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	1	42	0.14	0.71
Within-Subject Factors				
Study	1	42	6.56	0.46
Study × exposure group	1	42	0.69	0.42

Table C.13. ANOVA for Methanol Half-Life (log of minutes) from Toxicokinetic Study 2 vs. Study 3 vs. Study 4

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	1	17	6.85	0.02[a]
Within-Subject Factors				
Study	2	34	0.46	0.63
Study × exposure group	2	34	1.24	0.3

[a] Difference reached statistical significance.

Table C.14. ANOVA for Methanol Clearance (log of mL/min/kg) from Toxicokinetic Study 2 vs. Study 3 vs. Study 4

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	1	17	0.54	0.47
Within-Subject Factors				
Study	2	34	1.87	0.17
Study × exposure group	2	34	0.71	0.50

Table C.15. ANOVA for Methanol Distribution Volume (log of L/kg) from Toxicokinetic Study 2 vs. Study 3 vs. Study 4

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	1	17	1.91	0.18
Within-Subject Factors				
Study	2	34	1.87	0.01
Study × exposure group	2	34	0.22	0.80

Table C.16. Plasma Formate (mM) During Baseline Period

Source	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA				
Exposure group	3	42	0.63	0.60
Linear Contrasts				
Controls vs. all exposed	3	42	0.63	0.60
Controls vs. 200 ppm	1	42	0.23	0.63
Controls vs. 600 ppm	1	42	1.22	0.28
Controls vs. 1,800 ppm	1	42	0.00	0.95

Table C.17. ANOVA for Plasma Formate (mM) During Baseline and Prebreeding Periods

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	3	42	1.78	0.16
Within-Subject Factors				
Period[a]	1	555	7.79	0.005
Period × exposure group	3	555	2.43	0.06

[a] Period = methanol exposure period.

Table C.18. ANOVA for Plasma Formate (mM) During Prebreeding and Pregnancy Periods

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	3	33	0.59	0.63
Within-Subject Factors				
Period[a]	1	641	107.92	0.0001
Period × exposure group	3	641	0.82	0.48

[a] Period = methanol exposure period.

Table C.19. Serum Folate (ng/mL) During Baseline Period

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	39.37	3	13.12	0.85	0.47
Error	647.88	42	15.43		
Linear Contrasts					
Controls vs. all exposed	35.89	1	35.89	2.33	0.13
Controls vs. 200 ppm	35.83	1	35.83	2.32	0.13
Controls vs. 600 ppm	19.85	1	19.85	1.29	0.26
Controls vs. 1,800 ppm	18.90	1	18.90	1.23	0.27

Table C.20. ANOVA for Serum Folate (ng/mL) During Baseline and Prepregnancy Periods

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	3	42	0.76	0.52
Within-Subject Factors				
Period[a]	1	87	5.73	0.02
Period × exposure group	3	87	2.01	0.12

[a] Period = methanol exposure period.

Table C.21. ANOVA for Serum Folate (ng/mL) During Baseline and Prepregnancy Periods

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	3	33	1.20	0.32
Within-Subject Factors				
Period[a]	1	102	7.67	0.007
Period × exposure group	3	102	1.46	0.23

[a] Period = methanol exposure period.

Table C.22. Weight Gain (g) During Pregnancy

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	1285108.92	3	428369.64	1.51	0.23
Error	9356010.00	33	283515.45		
Linear Contrasts					
Controls vs. all exposed	133623.61	1	133623.61	0.47	0.50
Controls vs. 200 ppm	716005.56	1	716005.56	2.53	0.12
Controls vs. 600 ppm	51200.00	1	51200.00	0.18	0.67
Controls vs. 1,800 ppm	77743.22	1	77743.22	0.27	0.60
ANOVA (Cohort Interaction Model)					
Cohort	1079155.98	1	1079155.98	4.98	0.03
Exposure group	1437616.28	3	479205.43	2.21	0.11
Cohort × exposure group	1879245.54	3	626415.18	2.89	0.05
Error	6286428.33	29	216773.39		

Table C.23. Menstrual Cycle Length (days) During Baseline Period

Source	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA				
Exposure group	3	42	0.87	0.46
Linear Contrasts				
Controls vs. all exposed	1	42	1.16	0.28
Controls vs. 200 ppm	1	42	2.47	0.12
Controls vs. 600 ppm	1	42	0.31	0.58
Controls vs. 1,800 ppm	1	42	0.28	0.60

Table C.24. ANOVA for Menstrual Cycle Length (days) During Baseline and Prebreeding Periods

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	3	42	0.52	0.67
Within-Subject Factors				
Period[a]	1	271	1.17	0.28
Period × exposure group	3	271	0.89	0.45

[a] Period = methanol exposure period.

Table C.25. Number of Conceptions by Exposure Group[a]

Exposure Group	Conceived	Not Conceived	Total
Control	9 (81.82%)	2 (18.18%)	11
200 ppm	9 (75.00%)	3 (25.00%)	12
600 ppm	9 (81.82%)	2 (18.18%)	11
1,800 ppm	10 (83.33%)	2 (16.67%)	12
Total	37 (80.43%)	9 (19.57%)	46

[a] The *p* value of the Fisher's exact test of homogeneity among groups is 1.0, indicating that the conception rates were not significantly different across the four exposure groups. Values are presented as number (percentage of total for that exposure group).

Table C.26. Number of Complications During Pregnancy and Delivery by Exposure Group[a]

Exposure Group	No Complications	Some Complications	Total
Control	7 (77.78%)	2 (22.22%)	9
200 ppm	7 (77.78%)	2 (22.22%)	9
600 ppm	6 (66.67%)	3 (33.33%)	9
1,800 ppm	7 (70.00%)	3 (30.00%)	10
Total	27 (72.97%)	10 (27.03%)	37

[a] The *p* value of the Fisher's exact test of homogeneity among groups is 1.0, indicating that the rates of complications during pregnancy and delivery were not significantly different across the four exposure groups. Values are presented as number (percentage of total for that exposure group).

Table C.27. Number of Live-Born Deliveries by Exposure Group[a]

Exposure Group	Live-Born	Not Live-Born	Total
Control	8 (88.89%)	1 (11.11%)	9
200 ppm	9 (100.00%)	0 (0.00%)	9
600 ppm	8 (88.89%)	1 (11.11%)	9
1,800 ppm	9 (90.00%)	1 (10.00%)	10
Total	34 (97.89%)	3 (8.11%)	37

[a] The *p* value of the Fisher's exact test of homogeneity among groups is 1.0, indicating that the live-born delivery rates were not significantly different across the four exposure groups.

Table C.28. Duration of Pregnancy (days) for Live-Born Deliveries

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	332.51	3	110.84	3.39	0.03
Error	981.38	30	32.71		
Linear Contrasts					
Controls vs. all exposed	305.52	1	305.52	9.34	0.005
Controls vs. 200 ppm	297.07	1	297.07	9.08	0.005
Controls vs. 600 ppm	150.06	1	150.06	4.59	0.04
Controls vs. 1,800 ppm	190.60	1	190.60	5.83	0.02

Table C.29. Gestation Length (days) for Live-Born Offspring

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	332.51	3	110.84	3.39	0.03
Error	981.38	30	32.71		
Linear Contrasts					
Controls vs. all exposed	305.52	1	305.52	9.34	0.005
Controls vs. 200 ppm	297.07	1	297.07	9.08	0.005
Controls vs. 600 ppm	150.06	1	150.06	4.59	0.04
Controls vs. 1,800 ppm	190.60	1	190.60	5.83	0.02
ANOVA (Sex Interaction Model)					
Exposure group	375.77	3	125.26	4.42	0.01
Sex	57.82	1	57.82	2.04	0.17
Exposure group × sex	186.47	3	62.16	2.19	0.11
Error	737.47	26	28.36		
ANOVA (Sex Interaction Model—Reduced)					
Exposure group	350.63	3	116.88	3.67	0.02
Sex	57.43	1	57.43	1.80	0.19
Error	923.95	29	31.86		

Table C.30. Infant Birthweight (g) for Live-Born Offspring

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	3932.95	3	1310.98	0.42	0.74
Error	93028.82	30	3100.96		
Linear Contrasts					
Controls vs. all exposed	398.98	1	398.98	0.13	0.72
Controls vs. 200 ppm	2502.04	1	2502.04	0.81	0.38
Controls vs. 600 ppm	1.56	1	1.56	0.0005	0.98
Controls vs. 1,800 ppm	2.04	1	2.04	0.0006	0.98
ANOVA (Sex Interaction Model)					
Exposure group	8692.79	3	2897.60	0.98	0.42
Sex	4358.05	1	4358.05	1.48	0.24
Exposure group × sex	12076.25	3	4025.42	1.36	0.28
Error	76731.13	26	2951.20		
ANOVA (Sex Interaction Model—Reduced)					
Exposure group	5733.82	3	1911.27	0.62	0.61
Sex	4221.43	1	4221.43	1.38	0.25
Error	88807.39	29	3062.32		
ANOVA (Cohort Interaction Model)					
Cohort	5572.90	3	1857.63	1.07	0.38
Exposure group	15765.18	1	15765.18	9.07	0.006
Exposure group × cohort	32643.33	3	10881.11	6.26	0.002
Error	774.77	26	29.80		

Table C.31. Infant Crown–Rump Length (mm) for Live-Born Offspring

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	122.18	3	40.73	0.49	0.70
Error	2518.76	30	83.96		
Linear Contrasts					
Controls vs. all exposed	105.90	1	105.90	1.26	0.27
Controls vs. 200 ppm	105.88	1	105.88	1.26	0.27
Controls vs. 600 ppm	76.56	1	76.56	0.91	0.35
Controls vs. 1,800 ppm	40.99	1	40.99	0.49	0.49
ANOVA (Sex Interaction Model)					
Exposure group	182.06	3	60.69	0.81	0.50
Sex	30.94	1	30.94	0.41	0.53
Exposure group × sex	534.65	3	178.22	2.37	0.09
Error	1956.77	26	75.26		
ANOVA (Sex Interaction Model—Reduced)					
Exposure group	133.64	3	44.55	0.52	0.67
Sex	27.34	1	27.34	0.32	0.58
Error	2491.42	29	85.91		

Table C.32. Infant Head Circumference (mm) for Live-Born Offspring

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	65.25	3	21.75	0.80	0.50
Error	810.99	30	27.03		
Linear Contrasts					
Controls vs. all exposed	40.07	1	40.07	1.48	0.23
Controls vs. 200 ppm	61.79	1	61.79	2.29	0.14
Controls vs. 600 ppm	22.56	1	22.56	0.83	0.37
Controls vs. 1,800 ppm	9.35	1	9.35	0.35	0.56
ANOVA (Sex Interaction Model)					
Exposure group	63.13	3	21.04	0.70	0.56
Sex	1.36	1	1.36	0.04	0.83
Exposure group × sex	23.16	3	7.72	0.26	0.86
Error	786.03	26	30.23		
ANOVA (Sex Interaction Model—Reduced)					
Exposure group	60.32	3	20.11	0.72	0.55
Sex	1.80	1	1.80	0.06	0.80
Error	809.19	29	27.90		
ANOVA (Cohort Interaction Model)					
Cohort	61.72	3	20.57	1.20	0.33
Exposure group	188.69	1	188.69	11.01	0.003
Exposure group × cohort	172.23	3	57.41	3.35	0.03
Error	445.50	26	17.13		

Table C.33. Infant Head Length (mm) for Live-Born Offspring

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	4.40	3	1.47	0.34	0.79
Error	127.72	30	4.26		
Linear Contrasts					
Controls vs. all exposed	0.72	1	0.72	0.17	0.68
Controls vs. 200 ppm	2.94	1	2.94	0.69	0.41
Controls vs. 600 ppm	0.25	1	0.25	0.06	0.81
Controls vs. 1,800 ppm	0.01	1	0.01	0.003	0.96
ANOVA (Sex Interaction Model)					
Exposure group	4.22	3	1.41	0.29	0.83
Sex	0.004	1	0.004	0.0009	0.98
Exposure group × sex	0.63	3	0.21	0.04	0.99
Error	127.08	26	4.89		
ANOVA (Sex Interaction Model—Reduced)					
Exposure group	4.27	3	1.42	0.32	0.81
Sex	0.01	1	0.01	0.003	0.96
Error	127.71	29	4.40		

Table C.34. Infant Head Width (mm) for Live-Born Offspring

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	13.10	3	4.37	1.48	0.24
Error	88.43	30	2.95		
Linear Contrasts					
Controls vs. all exposed	10.79	1	10.79	3.66	0.07
Controls vs. 200 ppm	9.01	1	9.01	3.06	0.09
Controls vs. 600 ppm	10.56	1	10.56	3.58	0.07
Controls vs. 1,800 ppm	3.45	1	3.45	1.17	0.29
ANOVA (Sex Interaction Model)					
Exposure group	14.02	3	4.67	1.40	0.26
Sex	0.39	1	0.39	0.12	0.74
Exposure group × sex	1.71	3	0.57	0.17	0.92
Error	86.48	26	3.33		
ANOVA (Sex Interaction Model—Reduced)					
Exposure group	13.31	3	4.44	1.46	0.25
Sex	0.24	1	0.24	0.08	0.78
Error	88.19	29	3.04		

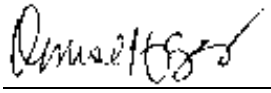
APPENDIX D. HEI Quality Assurance Report

The conduct of this study has been subjected to periodic audits by the Quality Assurance Officer from Primedica Corporation. The audits have included in-process monitoring of study activities and audits of the data. The dates of audits and nature of the visit are listed in the table below. The results of the inspections were reported to the Director of Research of the Health Effects Institute, who was responsible for transmitting the reports to the Principal Investigator.

Observations made during these visits indicate that the study is well documented, and that the report describes the methods used and reflects the raw data. The effect of deviations from the protocol and standard operating procedures on the results of the study have been considered and addressed, as appropriate, in the data or final report.

AUDITS BY QUALITY ASSURANCE OFFICER

Date	Procedure/Data Reviewed
August 12–13, 1991	Prestudy visit
January 21–22, 1993	Observe activities for Cohort 1
January 21 and 24, 1994	Interim data audit
June 22–23, 1995	Observe activities for Cohort 2 and interim data audit
June 16–17, 1997	Final data audit and review of final report
October 1998	Review of revised final report



Denise Hayes, HEI Quality Assurance Officer

ABOUT THE AUTHORS

Thomas M. Burbacher is an Associate Professor of Environmental Health in the Division of Toxicology at the University of Washington. He received his Ph.D. from the University of Washington in 1983. His research focuses on the effects of developmental exposure to environmental contaminants (metals, pesticides) on nervous system functions. Most recently his studies have examined the effects of prenatal exposure to methylmercury on the aging process in nonhuman primates. His research also includes follow-up studies of the animals from this investigation.

Doris Damian is a Ph.D. candidate in the Department of Biostatistics at the University of Washington. She received her M.Sc. in statistics in 1995 from the Hebrew University of Jerusalem. Her interests include spatial statistics and health effects of air pollution.

Stephen S. Ellis, III, worked for the Department of Environmental Health from 1991 until 1999. He was head of the data management and processing team for the methanol project. He received his M.S.W. from Walla Walla College in 1998 and is currently a child welfare social worker for the Department of Social and Human Services in the state of Washington.

Kimberly S. Grant is a Research Scientist in the Department of Environmental Health at the University of Washington. She received her Ph.D. from the University of Washington in 1990. She has worked with Dr. Burbacher over the past 10 years as the director of research for this and related projects. Her research expertise is in pediatric environmental health, particularly the assessment of behavioral development.

Noelle Liberato is a Research Supervisor with the Department of Environmental Health. She received her B.A. from the University of Washington in 1992. She was supervisor for behavioral testing for the methanol project. She currently supervises the follow-up studies of the animals from this investigation.

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Lianne Sheppard is a Research Assistant Professor in the Departments of Biostatistics and Environmental Health at the University of Washington. She received her Ph.D. from the University of Washington in 1992. Her biostatistical research interests emphasize estimation of health effects from environmental and occupational exposures and incorporating group information in epidemiologic studies. Her interest in applications is wide-ranging including diet and cancer, air pollution health effects, and occupational exposures.

ABBREVIATIONS AND OTHER TERMS

ANOVA	analysis of variance
AUC	area under the curve
CIIT	Chemical Industry Institute of Toxicology
CO_2	carbon dioxide
C-section	Cesarean section
CV	coefficients of variation
F	fractional absorption of methanol vapor via the lungs
FSH	follicle-stimulating hormone
GAC	General Analysis Corporation
GC	gas chromatography
HPLC	high-performance liquid chromatography
K_m	Michaelis constant
LH	luteinizing hormone
MTBE	methyl tertiary-butyl ether
NADH	nicotinamide adenine dinucleotide
NOAEL	no observable adverse effect level
RTDs	resistance temperature detectors
TWA	time-weighted average
V	true distribution volume
V/F	apparent distribution volume
V_{max}	maximum rate of methanol metabolism

Reproduction and Offspring Developmental Effects Following Maternal Inhalation Exposure to Methanol in Nonhuman Primates

Part II: Developmental Effects in Infants Exposed Prenatally to Methanol

Thomas Burbacher, Kimberly Grant, Danny Shen, Doris Damian, Stephen Ellis, and Noelle Liberato

ABSTRACT

A two-cohort study design utilizing 48 adult female *Macaca fascicularis* (24/cohort), 4 adult male *M. fascicularis* (2/cohort), and their offspring was used to investigate the reproductive and developmental toxicity of inhaled methanol vapor. Adult females (n = 6/group/cohort) were exposed to 0, 200, 600, or 1,800 ppm methanol vapor for 2 hours/day, 7 days/week prior to and during pregnancy. The average duration of methanol exposure prior to pregnancy was 180 days. Daily exposures produced blood concentrations of methanol from twice to 10 times background endogenous levels.

Thirty-four liveborn infants were delivered. Two infants, 1 in the control group and 1 in the 600 ppm–exposure group, were were delivered stillborn, and 1 fetus died in the 1,800 ppm–exposure group. No obvious birth defects were observed in any of the live-birth or stillborn infants. For the liveborn offspring, 5 of the methanol-exposed infants had to be delivered via Cesarean section (C-section)* because of complications at delivery. Four of these infants required oxygen therapy. The average gestation length of the methanol-exposed infants (~160 days) was significantly shorter than that of the controls (~168 days). Prenatal methanol exposure did not affect offspring size at birth. Results of the behavioral assessments did not indicate significant methanol exposure effects on early reflex responses, gross motor development, spatial and concept learning or memory and social behavior. Methanol exposure was associated with ratings of "low arousal" on the Neonatal Assessment scale. This effect may not be directly related to methanol exposure, independent of C-section delivery. Methanol exposure was also associated with a delay in early sensorimotor development for male infants, and visual recognition memory was also affected. Finally, prenatal methanol exposure was associated with a *wasting syndrome* in females after approximately 1 year of age. The syndrome was severe and resulted in euthanasia.

Since this is the first report of an association between low-level methanol exposure and offspring developmental effects, further investigations are needed to confirm these findings. Investigations should focus on evaluating possible changes in fine motor or somatosensory responses and the speed and accuracy of information processing. Long-term follow-up of physical growth during adolescence is also warranted.

INTRODUCTION

The principal dietary sources of prenatal exposure to methanol in humans are maternal consumption of adulterated alcoholic beverages, fruit, vegetables, and food or drinks that have been artificially sweetened with aspartame. In the case of adulterated beverage alcohol, disentangling the effects on the developing fetus of ingested methanol from those of ethanol is not possible. It is tempting to speculate that methanol's effects might be similar to ethanol's because the compounds are closely related and the placental transfer index of both is greater

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

This report is Part II of Health Effects Institute Research Report Number 89, which also includes a Part I, a Commentary by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Thomas M. Burbacher, University of Washington, Department of Environmental Health, Health Sciences Building, Room F461F, School of Public Health and Community Medicine, Box 357234, Seattle, WA 98195-7234.

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Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R824835 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review. Therefore, it may not reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private-party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

than one (Bissonnette et al. 1979). The effects of prenatal ethanol exposure are well documented, and fetal alcohol syndrome is one of the leading known causes of mental retardation in humans (Abel and Sokol 1987). The neurobehavioral effects of prenatal exposure to ethanol in humans are dose dependent and long lasting (Streissguth et al. 1994, 1995). In a series of studies by Streissguth and colleagues (1994, 1995), the effects of prenatal ethanol exposure were immediately observed in newborn behavior: Ethanol-exposed newborns habituated less to a repetitive stimulus, had poorer reflex responses, and exhibited "low arousal" when compared with control infants. At 8 months of age, ethanol-exposed infants exhibited decrements in both motor and mental development. At 4 to 7 years of age, prenatal ethanol exposure was associated with a loss of 4 to 7 IQ points on intelligence tests. Finally, evaluation at 14 years of age indicated deficits in attention and memory, problems with response inhibition, and spatial learning deficits.

There are no reported cases in the literature of developmental toxicity in humans following prenatal exposure to methanol. In one case of documented methanol poisoning during infancy, an infant was fed a mixture of formula and a windshield cleaner that contained methanol. The infant was hospitalized and recovered without apparent neurological sequelae (Brent et al. 1988).

Laboratory studies of prenatal methanol exposure using rodent animal models have reported numerous signs of teratogenicity in methanol-exposed pups. Pregnant rats exposed by inhalation to either methanol or ethanol, in concentrations ranging from 5,000 to 20,000 ppm, delivered offspring with an increased number of malformations (primarily extra cervical ribs and urinary or cardiovascular defects). At equivalent doses, the treatment-related effects were more pronounced in litters exposed to methanol than in litters exposed to ethanol (Nelson et al. 1985). Bolon and colleagues (1993) exposed pregnant mice to 5,000, 10,000, or 15,000 ppm methanol vapor; they reported an increased number of fetal resorptions and malformations, as well as reduced fetal weights. Depending on the gestational day of exposure, neural tube defects, ocular lesions, limb abnormalities, cleft palate, and hydronephrosis were also observed at the two higher levels of exposure. No such methanol-related effects were observed in the group exposed to the lowest level of methanol, 5,000 ppm. Rogers and colleagues (1993) exposed pregnant mice to concentrations of methanol ranging from 1,000 to 15,000 ppm. In pups exposed to 5,000 ppm methanol or higher, an increase in the number of exencephalies and cleft palate was observed. Some evidence for cervical rib malformations in offspring born to dams exposed to 2,000 ppm was also documented.

Neurobehavioral evaluations of rodents developmentally exposed to methanol are few in number. In a recent study by Weiss and colleagues (1996), pregnant rats were exposed via inhalation to either 4,500 ppm methanol or 0 ppm for 6 hours daily. Maternal exposure began on gestational day 6, and both pups and dams were exposed until postnatal day 21. Suckling behavior, odor discrimination, and motor activity were measured prior to weaning. Adult function was measured with two operant procedures, the fixed-ratio wheel running procedure and a stochastic spatial discrimination task. Although the behavioral effects in the exposed offspring were subtle in nature, it is important to consider that methanol exposure did influence a number of the functional endpoints studied. In the preweaning phase of testing, a treatment-related effect was observed on the motor activity test. On the operant measures, both tests showed evidence of performance decrements due to methanol exposure, but the differences were not robust. In an earlier study by Infurna and Weiss (1986), pregnant rats were exposed to methanol in drinking water at 20,000 ppm on either gestational days 15 to 17 or gestational days 17 to 19. Exposed pups displayed difficulties in suckling behavior and in finding nesting material from the home cage. Stanton and colleagues (1995) exposed pregnant rats to methanol via inhalation at a concentration of 15,000 ppm on gestational days 7 to 19. A broad-based battery of behavioral tests was given to the offspring, including motor activity, T-maze learning, olfactory learning, acoustic startle, and passive avoidance learning. The exposed pups did not show methanol-related impairments on any of the neurobehavioral measures. The only adverse effect of methanol exposure observed in this study was reduced birth weight.

As indicated above, consumption of aspartame-sweetened products results in methanol exposure. The release of methanol occurs when aspartame is absorbed and metabolized during digestion (Stegink et al. 1983). In a chapter reviewing the effects of aspartame ingestion during pregnancy, the authors were unable to comment definitively on the extent of prenatal methanol loading from maternal ingestion, although it is generally regarded to be insignificant (Pitkin 1984). In an attempt to investigate the effects of aspartame ingestion, infant stumptail macaques were fed high levels of aspartame in their formula for 9 months (Reynolds et al. 1984). There were no aspartame-related effects on parameters of development, such as physical growth, serum chemistry, urinalysis, hematology and brain-wave patterns. Later investigations of hearing and cognitive development in these animals

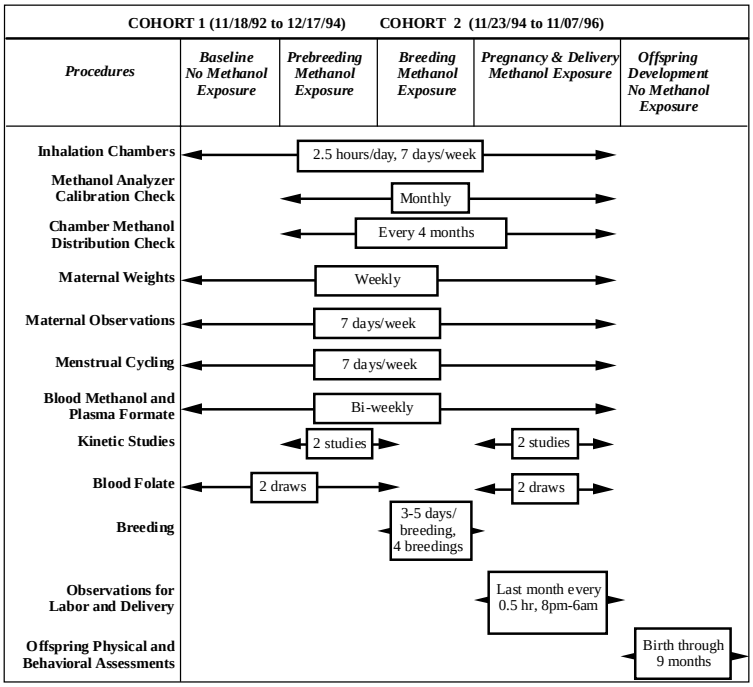

Figure 1. Experimental design and schedule of study.

did not indicate adverse effects attributed to early exposure to aspartame (Suomi 1984).

The present study is the first to attempt to evaluate the effects of maternal methanol exposure during pregnancy on infant development in nonhuman primates.

SPECIFIC AIMS

The specific aims of the project were to determine for methanol vapor, the exposure dose–response relationship in nonhuman primates for:

1. changes in methanol metabolism related to repeated exposures to methanol, defined by changes in methanol blood clearance, distribution kinetics, or both, in nonhuman primate females;
2. changes in methanol metabolism related to pregnancy, defined by changes in methanol blood clearance, distribution kinetics, or both, in nonhuman primate females;
3. toxic effects on maternal reproductive function, defined by decreased conceptions, increased complications of pregnancy, labor, or delivery, changes in pregnancy duration, or decreased live-birth deliveries in methanol-exposed nonhuman primate females; and
4. toxic effects on infant development, defined by alterations in the physical or neurobehavioral development, or both, of nonhuman primate infants exposed prenatally to methanol.

Results related to Specific Aims 1 through 3 are described in Part I of this report. Specific Aim 4 was addressed by examining the physical growth and neurobehavioral development of *M. fascicularis* infants exposed prenatally to methanol compared with growth and development in control infants. Many of the neurobehavioral tests used to address Specific Aim 4 were adapted from studies of normal, high-risk, and teratogen-exposed human infants (Jacobson et al. 1985; Fagan and Singer 1983; Rose 1983; Streissguth et al. 1981; Weisz and Zigler 1979). Similar response patterns on these tests have been observed for both human and nonhuman primate infants (for review, see Burbacher et al. 1990a).

METHODS

STUDY DESIGN AND TESTING SCHEDULE

Detailed descriptions of study design, procedures associated with maternal methanol exposures, and analyses of data related to Specific Aims 1 through 3 are included in Part I of this report. Briefly, a two-cohort study design utilized 48 adult female *M. fascicularis* monkeys (24/cohort), 4 adult male *M. fascicularis* (2/cohort), and their offspring

Table 1. Distribution of Male and Female Offspring Across Exposure Groups and Cohorts

	Cohort 1		Cohort 2		Cumulative		
Exposure Group	Males	Females	Males	Females	Males	Females	Total (*n*)
Control	2	3	1	2	3	5	8
200 ppm	4	1	1	3	5	4	9
600 ppm	2	2	1	3	3	5	8
1,800 ppm	1	4	1	3	2	7	9
Total (*n*)	9	10	4	11	13	21	34

(see Figure 1). Females were exposed to 0, 200, 600, or 1,800 ppm methanol vapor for 2.5 hours/day, 7 days/week, both prior to and during pregnancy.

At the end of each 2-hour exposure, the animals remained in the inhalation chamber for another 30 minutes while the methanol dissipated. The average duration of methanol exposure prior to pregnancy was approximately 180 days. Females were bred to unexposed males for 3 to 5 days and resultant pregnancies were confirmed via progesterone assay. Blood was obtained from all females on a biweekly basis throughout the study for methanol and formate analyses. Information concerning parental characteristics, weights, medications, and blood methanol and serum formate concentrations is summarized in Part I of this report.

A total of 34 live-born offspring were delivered (see Table 1). The 34 infants were evaluated during the first 9 months of life using a test battery that included procedures largely adapted from studies with human infants (see Table 2). In many cases, similar patterns of responses have been observed for infant human and nonhuman primates. In addition, many of the procedures have been used successfully in studies involving other environmentally relevant compounds (Burbacher et al. 1990a).

MATERNAL METHANOL EXPOSURE

The inhalation laboratory and exposure procedures used in the study are described in detail in Part I of this report. Briefly, eight inhalation chambers were used, with each chamber housing one animal in a cage. Air was delivered to the chambers from the room, and a second blower provided air exhaust for the chambers. Air flow to each chamber was set at a rate of 420 L/min. Chamber pressures were adjusted to –0.10 inches H_2O relative to room pressure. Methanol vapor was generated by passing compressed air through gas dispersion bottles filled with chromatographic-grade methanol (99.9% pure). The methanol was heated by placing

Table 2. Offspring Assessment Battery Used to Evaluate Developmental Effects of Prenatal Methanol Exposure

Offspring Assessment	Postnatal Age at Assessment
Newborn Size	Birth
Medical Treatments	Birth to 9 Months
Newborn Health Assessment	Birth
Neonatal Behavioral Scale	Days 1 to 13
Object Retrieval Test	Weeks 2 to 6
Visual Acuity	Weeks 1 to 12
Object Concept Test	Week 2 to 3½ Months
Recognition Memory	Days 190 to 220[a]
Social Observations	Week 2 to 7 Months
Physical Growth	Birth to 9 Months
Motor Milestones Observations	Week 2 to 7 Months
Spatial Discrimination and Reversal	Months 5 to 7
Nonmatch-to-Sample	Months 8 to 9

[a] Postconceptional days of age.

the bottles in a water bath. Methanol vapor was delivered to the chambers using eight independent generation and delivery systems, thus permitting independent operation of each chamber. To control the timing of methanol delivery to the chambers and air sampling from each chamber, computer programs were developed with use of LABVIEW software, National Instruments interface equipment, and a Macintosh IIci computer. Methanol was delivered to any given chamber for a 2-hour period. Methanol concentration was measured by withdrawing an air sample from each chamber to a General Analysis Corporation infrared analyzer. A 10-valve sampling system was constructed to allow flow from a particular chamber, the whole room, or a control filter to the analyzer. Each chamber (as well as the room and control filter) was sampled for 1 minute, with the initial sample from the chamber drawn 4 minutes after the onset of methanol flow. With this schedule, the sample rate per chamber was 1 sample/10 minutes. Values for methanol concentration were entered into an Excel file, which was copied to a 3.5-inch computer diskette for processing at the end of each day.

BLOOD METHANOL AND FORMATE ANALYSES

Blood sampling and analysis procedures are also described in detail in Part I. Briefly, blood was collected from all females every other week throughout the study for methanol and formate analyses. Blood draws occurred approximately 10 minutes after the females were removed from the inhalation chambers, or roughly 30 minutes after the end of methanol flow to the chambers. Approximately 3 mL blood was collected from the saphenous vein of each unanesthetized female. Methanol in whole blood was analyzed in duplicate by a direct headspace capillary gas-chromatographic procedure developed in our laboratory. The addition of 0.5 mL of 0.25 M sodium hydroxide (NaOH) to 0.5 mL of blood specimen solubilized blood proteins and enabled the use of high bath temperature (85°C) during headspace equilibration; this increased the sensitivity of the assay without producing the confounding problem of blood coagulation. Acetonitrile was added as an internal standard. Chromatographic resolution was achieved on a widebore carbowax column (J & W, DB-WAX, 30 m × 0.53 mm internal diameter, 1µm thickness) with detection afforded by a flame ionization detector. Plasma formate concentrations were measured with use of the enzymatic assay procedure of Buttery and Chamberlain (1988). This formate assay relies on the colorimetric measurement of reduced nicotinamide adenine dinucleotide (NADH) produced by the action of formate dehydrogenase on plasma formate.

OFFSPRING ASSESSMENTS

Many of the tests included in the battery utilized to assess effects of prenatal methanol exposure have been used in previous investigations of teratogen-exposed nonhuman primates (namely, infants exposed to methylmercury (Burbacher et al. 1986, 1990b), ethanol (Clarren et al. 1992), and zidovudine (AZT) (Ha et al. 1998). For the present study, separate categories, or domains, of physical and behavioral development were formulated, including prenatal and postnatal growth, newborn health, neonatal behavioral responses, and infant visual, sensorimotor, cognitive, and social behavioral development. A set of primary- and secondary-outcome variables was established prior to the beginning of the investigation in order to prioritize analyses. The primary-outcome variables were developed on the basis of previous reports of ethanol-related effects on offspring development (Streissguth et al. 1994) and on the past sensitivity of assessment procedures chosen to detect teratogen-induced developmental effects in nonhuman primates (Burbacher et al. 1990a). The separate domains of infant development, the individual assessment procedures, and the primary- and secondary-outcome variables used in the study are shown in Table 3. Details of the assessment procedures are provided later in this section.

Offspring Size at Birth

Females in the last month of pregnancy were observed via infrared camera every 30 minutes from 6 p.m. until 6 a.m. to detect onset of labor. When labor was detected, study personnel were notified in order to provide any needed assistance and to separate the infant from the mother immediately after delivery. Birth weight, crown–rump length, head circumference, head width, and head length were obtained for all infants within 1 hour after delivery. Weight was determined by placing the infant in a small carrier onto a platform scale with digital readout. The scale was set to zero with the carrier in place, and the weight was read and recorded. Crown–rump length was determined by placing the infant in a supine position on a metal sliding scale with its rump against the base of the scale and its crown against a sliding barrier. The distance between the top of the infant's head and the rump was measured. Head circumference was determined with use of a measuring tape that was placed around the infant's head below the brow ridge, along the nuchal crest and above the ears, thus covering the occiput. Head width and length were determined using spreading calipers. Head width was measured using landmarks just above and in front of the ears, which provided a measure of the two widest points of the skull. Head length was measured

Table 3. Outcome Variables Used to Evaluate Developmental Effects of Prenatal Methanol Exposure

Physical/Behavioral Domain	Assessment Procedure	Variable	
		Primary Outcome	Secondary Outcome
Newborn size	Weights and Anthropometrics	Birth weight	Crown–rump length; head width, length, and circumference
Medical treatments	Medical Log	Medications at birth and during first 9 months	Number of days medicated during each assessment procedure
Newborn health	Newborn Health Assessment	Total score for ratings of color, muscle tone, temperature, heart rate, and respiration 10 min after birth	Total score for ratings of color, muscle tone, temperature, heart rate, and respiration at 30 min after birth
Neonatal behavioral responses	Neonatal Behavioral Assessment Scale	Age at optimal score for behavioral state, responsitivity, reflexes, and muscle tone	
Infant sensorimotor development	Visually Directed Reaching Test	Age to retrieve small object	Age to climb or jump on ramp and shelves in playroom (motor milestone)
Infant visual acuity development	Preferential Looking Test	Development of visual acuity thresholds during first 12 weeks	
Infant spatial memory	Object Concept Test	Age to retrieve hidden object on screen test	Age to retrieve hidden object on Well Test and A-not-B Test
	Spatial Discrimination and Reversal Test		Number of nonbalk trials to criterion for spatial discrimination; percentage correct over six reversals
Infant recognition memory	Fagan Test of Infant Intelligence	Novelty scores, the percentage of time looking to novel stimulus	
	Nonmatch-to-Sample Test		Number of nonbalk trials to criterion for Nonmatch-to-Sample; percentage correct over five delays
Infant social behavioral development	Coding of behavior in playroom	Percentage time in passive, social, and nonsocial behaviors	
Physical growth	Weights and Anthropometrics	Weight gain during first 9 months	At nine months: weight; crown–rump length; and head width, length, and circumference. Teeth eruption

Table 4. Newborn Health Assessment Scale

	Rating Points		
Measure	0	1	2
Heart rate (beats/min)	None	≤ 150	> 151
Respiration rate (breaths/min)	None	≤ 60	> 60
Muscle tone	None	Some	Flexed
Activity		Passive	Active
Skin color	Gray	Somewhat gray	Pink
Rectal temperature (°C)	< 60	60–90	> 90

using the length from the area between the eyes to the occiput. All anthropometric measures were recorded in millimeters.

Offspring Medical Treatments

Offspring were observed daily for signs of illness by a trained veterinary technologist. Observations focused on detecting signs of dehydration, diarrhea, vomiting, and lethargy. If signs of illness were observed, appropriate medical treatment was begun. All medical treatments were recorded on a daily form by the veterinary staff. Treatments for specific infants were then transcribed onto the daily weight data sheets by study personnel. The number of days that each infant was medicated could then be summarized across specific testing periods to assess any relation to outcome variables of interest. All of the veterinary staff were blind to the methanol-exposure group of the offspring.

Newborn Health Status

A Newborn Health Assessment, which was modeled after the Apgar rating scale used with human infants (Apgar 1953), was performed as soon as possible after birth and again at approximately 30 minutes after birth. Heart rate, respiration rate, rectal temperature, muscle tone, activity, and skin color were evaluated (see Table 4). Heart rate was measured over a 15-second period using a standard stethoscope and a stop watch. Respiration rate was also measured over a 15-second period using a stop watch. Rectal temperature was measured via a digital thermometer; muscle tone, activity, and skin color were evaluated using predefined rating points from 0 to 2. In addition to the Newborn Health Assessment, a systematic examination of each newborn for major birth defects took place after the initial assessment.

Neonatal Behavioral Responses

An assessment procedure modeled after the Brazelton Neonatal Behavioral Assessment Scale (Brazelton 1973) was used to evaluate newborn reflexes and responses (see Table 5). The assessment began by observing the infant's state prior to testing (Item 1) and the position of limbs in a resting position (Item 2). The tester then removed the infant from its cage and wrapped the infant in a diaper, leaving the limbs exposed. The tester evaluated grasping responses of hands and feet by placing the index finger against the palms of the hands and soles of the feet (Item 3). Resistance of arms and legs to extension was then evaluated by gently pulling the limbs away from the body (Item 4). The tester touched the infant on the edge of the mouth and on the nose to elicit the rooting and snout responses (Items 5 and 6). Then, a metal lid was dropped onto a counter behind the infant to elicit a startle response (Item 7). Next, the infant was turned away from the tester, and the tester made a lipsmacking noise at the back of each of the infant's ears to elicit an orienting response (Item 8). The tester proceeded to hold a small plastic toy in front of the infant and move it in a side-to-side motion to examine visual orientation and following (Items 9 and 10). After that was done, the infant was held horizontally above the counter and moved quickly toward the countertop to elicit the placing response of feet and hands (Item 11). The infant's arms and legs were placed around the underside of a rolled-up diaper held horizontally to elicit a clasping response (Item 12). Finally, the infant was placed on its back to elicit the righting response (Item 13). After the infant was returned to its cage, the tester evaluated the overall behavior state of the infant during testing (Item 14) and assessed the overall irritability and consolability of the infant (Items 15 and 16). Neonatal assessments took place every other day during the first two weeks of life at approximately 9:00 a.m., halfway between the regular 2-hour feedings.

Table 5. Neonatal Behavioral Scale

Response	Points
Item 1: Behavioral State Prior to Testing	
Asleep	0
Passive (awake, but inactive)	1
Alert (awake and aware)	2[a]
Alert, but somewhat agitated	3
Extremely agitated (body jerk, screeching)	4
Item 2: Asymmetry in Resting Position	
Yes (limbs not symmetrically placed)	0
No (limbs symmetrically placed)	1[a]
Item 3: Grasping	
No grasp	0
Partial (digits not closed completely)	1
Weak (digits closed but loosely)	2
Strong (digits close tightly)	3[a]
Item 4: Resistance to Passive Movement	
Floppy, no resistance	0
Slight (doesn't pull back limb)	1
Some (pulls limb back sluggishly)	2
Resistant (pulls limb back quickly)	3[a]
Rigid (difficult to move limb)	4
Very rigid (can barely move limb)	5
Item 5: Rooting	
No rooting	0
Partial (doesn't move completely)	1
Weak (slow and/or intermittent move)	2
Strong (quick, vigorous move)	3[a]
Item 6: Snout	
None	0
Partial (mouth not open)	1
Slow (opens mouth), but sluggish	2[a]
Quick (full response, opens mouth)	3[a]
Item 7: Startle to Auditory Stimulus	
No startle	0
Ear twitch only	1
Eye blink	2[a]
Eye blink and ear twitch	3[a]
Eye blink and head jerk	4[a]
Whole body jerk	5[a]
Item 8: Orient to Auditory Stimulus	
No orient	0
Partial (looks to observer)	1
Full (looks in face of observer)	2[a]
Item 9: Orient to Visual Stimulus	
No orient	0
Head moves (difficult to observe contact)	1
Direct, brief contact	2[a]
Direct, prolonged contact	3[a]
Item 10: Follow Visual Stimulus	
No contact or following	0
Contact but no following	1
Starts to follow but stops	2[a]
Follows object completely	3[a]
Item 11: Placing Response of Limbs	
No placing or movement	0
Moves limbs (doesn't place)	1
Partial placing	2[a]
Slow (places limbs sluggishly)	3[a]
Quick (places limbs quickly)	4[a]
Item 12: Clasping	
No clasping (falls off diaper)	0
Loose (dangles somewhat)	1
Firm (held tightly against diaper)	2[a]
Climbs off	3
Item 13: Righting	
No movement	0
Thrashing, but no righting	1
Rolled onto side (no thrashing)	2
Thrashing and partial righting	3
Rights in > 15 seconds	4
Rights in 5–15 seconds	5
Rights in < 5 seconds	6[a]
Item 14: Behavioral State Predominant During Testing	
Asleep	0
Passive (awake, but inactive)	1
Alert (awake and aware)	2[a]
Alert, but somewhat agitated	3
Extremely agitated (body jerk, screeching)	4
Item 15: Irritability	
Drastic alterations in state	0
Some alterations in state	1[a]
No alteration in state	2[a]
Item 16: Consolability	
Cannot be consoled	0
Can be consoled, but with difficulty	1[a]
Easy to console	2[a]

[a] Optimal rating points for each item.

(Table continues next column)

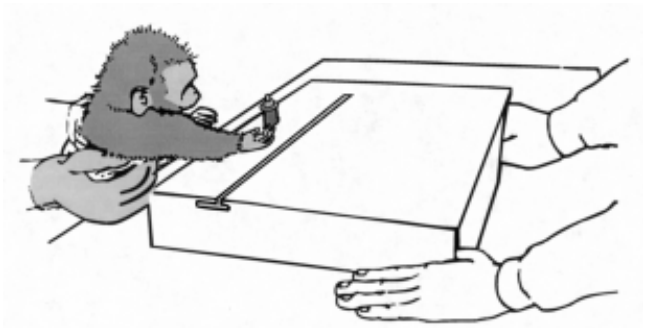

Figure 2. Visually Directed Reaching Test. Infant retrieving a small object with nipple attached to receive the applesauce reward.

Infant Sensorimotor Development

Infants were assessed using a Visually Directed Reaching Test to evaluate early sensorimotor development (for a detailed description of this test, see Burbacher et al. 1986). Briefly, infants were required to reach for and retrieve a small object in full view in order to receive an applesauce reward (see Figure 2). A small, brightly colored plastic toy with a rubber nipple attached to one end served as the stimulus. The nipple was placed in applesauce prior to each trial to provide the reward for the infant. Beginning at two weeks of age, infants were tested 4 days/week with 5 trials/day. Infants were held by one examiner while a second examiner began the trial by dipping the nipple end of the object in applesauce and allowing the infant to suck briefly on the nipple. The infant was then given a 15-second interval to visually orient to the object as it was placed within reach on a platform. A second 15-second interval followed to enable the infant to retrieve the object. Responses were scored as no orient, orient but no response, orient and reach but no retrieval, or orient, reach and retrieval. The testing criterion was achieved when the infant successfully retrieved the object on 8 of 10 consecutive trials over two testing sessions.

Sensorimotor responses were also evaluated over the first 9 months of life by observing infants in a 2.4 m × 1.9 m × 2.0 m playroom equipped with shelves, a ramp, toys, and a two-way observation window. Four separate shelves were located 48 to 96 cm above the playroom floor with an inclined wire-mesh ramp attached to one of the shelves (see Figure 3). Infants were observed 5 days/week, and the ages that they first successfully climbed up and down the ramp and climbed or jumped on and off the four separate shelves were recorded.

Infant Visual Acuity Development

A forced-choice preferential looking technique adapted from human infancy research (Dobson et al. 1986) was used to evaluate the development of visual acuity over the first 12 weeks of life (see Figure 4). The Preferential Looking Test relies on the strong visual preference for a pattern versus a gray field that is shown by human and nonhuman primate infants (Teller 1979, 1981). Acuity is determined by showing infants various black and white gratings (stripes) paired with a gray field of equal intensity. A tester must observe the visual orientation responses of the infant and indicate the location (left or right) of the stripes. If the infant is able to see the stripes, the observer will be able to determine the location of the stripes above the level indicated by random chance (> 75%). By varying the width of the stripes, patterns related to visual acuity from 0.5 to 28 cycles/degree (20/1400 to 20/23 Snellen) were tested over a 12-week period. Infants were tested at

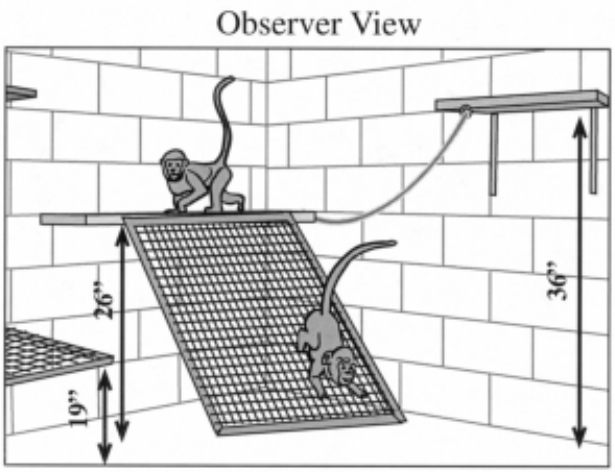

Figure 3. Playroom with ramp and shelves for assessment of social behaviors and motor milestones of infant monkeys.

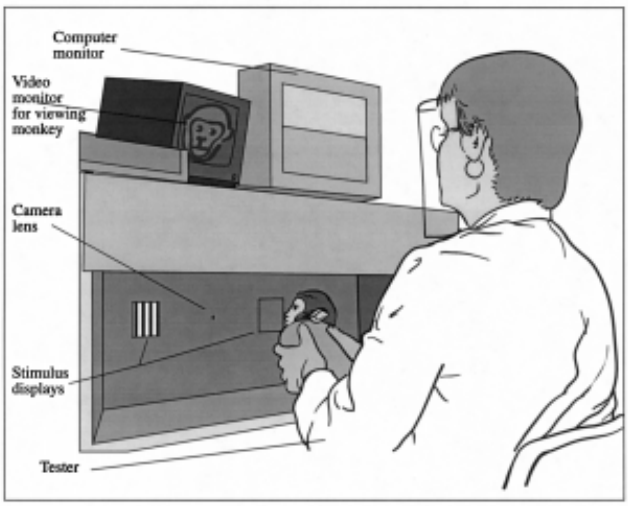

Figure 4. Preferential looking apparatus and example of stimuli for assessment of visual acuity. Visual fixations to the left and right stimuli are recorded via foot switches.

1, 4, 8, and 12 weeks of age. For each test, 5 different patterns increasing in difficulty were presented 30 times each (for a total of 150 trials) to determine a threshold acuity (75% correct) at each age. For all tests, the largest stripe pattern was considered the control stimulus. Performance on the control stripe had to exceed 83% correct for the results of the test to be considered reliable. If performance did not exceed 83% correct for the control stripe, infants were retested at the earliest opportunity. Infants were tested a maximum of 3 times at each age.

Infant Spatial Memory

According to Piaget (1954), object permanence develops through a sequence of six stages during the sensorimotor period and forms the basis for future cognitive development. Object permanence first emerges in the human infant during Stage 4 of the sensorimotor period, when the infant can successfully retrieve a fully hidden object. Object permanence, which develops in parallel stages in both human and macaque monkey infants, is considered a measure of early spatial memory (Mishkin and Appenzeller 1987; Diamond and Goldman-Rakic 1989). For this study, development of object permanence was assessed according to procedures described for human infants by Piaget (1954) and adapted for monkeys by Burbacher and colleagues (1986).

Performance of the Object Concept Test began after infants reached the criterion on the Visually Directed Reaching Test. A series of no-hiding, partial-hiding, and full-hiding tasks was presented using either a screen (2 days/week) or a well (2 days/week) to hide the object (see Figure 5a,b). The hiding tasks consisted of a 15-second orientation period and a 15-second response period. The orientation period was identical to that in the Visually Directed Reaching Test. During the response period of the Object Concept Test, however, the object was placed in front of the infant and was either (1) not hidden from view, (2) partially hidden from view, or (3) fully hidden from view. For the screen test, the object was placed on a platform and an opaque screen was moved horizontally either in back of the object (no hiding), half-way in front of the object (partial hiding), or completely in front of the object (full hiding). For the well test, the object was placed in a shallow well, and an opaque lid was placed either in back of the well (no hiding), half-way over the well (partial hiding), or completely over the well (full hiding). All hiding motions took place while the infant was visually orienting to the object, and 15 trials, 5/hiding condition, were presented in random order on each testing day. Infants were required to grasp and retrieve the object on 8 of 10 consecutive trials to reach criterion on each hiding condition. All hiding tasks were presented until criterion was reached on the full-hiding task. The A-not-B test began after infants reached criterion on the full-hiding condition of the screen and well tests. For the A-not-B test, a series of full hidings were presented using a 2-well presentation board (see Figure 5c). After the infant retrieved the object from 1 of the wells on 2 trials (either the right or the left), the object was hidden in the opposite well in full view of the infant. The tester then tapped on the top of the well in which the object had been previously hidden, and the infant was given 15 seconds to retrieve the object. Infants were required to grasp and retrieve the object from the opposite well for a successful trial. A total of 5 trials were presented/day, 5 days/week, and criterion was successful retrieval of the object on 8 of 10 consecutive trials.

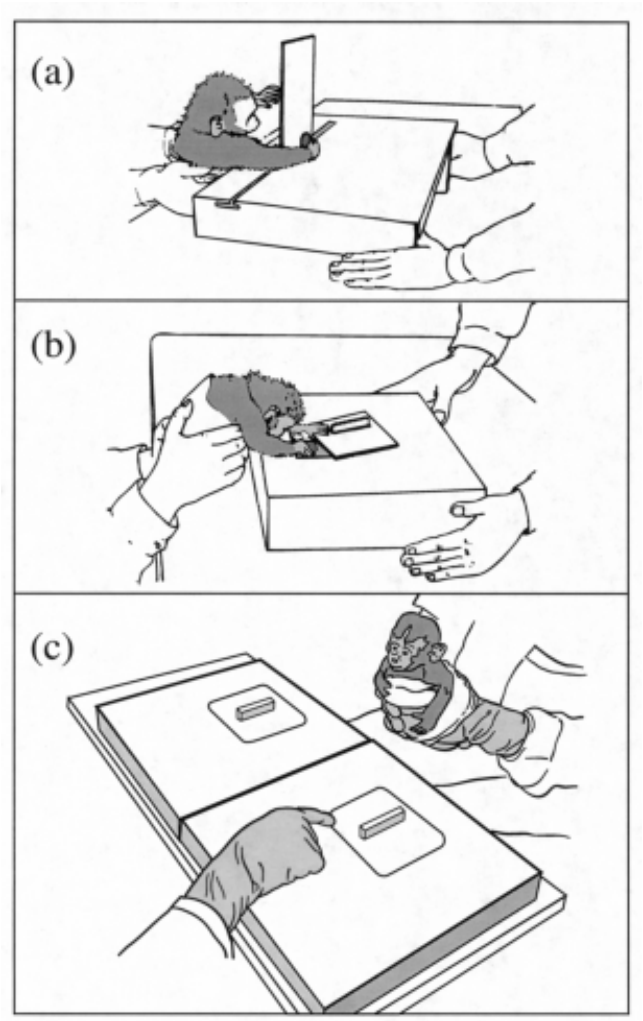

Figure 5. Object Concept Test: (a) screen task, (b) well task, and (c) A-not-B task.

Spatial learning and memory were also assessed with a Spatial Discrimination and Reversal Test (Schantz et al. 1989) that used the Wisconsin General Testing Apparatus (WGTA). The WGTA has a long history of use in research on learning and memory in nonhuman primates (Harlow and Bromer 1938; Harlow et al. 1960). For the WGTA tests used in this study, the infant was placed in a testing cage equipped with an opaque door that could be raised and lowered to allow access to a presentation board located in front of the cage. The presentation board for the Spatial Discrimination and Reversal Test had 2 shallow wells that could hold a food reward, 1 on the left side and 1 on the right side (see Figure 6). The tester, sitting behind a screen with the cage door lowered, placed a food reward in either the left or right well on the presentation board. Then the tester covered the wells, one with a large blue block and the other with a small red block. Different block sizes and colors were used to increase the difficulty of the test through inclusion of irrelevant cues. The tester raised the door to allow access to the presentation board. The infant was given 60 seconds to push 1 of the blocks off the well. If the side with the reward was chosen (that is, the correct response), the monkey retrieved the food reward, and the door was lowered for the next trial. If the side without the reward was chosen (the incorrect response), no reward was provided, and the door was lowered for the next trial. If the monkey did not respond during the 60-second period (a balk), the trial was terminated by lowering the door. A total of 25 trials were run/day, 5 days/week, with the location of the reward (left or right well) held constant. Criterion for the Spatial Discrimination Test was 23 correct responses out of the 25 daily trials. After reaching criterion on the Spatial Discrimination Test, the Spatial Reversal Test began. The procedure for the Reversal Test was the same as described above, with the exception that the reward was placed in the opposite well than the one used for the Discrimination Test. Criterion for the Spatial Reversal Test was 9 correct responses out of 10 trials. After reaching criterion on the initial reversal, the location of the reward was changed back to the original position for subsequent trials. Trials continued, using this revolving pattern of reward placement, for a total of 6 reversals. Infants were first tested at approximately 5 months of age.

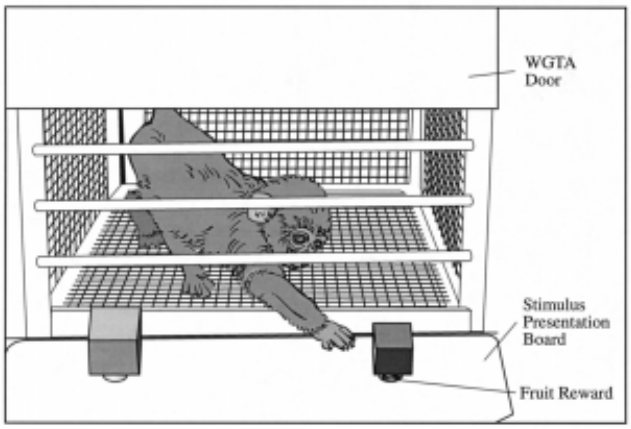

Figure 6. WGTA apparatus and example of stimulus presentation board for use in the Spatial Discrimination and Reversal Test. Different size and color stimuli were included as irrelevant cues.

Infant Recognition Memory

The Fagan Test of Infant Intelligence (Copyright 1981 by J. F. Fagan III) is a visual-recognition memory test based on the familiarization-novelty paradigm described by Fagan and Singer (1983) and adapted for monkeys by Gunderson and Sackett (1984). This paradigm makes use of the infant's proclivity to direct more visual attention to novel rather than to familiar stimuli. Recognition memory is inferred from performance on the novelty paradigm because some aspects of the familiar stimulus must be encoded in memory for the novelty response to occur. The ability to recognize previously seen stimuli from those that are new during infancy is substantially correlated in human children with performance on standardized intelligence tests (Rose and Wallace 1985). Using the preferential looking apparatus shown in Figure 4, testers presented infants with 11 different sets of problems over 3 sessions. Problems using abstract patterns for stimuli were presented on postconception days 190 (3 problems), 200 (2 problems), and 210 (2 problems). Problems using monkey faces as stimuli were presented on postconception days 200 (2 problems) and 210 (2 problems). The problems consisted of age-graded stimulus pairs so that the easiest (that is, the least complex, most dissimilar) were administered at the youngest age, and the most difficult (that is, most complex, most similar) were administered at the oldest age. All stimuli were presented in the form of 35-mm slides (for examples of stimuli, see Figure 7). For each problem, the monkey was initially presented with 2 identical patterns or faces during a familiarization period. Looking time toward the left and right stimuli was recorded until a predetermined amount of total looking time was reached (16 to 60 seconds, depending on the particular problem). The monkey was then given an immediate 2-part test trial in which the stimulus used during the familiarization period was paired with a new (or novel) stimulus. The positioning of the familiar and novel stimuli was reversed for the second part of the trial in order to control for side preferences. Looking times toward the familiar and the novel stimuli were recorded for a 10- to 20-second period, depending on the particular problem. Looking times were recorded from the first

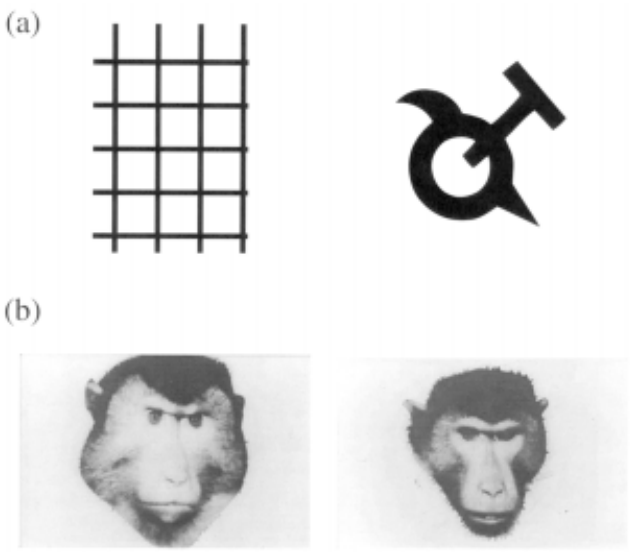

Figure 7. Example of test stimuli for assessment of recognition memory: (a) abstract patterns, and (b) social stimuli.

visual fixation to either stimulus. For each problem, the total looking time over the 2 trials was calculated for the familiar and the novel stimuli, as well as the duration of each look. The amount of time required to reach the predetermined looking time during familiarization was also calculated for each problem.

The Nonmatch-to-Sample Test requires a conceptually based solution and provides a measure of complex learning abilities (King and Fobes 1982). The trial-unique procedure used in this study required that the infant solve the task by learning the concept of nonmatching (that is, response to the object that is different than the one just seen). Monkeys were tested using the WGTA apparatus. The 3-well presentation board for the Nonmatch-to-Sample Test had one shallow well on the left side, one on the right side, and one in the middle, all of which could hold a food reward. To begin a trial, the tester placed a food reward in the left or right well. A sample object was then placed over the middle well and 2 choice objects, 1 identical to the sample object and 1 different, were placed over the left and right wells. The choice object that was different from the sample object (the nonmatch) was always placed over the well with the food reward. The tester then raised the first door, which provided access to the sample object (see Figure 8a). Infants were given 60 seconds to push the sample object off the middle well. After the infant displaced the sample object, the tester removed it from the board and raised a second door to present the 2 choice stimuli (see Figure 8b). If the nonmatching object was chosen (the correct response), the infant retrieved the food reward, and the door was lowered for the next trial. If the matching object was chosen (the incorrect response), no reward was provided, and the door was lowered for the next trial. If the monkey did not respond during the 60-second period (a balk), the trial was terminated by lowering the door. A total of 25 trials were run/day, 5 days/week. Criterion for the Nonmatch-to-Sample Test was 23 correct responses of the 25 daily trials. After reaching criterion on the Nonmatch-to-Sample Test, delay periods were introduced between the initial stimulus presentation and the choice presentation. Five delay periods (0, 10, 20, 40, and 60 seconds) were randomly presented 5 times/day for a total of 15 days. Infants were tested on the Nonmatch-to-Sample Test at approximately 8 to 9 months of age.

Infant Social Behavioral Development

Social behaviors in mixed-sex play groups were coded in real time by a trained observer 3 days/week throughout the study. Infants were placed in a playroom (see Figure 3) for 30 minutes, and each infant's behaviors were scored for 5 minutes using a 4-digit mutually exclusive coding system (Burbacher et al. 1990b, see Table 6). The code numbers (1) identify individual animals involved in interactions with the focal infant being scored; (2) measure major classes of behavior such as play, fear, aggression, grooming, and exploration; (3) identify infants that are initiators of interactions; and (4) identify objects and parts of the playroom environment involved in each behavior. The sequence of focal observations was randomized across infants, and the behaviors of other infants in the group were coded only during interactions with the focal infant. Behaviors were recorded whenever a change occurred in any aspect of the 4 scoring dimensions.

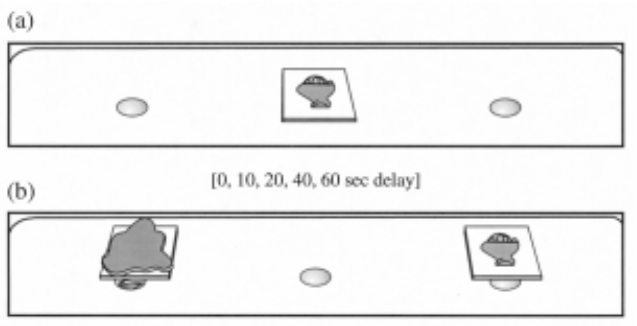

Figure 8. Example of the 3-well stimulus presentation board used in the Nonmatch-to-Sample Test. Infants were required to displace a sample object (a) prior to presentation of the match and nonmatch choice objects and (b) after presentation of the choice objects (which were presented immediately or 10, 20, 40, or 60 seconds after the sample object was displaced).

Infant Physical Growth

Physical growth was assessed during the first 9 months of life by recording the weights of all infants on a daily basis (until 147 postnatal days of age) and then weekly, and by collecting anthropometric measures every other week (until 84 postnatal days of age) and then monthly. Procedures to evaluate weight, crown–rump length, and head size were identical to those described as part of assessment at birth. Finally, infants were examined for the eruption of deciduous teeth. Infants were observed on a daily basis until all incisors and canines had erupted. The first molar eruption was detected by weekly observations, which were followed by daily observations until the remaining molars had erupted.

Quality Control Procedures for Offspring Assessment

The quality control program for offspring assessment included standard procedures for determining reliability across all testers, weekly checks of all completed data forms for detecting errors and omissions, a two-step data editing procedure, and frequent graphical summaries of the data for detecting outliers. The most important aspect of our quality control procedures was the reliability testing. The tests were performed before Cohort 1 offspring were delivered and again just prior to the delivery of Cohort 2 offspring. For these tests, one coinvestigator on the project (KG), who was blind to the exposure history of the females, conducted an initial reliability test with a predesignated primary tester. When the primary tester demonstrated reliability in testing (a score of 90% or above), he/she then conducted tests with other individuals who would routinely perform the procedures. After scoring 90% or above on the reliability tests, these individuals were then eligible to test the offspring. Typically, the testers who routinely performed the procedures were blind to the exposure history of the mothers.

Table 6. Four-Digit, Four-Dimensional Coding System for Studying Social Interactions of Focal Infant Tested in a Mixed-Sex Infant Group (Five-Minute Trials)

	Digit Position in Test Result Code[a]			
Code Number	1 Role of Focal Individual	2 Behavior of Focal Individual	3 Interaction Behavior	4 Subject ID Direction
0	Nonsocial	Passive	Passive	Nonsocial
1	Initiate with physical contact	Explore	Explore	Monkey 1[b]
2	Initiate with no physical contact	Withdraw	Withdraw	Monkey 2
3	Reciprocate with physical contact	Disturbance, fear	Disturbance, fear	Monkey 3
4	Reciprocate with no physical contact	Rock, huddle, self-clasp	Rock, huddle, self-clasp	Monkey 4
5	Ignore with physical contact	Stereotypical	Stereotypical	Self
6	Ignore with no physical contact	Play	Play	Toy
7		Sex	Sex	Ladder, shelf
8		Threat, aggression	Threat, aggression	Window
9			No response[c]	

[a] A test result code of 0037 would represent a nonsocial, passive infant that showed signs of disturbance or fear while located on the ladder or shelf of the playroom.

[b] The monkeys in the group are arbitrarily labeled with subject ID codes 1 through 4.

[c] The no-response category for the third-digit, Interaction Behavior (Code 9) refers to social interactions initiated by the focal subject that produce no change in the potential interaction from that occurring before the initiation (adapted from Burbacher et al. 1990).

STATISTICAL ANALYSES

Raw data were transferred as ASCII files from the study database to computers in the Departments of Environmental Health and Biostatistics at the University of Washington. The statistical analyses were performed using Systat, SAS, or Splus. The individual statistical models were chosen based on the nature of the data sets. For each of the 10 specified domains of infant development, the general approach was first to assess whether there was an exposure effect globally, and then to assess whether there was one specifically on the primary-outcome variable(s) (see Table 3). A global *F* test (or equivalent) was used for assessing whether there were detectable differences in mean values among the 4 methanol-exposed groups. Because this test has less power than specific alternatives, linear contrast tests based on the analysis of variance (ANOVA) models were used to compare the control group to all methanol-exposure groups combined and then to each of the individual methanol-exposure groups. The impact of controlling for sex and cohort of the offspring was assessed in separate mean models. These models are reported, however, only if there was a change in the results pertaining to methanol exposure. For some outcomes, the impact of controlling for a covariate was assessed in mean models (namely, length of gestation for the Visually Directed Reaching Test). These models are also reported only if there was a change in the results pertaining to methanol exposure. Similar analyses were then performed on the secondary-outcome variables. No correction was used for multiple testing.

One-way ANOVA models were used to analyze the primary-outcome variables for birth size of offspring, neonatal responses, infant sensorimotor development, spatial and recognition memory, and physical growth. Repeated measures ANOVA models were used to examine infant social behavioral development and secondary-outcome variables from the Spatial Memory and Nonmatch-to-Sample Tests. For the repeated measures ANOVAs, the focus of the global and specific models was on assessing the interaction of methanol exposure and the repeated measures factor (namely, age, delays). Fisher's exact tests were used to examine the discrete outcome measures (frequency of use of oxygen therapy at birth, total score on the Newborn Health Assessment). Only descriptive statistics are provided for the visual acuity assessment due to the small number of infants that completed the test over the 12-week testing period.

Goodness-of-fit of all linear models was assessed through examination of residuals. Theoretically, for inference in ANOVA the residuals are assumed to be random observations from a normal distribution with fixed variance. Whenever the empirical distribution of the residuals was too skewed to be considered normal, a log transformation was used. Individual observations for which a model did not seem to fit well, or that were considered potentially influential, were removed from the model and the model was refit without them. In no case were there impacts on the conclusions due to these removals; thus, only the results from the original fit (done to the full data set) are reported.

RESULTS

MATERNAL METHANOL EXPOSURE

Results of the daily methanol exposures during pregnancy are shown in Figure 9. The mean methanol concentration shown for each female for each day was calculated by using the 11 methanol samples taken from 14 minutes after the onset of methanol flow until 6 minutes prior to the end of methanol flow (see Part I for details). The average daily chamber methanol concentrations during pregnancy were stable and within 10% of the target concentrations.

BLOOD METHANOL AND FORMATE ANALYSES

The mean blood methanol concentrations during pregnancy were approximately 3 µg/mL for the controls, 5.5 µg/mL for the 200 ppm–exposure group, 11 µg/mL for the 600 ppm–exposure group, and 35 µg/mL for the 1,800 ppm–exposure group (see Figure 10). Plasma formate concentrations remained low during pregnancy, with values between 0.02 and 0.76 mM. The mean plasma formate concentrations for all the females in the exposure groups were below 0.27 mM during pregnancy (see Figure 11).

OFFSPRING ASSESSMENTS

A total of 37 infants were delivered from the 46 females, with 2 infants delivered stillborn (1 in the control group and 1 in the 600 ppm–exposure group) and 1 fetal death in utero (in the 1,800 ppm–exposure group). Of the remaining 34 live-birth deliveries, 5 of the methanol-exposed infants (2 in the 200 ppm–exposure group, 2 in the 600 ppm–exposure group, and 1 in the 1,800 ppm–exposure group) were delivered via C-section due to vaginal bleeding (4 infants) or nonproductive labor (1 infant). The bleeding was presumed to be due to premature placental detachment.

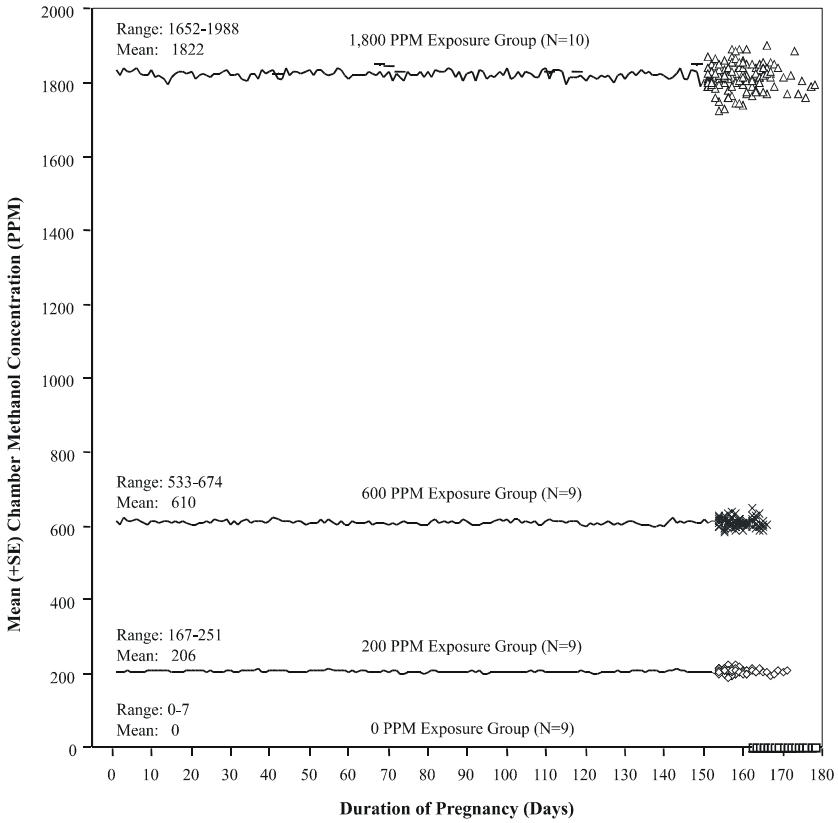

Figure 9. Mean (± SE) methanol concentrations in chambers during pregnancy. Scatterplots shown when entire group not represented due to delivery of offspring.

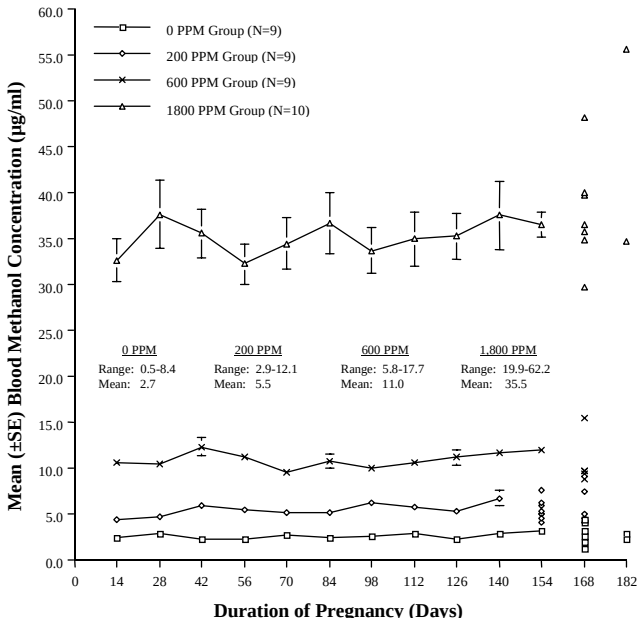

Figure 10. Mean (± SE) maternal blood methanol concentrations 30 minutes after exposure during pregnancy. Scatterplots shown when entire group not represented due to delivery of offspring.

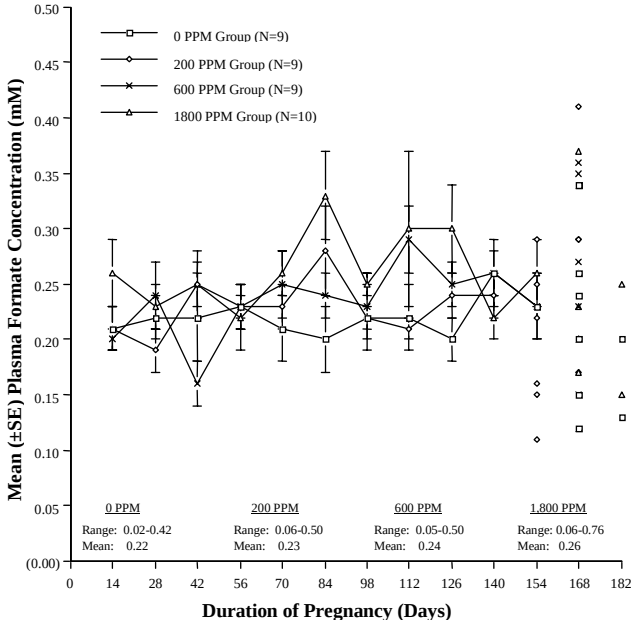

Figure 11. Mean (± SE) maternal plasma formate concentrations 30 minutes after exposure during pregnancy. Scatterplots shown when entire group not represented due to delivery of offspring.

Table 7. Size of Live-Born Offspring at Birth

	Newborn Characteristic[a]				
Exposure Group	Birth Weight (g)	Crown–Rump Length (mm)	Head Circumference (mm)	Head Length (mm)	Head Width (mm)
Control (n = 8)	369 ± 14 (295–425)	180 ± 3 (167–192)	182 ± 1 (177–186)	63 ± 1 (60–64)	49 ± 0 (47–50)
200 ppm (n = 9)	344 ± 14 (290–420)	175 ± 3 (165–188)	179 ± 1 (174–183)	62 ± 1 (59–65)	48 ± 1 (45–50)
600 ppm (n = 8)	368 ± 025 (280–475)	176 ± 4 (165–196)	180 ± 3 (170–191)	62 ± 1 (58–67)	48 ± 1 (45–51)
1,800 ppm (n = 9)	369 ± 21 (260–465)	177 ± 3 (158–189)	181 ± 2 (170–188)	63 ± 1 (59–65)	48 ± 1 (45–51)

[a] Each value is presented as mean ± SE (range).

Offspring Size at Birth

The primary-outcome variable used to assess the effects of methanol exposure on offspring size at birth was birth weight. Because the infants delivered via C-section were not considered outliers, all 34 live-born infants were included in the statistics. The average birth weights of the control group and the 3 methanol-exposure groups were very similar (ranging from 344 g to 369 g, see Table 7). Results of the ANOVA and linear contrast tests did not indicate a significant difference in birth weight due to methanol exposure ($p > 0.37$, see Table A.1).

Similar analyses of the other birth characteristics (crown–rump length, head circumference, head length, and head width, see Table 7) were consistent with the birth-weight results and did not indicate a methanol-exposure effect ($p > 0.74$, see Tables A.2 through A.5).

Offspring Medical Treatments

Several of the methanol-exposed infants required medical treatment at or soon after birth (see Table 8). Of the 5 infants delivered by C-section, 4 exhibited signs of respiratory distress, cardiac depression, or both, at birth and were given oxygen. One infant in the 1,800 ppm–exposure group exhibited signs of prematurity (irregular respiration and temperature, feeding difficulties) and also received oxygen. One vaginally delivered infant in the control group exhibited signs of postmaturity, including meconium staining and hyperemia. This infant, however, did not require treatment. Fisher's exact tests were used to examine whether or not the frequency of oxygen treatment varied by presence or level of methanol exposure. To this end, the distribution of oxygen treatments was examined across the 4 methanol-exposure groups and then again across the controls and all exposed offspring (see Table A.6). The results of the Fisher's exact tests did not indicate a significant difference in distribution of oxygen treatment across the 4 methanol-exposure groups ($p = 0.65$) or between control and all methanol-exposed offspring ($p = 0.24$).

To examine the potential impact of illness, medications, or both on the performance of offspring on the various assessment procedures, the total number of days that the offspring were medicated during each testing period was examined. ANOVA models that compared the number of days medicated across the 4 methanol-exposure groups indicated a difference for only the Object Concept Test ($p = 0.02$). Therefore, the number of days medicated was used as a covariate in ANOVA models for this test procedure.

Table 8. Offspring Requiring Oxygen Therapy At or Soon After Birth

Exposure Group	Cohort	Sex	Age (Days)	Condition
200 ppm	2	Female	Birth–3	C-section delivery
200 ppm	2	Male	Birth–2	C-section delivery
600 ppm	1	Male	Birth	C-section delivery
600 ppm	2	Female	Birth	C-section delivery
1,800 ppm	1	Female	3–11	Premature delivery

Table 9. Distribution of Total Scores for Initial Newborn Health Assessment

Exposure Group	Total Score on Newborn Assessment[a]				
	8	9	10	11	12
Initial Assessment					
Control (n = 6)	0 (0)	0 (0)	0 (0)	1 (17)	5 (83)
200 ppm (n = 7)	0 (0)	0 (0)	2 (29)	4 (57)	1 (14)
600 ppm (n = 7)	1 (14)	0 (0)	0 (0)	3 (43)	3 (43)
1,800 ppm (n = 7)	0 (0)	0 (0)	1 (14)	3 (43)	3 (43)
Follow-Up Assessment					
Control (n = 7)	0 (0)	0 (0)	2 (29)	4 (57)	1 (14)
200 ppm (n = 7)	0 (0)	0 (0)	1 (14)	5 (71)	1 (14)
600 ppm (n = 8)	1 (12)	0 (0)	2 (25)	5 (62)	0 (0)
1,800 ppm (n = 7)	1 (14)	0 (0)	2 (28)	3 (42)	1 (14)

[a] Values are numbers of animals with this score (percentage of all animals).

Newborn Health Status

The primary-outcome variable used to assess the effects of methanol exposure on newborn health status was the total score that infants received on the initial Newborn Health Assessment. Each of the 6 items on the Newborn Health Assessment was scored with 0 to 2 points using a previously defined scoring system (see Table 4). The scores for the 6 items were added together, and the total score (0–12) was used in the analyses. Of the 34 live-born infants, 7 were not tested on the initial Newborn Health Assessment. Of these, 4 infants were not tested because they were delivered via C-section, and the tester was not available at time of surgery. Three additional infants were not tested due to equipment or tester error. The results of the Newborn Health Assessments are shown in Table 9. The health status of the majority of newborns was good. Nearly all of the infants tested received a total score of 10 or greater on the initial Assessment. Fisher's exact tests were used to examine whether or not the distribution of total scores for the Newborn Health Assessment varied due to methanol exposure. The Fisher's exact test that compared the distribution of the total scores across the 4 methanol-exposure groups did not indicate a significant difference due to methanol exposure for the initial Assessment (p = 0.25, see Table A.7).

Results for the follow-up Newborn Health Assessment were similar. A total of 5 infants were not tested on the follow-up Assessment due to equipment or tester error. A Fisher's exact test that compared the distribution of the total scores across the 4 methanol-exposure groups did not show a significant difference due to methanol exposure for the follow-up Assessment (p = 0.97, see Table A.8).

Neonatal Behavioral Responses

The primary-outcome variable used to assess the effects of methanol exposure on neonatal behavioral responses was the age at which infants received their optimal scores on items from the Neonatal Behavioral Scale (see Table 5). Optimal point scores were defined for each of the items included in the Scale, and the age at which each infant exhibited optimal scores was recorded. The 9 reflex- and 7 behavioral-response items were used to develop 4 independent factors in a manner similar to that used in previous studies (Streissguth et al. 1983; Jacobson et al. 1984). The 4 factors developed for this study were Behavioral State,

Table 10. Distribution of Test Items for Neonatal Behavioral Scale

Factor	Test Items			
Behavioral state	Prior state	Irritability	Consolability	Predominant state
Reflexes	Rooting	Righting	Snout	Placing
Muscle tone	Asymmetry	Resistance	Grasping	Clasping
Responsivity	Startle auditory	Orient auditory	Orient visual	Follow visual

Table 11. Results of Neonatal Behavioral Scale

Exposure Group	Factors[a]			
	Behavioral State	Reflexes	Muscle Tone	Responsivity
Control (n = 8)	1.8 ± 0.2 (1.0–3.0)	2.8 ± 0.9 (1.4–5.8)	1.0 ± 0.0 (1.0–1.0)	1.8 ± 0.2 (1.0–2.5)
200 ppm (n = 9)	2.3 ± 0.2 (1.5–3.5)	2.4 ± 0.3 (1.4–3.8)	1.2 ± 0.2 (1.0–2.5)	2.6 ± 0.4 (1.0–5.0)
600 ppm (n = 8)	3.3 ± 0.5 (1.5–5.3)	1.8 ± 0.2 (1.0–2.6)	1.3 ± 0.2 (1.0–2.0)	2.6 ± 0.4 (1.0–1.8)
1,800 ppm (n = 9)	2.6 ± 0.4 (1.0–2.5)	2.4 ± 0.4 (1.0–5.0)	1.1 ± 0.1 (1.0–5.0)	1.8 ± 0.4 (1.0–3.0)

[a] Each value is mean ± SE (range) postnatal age (in days) at optimal score.

Reflexes, Muscle Tone, and Responsivity (see Table 10). Of the items included in the assessment, 3 (suck, response strength, response speed) were not used to develop factors because several infants did not receive optimal scores on these items during the 2 weeks of testing. Mean ages for attaining optimal scores were calculated for each of the 4 factors using the results from each of the items included in the individual factors.

A summary of the results for the 4 factors assessed with the Neonatal Behavioral Scale is shown in Table 11. For the analyses, the logs of the mean ages to attain optimal scores were used due to asymmetric distributions. The results of the ANOVA models, which compared the logs of mean age across the 4 methanol-exposure groups, did not indicate a significant effect of methanol exposure for any of the 4 factors ($p > 0.08$, see Tables A.9 through A.12). For the Behavioral State, however, linear contrast tests based on these models indicated a significant difference when control infants were compared with all methanol-exposed offspring combined ($p = 0.03$), as well as when controls were compared with the infants in the 600 ppm–exposure group ($p = 0.01$). The mean age to attain the optimal score for the Behavioral State Factor for the control infants was 1.8 days, as compared with 2.3 to 3.3 days for the infants in the 3 methanol-exposed groups.

Infant Sensorimotor Development

The primary-outcome variable used to assess the effects of methanol exposure on infant sensorimotor responses was the age at which infants reached criterion on the Visu-

Table 12. Results of Visually Directed Reaching Test

Exposure Group	Infant Groups[a]		
	All	Males	Females
Control (n = 8)	30.3 ± 2.7 (17–39)	23.7 ± 4.8 (17–33) (n = 3)	34.2 ± 1.8 (28–39) (n = 5)
200 ppm (n = 9)	32.7 ± 2.5 (24–45)	32.4 ± 4.1 (24–45) (n = 5)	33.0 ± 2.9 (27–41) (n = 4)
600 ppm (n = 8)	33.3 ± 4.1 (21–58)	42.7 ± 8.0 (31–58) (n = 3)	27.6 ± 2.7 (21–36) (n = 5)
1,800 ppm (n = 9)	40.1 ± 3.7 (28–57)	40.5 ± 12.5 (28–53) (n = 2)	40.0 ± 4.0 (32–57) (n = 7)

[a] Each value is mean ± SE (range) postnatal age (in days) to retrieve object.

Table 13. Results of Motor Milestones Observations in Playroom

	Infant Groups[a]		
Exposure Group	All	Males	Females
Control (n = 8)	126.3 ± 8.5 (90–154)	102.3 ± 7.2 (90–115) (n = 3)	140.8 ± 6.7 (117–154) (n = 5)
200 ppm (n = 9)	101.1 ± 6.3 (75–131)	101.4 ± 7.7 (81–121) (n = 5)	100.8 ± 11.7 (75–131) (n = 4)
600 ppm (n = 8)	105.4 ± 11.3 (63–144)	137.0 ± 6.5 (124–144) (n = 3)	92.4 ± 9.9 (63–116) (n = 5)
1,800 ppm (n = 9)	110.4 ± 10.3 (76–155)	81.5 ± 5.5 (76–87) (n = 2)	120.6 ± 10.2 (82–155) (n = 7)

[a] Each value is mean ± SE (range) postnatal age (in days) to climb or jump on ramp and all four shelves in playroom (see Figure 3).

ally Directed Reaching Test. In general, the age at achievement of criterion on the Visually Directed Reaching Test increased with the increasing level of methanol exposure (Table 12). For the analyses, the log of the age at which the infant reached criterion (that is, successfully retrieved the object on 8 of 10 consecutive trials over 2 days of testing) was used due to asymmetric distributions. The results of the ANOVA model that compared the log of age at criterion across the 4 methanol-exposure groups did not indicate a significant effect due to methanol exposure (p = 0.20, see Table A.13). Linear contrast tests based on this ANOVA, however, indicated a significant difference between control infants compared with infants of the 1,800 ppm–exposure group (p = 0.04). A post-hoc test for linearity of scores across the 4 methanol-exposure groups was also significant (p = 0.04). The ANOVA model controlling for sex of offspring indicated a significant interaction between methanol-exposure group and sex of offspring (p = 0.03, see Table A.14). Sex-specific linear contrast tests based on this ANOVA indicated a significant difference between control infants compared with all methanol-exposed offspring combined, as well as with the 600 ppm– and the 1,800 ppm–exposure groups for males ($p < 0.04$). The comparison between the control group and the 200 ppm–exposure group was nearly significant (p = 0.09). No significant differences were obtained for female infants ($p > 0.15$). Since the control infants had significantly longer gestation lengths than the methanol-exposed infants, a covariate ANOVA model controlling for gestation length at birth was run (see Tables A.15 and A.16). Significant differences for male offspring were still obtained. The mean postnatal age at criterion on the Visually Directed Reaching Test for the control male infants was approximately 24 days, compared with 32, 43, and 41 days for males in the methanol-exposed groups. If one calculates the mean postconception age at criterion, the controls and the 200 ppm–exposure group are identical (192 days). The postconception age at criterion for the 600 ppm– and 1,800 ppm–exposure groups, however, are still nearly 2 weeks later, 205 and 203 days, respectively.

The results of the secondary-outcome variable, observations of motor milestones in the playroom, did not indicate a methanol-exposure effect on gross sensorimotor responses. Data from observations were summarized to examine potential differences in the ages of infants when they were able to use the ramp and all 4 of the shelves (see Table 13). Due to asymmetric distributions, the log of age was used in the analyses. The results of the ANOVA model that compared the log of age at criterion across the 4 methanol-exposure groups did not indicate a significant effect due to methanol exposure (p = 0.57, see Table A.17). The ANOVA model controlling for sex of offspring, however, indicated a significant interaction effect between methanol-exposure group and sex of offspring (p = 0.001, see Table A.18). Sex-specific linear contrast tests based on this ANOVA indicated a significant difference between control infants compared with all methanol-exposed offspring combined, as well as the female offspring of the 200 ppm– and 600 ppm–exposure groups ($p < 0.01$). Significant effects were also observed when control infants were compared with 600 ppm–exposure group male offspring (p = 0.02). Overall, the mean age at criterion for the control infants was approximately 126 days, compared

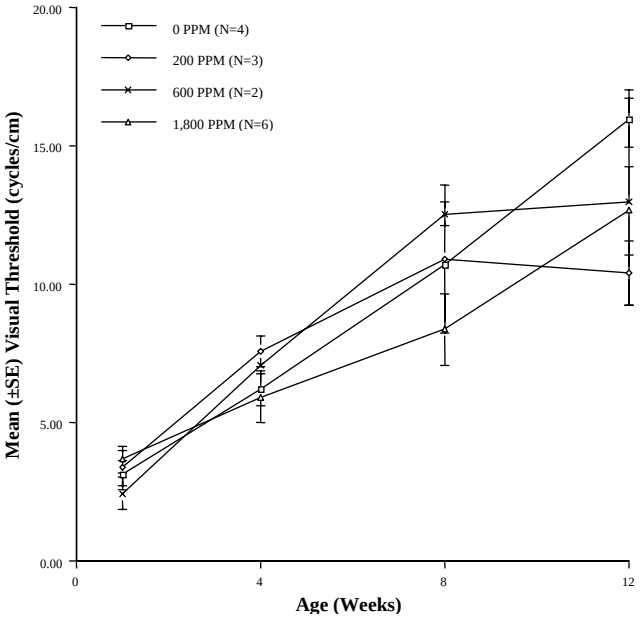

Figure 12. Summary of assessment of infant visual acuity. Graph includes only those infants that passed assessments at all 4 ages tested.

with 101 to 110 days for the infants of the methanol-exposed groups. For females, the mean age at criterion for the controls was approximately 141 days, compared with 92 to 121 days for the infants in the methanol-exposed groups.

Infant Visual Acuity Development

The results of the visual acuity assessment (Preferential Looking Test) are shown in Figure 12. Several tests that were run across the 12-week period were considered unreliable because the percentage of correct responses for the control stripe did not exceed 83%. Visual threshold values could not be calculated for 5 infants for the Week-1 test, 10 infants for the Week-4 test, 11 infants for the Week-8 test, and 13 infants for the Week-12 test. Only 15 of the 34 infants had visual threshold values for all ages. Because of the high failure rate of this test, no analyses were performed to evaluate methanol-exposure effects on visual acuity development. In general, the results indicate an improvement in visual acuity with increasing age for all exposure groups. However, there seems to be an apparent lack of improvement from 8 to 12 weeks of age for the methanol-exposed infants (see Figure 12).

Infant Spatial Memory

The primary-outcome variable used to assess the effects of methanol exposure on infant spatial memory was the age at which infants displayed object permanence on the screen task of the Object Concept Test. The age at which each infant achieved criterion (that is, successfully retrieved the object on 8 of 10 consecutive trials over 2 days of testing in the full-hiding condition) was recorded (see Table 14). The logs of ages at criterion were used in analyses due to asymmetric distributions. The results of the ANOVA models that compared the log of age at criterion across the 4 methanol-exposure groups did not indicate a significant effect of methanol exposure on the development of object permanence as assessed with the screen task ($p = 0.55$, see Table A.19). Similar analyses of the secondary-outcome variables from the Object Concept Test (ages at criterion on the full-hiding well and A-not-B tasks) were consistent with the results from the screen task and did not indicate a methanol-exposure effect ($p >$

Table 14. Results of Object Concept Test

	Object Concept Factors[a]		
Exposure Group	Screen	Wall	A-Not-B
Control ($n = 8$)	66.0 ± 7.1 (46–97)	87.9 ± 16.2 (138–158)	97.0 ± 15.8[b] (52–161)
200 ppm ($n = 9$)	64.1 ± 2.9 (47–75)	70.7 ± 10.4 (35–124)	88.2 ± 7.6 (63–129)
600 ppm ($n = 8$)	57.0 ± 8.8 (33–110)	64.8 ± 12.0 (31–129)	84.8 ± 10.3 (54–132)
1,800 ppm ($n = 9$)	63.7 ± 5.7 (40–85)	76.4 ± 8.5 (41–117)	96.3 ± 8.8 (54–135)

[a] Each value is mean ± SE (range) postnatal age (in days) to retrieve fully hidden object.

[b] For controls, $n = 7$, because one infant did not reach criterion on A-not-B test and is not included for this factor.

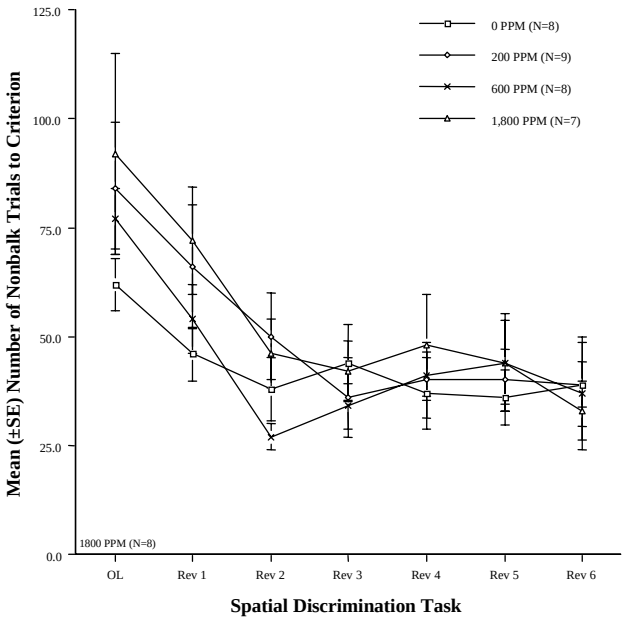

Figure 13. Results of Infant Spatial Discrimination and Reversal Test. One infant in the 1,800 ppm–exposure group did not reach criterion on original discrimination task and is not included in the original discrimination and reversal data. One additional infant in the 1,800 ppm–exposure group is not included in the reversal data due to tester error.

0.57, see Tables A.20 and A.21). Finally, preliminary analyses of the numbers of days medicated during Object Concept assessment did indicate a significant difference across the exposure groups for this measure. Thus, the number of days medicated was added to the above models; the results were consistent with those described above. Object permanence was displayed by the infants in all exposure groups by approximately 2 months of age.

The results of the other spatial memory test, the Spatial Discrimination and Reversal Test, were consistent with the results for the Object Concept Test; they did not indicate a methanol-exposure effect on spatial memory (see Figure 13). The number of nonbalk trials to reach criterion (23 correct responses of the 25 daily trials) on the Spatial Discrimination Test varied markedly within each exposure group. The range of nonbalk trials to reach criterion was 44 to 94 for the control infants, 25 to 160 for the infants in the 200 ppm–exposure group, 48 to 112 for the infants in the 600 ppm–exposure group, and 24 to 241 for the infants in the 1,800 ppm–exposure group. Data from 1 infant in the 1,800 ppm–exposure group were not included in the analysis due to tester error. For the analyses, the log of the number of nonbalk trials to reach criterion was used due to asymmetric distributions. The results of the ANOVA models did not indicate a significant effect of methanol exposure on the number of nonbalk trials to reach criterion ($p = 0.75$, see Table A.22).

Repeated measures ANOVA models were used to analyze the data from the Spatial Reversal Test. For these analyses, the log of the number of nonbalk trials to reach criterion (9 correct responses out of 10 daily trials) was used due to asymmetric distributions. ANOVA models excluded 2 infants from the 1,800 ppm–exposure group, the infant described previously and 1 infant that was dropped due to tester error on the Reversal Test. The results of the repeated measures ANOVA models did not indicate a significant effect of methanol exposure on the number of nonbalk trials to reach criterion over the 6 reversals ($p = 0.65$, see Tables A.23 and A.24). In general, the number of nonbalk trials to criterion for all of the exposure groups decreased on the second reversal but did not decrease on subsequent reversals (see Figure 13).

Infant Recognition Memory

Novelty scores, or the mean percentage of time looking at novel stimuli on the Fagan Test of Infant Intelligence, were used as the primary-outcome variable to assess the effects of methanol exposure on infant recognition memory (see Tables 15 and 16). Novelty scores for each infant were calculated across the 7 abstract problems and the 4 social problems tested. Using *t* tests, the novelty scores for each methanol-exposure group were compared with results expected due to random chance (50%) to indicate whether or not a significant novelty preference was observed for the group (Fagan and Singer 1983). Following these analyses, ANOVA models were used to examine whether exposure-group differences in novelty scores were observed (see Tables A.25 through A.29). Data from 1 infant in the control group were not used in the analyses due to tester error (that is, the infant was tested at a later age than the rest in the group).

Abstract Patterns Test The results of initial *t* tests for the abstract patterns problems indicated that only the 600 ppm–exposure group failed to exhibit a significant novelty preference ($p > 0.10$; see Table A.25). The results of the ANOVA model that compared novelty scores across the 4 methanol-exposure groups did not indicate a significant effect due to methanol exposure ($p = 0.18$, see Table A.26). Linear contrast tests based on this ANOVA model, however, indicated a significant difference when control infants were compared with infants in the 600 ppm–exposure group ($p = 0.03$). The ANOVA model controlling for offspring cohort indicated both a significant effect due to methanol exposure ($p = 0.05$) and a significant interaction effect of methanol exposure and cohort ($p = 0.009$, see

Table 15. Results of Recognition Memory Assessment Using Abstract Patterns[a]

Exposure Groups	Infant Groups		
	All	Cohort 1	Cohort 2
Control (n = 7)	0.62 ± 0.04[b] (0.46–0.79)	0.55 ± 0.04 (0.46–0.64) (n = 4)[c]	0.70 ± 0.05 (0.61–0.79) (n = 3)
200 ppm (n = 9)	0.59 ± 0.02[b] (0.52–0.66)	0.57 ± 0.02 (0.52–0.63) (n = 5)	0.61 ± 0.02 (0.56–0.66) (n = 4)
600 ppm (n = 8)	0.54 ± 0.02 (0.42–0.63)	0.58 ± 0.02 (0.51–0.63) (n = 4)	0.50 ± 0.03 (0.42–0.55) (n = 4)
1,800 ppm (n = 9)	0.59 ± 0.02[b] (0.52–0.68)	0.57 ± 0.03 (0.52–0.68) (n = 5)	0.60 ± 0.01 (0.58–0.64) (n = 4)

[a] Each value is mean ± SE (range) novelty preference (percentage of time looking toward novel stimuli) using Fagan Test of Infant Intelligence.

[b] Significant novelty preference for controls, 200 ppm, and 1,800 ppm groups.

[c] Data for one infant in control group not included due to tester error.

Table A.27). Linear contrast tests based on this ANOVA model indicated a significant difference when control infants were compared with all methanol-exposed offspring combined, as well as with the the offspring of the 600 ppm–exposure group ($p < 0.005$). Cohort-specific linear contrast tests based on this ANOVA model indicated a significant difference when control infants were compared with all methanol-exposed offspring combined, as well as with each of the 3 methanol-exposed groups for Cohort 2 ($p < 0.05$). No significant effects were obtained for Cohort 1 ($p > 0.50$): Overall, the mean novelty score for controls was 62%, compared with 54% to 59% for the 3 methanol-exposed groups. For Cohort 2 offspring, the mean novelty score for the controls was 70%, compared with 50% to 61% for the 3 methanol-exposed groups (see Table 15).

Table 16. Results of Recognition Memory Assessment Using Social Stimuli

Exposure Group	Novelty Preference[a]
Control (n = 7)[b]	0.62 ± 0.05[c] (0.43–0.80)
200 ppm (n = 9)	0.53 ± 0.03 (0.39–0.69)
600 ppm (n = 8)	0.49 ± 0.06 (0.23–0.74)
1,800 ppm (n = 9)	0.57 ± 0.05 (0.25–0.78)

[a] Each value is mean ± SE (range) novelty preference (percentage of time looking toward novel stimuli) using Fagan Test of Infant Intelligence.

[b] Data for one infant in control group not included due to tester error.

[c] Significant novelty preference for controls only.

Social Stimuli Test The results of initial t tests for the social stimuli problems indicated that the control group exhibited a significant preference for novel stimuli (62%), whereas the 3 methanol-exposed groups did not (49% to 57%) (Tables 16 and A.28). The ANOVA model that compared novelty scores across the 4 methanol-exposure groups, however, did not indicate a significant effect due to methanol exposure (p = 0.38, see Table A.29).

For both sets of problems, the average time it took infants to reach a predetermined amount of looking during the familiarization period was calculated for each methanol-exposure group as a measure of attention. In addition, the average duration of the fixations to test stimuli was calculated for each group. The results of the ANOVA model that compared these measures across the 4 methanol-exposure groups did not indicate a significant effect due to methanol exposure (data not shown).

Nonmatch-to-Sample Test This measure was used as the secondary-outcome variable for infant recognition memory. Infants were required to learn the concept of nonmatching, or novel, to successfully master the test. The results of the Nonmatch-to-Sample Test are summarized in Table 17. Two offspring (1 infant from the control group and 1 infant from the 200 ppm–exposure group) refused to respond on this test and were dropped from testing. For the remaining infants, the number of nonbalk trials to reach criterion (23 correct responses of the 25 daily trials) varied markedly within each exposure group. The range of nonbalk trials was 238 to 836 for the control

Table 17. Results of Nonmatch-to-Sample Test for Infant Recognition Memory

Exposure Group	Nonbalk Trials to Criterion[a]
Control (n = 7)[b]	435.7 ± 83.1 (238–836)
200 ppm (n = 8)[b]	540.8 ± 122.7 (224–1,325)
600 ppm (n = 8)	372.1 ± 53.7 (190–634)
1,800 ppm (n = 9)	399.3 ± 70.7 (259–871)

[a] Each value is mean ± SE (range) number of nonbalk trials to criterion.

[b] One control infant and one infant exposed to 200 ppm methanol did not reach criterion and are not included.

group, 224 to 1,325 for the 200 ppm–exposure group, 199 to 634 for the 600 ppm–exposure group, and 259 to 871 for the 1,800 ppm–exposure group. The log of the number of nonbalk trials to reach criterion was used in the ANOVA models due to asymmetric distributions. The results of the ANOVA model that compared the number of nonbalk trials to criterion across the 4 methanol-exposure groups did not indicate a significant effect due to methanol exposure (p = 0.53, see Table A.30).

Repeated measures ANOVA models were used to analyze performances on the Delayed Nonmatch-to-Sample Tests. The overall percentages of correct responses for the 5 delay periods (0, 10, 20, 40, 60 seconds) were used in the analyses. The results of the ANOVA model that compared the percentage of correct responses across the 4 methanol-exposure groups over the 5 delay periods did not indicate either a significant effect due to methanol exposure (p = 0.48) or an interaction between methanol exposure and delay (p = 0.37, see Tables A.31 and A.32). In general, results indicated a linear decline in performance with increasing delay periods for all exposure groups (see Figure 14).

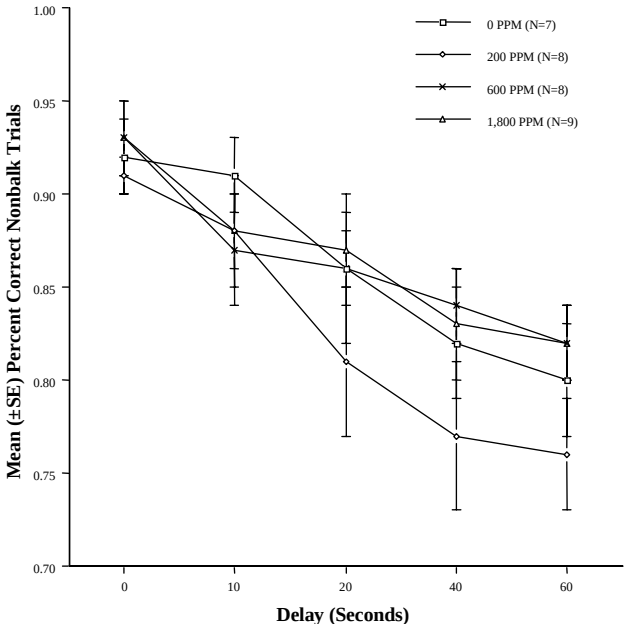

Figure 14. Results of delayed Nonmatch-to-Sample Test. One infant in the control group and one in the 200 ppm–exposure group did not reach criterion and are not included.

Infant Social Behavioral Development

Results of the Social Observations are shown in Figure 15. The social behavioral data were summarized for each month for months 1 through 7. Data summaries yielded 3 behavioral categories: passive behaviors, social behaviors, and nonsocial behaviors (see Table 18). Separate repeated measures ANOVA models were used to analyze the percentage of time in each of the 3 behavioral categories, using data from all 7 months of testing. Data from 5 infants (2 controls, as well as 1 in each methanol-exposed group) were missing for 1 of the 7 months of testing. Four infants did not have data for month 1, either because social partners were not available or because of sickness. The additional infant did not have data for month 2 due to sickness. The results of the ANOVA models that compared percentage of time in each of the behavioral categories across the 4 methanol-exposure groups and across age did not indicate a significant effect either due to methanol exposure ($p > 0.50$) or an interaction of methanol exposure and age ($p > 0.60$, see Tables A.33 through A.38). The percentage of time in passive and nonsocial behaviors decreased with increasing age, while the percentage of time in social behaviors increased with age for all groups (see Figure 15).

Infant Physical Growth

The primary-outcome variable used to assess the effects of methanol exposure on postnatal physical growth was the rate of weight gain during the first 9 months of life. The average rate of weight gain for the control group and the 3 methanol-exposed groups was very similar, at approximately 4 g/day (see Table 19). The slope of the weights of each infant over the first 9 months of life was calculated. The value for the slope was then used in the analyses. The results of the ANOVA model that compared the slopes of the weights during the first 9 months of life across the 4 methanol-exposure groups (see Table A.39) did not indicate a significant effect due to methanol exposure (p = 0.99).

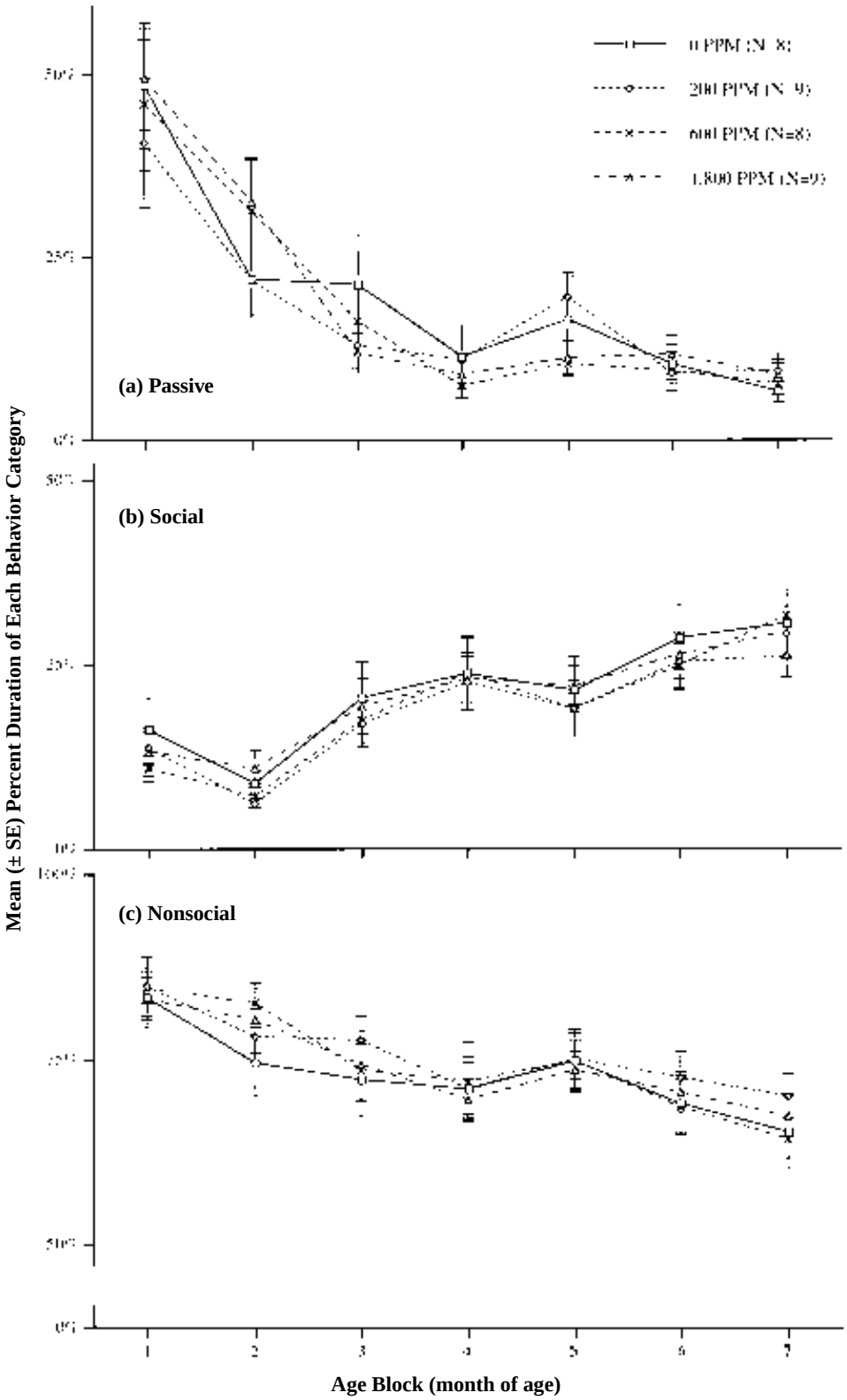

Figure 15. Results of playroom Social Observations. Categories of passive behavior (a), social behavior (b), and nonsocial behavior (c). For Age Block 1, 4 infants (1 in each group) are not included due to insufficient number of sessions. For Age Block 2, 1 control infant is not included due to insufficient number of sessions.

Table 18. Behavioral Categories Derived from Four-Digit Coding System for Social Observations

Category	Behavior	Description of Behavior
Passive	Nonsocial passive	Sleeping or nondirected looking while sitting, standing, or walking
	Social passive	Sitting in contact with peer, mutual huddle, or embrace
Nonsocial	Self explore	Visual, oral, or manual self-inspection or self-grooming
	Environmental explore	Directed visual, oral, or manual inspection of objects or environment
	"Normal" fear, disturbance	Physical withdrawal, vocalization, fear grimace, pilo-erection, or convulsive jerking in response to objects or environment
	"Abnormal" fear, disturbance	Self-clasping, self-biting, rocking, or stereotypic movements (pacing, back-flipping) in response to objects or environment
	Play	Solitary bouncing, leaping, rolling, or "play-faces" directed toward objects or environment
	Aggressive	Stiff four-point stance, "threat face," or barking directed toward objects or environment
Social	Explore	Visual, oral, or manual self-inspection or self-grooming
	"Normal" fear, disturbance	Physical withdrawal, vocalization, fear grimace, pilo-erection, or convulsive jerking in response to peer
	"Abnormal" fear, disturbance	Self-clasping, self-biting, rocking, or stereotypic movements (pacing, back-flipping) in response to peer
	Play	"Rough and tumble" wrestling, bouncing, or chasing with peer; mounting or sexual presenting
	Aggressive	Stiff four-point stance, "threat face," barking, pilo-erection, or physical attack (biting, pulling) directed toward peer

Analyses of secondary-outcome variables to assess physical growth utilized measures of offspring size at 9 months of age (weight, crown–rump length, head circumference, head length, and head width, see Table 20). The results of these analyses were consistent with the results for weight gain; they did not indicate a methanol-exposure effect ($p > 0.75$, see Tables A.40 through A.44). Finally, the mean age at eruption for each type of deciduous tooth (namely, central incisors, lateral incisors, canines, first molar, second molar) was calculated for each infant as a measure of skeletal growth (see Figure 16). The mean ages were then used in a repeated measures ANOVA model. The results of the ANOVA model, which compared the age at eruption for the 5 different deciduous teeth across the 4 methanol-exposure groups, did not indicate a significant effect due to methanol exposure or a significant interaction effect of methanol exposure and tooth type ($p > 0.30$, see Tables A.45 and A.46).

Long-Term Survival of Offspring

Although not part of the original design, follow-up observations of all offspring continue to take place. Thus far, two Cohort 1 females from the 1,800 ppm–methanol exposure group required euthansia due to a chronic wasting syndrome that became evident after project testing was completed. The first female began to show ini-

Table 19. Slope of Weight Gain of Infants over First 9 Months of Life

Exposure Group	Weight Gain[a]
Control ($n = 8$)	4.0 ± 0.2 (3.3–5.1)
200 ppm ($n = 9$)	4.0 ± 0.2 (3.2–5.1)
600 ppm ($n = 8$)	4.1 ± 0.2 (3.4–4.7)
1,800 ppm ($n = 9$)	4.0 ± 0.1 (3.2–4.4)

[a] Each value is mean ± SE (range) in g/day.

Table 20. Offspring Size at Nine Months of Age

Exposure Group	Physical Growth: Secondary Assessment Factors[a]				
	Weight (kg)	Crown–Rump Length (mm)	Head Circumference (mm)	Head Length (mm)	Head Width (mm)
Control (n = 8)	1.49 ± 0.61 (1.30–1.85)	300.8 ± 4.2 (290–325)	220.7 ± 1.9 (214–230)	75.3 ± 0.7 (73–79)	59.5 ± 0.8 (57–64)
200 ppm (n = 9)	1.48 ± 0.62 (1.20–1.89)	299.7 ± 3.7 (284–323)	219.3 ± 1.6 (213–225)	75.2 ± 0.5 (73–78)	59.1 ± 0.8 (56–63)
600 ppm (n = 8)	1.55 ± 0.75 (1.22–1.83)	306.3 ± 5.1 (288–324)	219.9 ± 3.2 (204–229)	75.9 ± 1.3 (70–80)	58.9 ± 1.1 (53–63)
1,800 ppm (n = 9)	1.46 ± 0.55 (1.11–1.66)	298.8 ± 3.8 (274–316)	220.2 ± 2.0 (210–232)	75.1 ± 0.9 (69–79)	59.7 ± 0.7 (57–63)

[a] Each value is mean ± SE (range).

tial signs of growth retardation at approximately 12 months of age. The growth retardation continued even though her food intake was normal. By approximately 20 months of age she was very weak and was euthanized. The second female began to show similar signs of growth retardation beginning at approximately 17 months of age. Again, the growth retardation continued even though her food intake was normal. Assays for viral infection, blood chemistry, complete blood count, and liver, kidney, thyroid, and pancreatic function were run in an attempt to diagnose the cause of the problem. The laboratory results were all within the normal range for macaque monkeys. At approximately 36 months of age, she was euthanized. The only remarkable findings on the necropsy reports for both offspring were signs of severe malnutrition and gastroenteritis. This syndrome was not observed in any of the Cohort 2 offspring.

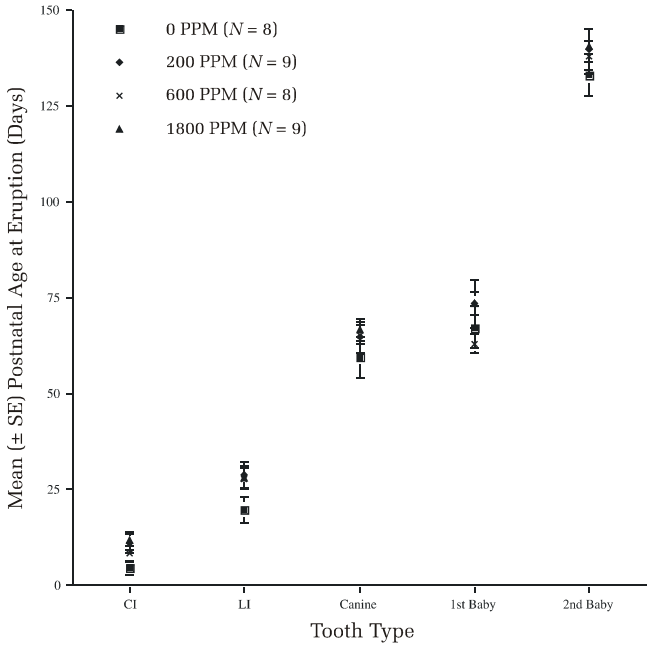

Figure 16. Summary of observations for the eruption of the deciduous teeth. The types of teeth represented include central incisors (CI), lateral incisors (LI), canine, first molars (1st Baby), and second molars (2nd Baby).

DISCUSSION AND CONCLUSIONS

Adult female *M. fascicularis* monkeys were exposed to 0 ppm, 200 ppm, 600 ppm, or 1,800 ppm methanol vapor for approximately 2.5 hours/day, 7 days/week, both prior to and during pregnancy. Blood concentrations of methanol during pregnancy varied from 2 to 10 times the background endogenous levels, depending on the specific level of methanol exposure. Offspring from these females were tested on a series of assessments in order to evaluate the effects of prenatal methanol exposure on physical and behavioral development during the first 9 months of life. Assessment procedures evaluated possible methanol-exposure effects on fetal mortality and malformations, offspring size at birth, newborn health, neonatal behavioral responses, infant sensorimotor and visual acuity development, infant spatial and recognition memory, infant social behavioral development, and postnatal physical growth. A discussion of the results pertaining to each of these outcomes follows.

FETAL MORTALITY AND MALFORMATIONS

The results of the study did not indicate an increase in fetal mortality or malformations due to maternal methanol

exposure. The 46 adult females delivered 34 live-born infants (8 of 11 control females, 9 of 12 females exposed to 200 ppm methanol vapor, 8 of 11 females exposed to 600 ppm methanol vapor, and 9 of 12 females exposed to 1,800 ppm methanol vapor). Two infants, 1 in the control group and 1 in the 600 ppm–exposure group, were delivered stillborn; in addition, there was 1 fetal death in the 1,800 ppm–exposure group. Although there were no obvious birth defects observed in any of the live-born or stillborn infants, an autopsy on the fetus that had died in utero indicated the presence of hydrocephalus and significant autolysis in all major organs. Autopsies on the 2 stillborn infants indicated that the lungs were not inflated and that both had died close to or during delivery. The cause of death for both infants was asphyxiation. In general, the results are consistent with previous studies of methanol exposure that used rodent animal models. These studies reported increases in fetal mortality or malformations following prolonged daily exposures at high concentrations of methanol vapor, over 10,000 ppm methanol for rats (Nelson et al. 1985; Infurna and Weiss 1986; Stanton et al. 1995) and over 2,000 ppm methanol for mice (Rogers et al. 1993). The 2-hour exposure period used in the present study was most likely too brief to cause increases in maternal or fetal mortality, even at the level of 1,800-ppm methanol exposure.

OFFSPRING SIZE AT BIRTH

Although methanol exposure was associated with shortened gestation lengths (for a discussion of the effects on length of pregnancy, see Part I of this report), exposure did not affect the size of the offspring at birth. Several physical measures were used to evaluate neonatal size (namely, birth weight, crown–rump length, head size). For all of the measures, the results varied little across the 4 methanol-exposure groups. Reduced fetal weights have been reported in studies of rats and mice after prolonged maternal exposure to methanol at levels of 10,000 ppm and above (Nelson et al. 1985; Rogers et al. 1993). Previous reports using rodent models, however, have not indicated an increase in premature deliveries following methanol exposure during pregnancy (Infurna and Weiss 1986; Stanton et al. 1995; Weiss et al. 1996). Early reports in humans of effects of alcohol (ethanol) ingestion during pregnancy did indicate increases in premature deliveries. Later studies, however, have shown that low birth weight, not prematurity, is associated with alcohol ingestion during pregnancy (Virji 1991; Sampson et al. 1994; Windham et al. 1995).

The current results indicate that methanol exposure during pregnancy may influence hormonal control over the onset of labor (see Part I of this report) at exposure concentrations that do not themselves affect overall fetal growth. Future studies should include procedures to define the possible mechanism(s) associated with this effect since the current study did not evaluate this issue.

OFFSPRING ASSESSMENTS

Newborn Health Status

The results of the present study did not indicate a significant increase in newborn morbidity due to methanol exposure, although several problems were observed in methanol-exposed offspring soon after birth. Of the live-born infants, 5 methanol-exposed offspring were delivered via C-section due to complications of labor and delivery (see Part I of this report). Of these newborns, 4 of the 5 exhibited signs of respiratory depression, cardiac depression, or both, soon after birth and received oxygen therapy. The fifth infant, a female in the 1,800 ppm–exposure group, exhibited signs of prematurity (irregular respiration and temperature, feeding difficulties) and received oxygen. All of the infants responded well to oxygen ther- apy and required treatment for only a few days. Although the number of methanol-exposed infants receiving oxygen was not significantly increased or dose related, none of the control infants were treated and a prevalence of nearly 20% (5 out of 26) is high for these animals (Burbacher et al. 1988). No significant differences due to methanol exposure were found in the distribution of total rating points on the Newborn Health Assessment, which included measures of heart and respiration rates, muscle tone, temperature, and color. There were procedural problems, however, with the testing of all infants on this assessment and, unfortunately, very few of the infants delivered via C-section were tested. Only 1 of the C-section newborns, a female from the 600 ppm–exposure group, was tested on the initial assessment (conducted approximately 10 minutes after birth). This newborn was the only one to receive a score of 0 on any test item (skin color), and she was also the only one to receive a total score of less than 10. Two newborns received scores of less than 10 on the follow-up assessment, conducted at approximately 30 minutes after birth. One was a male from the 600 ppm–exposure group, and the other was a female from the 1,800 ppm–exposure group. Both were delivered via C-section and had not been tested on the initial assessment. Therefore, a clear relationship was found between C-section delivery and lower scores on the Newborn Health Assessment. These findings are consistent with reports of human newborns delivered via C-section (Burt et al. 1988). Although the lower scores for the new-

borns delivered by C-section cannot be attributed directly to methanol exposure, it is likely that the scores of the methanol-exposed groups on the Newborn Health Assessment would have been even lower if all of the newborns delivered by C-section had been included in the analysis. Thus, the overall health status of the infants in the methanol-exposed groups soon after birth was most likely even poorer than indicated by the scores from the Newborn Health Assessment.

Neonatal Behavioral Responses

Evaluation of neonatal responses during the first 2 weeks of life using the Neonatal Behavioral Scale did not indicate an effect of methanol exposure on reflexes and behavioral responses. Methanol exposure was associated, however, with a significant delay in the mean age at which the infants were scored as "alert, awake, and aware" on the 4 items that made up the Behavior State factor for this assessment. A significant effect was observed on the linear contrast tests when the controls were compared with all methanol-exposed offspring combined and when the control group was compared with the 600 ppm–exposure group. An examination of the scores given to the methanol-exposed and control infants for the individual items in this factor revealed that the primary difference was in the behavioral state of the infants prior to testing. Methanol-exposed infants were more likely to be scored as asleep or passive on this item than were controls. In addition, within the methanol-exposed groups, the infants delivered via C-section were typically the last to receive the optimal score for this factor. Thus, the effect on behavior state cannot be attributed directly to methanol exposure. Prenatal ethanol exposure in humans has been associated with lower scores on the Brazelton Neonatal Behavioral Assessment Scale. Abnormal reflexes, poor habituation responses, and low arousal have all been reported (Streissguth et al. 1994). Unlike the results of the present study, these effects were independent of C-section delivery.

Infant Sensorimotor Development

For infant macaques, early sensorimotor development can be evaluated over the first month of life by assessing the ability of the infant to grasp and retrieve a small object (Burbacher et al. 1986). For human infants, visually directed reaching typically develops between the third and fifth months of life (Bushnell 1985). Studies using animal models have indicated that this response is largely dependent on the development of the prefrontal cortex (Diamond 1990). The results of the Visually Directed Reaching Test for the present study indicate an association between prenatal methanol exposure and retarded sensorimotor development. The sensorimotor effect was observed primarily for male offspring. The delay for males was dose related and ranged from approximately 9 days for the 200 ppm–methanol exposure group to more than 2 weeks for the 600 ppm– and 1,800 ppm–methanol exposure groups.

Gross motor dysfunction due to methanol exposure was not observed in the present study on the basis of results of playroom observations of motor milestones. In general, the results were inconsistent, indicating a delay in milestones for males in the 600 ppm–exposure group and an acceleration in milestones for females in the 200 ppm– and 600 ppm–exposure groups.

Motor dysfunction, in the context of a Parkinsonian-like syndrome, has been reported in humans as a permanent complication of methanol toxicity (Riegel and Wolf 1966; Guggenheim et al. 1971) and an increased incidence of tremor is associated with this disorder (Nakashima et al. 1993; Staude et al. 1995). Although motor effects are typically observed only after episodes of acute methanol poisoning, it is noteworthy that a relationship between motor functioning and methanol exposure was recently reported in rodents after developmental exposure to 4,500 ppm methanol, a level below that required to produce signs of clinical toxicity (Weiss et al. 1996).

The results of the present study indicate that prenatal methanol exposure is associated with a delay in early sensorimotor development as measured by the infant's ability to reach for, grasp, and retrieve a small object during the first month of life. This effect was observed in male infants only. Future studies should focus on the possible long-term effects of prenatal methanol exposure on fine sensorimotor skills. Other studies of macaques indicated that delays in Visually Directed Reaching due to prenatal methylmercury exposure were associated with increased tremor, a motor effect (Rice 1989), or decreased vibration sensitivity, a somatosensory effect (Rice and Gilbert 1995). Follow-up studies using these assessment procedures are warranted.

Infant Visual Acuity Development

The visual system is known to be affected by high-level methanol exposure (Kavet and Nauss 1990). In humans, retinal and optic-nerve toxicity are the hallmark features of adult methanol intoxication (Benton and Calhoun 1952; Eells 1992). Both acute and chronic methanol exposure have been shown to produce retinal and optic-nerve toxicity in humans (Benton and Calhoun 1952; Ruedeman 1961; Eells 1992), nonhuman primates (Ingemansson 1983), and in methanol-sensitive rodent models (Eells 1991; Lee et al. 1994; Eells et al. 1996). Human studies

have also shown that chronic, low-level exposures may impair visual function (namely, produce blurred vision) (Frederick et al. 1984; Andrews et al. 1987). The effects of methanol on the visual system have been largely attributed to increases in methanol-derived formate following acute, high-level exposures. Although our study design purposely excluded methanol doses that would raise maternal formate concentrations, it is not known whether fetal concentrations of formate were increased over background levels. Previous studies have also indicated that local (namely, intraretinal) metabolism of methanol to formate occurs following prolonged methanol exposure (Garner et al. 1995). Whether this process can occur in the fetus is also unknown. Although there is no direct evidence that prenatal methanol exposure affects visual function, visual disturbances are commonly observed after prenatal ethanol exposure (Stromland 1985; Stromland and Pinazo-Duran 1994). Visual effects include loss of acuity, strabismus, ptosis, and optic-nerve hypoplasia. For these reasons, an early evaluation of visual acuity development was considered a very important component of the study's test battery. The procedure that was chosen, the Preferential Looking Test, has a long history of use in human infancy research (Teller 1979) and has been previously used in studies of macaque visual development (Teller 1981). For the present study, this procedure did not provide reliable data for the majority of infants. Visual acuity thresholds could only be calculated across all ages for 15 of the 34 infants. The results from these infants indicated lower visual acuity thresholds for all 4 methanol-exposure groups at 12 weeks of age. The Week-12 test was the last in the series, so whether this pattern continues is unknown. Because most of the infants did not provide reliable data for this test, the results of the few infants who did cannot be considered representative of the entire group. Previous studies of adult macaques have used operant procedures to measure visual-contrast sensitivity functions (Rice and Gilbert 1990). Because the current study did not provide an adequate evaluation of the potential effects of prenatal methanol exposure on visual function, further evaluation of these offspring using the contrast sensitivity procedure is warranted.

Infant Spatial Memory

The results of the present study did not indicate a methanol-exposure effect on spatial memory as measured by the Object Concept or Spatial Discrimination and Reversal Tests. Object permanence, which develops in parallel stages in both human and macaque infants, is considered a measure of early spatial memory (Mishkin and Appenzeller 1987; Diamond and Goldman-Rakic 1989). In *M. fascicularis* infants, object permanence is established by approximately 2 months of age (Burbacher et al. 1986). The results of the current study are consistent with previous results; the mean age for developing object permanence for all 4 of the exposure groups was approximately 60 days. The results of the Spatial Discrimination and Reversal Test, when compared with previous studies in macaques (Rice 1985; Schantz et al. 1989), suggest that this assessment may have been too difficult for 5-month-old infants. The number of trials to learn the initial spatial discrimination was much higher and the variation in the performance of the infants much greater than in previous studies that used this procedure. For the Reversal Test, all of the exposure groups showed little improvement in performance over the 6 reversals. The performance of the methanol-exposed infants on these tests, however, did not provide evidence for a methanol-exposure effect on early spatial memory.

Infant Recognition Memory

The Fagan Test of Infant Intelligence was designed to provide an early indicator of information processing, attention, and visual memory capabilities in human infants (Fagan and Detterman 1992). As such, the test is said to provide an early evaluation of possible alterations in hippocampal and temporal cortical function (Bachevalier 1990). Numerous studies with human and nonhuman primate infants have reported consistent findings related to infants' preference for novelty. Both human and nonhuman primate infants have consistently shown novelty scores at or above 60% (Burbacher et al. 1990a). The ability to recognize previously seen stimuli from those that are new during infancy is substantially correlated in humans with performance on standardized childhood intelligence tests (Rose and Wallace 1985). Studies have indicated that prenatal exposure to environmental contaminants such as polychlorinated biphenyls and methylmercury lowers, and sometimes eliminates, novelty preference in infants that are tested (Jacobson et al. 1985; Gunderson et al. 1986, 1988). Exposure effects on the novelty response are said to be due to alterations in information processing, memory for objects, or both.

For the present study, the results of the Fagan Test indicate that prenatal methanol exposure is associated with deficits in recognition memory. The control infants exhibited a significant novelty response for both the abstract patterns and social stimuli that was consistent with previous reports for normal macaque and human infants (Burbacher et al. 1990a). The results of the abstract patterns test varied greatly by cohort and no consistent pattern of results were observed. For the Social Stimuli Test,

all 3 of the methanol-exposed groups failed to show a significant preference for novel stimuli. The failure of the methanol-exposed infants to show a significant novelty response with social stimuli is consistent with results from a previous study of macaque infants exposed prenatally to methylmercury (Gunderson et al. 1988). Although no significant differences in the mean novelty scores were observed across groups in either study, the exposed groups in both studies failed to exhibit significant novelty scores to relatively complex (social) stimuli.

The methanol-exposure effects observed in testing of recognition memory were most likely not related to a deficit in object memory. The performance of the methanol-exposed infants on the Nonmatch-to-Sample Test did not indicate a methanol-exposure effect. This assessment may have been too difficult for these young animals. The number of trials to learn the initial nonmatch concept was much higher, and the variation in performance of infants much greater, than in the previous studies that used this procedure with older macaques (Bachevalier and Mishkin 1984). However, no methanol-exposure effects were observed for the original nonmatch procedure. In addition, the decline in performance observed across the delay periods was similar for all 4 methanol-exposure groups. Future studies investigating the potential source of the recognition-memory effect should focus on assessments of information processing abilities.

Studies of human infants exposed prenatally to ethanol have not reported effects on the novelty preference on the basis of results of the Fagan Test. Differences in the duration of visual fixations on the recognition memory test, however, have been reported (Jacobson et al. 1993). Visual fixations and visual attention were examined in the present study, in addition to the novelty preference. The mean duration of visual fixations was calculated by dividing the total duration of looking time by the number of looks. Visual attention was measured by calculating the time for infants to accumulate the predetermined amount of looking time during the familiarization period. No differences in visual fixations or attention due to methanol exposure were observed.

Infant Social Behavioral Development

The results of the Social Observations revealed a pattern of social behaviors consistent with those found in previous studies (Burbacher et al. 1990b). Although no effects were associated with methanol exposure, the typical pattern of increasing frequency of social behaviors and decreasing frequency of passive and nonsocial behaviors with age was evident. In other studies of macaques, changes in social behaviors have been observed following exposure to methylmercury or 2,3,7,8-tetrachloridibenzo-*p*-dioxin (TCDD) (Burbacher et al. 1990b; Schantz et al. 1992). The methylmercury effects were observed using the identical coding system utilized in the present study. Studies of prenatal ethanol exposure in other animal models have reported effects on social behavior (Meyer and Riley 1986; Markel et al. 1995). In rats, ethanol exposure has been associated with a reduction in the frequency and duration of social interactions, as well as a reduction in the amount of play-fighting among males. The results of the present study, however, do not indicate that prenatal exposure to methanol affects social behavioral development during the first 7 months of life.

Infant Physical Growth

Prenatal methanol exposure did not retard growth rates during the first 9 months of life. Our later studies, however, indicate that pubertal growth retardation in females may be a delayed effect of high-dose methanol exposure. This effect was not observed as a general decrease in the growth of females as a group, but rather as a severe wasting syndrome in 2 female offspring in the 1,800 ppm–exposure group, with onset after 1 year of age. Since the syndrome was similar to that observed in reports of retroviruses in macaques (Lackner et al. 1990), assays for SRV, SIV, and STLV were performed. The results of these assays did not indicate a simian retrovirus as the etiology of the syndrome. All assays were negative. Results of assays for blood chemistry, complete blood count, and liver, kidney, thyroid, and pancreatic function were also unremarkable. Autopsies were conducted on the 2 females after euthanasia. The only remarkable findings were severe malnutrition and gastroenteritis. Prenatal ethanol exposure has been associated with growth retardation and delayed pubertal growth (Hannigan and Bellisario 1990; Breese et al. 1993), with growth effects typically observed as a general decrease in birth weight and postnatal growth. The observed ethanol effects have been generally related to changes in growth hormone, thyroxin, and insulin-like growth factor-1 (IGF-1). Prepubertal ethanol treatment has also been shown to affect hypothalamic and pituitary hormones such as growth hormone and luteinizing hormone (Dees et al. 1990). Assays on blood from 1 of the 2 females that exhibited the wasting syndrome were within normal limits for growth hormone and thyroxin. Whether IGF-1 or other hormones were affected is unknown. Future studies should include procedures to evaluate prenatal and postnatal hormonal development to investigate this effect further.

SUMMARY

The results of the present study indicate that, for this nonhuman primate model, daily 2.5 hour-long exposures to methanol vapor from levels of 200 ppm to 1,800 ppm produce blood concentrations of methanol from 2 to 10 times background endogenous levels. Chronic exposures up to 1 year at these levels did not cause overt maternal toxicity in *M. fascicularis* females. The menstrual cycle and ability to conceive were also unaffected. Methanol exposures, however, were associated with a reduction in the length of pregnancy in this animal model, thus shortening the gestation length of the offspring. These results are discussed in detail in Part I of this report.

The results of behavioral assessments of offspring did not indicate significant methanol-exposure effects on most domains of early behavioral development. No consistent effects due to methanol exposure were observed on early reflex responses, gross motor development, spatial and concept learning and memory, and social behavior.

Methanol exposure was associated with ratings of "low arousal" on the Neonatal Behavioral Scale. The effect was observed when all of the methanol-exposed infants were compared with controls. Further comparisons, however, did not indicate that the effect was dose dependent. In addition, many of the methanol-exposed infants that were the last to receive optimal scores for the arousal items had been delivered via C-section. Thus, this effect may not be directly related to methanol exposure independent of mode of delivery.

Methanol exposure was also associated with a delay in early sensorimotor development for male infants. The effect was observed after controlling for the shortened gestation length observed for the 3 methanol-exposed groups. The delay was dose dependent and ranged from 9 days for the 200 ppm–exposure group to over 2 weeks for the 600 ppm– and 1,800 ppm–exposure groups.

The results of the Fagan Test of Infant Intelligence indicated a possible effect of methanol exposure on visual recognition memory when complex stimuli (social problems) were used in testing. Although there were no mean group differences in the novelty scores across the 4 exposure groups, only the control group exhibited a significant novelty preference for the social stimuli.

Finally, prenatal methanol exposure was associated with the occurrence of a wasting syndrome in females after approximately 1 year of age. The wasting syndrome was observed in 2 of the 7 females in the 1,800 ppm–exposure group. The syndrome was severe and resulted in euthanasia of both of the females. The results of clinical blood tests and autopsy examinations did not provide evidence as to the etiology of the syndrome. Because this is the first report of an association between low-level methanol exposure and offspring developmental effects, further investigations are needed to confirm these findings. Investigations should focus on evaluating possible changes in fine motor or somatosensory responses. Studies to measure the speed and accuracy of information processing are also warranted. Finally, future studies should include a long-term follow-up assessment of methanol-exposed infants to determine the possible effects on physical growth during adolescence.

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APPENDIX A. Statistical Analysis Tables

Table A.1. Birth Weight (g) for Live-Born Offspring

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	3932.95	3	1310.98	0.42	0.74
Error	93028.82	30	3,100.96		
Linear Contrasts[a]					
Controls (n = 8) vs. all exposed (n = 26)	398.98	1	398.98	0.13	0.72
Controls vs. 200 ppm (n = 9)	2502.04	1	2502.04	0.81	0.38
Controls vs. 600 ppm (n = 8)	1.56	1	1.56	0.0005	0.98
Controls vs. 1,800 ppm (n = 9)	2.04	1	2.04	0.0006	0.98

[a] The n values given in column one are applicable throughout the tables in this appendix unless otherwise noted.

Table A.2. Crown–Rump Length (mm) at Birth for Live-Born Offspring

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	122.18	3	40.73	0.49	0.7
Error	2518.76	30	83.96		
Linear Contrasts					
Controls vs. all exposed	105.90	1	105.90	1.26	0.27
Controls vs. 200 ppm	105.88	1	105.88	1.26	0.27
Controls vs. 600 ppm	76.56	1	76.56	0.91	0.35
Controls vs. 1,800 ppm	40.99	1	40.99	0.49	0.49

Table A.3. Head Circumference (mm) at Birth for Live-Born Offspring

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	65.25	3	21.75	0.80	0.50
Error	810.99	30	27.03		
Linear Contrasts					
Controls vs. all exposed	40.07	1	40.07	1.48	0.23
Controls vs. 200 ppm	61.79	1	61.79	2.29	0.14
Controls vs. 600 ppm	22.56	1	22.56	0.83	0.37
Controls vs. 1,800 ppm	9.35	1	9.35	0.35	0.56

Table A.4. Head Length (mm) at Birth for Live-Born Offspring

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	4.40	3	1.47	0.34	0.79
Error	127.72	30	4.26		
Linear Contrasts					
Controls vs. all exposed	0.72	1	0.72	0.17	0.68
Controls vs. 200 ppm	2.94	1	2.94	0.69	0.41
Controls vs. 600 ppm	0.25	1	0.25	0.06	0.81
Controls vs. 1,800 ppm	0.01	1	0.01	0.003	0.96

Table A.5. Head Width (mm) at Birth for Live-Born Offspring

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	13.10	3	4.37	1.48	0.24
Error	88.43	30	2.95		
Linear Contrasts					
Controls vs. all exposed	10.79	1	10.79	3.66	0.07
Controls vs. 200 ppm	9.01	1	9.01	3.06	0.09
Controls vs. 600 ppm	10.56	1	10.56	3.58	0.07
Controls vs. 1,800 ppm	3.45	1	3.45	1.17	0.29

Table A.6. Frequency of Oxygen Treatment of Newborns[a]

Exposure Group	Oxygen	No Oxygen
Methanol Exposure Groups[b]		
Control (n = 8)	0 (0)	8 (100)
200 ppm (n = 9)	2 (22)	7 (78)
600 ppm (n = 8)	2 (25)	6 (75)
1,800 ppm (n = 9)	1 (11)	8 (89)
Methanol Exposure[c]		
Control (n = 8)	0 (0)	8 (100)
Methanol-exposed (n = 26)	5 (19)	21 (81)

[a] Values are n (%).

[b] Fisher's exact test (two-tailed) p value = 0.65, indicating that the frequency of oxygen treatment across the four groups was not significantly different.

[c] Fisher's exact test (two-tailed) p value = 0.24, indicating that the frequency of oxygen treatment for controls and for all methanol-exposed infants was not significantly different.

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Table A.7. Distribution of Total Scores for Initial Newborn Health Assessment[a]

	Total Score on Newborn Assessment				
Exposure Group	8	9	10	11	12
Control (n = 6)	0 (0)	0 (0)	0 (0)	1 (17)	5 (83)
200 ppm (n = 7)	0 (0)	0 (0)	2 (29)	4 (57)	1 (14)
600 ppm (n = 7)	1 (14)	0 (0)	0 (0)	3 (43)	3 (43)
1,800 ppm (n = 7)	0 (0)	0 (0)	1 (14)	3 (43)	3 (43)

[a] Values are n (%). Fisher's exact test (two-tailed) p value = 0.25, indicating that the distribution of total scores for initial newborn health assessment across the four exposure groups was not significantly different.

Table A.8. Distribution of Total Scores for Follow-up Newborn Health Assessment[a]

	Total Score on Newborn Assessment				
Exposure Group	8	9	10	11	12
Control (n = 7)	0 (0)	0 (0)	2 (29)	4 (57)	1 (14)
200 ppm (n = 7)	0 (0)	0 (0)	1 (14)	5 (71)	1 (14)
600 ppm (n = 8)	1 (12)	0 (0)	2 (25)	5 (63)	0 (0)
1,800 ppm (n = 7)	1 (14)	0 (0)	2 (29)	3 (43)	1 (14)

[a] Values are n (%). Fisher's exact test (two-tailed) p value = 0.97, indicating that the distribution of total scores for follow-up newborn health assessment across the four exposure groups was not significantly different.

Table A.9. Neonatal Behavioral Scale: Log of Age (Days) at Optimal Score for Behavior State Factor

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	1.22	3	0.41	2.37	0.09
Error	5.13	30	0.17		
Linear Contrasts					
Controls vs. all exposed	0.83	1	0.83	4.88	0.03
Controls vs. 200 ppm	0.25	1	0.25	1.45	0.24
Controls vs. 600 ppm	1.19	1	1.19	6.96	0.01
Controls vs. 1,800 ppm	0.44	1	0.44	2.55	0.12

Table A.10. Neonatal Behavioral Scale: Log of Age (Days) at Optimal Score for Reflex Factor

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.61	3	0.20	1.15	0.35
Error	5.31	30	0.18		
Linear Contrasts					
Controls vs. all exposed	0.28	1	0.28	1.59	0.22
Controls vs. 200 ppm	0.03	1	0.03	0.20	0.66
Controls vs. 600 ppm	0.55	1	0.55	3.11	0.09
Controls vs. 1,800 ppm	0.14	1	0.14	0.80	0.38

Table A.11. Neonatal Behavioral Scale: Log of Age (Days) at Optimal Score for Muscle Tone Factor

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.14	3	0.05	0.74	0.54
Error	1.90	30	0.06		
Linear Contrasts					
Controls vs. all exposed	0.12	1	0.12	1.95	0.17
Controls vs. 200 ppm	0.09	1	0.09	1.44	0.24
Controls vs. 600 ppm	0.12	1	0.12	1.89	0.18
Controls vs. 1,800 ppm	0.05	1	0.05	0.77	0.39

Table A.12. Neonatal Behavioral Scale: Log of Age (Days) at Optimal Score for Responsivity Factor

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.80	3	0.27	1.22	0.32
Error	6.59	30	0.22		
Linear Contrasts					
Controls vs. all exposed	0.25	1	0.25	1.16	0.29
Controls vs. 200 ppm	0.36	1	0.36	1.62	0.21
Controls vs. 600 ppm	0.42	1	0.42	1.91	0.18
Controls vs. 1,800 ppm	0.00001	1	0.00001	0.00006	0.99

Table A.13. Visually Directed Reaching Test: Log of Age (Days) at Criterion

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.38	3	0.13	1.66	0.20
Error	2.26	30	0.08		
Linear Contrasts					
Controls vs. all exposed	0.14	1	0.14	1.85	0.18
Controls vs. 200 ppm	0.03	1	0.03	0.44	0.51
Controls vs. 600 ppm	0.03	1	0.03	0.35	0.56
Controls vs. 1,800 ppm	0.34	1	0.34	4.51	0.04
Linearity	0.36	1	0.36	4.73	0.04

Table A.14. Visually Directed Reaching Test: Log of Age (Days) at Criterion (Males and Females)

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.38	3	0.13	2.02	0.14
Sex	0.0004	1	0.0004	0.006	0.94
Exposure group × sex	0.64	3	0.21	3.38	0.03
Error	1.63	26	0.06		
Linear Contrasts for Males					
Controls vs. all exposed	0.52	1	0.52	8.25	0.008
Controls vs. 200 ppm	0.20	1	0.20	3.12	0.09
Controls vs. 600 ppm	0.53	1	0.53	8.47	0.007
Controls vs. 1,800 ppm	0.33	1	0.33	5.31	0.03
Linearity	0.22	1	0.22	3.51	0.07
Linear Contrasts for Females					
Controls vs. all exposed	0.007	1	0.007	0.12	0.73
Controls vs. 200 ppm	0.003	1	0.003	0.06	0.81
Controls vs. 600 ppm	0.13	1	0.13	2.05	0.16
Controls vs. 1,800 ppm	0.05	1	0.05	0.85	0.36
Linearity	0.11	1	0.11	1.69	0.20

Table A.15. Visually Directed Reaching Test: Log of Age (Days) at Criterion (Gestational Length)

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Gestation Length	0.17	1	0.17	2.39	0.13
Exposure group	0.27	3	0.09	1.26	0.31
Error	2.09	29	0.07		
Linear Contrasts					
Controls vs. all exposed	0.02	1	0.02	0.21	0.65
Controls vs. 200 ppm	0.002	1	0.002	0.02	0.88
Controls vs. 600 ppm	1×10^{-8}	1	1×10^{-8}	1×10^{-7}	1.00
Controls vs. 1,800 ppm	0.13	1	0.13	1.86	0.18
Linearity	0.23	1	0.23	3.21	0.08

Table A.16. Visually Directed Reaching Test: Log of Age (Days) at Criterion (Sex Covariate Model)

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Gestation length	0.03	1	0.03	0.47	0.50
Exposure group	0.30	3	0.10	1.59	0.22
Sex	0.0008	1	0.0008	0.01	0.91
Exposure group × sex	0.49	3	0.16	2.54	0.08
Error	1.60	25	0.06		
Linear Contrasts for Males					
Controls vs. all exposed	0.34	1	0.34	5.31	0.03
Controls vs. 200 ppm	0.11	1	0.11	1.80	0.19
Controls vs. 600 ppm	0.30	1	0.30	4.76	0.04
Controls vs. 1,800 ppm	0.29	1	0.29	4.59	0.04
Linearity	0.22	1	0.22	3.41	0.08
Linear Contrasts for Females					
Controls vs. all exposed	0.02	1	0.02	0.35	0.56
Controls vs. 200 ppm	0.02	1	0.02	0.30	0.59
Controls vs. 600 ppm	0.14	1	0.14	2.25	0.15
Controls vs. 1,800 ppm	0.02	1	0.02	0.33	0.57
Linearity	0.08	1	0.08	1.28	0.27

Table A.17. Observations of Motor Milestones in Playroom: Log of Age (Days) to Reach all Shelves and Ramp

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.24	3	0.08	0.68	0.57
Error	3.57	30	0.12		
Linear Contrasts					
Controls vs. all exposed	0.16	1	0.16	1.37	0.25
Controls vs. 200 ppm	0.24	1	0.24	2.04	0.16
Controls vs. 600 ppm	0.07	1	0.07	0.55	0.46
Controls vs. 1,800 ppm	0.06	1	0.06	0.53	0.47

Table A.18. Observations of Motor Milestones in Playroom: Log of Age (Days) to Reach all Shelves and Ramp (Male and Female Model)

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.30	3	0.10	1.35	0.28
Sex	0.01	1	0.01	0.20	0.66
Exposure group × sex	1.62	3	0.54	7.16	0.001
Error	1.96	26	0.08		
Linear Contrasts for Males					
Controls vs. all exposed	0.05	1	0.05	0.67	0.42
Controls vs. 200 ppm	0.02	1	0.02	0.32	0.58
Controls vs. 600 ppm	0.47	1	0.47	6.31	0.02
Controls vs. 1,800 ppm	0.06	1	0.06	0.81	0.38
Linear Contrasts for Females					
Controls vs. all exposed	0.62	1	0.62	8.25	0.008
Controls vs. 200 ppm	0.54	1	0.54	7.16	0.01
Controls vs. 600 ppm	0.74	1	0.74	9.80	0.004
Controls vs. 1,800 ppm	0.10	1	0.10	1.30	0.26

Table A.19. Object Concept Test: Log of Age (Days) at Criterion on Screen Test for Full-Hiding Condition

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*P* Value
ANOVA					
Exposure group	0.18	3	0.06	0.72	0.55
Error	2.46	30	0.08		
Linear Contrasts					
Controls vs. all exposed	0.03	1	0.03	0.34	0.57
Controls vs. 200 ppm	0.00004	1	0.00004	0.0004	0.98
Controls vs. 600 ppm	0.12	1	0.12	1.51	0.23
Controls vs. 1,800 ppm	0.003	1	0.003	0.04	0.84

Table A.20. Object Concept Test: Log of Age (Days) at Criterion on Well Test for Full-Hiding Condition

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.42	3	0.14	0.68	0.57
Error	6.19	30	0.21		
Linear Contrasts					
Controls vs. all exposed	0.21	1	0.21	1.04	0.32
Controls vs. 200 ppm	0.14	1	0.14	0.70	0.41
Controls vs. 600 ppm	0.36	1	0.36	1.76	0.19
Controls vs. 1,800 ppm	0.02	1	0.02	0.12	0.74

Table A.21. Object Concept Test: Log of Age (Days) at Criterion on A-not-B Test

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.09	3	0.03	0.29	0.84
Error	3.13	29	0.11		
Linear Contrasts					
Controls vs. all exposed	0.007	1	0.007	0.06	0.80
Controls vs. 200 ppm	0.007	1	0.007	0.06	0.81
Controls vs. 600 ppm	0.04	1	0.04	0.37	0.55
Controls vs. 1,800 ppm	0.006	1	0.006	0.06	0.82

Table A.22. Spatial Discrimination Test: Log of Nonbalk Trials to Criterion

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.31	3	0.10	0.41	0.75
Error	7.46	29	0.26		
Linear Contrasts					
Controls vs. all exposed	0.28	1	0.28	1.09	0.30
Controls vs. 200 ppm	0.12	1	0.12	0.45	0.51
Controls vs. 600 ppm	0.19	1	0.19	0.75	0.39
Controls vs. 1,800 ppm	0.27	1	0.27	1.05	0.31

Table A.23. Spatial Reversal Test: Log of Nonbalk Trials to Criterion Across Six Reversals

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
Between-Subjects Factors					
Exposure group	0.89	3	0.30	0.31	0.82
Error	26.90	28	0.96		
Within-Subject Factors					
Reversal[a]	5.83	5	1.17	5.59	0.0001
Reversal × exposure group	2.57	15	0.17	0.82	0.65
Error	29.21	140	0.21		

[a] Reversal = Spatial Discrimination Reversal Number (1–6).

Table A.24. Test for Parallel Profiles for Spatial Reversal Test: Log of Nonbalk Trials to Criterion Across Six Reversals

Source	Degrees of Freedom for *F* Test Numerator[a]	Degrees of Freedom for *F* Test Denominator[a]	*F* Value	*p* Value
Controls vs. all exposed	5	140	0.83	0.53
Controls vs. 200 ppm	5	140	0.63	0.68
Controls vs. 600 ppm	5	140	1.07	0.38
Controls vs. 1,800 ppm	5	140	0.77	0.58

[a] Refer to Table A.23.

Table A.25. *t* Test for Recognition Memory Assessment: Novelty Scores (Percentage of Time Looking Toward Novel Stimuli for Abstract Patterns Test)

Source	Difference (Mean ± SD)	*t* Value	Degrees of Freedom	*p* Value
Controls vs. chance	0.12 ± 0.11	2.74	6	0.03
200 ppm vs. chance	0.09 ± 0.05	5.61	8	0.005
600 ppm vs. chance	0.04 ± 0.06	1.67	7	0.14
1,800 ppm vs. chance	0.09 ± 0.05	5.23	8	0.0008

Table A.26. Recognition Memory Assessment: Novelty Scores (Percentage of Time Looking Toward Novel Stimuli for Abstract Patterns Test)

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.03	3	0.008	1.75	0.18
Error	0.14	29	0.005		
Linear Contrasts					
Controls vs. all exposed	0.01	1	0.01	2.69	0.11
Controls vs. 200 ppm	0.004	1	0.004	0.88	0.35
Controls vs. 600 ppm	0.02	1	0.02	5.05	0.03
Controls vs. 1,800 ppm	0.004	1	0.004	0.83	0.37

Table A.27. Recognition Memory Assessment: Novelty Scores (Percentage of Time Looking Toward Novel Stimuli for Abstract Patterns Test [Cohort Model])

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.03	3	0.01	3.09	0.05
Cohort	0.009	1	0.009	2.75	0.11
Exposure group × cohort	0.05	3	0.02	4.74	0.009
Cohort	0.09	25	0.003		
Linear Contrasts for All Animals					
Controls vs. all exposed		2/25[a]		6.50	0.005
Controls vs. 200 ppm		2/25[a]		2.37	0.11
Controls vs. 600 ppm		2/25[a]		10.82	0.0004
Controls vs. 1,800 ppm		2/25[a]		2.68	0.09
Linear Contrasts for Cohort 1					
Controls vs. all exposed	0.001	1	0.001	0.30	0.59
Controls vs. 200 ppm	0.0003	1	0.0003	0.11	0.74
Controls vs. 600 ppm	0.001	1	0.001	0.30	0.59
Controls vs. 1,800 ppm	0.0008	1	0.0008	0.24	0.63
Linear Contrasts for Cohort 2					
Controls vs. all exposed	0.04	1	0.04	12.70	0.002
Controls vs. 200 ppm	0.02	1	0.02	4.63	0.04
Controls vs. 600 ppm	0.07	1	0.07	21.34	0.0001
Controls vs. 1,800 ppm	0.02	1	0.02	5.12	0.03

[a] Degrees of freedom for *F* test numerator/denominator.

Table A.28. *t* Test for Recognition Memory Assessment: Novelty Scores (Percentage of Time Looking Toward Novel Stimuli for Social Stimuli Test)

Source	Difference (Mean ± SD)	*t* Value	Degrees of Freedom	*p* Value
Controls vs. chance	0.12 ± 0.13	2.41	6	0.05
200 ppm vs. chance	0.03 ± 0.10	0.98	8	0.35
600 ppm vs. chance	0.01 ± 0.16	0.12	7	0.91
1,800 ppm vs. chance	0.07 ± 0.16	1.41	8	0.20

Table A.29. Recognition Memory Assessment: Novelty Scores (Percentage of Time Looking Toward Novel Stimuli for Social Stimuli Test)

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.06	3	0.02	1.07	0.38
Error	0.57	29	0.02		
Linear Contrasts					
Controls vs. all exposed	0.04	1	0.04	1.94	0.17
Controls vs. 200 ppm	0.03	1	0.03	1.38	0.25
Controls vs. 600 ppm	0.06	1	0.06	2.85	0.10
Controls vs. 1,800 ppm	0.007	1	0.007	0.39	0.54

Table A.30. Nonmatch-to-Sample Test: Number of Nonbalk Trials to Criterion

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	132520.42	3	44173.47	0.75	0.53
Error	1654241.80	28	59080.06		
Linear Contrasts					
Controls vs. all exposed	15.58	1	15.58	0.0003	0.99
Controls vs. 200 ppm	41188.00	1	41188.00	0.70	0.41
Controls vs. 600 ppm	15096.10	1	15096.10	0.26	0.62
Controls vs. 1,800 ppm	5211.57	1	5211.57	0.09	0.77

Table A.31. Delayed Nonmatch-to-Sample Test: Percentage of Correct Nonbalk Trials Across Five Delay Periods

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
Between-Subjects Factors					
Exposure group	0.05	3	0.02	0.86	0.48
Error	0.51	28	0.02		
Within-Subject Factors					
Delay[a]	0.31	4	0.08	46.23	1×10^{-15}
Delay × exposure group	0.02	12	0.002	1.09	0.37
Error	0.19	112	0.002		

[a] Delay = 0, 10, 20, 40, or 60 seconds.

Table A.32. Test for Parallel Profiles for Delayed Nonmatch-to-Sample Test: Percentage of Correct Nonbalk Trials Across Five Delay Periods

Source	Degrees of Freedom for *F* Test Numerator[a]	Degrees of Freedom for *F* Test Denominator[a]	*F* Value	*p* Value
Controls vs. all exposed	4	112	0.87	0.49
Controls vs. 200 ppm	4	112	0.76	0.56
Controls vs. 600 ppm	4	112	1.39	0.24
Controls vs. 1,800 ppm	4	112	0.78	0.54

[a] Refer to Table A.31.

Table A.33. Passive Behavior Category: Log of Percentage of Time (min) Engaged in Passive Behaviors

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	3	29	0.03	0.99
Within-Subject Factors				
Age[a]	6	169	36.98	0.0001
Age × exposure group	18	169	0.85	0.64

[a] Age = month of age.

Table A.34. Test for Parallel Profiles for Passive Behavior Category: Log of Percentage of Time (min) Engaged in Passive Behaviors

Source	Degrees of Freedom for *F* Test Numerator[a]	Degrees of Freedom for *F* Test Denominator[a]	*F* Value	*p* Value
Controls vs. all exposed	6	169	0.28	0.95
Controls vs. 200 ppm	6	169	0.84	0.54
Controls vs. 600 ppm	6	169	0.08	1.00
Controls vs. 1,800 ppm	6	169	0.71	0.64

[a] Refer to Table A.33.

Table A.35. Social Behavior Category: Percentage of Time (min) Engaged in Social Behaviors

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	3	30	0.16	0.92
Within-Subject Factors				
Age[a]	6	174	41.40	0.0001
Age × exposure group	18	174	0.62	0.88

[a] Age = month of age.

Table A.36. Test for Parallel Profiles for Social Behavior Category: Percentage of Time (min) Engaged in Social Behaviors

Source	Degrees of Freedom for *F* Test Numerator[a]	Degrees of Freedom for *F* Test Denominator[a]	*F* Value	*p* Value
Controls vs. all exposed	6	174	0.33	0.92
Controls vs. 200 ppm	6	174	0.13	0.99
Controls vs. 600 ppm	6	174	0.57	0.75
Controls vs. 1,800 ppm	6	174	0.70	0.65

[a] Refer to Table A.35

Table A.37 Nonsocial Behavior Category: Percentage of Time (min) Engaged in Nonsocial Behaviors

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	3	30	0.74	0.53
Within-Subject Factors				
Age[a]	6	174	19.74	0.0001
Age × exposure group	18	174	0.42	0.98

[a] Age = month of age.

Table A.38. Test for Parallel Profiles for Nonsocial Behavior Category: Percentage of Time (min) Engaged in Nonsocial Behaviors

Source	Degrees of Freedom for *F* Test Numerator[a]	Degrees of Freedom for *F* Test Denominator[a]	*F* Value	*p* Value
Controls vs. all exposed	6	174	0.38	0.89
Controls vs. 200 ppm	6	174	0.35	0.91
Controls vs. 600 ppm	6	174	0.38	0.89
Controls vs. 1,800 ppm	6	174	0.49	0.81

[a] Refer to Table A.37

Table A.39. Slope of Weight Gain (g/day) over First Nine Months of Life

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	0.03	3	0.009	0.04	0.99
Error	6.58	30	0.22		
Linear Contrasts					
Controls vs. all exposed	0.01	1	0.01	0.06	0.81
Controls vs. 200 ppm	0.006	1	0.006	0.03	0.87
Controls vs. 600 ppm	0.03	1	0.03	0.12	0.73
Controls vs. 1,800 ppm	0.002	1	0.002	0.01	0.92

Table A.40. Weight (g) at Nine Months of Age

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	35424.35	3	11808.12	0.35	0.79
Error	1012176.39	30	33739.21		
Linear Contrasts					
Controls vs. all exposed	224.86	1	224.86	0.007	0.94
Controls vs. 200 ppm	816.99	1	816.99	0.02	0.88
Controls vs. 600 ppm	13806.25	1	13806.25	0.41	0.53
Controls vs. 1,800 ppm	3011.76	1	3011.76	0.09	0.77

Table A.41. Crown–Rump Length (mm) at Nine Months of Age

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	279.56	3	93.19	0.63	0.60
Error	4472.06	30	149.07		
Linear Contrasts					
Controls vs. all exposed	4.06	1	4.06	0.03	0.87
Controls vs. 200 ppm	4.97	1	4.97	0.03	0.86
Controls vs. 600 ppm	121.00	1	121.00	0.81	0.37
Controls vs. 1,800 ppm	16.47	1	16.47	0.11	0.74

Table A.42. Head Circumference (mm) at Nine Months of Age

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	8.84	3	2.95	0.07	0.98
Error	1279.40	30	42.65		
Linear Contrasts					
Controls vs. all exposed	5.11	1	5.11	0.12	0.73
Controls vs. 200 ppm	8.42	1	8.42	0.20	0.66
Controls vs. 600 ppm	2.64	1	2.64	0.06	0.81
Controls vs. 1,800 ppm	1.15	1	1.15	0.03	0.87

Table A.43. Head Length (mm) at Nine Months of Age

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	3.29	3	1.10	0.17	0.92
Error	195.57	30	6.52		
Linear Contrasts					
Controls vs. all exposed	0.02	1	0.02	0.003	0.96
Controls vs. 200 ppm	0.09	1	0.09	0.01	0.91
Controls vs. 600 ppm	1.27	1	1.27	0.19	0.66
Controls vs. 1,800 ppm	0.28	1	0.28	0.04	0.84

Table A.44. Head Width (mm) at Nine Months of Age

Source	Sum of Squares	Degrees of Freedom	Mean Square	*F* Value	*p* Value
ANOVA					
Exposure group	3.51	3	1.17	0.19	0.90
Error	184.50	30	6.15		
Linear Contrasts					
Controls vs. all exposed	0.42	1	0.42	0.07	0.80
Controls vs. 200 ppm	0.84	1	0.84	0.14	0.71
Controls vs. 600 ppm	1.27	1	1.27	0.21	0.65
Controls vs. 1,800 ppm	0.21	1	0.21	0.03	0.85

Table A.45. Eruption of Deciduous Teeth: Postnatal Age (Days) at Eruption of All Deciduous Teeth

Source	Degrees of Freedom for *F* Test Numerator	Degrees of Freedom for *F* Test Denominator	*F* Value	*p* Value
Between-Subjects Factors				
Exposure group	3	30	1.28	0.30
Within-Subject Factors				
Tooth[a]	4	120	1253.40	0.0001
Tooth × Exposure group	12	120	0.61	0.83

[a] Tooth = tooth type.

Table A.46. Test for Parallel Profiles for Eruption of Deciduous Teeth: Postnatal Age (Days) at Eruption of All Deciduous Teeth

Source	Degrees of Freedom for *F* Test Numerator[a]	Degrees of Freedom for *F* Test Denominator[a]	*F* Value	*p* Value
Controls vs. all exposed	4	120	0.39	0.82
Controls vs. 200 ppm	4	120	0.13	0.97
Controls vs. 600 ppm	4	120	1.30	0.27
Controls vs. 1,800 ppm	4	120	0.04	1.00

[a] Refer to Table A.45.

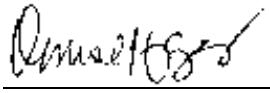
APPENDIX B. HEI Quality Assurance Report

The conduct of this study has been subjected to periodic audits by the Quality Assurance Officer from Primedica Corporation. The audits have included in-process monitoring of study activities and audits of the data. The dates of audits and nature of the visit are listed in the table below. The results of the inspections were reported to the Director of Research of Health Effects Institute, who was responsible for transmitting the reports to the Principal Investigator.

Observations made during these visits indicate that the study is well documented, and that the report describes the methods used and reflects the raw data. The effect of deviations from the protocol and standard operating procedures on the results of the study have been considered and addressed, as appropriate, in the data or final report.

Audits by Quality Assurance Officer

Date	Procedure/Data Reviewed
August 12–13, 1991	Pre-study visit
January 21 and 24, 1994	Observe testing of nonhuman primate infants
June 22–23, 1995	Audit of infant testing data
September 1998	Review of final report



Denise Hayes, HEI Quality Assurance Officer

APPENDIX C. Information on Test Animals and Raw Data

The following parts of Appendix C to Part II of this Research Report may be obtained by contacting the Health Effects Institute by mail (955 Massachusetts Avenue, Cambridge, MA 02139), fax (617-876-6709), or e-mail (pubs@healtheffects.org). Please provide both the Investigators' Report title and the appendix title when requesting appendices.

1. Parental Information
2. Infant Birth Characteristics
3. Newborn Assessment
4. Nursery Assessment
5. Pick-Up Assessment
6. Motor Milestones Assessment
7. Visual Acuity Assessment
8. Object Concept Assessment
9. Spatial Discrimination and Reversal Assessment
10. Recognition Memory Assessment
11. Nonmatch Assessment
12. Social Behavior Assessment
13. Infant Growth Assessment

ABBREVIATIONS AND OTHER TERMS

ANOVA	analysis of variance
C-section	Cesarean section
WGTA	Wisconsin General Testing Apparatus

INTRODUCTION

The Preface of this Research Report discusses the potential health concerns associated with the expanded use of methanol as an alternative fuel. Although methanol is a natural constituent of plant, animal, and human tissues, it is a dangerous toxic substance when sufficient quantities are ingested (HEI 1987; Kavet and Nauss 1990; WHO 1997). Usually, methanol poisoning is associated with accidental or intentional ingestion and occurs when the body's ability to metabolize methanol and convert it to nontoxic metabolites is overwhelmed. In contrast to our understanding of the toxic effects of such short-term, high-level exposures, little is known about the consequences of long-term exposures to low levels of methanol. Nor is it known whether such effects, if they exist, are mediated by the same pathways that cause the characteristic methanol poisoning syndrome. Addressing these information gaps is important because if methanol is widely adopted as a fuel, environmental exposures will increase through ingestion of contaminated drinking water, inhalation of vapors from evaporative or other emissions, and dermal contact.

As part of HEI's methanol research program, the Institute funded Dr. Burbacher and colleagues to evaluate the toxic and reproductive effects of inhaled methanol vapors in adult female nonhuman primates (*Macaca fascicularis*) and the neurobehavioral effects in their infant offspring who were prenatally exposed to methanol. Burbacher's study was one of four HEI-supported research projects funded to develop information that would help in assessing risks of environmental exposures to methanol, especially in ambient air. Because of the known sensitivity of the developing fetus to ethanol and other toxins, this study focused on developmental effects in a species that most closely resembles humans in the way it metabolizes methanol and in its response to neurotoxicants.

The investigators submitted two draft reports for review by HEI.* These reports underwent peer review by scientists with expertise in methanol metabolism, toxicology, neurobehavioral assessment, and biostatistics, as well as by the HEI Health Review Committee. During the review process, the Review Committee and the investigators had opportunities to exchange comments and to clarify issues. The Committee's Commentary on the Investigators' Report is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the investigators' results into scientific and regulatory perspective.

GOALS AND SPECIFIC AIMS

The goals of Dr. Burbacher's study were to determine the effects of long-term exposure to methanol vapors (0, 200, 600, and 1,800 ppm)† on methanol pharmacokinetics and reproduction in adult female nonhuman primates (*M. fascicularis*) and on the neurobehavioral and developmental measures in their offspring who had been exposed to methanol in utero.

The investigators' specific aims were to determine whether

- repeated exposure to methanol alters methanol metabolism in adult female monkeys;
- pregnancy alters methanol metabolism;
- long-term exposure to methanol produces overt adult toxicity, or reproductive toxicity, or both; and
- long-term exposure to methanol in utero affects offspring development, especially neurobehavioral development.

The investigators successfully completed all of their specific aims. The results for Specific Aims 1 through 3 are found in Part I of the Investigators' Report; the results for Specific Aim 4 are in Part II.

STUDY DESIGN AND CONDUCT

Studies of long-term inhalation exposure, especially those that involve primates, are difficult to perform for a variety of reasons. The exposure protocol requires rigorous quality assurance procedures in order to deliver reproducible concentrations of methanol vapors over an extended period of time. If animals are housed in individual cages (as in this study), the necessary repetitions of daily exposures limits sample size. Moreover, realistic exposure protocols are likely to cause subtle biologic effects, necessitating examination of a relatively large

* Reproductive and Offspring Developmental Effects Following Maternal Inhalation Exposure to Methanol in Nonhuman Primates: Part I. Methanol Disposition and Reproductive Toxicity in Adult Females; Part II. Developmental Effects in Infants Prenatally Exposed to Methanol.

† Conversion factor: 1 ppm = 1.31 mg/m^3.

number of animals. In addition, the behavioral testing strategies used to assess neurobehavioral toxicity include complex tests that require technical expertise and are labor intensive. The investigators are to be commended for their comprehensive and careful approach to the design and conduct of this study, including cohort and exposure group assignments, the methanol exposure protocol, and observational measurements.

As with any study involving live subjects, unexpected problems can and did lead to some departures from the original study design. All such changes were discussed with the HEI Health Research Committee and staff and are well documented in the Investigators' Reports. This attests to the rigorous quality assurance and quality control procedures employed by the investigators throughout the course of this study. The raw data were presented in detail in the draft reports, which allowed the external reviewers and the HEI Health Review Committee to evaluate the results independently and to arrive at their own conclusions.

SUBJECTS AND COHORTS

Burbacher and colleagues used *M. fascicularis*—a nonhuman primate—as the test species. The investigators employed a two-cohort study design utilizing a total of 48 adult females (24/cohort) and 4 adult males (2/cohort). All the males and the females in Cohort 1 were born in the wild and imported to the United States, shipped to the University of Washington in 1991, and quarantined at the University of Washington Primate Center. Cohort 1 monkeys were exposed to methanol from November 1992 through March 1994. The females in Cohort 2, a mixed group consisting of animals born in the wild and animals born in primate colonies located in the United States, were assigned to the project in 1994. These animals were exposed to methanol during the period of November 1994 through February 1996.

The investigator selected a two-cohort study design in order to minimize the number of subjects tested simultaneously while providing a sufficient sample size to detect small methanol-induced effects. Although the two-cohort design was necessary for practical reasons, it did introduce variability into the study that may be important. As discussed in the Results section, the effects observed in this study were small and, in some cases, the differences between cohorts appear to be as great as or greater than the treatment-related effects. Thus, the reported results must be considered in the light of possible cohort effects.

The study period for each cohort included (1) a baseline period (no exposures) of approximately four months, during which time the investigators characterized at least three menstrual cycles, measured selected biochemical parameters, and trained the animals; (2) a prebreeding period of four months, during which females were exposed daily to either filtered air or to one of three concentrations of methanol; (3) breeding and pregnancy periods during which daily exposures continued; and (4) a nine-month period after birth (no exposures) when the investigators assessed infant development. With the assistance of additional funding from the U.S. Environmental Protection Agency (EPA)*, the infants were maintained in the primate facility at the University of Washington after the HEI-funded study ended in order to monitor their growth and overt toxicity after nine months of age. The investigators have also obtained support from the National Institute of Environmental Health Sciences to conduct additional testing on animals one year of age and older.

The investigators originally estimated that each adult female would produce one offspring, providing 12 subjects in each exposure group. However, the conception rate for all groups was lower than anticipated (they were slightly lower in Cohort 2 than Cohort 1). Different males were used for breeding in the two Cohorts. The final number of offspring in each exposure group was as follows:

- Control (0 ppm) — 8 infants (5 in Cohort 1; 3 in Cohort 2)
- Methanol (200 ppm) — 9 infants (5 in Cohort 1; 4 in Cohort 2)
- Methanol (600 ppm) — 8 infants (4 in Cohort 1; 4 in Cohort 2)
- Methanol (1,800 ppm) — 9 infants (5 in Cohort 1; 4 in Cohort 2)

In addition to these live births, two infants (one in the control group [Cohort 1] and one in the 600 ppm group [Cohort 1]) were stillborn, and one fetal death occurred in the 1,800 ppm group (Cohort 1). The neurobehavioral analyses were performed on 34 offspring.

The number of animals in each group (8 to 9) was small when compared to the numbers used in studies of children exposed to lead, ethanol, or other neurotoxicants. Although this presents a challenge for identifying subtle exposure-related effects, the number of subjects is greater than that used in most nonhuman primate studies.

EXPOSURE

For the first two years of this study, the investigators devoted most of their time to setting up an inhalation laboratory specially adapted for the animal exposures. The

* A list of abbreviations and other terms appears at the end of each part of the Investigators' Report for your reference.

inhalation laboratory contained standard systems for filtering and maintaining temperature and humidity control of incoming air. Eight inhalation chambers were constructed; each chamber housed one animal in a cage designed to permit free flow of air or methanol. The investigators developed and tested procedures for generating methanol vapors and measuring methanol concentrations at various locations in the chambers. HEI consultants and staff verified that all inhalation and monitoring systems were functioning properly before any animals were exposed.

Burbacher and colleagues generated methanol vapors by passing compressed air through gas dispersion bottles filled with chromatographic grade (99% pure) methanol that had been heated to 36°C. The investigators did not provide information on either the number of batches of methanol they used or any attempts to verify purity or identify impurities during the study. Although these issues do not necessarily impact the outcome of the study, better characterization and reporting of the source material would have provided information needed to replicate the study. The investigators used eight independent generation and delivery systems and varied the flow rate of compressed air to deliver the various concentrations of methanol to the inhalation chambers. The investigators monitored the levels of methanol and carbon dioxide and the dew point in each inhalation chamber during the exposures. As evidenced by the data presented in Part I (Figures 5 and 6 of the Investigators' Report), the inhalation facility and methanol delivery system provided accurate and reproducible exposures to methanol vapor at or near the target concentrations.

Burbacher, in consultation with HEI, selected the methanol exposure concentrations (0, 200, 600, or 1,800 ppm) and an exposure period of 2.5 hours (methanol vapors were delivered to the chambers for 2 hours and the animals remained in the chamber for 30 additional minutes while the methanol dissipated) to cover worst-case environmental and occupational situations. An important factor in selecting these exposure concentrations were projections of the environmental exposures that might be expected if motor vehicles were powered by methanol fuel. Methanol levels in ambient air (1 to 10 ppb in remote areas and 4 to 100 ppb in cities) are currently orders of magnitude lower than the exposure concentrations used in the Burbacher study (EPA 1993; WHO 1997). Simulation models (based on limited emissions data) predict that if all vehicles in the United States were converted to 100% methanol fuel, the levels of methanol in ambient air could increase to between 1 and 10 ppm in urban air and, in isolated situations (such as enclosed spaces), could reach 200 ppm (HEI 1987; Kavet and Nauss 1990). Recommended occupational exposure limits are 200 ppm (National Institute of Occupational Safety and Health 1976; American Conference of Governmental Hygienists 1999). The investigators included a high dose (1,800 ppm) to maximize the potential for observing effects in the developing fetus without causing maternal toxicity. Having three methanol exposure doses also provided an opportunity to evaluate exposure responses.

The investigators exposed adult female monkeys to methanol vapors for 2.5 hours/day, seven days a week during three exposure periods: prebreeding (approximately four months), breeding (this period included 1 to 4 breeding cycles and varied from 3 days to 8 months), and pregnancy (approximately 5.5 months). Therefore, the adult females were exposed to methanol for 9.5 to 17.5 months. The adult males were not exposed to methanol. The infants were exposed to methanol in utero for the duration of the pregnancy.

PHARMACOKINETIC ANALYSES AND TOXICITY ASSESSMENT

Burbacher and colleagues used a multidisciplinary approach to assess the effects of exposure to methanol vapor on the following parameters.

Methanol pharmacokinetics. The investigators evaluated the influence of repeated methanol exposures and pregnancy on methanol disposition. (*Disposition*, a term used by toxicologists and pharmacologists to describe the fate of a compound in the body; includes absorption, distribution, metabolism, and excretion.)

Adult toxicity. The effects of methanol exposures on adult female monkeys were routinely monitored.

Reproductive toxicity. Maternal reproductive toxicity was assessed by evaluating conception rates, complications during pregnancy and delivery, pregnancy duration, and live-birth rate.

Developmental neurotoxicity. The neurobehavioral development of the offspring was monitored by a standard battery of tests administered throughout the first nine months of life.

One of the strengths of this study is that the investigators designed their experiments to evaluate exposure-dose-response relations. Blood methanol concentrations provide a measure of internal dose that is far superior to external exposure concentrations for estimating human risks. Because such exposure-dose-response information for methanol is not available for humans, data from relevant animal models are critical in estimating safe exposure levels.

QUALITY ASSURANCE

Burbacher and colleagues employed rigorous internal quality control procedures throughout the study. They calibrated their equipment and instruments at regularly scheduled intervals and paid particular attention to verifying the accuracy of the methanol delivery system and characterizing the methanol concentrations in the exposure chamber. The investigators employed internal quality control procedures for their blood methanol and plasma formate analyses at appropriate intervals. In addition, Burbacher's group participated in an interlaboratory evaluation of the blood methanol analysis with other investigators in HEI's methanol program to determine the extent of variability among the laboratories in the analytical results. Four HEI-funded investigators conducted three comparisons of their blood methanol concentrations during a three-year period. In general, the results from the three laboratories agreed, with a coefficient of variation ranging from 3% to 25%. Burbacher's quality control procedures for infant neurobehavioral assessment focused on the reliability of the testers and were performed every three to four months. Reliability criteria for nearly all of the procedures exceeded 90%.

In addition to the investigators' internal quality control procedures, HEI also engaged an external quality assurance specialist. The purpose of this oversight was to assure that the data were collected under defined conditions, were reliable, and were properly recorded. The quality assurance auditors made site visits at regularly scheduled intervals to observe the conduct of different aspects of the study, audited the data, and reviewed the Investigators' Reports. As indicated in the Quality Assurance Reports (Appendix D in Part I and Appendix B in Part II of the Investigators' Report), the study was well documented and the Investigators' Reports accurately reflect the raw data.

METHODS, RESULTS, AND INTERPRETATION

METHANOL METABOLISM AND PHARMACOKINETICS

Burbacher and colleagues collected blood from all female subjects every two weeks throughout the study to monitor methanol and formate concentrations. For these studies, they drew the blood samples approximately 30 minutes after the cessation of exposure to methanol. The investigators determined methanol concentrations in whole blood samples by capillary gas chromatographic analysis utilizing acetronitrile as an internal standard. This reliable and sensitive analytical procedure yields reproducible results. In addition to the biweekly monitoring of blood methanol and formate levels, they also conducted full pharmacokinetic profiles (changes in the blood clearance, distribution volume, and elimination half-life of methanol) at the following time intervals: (1) after the first exposure to methanol, (2) after three months of methanol exposure, and (3) twice during pregnancy. For the pharmacokinetic studies, the investigators collected blood 30 minutes prior to the beginning of a daily exposure and at intervals from 30 to 360 minutes after termination of exposure.

The endogenous or background blood methanol concentrations in the adult female monkeys ranged from 0.7 to 5.7 µg/mL and are comparable to those reported in humans (1 to 3 µg/mL) (Sedivec et al. 1981; Cook et al. 1991; Chuwers et al. 1995). As expected, a single exposure to methanol vapors elevated blood methanol concentrations. For the lowest exposure dose (200 ppm), blood methanol concentrations were 2 to 9.4 µg/mL at 30 minutes after exposure. At the same time point, blood methanol concentrations were approximately 5 to 22 µg/mL in the monkeys exposed to 600 ppm and 19 to 60 µg/mL in those exposed to 1,800 ppm. The pharmacokinetic studies indicated that for all exposure groups, the peak concentrations of blood methanol were observed 30 minutes after the methanol exposures ended. Blood methanol had returned to background levels by 120 minutes after the exposure ended for the monkeys exposed to 200 ppm methanol, and by 240 and 360 minutes for the 600 and 1,200 ppm–exposure groups, respectively.

The blood methanol concentrations reported by Burbacher and colleagues for a single 2.5-hour exposure of *M. fascicularis* to methanol vapors are comparable to those obtained by Horton and colleagues (1992) for adult male rhesus monkeys exposed to 200 ppm methanol for 6 hours. In healthy human subjects, increases in blood methanol levels of 1.3 µg/mL (Cook et al. 1991) and 6.5 µg/mL above baseline (Osterloh et al. 1996) have been reported after inhalation of 200 ppm methanol vapors for 75 minutes or 4 hours, respectively. Burbacher's data indicate that following single 2.5-hour exposures to 200 ppm methanol, blood methanol concentrations were elevated 2-fold. For the 600 and 1,800 ppm–exposure groups, elevations in blood methanol concentrations above endogenous levels were approximately 3- to 4-fold and 13- to 16-fold, respectively.

Burbacher reported no measurable increase in plasma formate concentrations above endogenous levels after either single or repeated exposures to 200, 600, or 1,800 ppm methanol. This important finding suggests that either

single or repeated 2.5-hour exposures to methanol vapors—even at concentrations as high as 1,800 ppm—are unlikely to overwhelm the body's capacity to metabolize this toxic intermediate (plasma formate). These results are consistent with those obtained by Horton and colleagues (1992) in male rhesus monkeys after single exposures to similar amounts of methanol that achieved similar blood methanol concentrations. They also agree with the finding that formate does not accumulate in the blood of healthy human subjects after a single exposure to 200 ppm methanol (Cook et al. 1991; Lee et al. 1992; d'Alessandro et al. 1994; Chuwers et al. 1995). It should be noted that although Burbacher found that formate did not accumulate in maternal blood, the present study does not resolve the issue of possible formate accumulation in fetal tissues. Because the metabolic capacity of fetal liver and other tissues is limited compared with maternal tissues, the fetal tissues possibly could be exposed to higher concentrations of formate than maternal tissues.

Burbacher and colleagues also conducted pharmacokinetic studies to determine whether the disposition of methanol changes during repeated methanol exposures and to assess pregnancy-related changes. An additional implicit aim of the study was to assess the effects of different methanol exposure concentrations on methanol disposition. These studies demonstrate that methanol disposition differed between the 600 ppm and 1,800 ppm exposure conditions, presumably due to saturation of methanol metabolism. This outcome is what one might expect because nonlinear methanol elimination has been documented in nonhuman primates (Makar et al. 1968; Horton et al. 1992), but it was uncertain that the 1,800 ppm exposures would be high enough to produce blood methanol concentrations that exceeded the apparent in vivo Michaelis-Menten constant (K_m) for methanol metabolism. Indeed, the investigators selected the 1,800 ppm exposure level to avoid the substantial accumulation of methanol that might occur if the K_m was exceeded during individual exposure sessions. The investigators utilized both linear and nonlinear kinetic models to describe the disposition of methanol after 600 ppm (linear only) and 1,800 ppm (saturable, Michaelis-Menten) methanol exposures. Blood methanol concentrations did not increase sufficiently in the animals exposed to 200 ppm methanol to allow inclusion of these data in the pharmacokinetic analysis. As noted elsewhere in this Commentary, formate concentrations were not elevated sufficiently in any of the exposure conditions to allow kinetic analysis of this methanol metabolite.

The pharmacokinetic studies suggest that repeated or long-term exposures to methanol increased the rate of methanol clearance, although the precise mechanism could not be ascertained. This change in methanol disposition during long-term exposure is a novel observation and, as suggested by the investigators, is likely due to an increased rate of methanol metabolism. Similar observations have been made for ethanol, presumably due to substrate induction of the enzymes involved in its metabolism (Kunitoh et al. 1993). If methanol does induce metabolic enzymes in the pregnant monkey, enzyme induction in fetal liver may also occur, as has been reported for ethanol (Carpenter et al. 1997). Alternatively, the decrease in blood methanol concentration during long-term exposure may be a function of decreased efficiency of pulmonary absorption, as has been reported in the rat (Perkins et al. 1996). Comparison of the distribution of methanol with that of ethanol may help to define the mechanism underlying the time-dependent changes in methanol disposition.

The differences in kinetics of the enzyme systems responsible for methanol metabolism in rodents and primates prompted examination of methanol disposition in the pregnant monkey. Previous studies have shown that the rate of methanol metabolism is decreased, albeit to a modest degree, in pregnant mice and rats (Pollack et al. 1996; Ward and Pollack 1996; Ward et al. 1997). Decreases in the rate of methanol elimination during pregnancy could lead to unanticipated accumulation of the alcohol during long-term exposure, particularly as blood concentrations approach the K_m for metabolism. In contrast to what has been observed in rodents, the results of this study indicate that pregnancy is not associated with significant changes in methanol disposition in monkeys. No measurable accumulation of formate was observed in the pregnant or nonpregnant animals. Thus, accumulation of methanol, or formate, or both during long-term exposure would not be expected to be higher in pregnant primates compared with their nonpregnant counterparts.

Burbacher and colleagues measured serum folate levels (using a commercial radioimmune assay) at five time points during the study. (*Folate* is a generic term for a family of vitamins that includes folic acid and related compounds with related biologic activity.) The rationale for inclusion of the folate analyses was predicated on substantial evidence that pregnancy can induce folate deficiency and the possibility that such deficiency may be exacerbated by long-term methanol exposure. Burbacher reported no changes in serum folate levels in adult female monkeys associated with methanol exposure, pregnancy, or the two conditions combined. These data suggest that in well-nourished nonhuman primates, pregnancy in combination with long-term methanol exposure does not produce overt folate deficiency. Although these results are

reassuring, serum folate measurements are not the most sensitive indicator of folate deficiency. Measurements of red blood cell folate would have provided more accurate estimates of tissue folate stores (Scott et al. 1994).

MATERNAL TOXICITY ASSESSMENT

This study assessed adult maternal methanol toxicity with observational techniques designed to detect labored respiration and deficits in visual and motor performance. These procedures provide a reasonable, although qualitative, measure of overt methanol toxicity. Detailed assessments of retinal and optic nerve function might have identified subtle functional changes associated with methanol exposure, but such studies would have added considerably to the complexity and cost of the study. Given that the primary focus of the study was on developmental effects in the exposed offspring, the procedures used to assess adult methanol toxicity were appropriate. The investigators' maternal toxicity assessments indicated that long-term methanol exposure at concentrations as high as 1,800 ppm did not affect weight gain or the apparent health of the female monkeys prior to or during pregnancy.

REPRODUCTIVE TOXICITY

Screening for reproductive methanol toxicity has always been an important component of toxicity testing for human health risk, but in recent years the scope of testing has included additional endpoints associated with the developing organism and adult reproductive parameters (EPA 1998; International Life Sciences Institute 1998).

Burbacher and colleagues used standard measures of reproductive performance to assess reproductive methanol toxicity, including menstrual cyclicity, conception rate, duration of pregnancy (mean gestation length), delivery rate, live-birth index, and size and weight of the offspring. The onset and duration of menstruation were assessed every day as the animals were transferred to and from the inhalation laboratory. Timed matings were conducted by breeding females with one of two nonexposed males for four hours each day on the 11^{th}, 12^{th}, and 13^{th} day of the cycle after the animals had exhibited a minimum of seven menstrual cycles (three prior to methanol exposure and four after exposure began). The investigators used semen detection as evidence of mating; pregnancy was confirmed by blood analysis of progesterone. Females who did not conceive were bred again to the same male during successive months for a maximum of four breeding cycles.

The investigators reported no effects of methanol exposure on the menstrual cycle, conception rates, rates of complications during pregnancy or delivery, or live-birth delivery rates (Table 1). Two animals (one in the control group and one in the 600 ppm group) delivered stillborn infants and one animal in the 1,800 ppm–exposure group required a Cesarean section to deliver a dead fetus. One infant in the control group was born prematurely. The number of animals requiring a Cesarean section were zero in the 0 ppm group, two in the 200 ppm group, two in the 600 ppm group, and one in the 1,800 ppm group.

The only reported adverse effect of methanol exposure on reproductive performance was an apparent shortening of the average duration of pregnancy (for live-born deliveries) by about six to eight days (analysis of variance [ANOVA], $p = 0.03$). No evidence suggested a dose-response relationship for this effect, nor was the reduced gestation length in methanol-exposed monkeys accompanied by any observed effect on neonatal size, weight, or other physiologic parameters.

Two questions remain: Is methanol responsible for the apparent shortening of the gestation period and, if so, what is the mechanism of this effect? Excursions outside the normal range for reproductive parameters can occur as a result of exposure to a reproductive toxicant or by chance. Reference to control data for a given species—both in the investigators' laboratory and in other laboratories—can be helpful in interpreting such data. The length of gestation reported for *M. fascicularis* in several colonies is 155 to 165 days (Hendrickx and Dukelow 1995). In Burbacher's study, 5 of 8 animals in the 0 ppm–exposure group had gestation lengths greater than 165 days compared with 1 of 9 animals in the 200 ppm group, 2 of 8 in the 600 ppm group, and 4 of 9 animals in the 1,800 ppm group. In contrast, no animal in the control group had a gestation length less than 155 days. The numbers of animals in the methanol-exposed groups with gestation lengths less than 155 days were one in the 200 ppm group, one in the 600 ppm group, and two in the 1,800 ppm group. It is unclear whether the apparent effect of inhaled methanol vapors on the duration of pregnancy was due to an unexplained extended gestation for the control animals or to methanol-related toxicity in the exposed monkeys.

Hormonal imbalance is one mechanism by which inhaled methanol could influence gestation length. In many species, a sudden increase in the secretion of cortisol by the fetal adrenal gland is a critical factor in the initiation of labor and parturition. Methanol exposure of the mother may somehow cause precocious development of the fetal hypothalamus, anterior pituitary, or adrenal cortices that initiates premature expulsion of the fetus. This hypothesis

Table 1. Prenatal Methanol Exposure Associated with Reproduction and Infant Growth in Nonhuman Primates

Outcome	Methanol-Induced Effects[a]	Comment
Maternal Measures		
Menstrual cycle	N	
Conception rate	N	
Complications during pregnancy and delivery	N	Cesarean sections performed on two animals exposed to 200 ppm methanol and two exposed to 600 ppm
Live-birth index	N	One dead fetus (1,800 ppm) and two stillborn deliveries (0 ppm and 600 ppm)
Gestation length	Y	6- to 8-day reduction in duration of pregnancy in methanol-exposed animals; no dose–response relation
Infant Measures		
Birth weight	N	
Other physical parameters (head size, crown–rump length)	N	
Growth rate		
0–9 months	N	
9 months up to 6 years	Y	Wasting occurred in 2 of 4 female infants in Cohort 1 exposed to 1,800 ppm methanol; wasting was not observed in Cohort 2 animals

could be tested by determining plasma adrenocorticotropic hormone or cortisol levels in the offspring immediately after delivery. This is a suggestion that can only be made in hindsight because it was not known at the outset of the study that a shortened gestation period would be encountered in the methanol-exposed mothers.

INFANT GROWTH AND SURVIVAL

Growth retardation is one indicator of developmental toxicity, although it is not as sensitive as other measures. Despite the shorter gestation period, methanol exposure in utero did not affect body weight of the newborns, nor did it affect other birth characteristics such as crown–rump length, head circumference, head length, or head width. Prenatal methanol exposure also did not affect infant growth rates during the first nine months of life. After one year of age, however, two female offspring in Cohort 1 that were prenatally exposed to 1,800 ppm methanol developed a wasting syndrome. The cause of this wasting is not known. Routine clinical assays and autopsy examination did not reveal the causes of death, nor were any changes in blood levels of growth hormone or thyroxine observed. Wasting of the sort described in these two animals is rare in primate colonies in the United States and thus should be viewed as an important finding. As of September 1999, no other cases of wasting syndrome had been observed in the remaining animals in Cohort 1 (5.5 to 6 years of age) or in any animals in Cohort 2 (3.5 to 4 years of age).

DEVELOPMENTAL NEUROTOXICITY

The use of nonhuman primates to characterize the developmental effects of potentially toxic compounds offers many advantages over rodents (Paule 1998). Placental structure and function are similar in pregnant human and nonhuman primates. Moreover, the nonhuman primate infant is similar to humans in complexity and maturity of brain function at birth and shows similar milestones in the development of cognitive function (Golub 1990). Neurobehavioral test batteries provide a tool for documenting clinical effects, some of which may not be apparent until later in life (Fiedler et al. 1996). Many of the tests used in children have similar counterparts for assessing developmental toxicity in nonhuman primates (Evans 1990; Golub 1990; Overman et al. 1992). In her review of neurobehavioral test batteries, Golub (1990) noted that developmental studies with nonhuman primates can produce valuable health information. She cautioned, however, that particular attention needs to be paid to experimental design and statistical analyses because the studies tend to have small sample sizes and include a large number of endpoints. In the case of methanol, nonhuman primates offer the additional advantage that the rate of methanol metabolism is similar to that in

humans and researchers can address the impact of methanol exposure and pregnancy on folate status and, ultimately, on formate accumulation.

Burbacher and colleagues conducted an exceptionally thorough analysis of neonatal and infant behavior in nonhuman primates exposed to methanol in utero. The behavioral responses they measured can be divided into three categories: neonatal responses, infant sensory and cognitive function, and infant social behavior. These encompass all the measures traditionally used to assess the neurobehavioral effects of chemical exposures (Kulig 1996). The investigators' results are summarized in Table 2.

Because of the small number of offspring available for statistical analysis (a total of 34) and the large number of outcome variables (10 specific domains), the investigators designed their statistical analysis to detect, with as much power as possible, the differences between the control and methanol-exposed groups. Within each domain they first identified primary variables (Table 3 in Part II of the Investigators' Report). They then performed a global ANOVA test separately for each primary variable to identify any differences across the four exposure groups. This test has poor statistical power, so it was supplemented with a test contrasting the control group with the combined methanol exposure groups and then also with tests contrasting the control group with each of the specific methanol groups. These latter two sets of tests (identified by Burbacher as linear contrast tests) were not adjusted for multiple testing and so are liberal; that is, they may declare too many differences as statistically significant. If such tests identify results with low p levels (for example, $p < 0.01$) and these results also display consistency across the methanol groups, then it is reasonable to consider them methanol-related effects. However, if they identify statistically significant differences of the control group with only one methanol group and these differences are not consistent with the other methanol groups, then they should be viewed as probable random fluctuations (that is, chance findings). At best, they can be considered useful for generating hypotheses that can be confirmed or refuted in later studies.

In addition to the above set of statistical procedures, Burbacher and colleagues performed tests controlling for sex and cohort. They also performed post hoc tests of trends across the control and methanol groups. Such procedures offer insight into the data, but any interpretations from them should be viewed with caution because they represent extensive statistical analyses without appropriate control for misidentifying random fluctuations as statistically significant. Burbacher and colleagues also performed analyses on the secondary variables identified in Table 3 of Part II of the Investigators' Report. Results of these analyses can be used to help explain what was seen with the primary variables, but statistical significance based solely on these analyses is not justified.

Although the statistical procedures selected by Burbacher have the problem of potentially identifying random fluctuations in the data as statistically significant, they are appropriate for such a large set of outcome variables and small number of subjects. However, for the reasons described above, great care must be taken in interpreting the results of the different analyses.

Neonatal Behavior

Neonatal behavior was assessed every other day during the first two weeks of life using a modification of the behavioral assessment scale developed by Brazelton for evaluating multiple aspects of neonatal nervous system function in human infants (Brazelton 1973). The investigators scored the animals' responses for nine reflex tests and seven behavioral tests and determined the age at which infants received the optimal scores. They then collapsed the test results into four categories: behavioral state, reflexes, muscle tone, and responsivity. Data for three of the 16 tests—sucking, response strength, and response speed—were not used in the final analysis because several infants did not receive optimal scores during the two weeks of testing. Analysis of the data using ANOVA models did not indicate any effect of methanol exposure for any of the four categories. For the behavior state factor (animals scored as alert, awake, or aware), linear contrast tests suggested that animals in the methanol-exposed groups took longer (2.3 to 3.3 days) to achieve their best score than those in the control group (1.8 days). The investigators attributed this effect to the influence of Cesarean-section delivery rather than to methanol exposure because the offspring delivered by Cesarean section were the last to receive the optimal score for the state factor. (No infants in the control group and five infants in the methanol-exposed groups were birthed by Cesarean section.)

Infant Sensorimotor Development

Burbacher used two tests to evaluate sensorimotor development: the primary outcome variable was the Visually Directed Reaching Test and the secondary outcome variable was the Gross Motor Milestones Test. The endpoint for the Visually Directed Reaching Test was the postnatal age at which the infants successfully retrieved a brightly colored object on 8 of 10 consecutive trials (beginning at two weeks of age) over two testing periods. Analysis of the results using the ANOVA model revealed no significant effects of methanol exposure on the acquisition of this skill

Table 2. Effects of Prenatal Methanol Exposure on Measures of Infant Neurobehavior (2 Weeks to 9 Months)

	Results of Statistical Significance for Methanol-Related Effects[a]			
Behavioral Tests[b]	ANOVA	*t* Test	Linear Contrast Test (Group)	Linear Contrast Test (Cohort/Sex)
Neonatal Behavior				
Neonatal Behavioral Scale[c]	N	—	N	—
Sensory Function				
Visually Directed Reaching Test	N	—	Response delayed in monkeys exposed to 1,800 ppm ($p = 0.04$) Positive test for linearity ($p = 0.04$)	Response delayed in males exposed to methanol ($p = 0.008$) Statistically significant differences for male monkeys exposed to 600 ppm ($p = 0.007$) and 1,800 ppm methanol ($p = 0.03$)[d]
Gross Motor Milestones Test	N	—	N	Gross motor development slower in males exposed to 600 ppm methanol and faster in females exposed to 200 or 600 ppm methanol
Preferential Looking Test	—	—	—	—
Cognitive Function				
Object Concept Test—Screen	N	—	N	—
Object Concept Test—Well	N	—	N	—
Spatial Discrimination and Reversal Test		—	N	—
Fagan Test for Infant Intelligence Abstract Patterns	N	All groups (except 600 ppm) showed significant novelty preference	Monkeys exposed to 600 ppm methanol did not show significant novelty preference ($p = 0.03$)	ANOVA model controling for cohort indicated difference between controls and all methanol-exposed groups ($p = 0.005$) Methanol-exposed monkeys in Cohort 2 spent less time looking at novel stimuli ($p = 0.05$) No effect of methanol in Cohort 1
Fagan Test for Infant Intelligence Social Patterns	N	Methanol-exposed groups did not show significant novelty preference	N	N
Nonmatch-to-Sample Test	N	—	N	N
Infant Social Behavior				
Passive	N	—	N	—
Social	N	—	N	—
Nonsocial	N	—	N	—

[a] N = no effect of methanol exposure; — = analysis not reported.

[b] Italic type = secondary variables (all others are primary variables).

[c] Some suggestion that methanol-exposed animals were less alert; attributed to birth by Cesarean section.

[d] Results based on a small number of male animals ($n = 2–3$/group).

($p = 0.2$). Linear contrast tests, however, indicated that the difference in the mean postnatal age to successfully retrieve an object between the control group (30 days) and the 1,800 group (40 days) was statistically significant ($p = 0.04$). When the analysis was further broken down by sex, the test for linearity was statistically significant for male infants ($p = 0.07$) and the results for male infants exposed to 600 ppm ($p = 0.007$) and 1,800 ppm methanol ($p = 0.03$) were significantly different from the male controls. However, the latter results should be interpreted cautiously as they are based on three males in the 600 ppm–exposure group and two in the 1,800 ppm–exposure group and may have been influenced by the low mean age reported for male control monkeys to successfully retrieve an object (Table 12 in Part II of the Investigators' Report). Also, no effects of methanol on the Visually Directed Reaching Test were reported for the female monkeys. The fact that the methanol-exposed animals had a shorter gestational age than the controls is also a consideration. When Burbacher and colleagues took this factor into account in the analyses, however, they obtained the same results.

The secondary variable used to measure sensorimotor development over the first nine months of life was the Gross Motor Milestones Test. Again, using the ANOVA model, methanol exposure had no significant effect on the age at which animals in the four groups were able to accomplish the climbing tasks. Sex-specific linear contrast tests indicated that females in the 200 ppm and 600 ppm–exposure groups achieved the milestones earlier (101 and 92 days, respectively) than the female monkeys in the control group (141 days). Males in the 600 ppm–exposure group achieved the milestones later (137 days) than control male animals (102 days). Although all of these results were statistically significant, the fact that the effect was in the opposite direction for males and females and did not show an exposure response raises the question as to whether this is a meaningful result.

Infant Visual Acuity

The investigators measured visual acuity (a measure of sensory function) over the first 12 weeks of life by using forced-choice preferential looking procedures in which the infants were presented with a series of vertical black and white stripes that were paired with a gray field of equal intensity. Monkeys are known to fixate preferentially on the stripes. By alternating stripe width and determining the smallest width fixated, the testers estimated visual acuity. For reasons that are not clear, both the control and the methanol-exposed monkeys had a high failure rate for this test. Because of the small number of animals that successfully performed the test, the investigators did not evaluate methanol exposure effects on this outcome. This is unfortunate because one of the hallmarks of acute methanol toxicity in humans is damage to the visual system.

Infant Cognitive Function

Burbacher and colleagues assessed the effect of methanol exposure on cognitive function development by using two tests of spatial memory (the Object Concept Test and the Spatial Discrimination and Reversal Test) and two tests of recognition memory (the Fagan Test of Infant Intelligence and the Nonmatch-to-Sample Test).

The Object Concept Test (sometimes called the Object Permanence Test) evaluates the infant's ability to recognize that an object placed out of sight is still present. Methanol-exposed monkeys took the same amount of time as the control animals to retrieve objects hidden behind a screen or in a covered well. The Spatial Discrimination and Reversal Test is a test of learning and memory that involves training the infants to a visual stimulus and, after they have learned the task, reversing the previously correct stimulus. Infants were tested beginning at approximately five months of age. For all animals, the number of trials required to successfully learn this task ranged widely; however, no effects of methanol exposure were observed. Thus, for these two tests, the investigators observed no effect of methanol exposure on the development of infant spatial memory.

The infants were also tested on a series of visual recognition tasks beginning at 190 days after conception using the primate version of the Fagan Test of Infant Intelligence. In these tests, the preference of the infant for a novel stimulus versus a familiar stimulus is interpreted as indicating the development of memory (Fagan and Singer 1983; Paule 1998). The investigators recorded novelty preference scores for seven abstract stimuli and four social stimuli for each infant. Novelty preference was measured by recording the percentage of time spent looking at the novel target when it was paired with the familiar target. Novelty preference is indicated if the subject's time spent looking at the novel target exceeds chance (that is, significantly greater than 50%). When an ANOVA test was employed, methanol exposure did not correlate with the degree of novelty preference (that is the percentage of time spent looking at novel stimuli) for either abstract patterns or social stimuli. However, further testing revealed two statistically significant findings. First, Burbacher and colleagues reported that all exposure groups showed a significant novelty preference for abstract patterns; however, none of the three methanol-exposed groups showed a novelty preference for social stimuli, whereas the control

group did exhibit such a preference. Second, the linear contrast analysis suggested that monkeys exposed to 600 ppm methanol spent less time looking at novel abstract patterns than did control monkeys or monkeys exposed to 200 ppm or 1,800 ppm methanol. Burbacher also analyzed the novelty preference data using the abstract patterns separately for the two cohorts and found that for Cohort 2 (2 to 4 animals/group), the percentage of time that animals spent looking at novel stimuli was significantly lower for methanol-exposed groups (70% for controls vs. 50% to 61% for exposed groups).

The secondary outcome variable for recognition memory was the Nonmatch-to-Sample Test in which the infants (approximately 7 months of age) were trained on a complex task: responding to a new cue to obtain a reward. For all animals, the number of trials required to learn the tasks varied widely and methanol exposure had no significant effect.

Infant Behavior

To assess the development of infant social behavior over the first seven months of life, the investigators observed groups of infant monkeys in a playroom for 30 minutes a day, three days a week. They scored the animals' behavior on the time spent in three categories: passive behaviors, social behaviors, and nonsocial behaviors. Methanol exposure did not correlate with any of these scores.

Discussion of Developmental Neurotoxicity

Clinical case reports and epidemiologic studies have identified a number of drugs, chemicals, and other substances that affect the developing nervous system in humans (Slikker and Chang 1998). Depending on the substance and the dose, these effects may be readily observable or subtle, permanent or reversible; some effects occur early in development and then disappear; others only become apparent late in life. Because of the complexity of the nervous system and the fact that toxic effects can be expressed on many different levels, no single neurobehavioral test can be used to assess the broad range of potential effects. Instead, researchers use multiple outcome measures that encompass diverse neurological functions (Russell et al. 1990; Kulig 1996). The tests include observational methods to document changes in physical growth or overt signs of neurotoxicity, as well as behavioral tests that measure sensory functions (visual, auditory, and somatosensory), motor function, cognitive functions (learning and memory), and social behavior. Some tests provide information about complex processes that involve integration of motor, sensory, and cognitive skills. Because neurobehavioral screening batteries typically provide a wealth of data on a large number of outcomes, the statistical analysis must be carefully designed to provide adequate power to detect effects while controlling for multiple testing. In the case of nonhuman primate studies, the task is further complicated by the relatively small sample size typical for this type of study.

For ethical reasons, clinical studies of developmental neurotoxicity in humans are not possible, but two studies that examined the neurobehavioral effects of single exposures to low levels of methanol vapors in adult human volunteers illustrate the problem of detecting and interpreting small effects when the test outcomes have inherently large variability and the number of subjects is small. In their HEI-funded pilot study, Cook and colleagues (1991) exposed young male subjects to either filtered air or methanol (192 ppm) for 75 minutes. Methanol exposure had no detectable effect on most of the 20 tests used to evaluate sensory, behavioral, and reasoning performance. Performance was slightly impaired in one test measuring memory and concentration and in another test of reaction time. In a similar study (men and women exposed to 200 ppm methanol for 4 hours), Chuwers and colleagues (1995) reported that overall visual, neurophysiological, and neurobehavioral test outcomes were not significantly affected by methanol exposure. Slight effects were noted on two tests when certain between-subject variables were considered. In both studies, the effects observed were minor and within the range of normal values. The possibility that these effects were due to chance is supported by the lack of positive findings in related tests.

Behavioral assessment in Burbacher's study revealed no significant effects of prenatal methanol exposure on most domains of early neurobehavioral development (Table 2): neonatal behavior, early reflex responses, infant gross motor development, spatial memory, and social behavior. When the investigators undertook sex-specific and cohort-specific analysis of the test results, some possible differences emerged. Most notably, in a test of early sensorimotor development, male infants exposed to 600 ppm and 1,800 ppm methanol were slower to learn how to reach for brightly colored objects than males exposed to 0 ppm. The Fagen Test of Infant Intelligence suggested a possible effect of methanol exposure on recognition memory (the ability to recognize novel stimuli).

These apparent effects of in utero exposure to methanol should be interpreted cautiously. First, the number of animals in each exposure group was small, especially for the sex and cohort analyses in which most statistically significant effects emerged. Second, the positive results were reported for statistical analyses that were not adjusted for

multiple testing. Third, no dose response was generally noted for the methanol-related effects. Although the lack of a dose response may reflect the wide interindividual variability in the animals' response to methanol, evidence for a dose response is one important criterion in establishing causation (Kimmel 1998). Fourth, patterns of cognitive impairment were not consistent in methanol-exposed animals. For example, although a statistically significant effect of methanol exposure was reported for some measures of the Fagan Test for Infant Intelligence, methanol exposure had no effect on the secondary variable (Nonmatch-to-Sample Test) used to evaluate the same cognitive function. Finally, the lack of a relation between the reported positive effects and maternal blood methanol concentration is difficult to explain. In some cases a suggestive effect appeared to be present at the lowest methanol exposure dose (200 ppm) even though maternal blood methanol concentrations were increased only slightly compared with unexposed controls. These results may indicate sensitivity to even small increases in maternal blood methanol, or they may indicate random findings.

Burbacher's results, together with observations of cognitive deficits in children prenatally exposed to ethanol, suggest focusing future research on specific outcomes such as learning and memory. Research on alcohol teratogenicity indicates that in addition to the fetal alcohol syndrome that can result from consumption of high or intoxicating levels of ethanol during pregnancy, a variety of neurobehavioral abnormalities occur in children exposed prenatally to moderate amounts of ethanol (Mattson and Riley 1998; Nulman et al. 1998). These include deficiencies in intelligence, learning, motor function, memory, and visuospatial activity. The findings among studies have not always been consistent, and over two decades of research in many laboratories using a large number of subjects were required to yield studies sufficiently robust to identify the specific neurobehavioral deficits associated with prenatal ethanol exposure. Complementary animal studies have provided important information on the effects of the timing and pattern of ethanol exposure but have utilized relatively high doses of ethanol, producing large elevations in blood ethanol concentrations. For example, the blood ethanol concentrations associated with changes in activity patterns, motor development, and cognitive development in the offspring of nonhuman primates (*Macaca nemestrina*) exposed weekly to an intoxicating dose of ethanol were 2,000 μg/mL (Clarren et al. 1992).

The extensive research efforts needed to understand the consequences of prenatal and infant exposure to ethanol and other neurotoxicants illustrate the challenge for investigators studying methanol. All humans are exposed to low doses of methanol from normal metabolism, food, and fermented beverages. Exposures from ambient air are currently very low and represent only a small fraction of exposure from these other sources. Thus, most environmental exposures to methanol vapors will produce only small transient elevations in blood methanol above baseline concentrations. Information on subtle neurobehavioral effects of methanol exposure will probably not be obtainable in the general population, as has been done for ethanol or lead; and instances of widespread environmental exposures to methanol (such as occurred for methyl mercury) that would provide an opportunity to conduct epidemiologic studies of methanol's neurotoxic potential are unknown. Thus, animal models will continue to play an important role in evaluating the potential reproductive and developmental effects of prenatal methanol exposure, and nonhuman primates will provide the most relevant information for human risk assessment.

The small methanol-related effects that emerged from the study reported here need to be replicated and warrant further investigation. Fortunately, the infant monkeys used in this study are being maintained at the University of Washington and represent a valuable resource for continuing studies of methanol health effects. The investigators are currently conducting studies to determine whether the cognitive outcomes they observed in infants persist in young monkeys and whether neurobehavioral or other effects emerge later in life.

SUMMARY AND CONCLUSIONS

This study, designed to identify the effects of prolonged prenatal methanol exposure on maternal reproduction of nonhuman primates and the neurobehavioral development of their infant offspring, included extensive pharmacokinetic experiments on the disposition of methanol during repeated or long-term methanol exposure and pregnancy. The study was complex and labor intensive; the experiments were well designed and executed with appropriate quality control and quality assurance procedures. Thus, one can have confidence in the data. Moreover, because nonhuman primates are the best surrogate for people to study methanol toxicity and neurobehavioral development, the results are highly relevant for risk assessment.

The investigators exposed two cohorts of adult female *M. fascicularis* to one of four concentrations of methanol vapors (0, 200, 600, or 1,800 ppm) for 2.5 hours/day, seven days/week prior to breeding, during the breeding cycle, and during pregnancy. (Even the lowest methanol expo-

sure concentration is approximately 10,000 times higher than current methanol levels in ambient air; occupational exposures to methanol generally do not exceed 200 ppm, although occasional higher levels have been reported.)

A single 2.5-hour exposure to methanol resulted in elevations in blood methanol concentrations above normal background levels that were proportional to the exposure concentration for the two lower exposure levels and nonlinear between the 600 ppm and the 1,800 ppm exposure levels. Blood methanol levels returned to baseline within 1 to 6 hours after the exposure ended. Repeated exposures to methanol caused blood methanol concentrations to decline slightly over the first month of regular daily exposure and thereafter remain constant for at least three months. Furthermore, methanol disposition remained stable during pregnancy. The concentrations of plasma formate (the toxic intermediate) remained at basal levels during the entire study in all exposure groups. Thus, any formate derived from long-term exposure to methanol vapors contributed only a small fraction of the total circulating formate levels. These results support the conclusion that long-term exposure to methanol (200 to 1,800 ppm) by inhalation would not result in elevated blood formate concentrations in humans who have adequate folate stores. The study did not provide information on accumulation of methanol or formate in fetal or placental tissues.

An important observation was that long-term exposure to methanol vapors at concentrations up to 1,800 ppm did not affect the health of the monkeys prior to or during pregnancy. Also of significance is the lack of apparent effects of methanol on the menstrual cycle, conception rate, and live-birth delivery rate. However, a decrease (6 to 8 days) in the duration of pregnancy was observed in all methanol-exposed animals. It is not clear if this effect was associated with methanol exposure because no dose-response relation was noted, the birth weight and other physical parameters of the methanol-exposed offspring were comparable to those of the control offspring, and the mean gestation period of the control group was slightly shorter than that reported for *M. fascicularis* bred in other colonies.

Prenatal maternal exposures to 200 or 600 ppm methanol did not adversely affect infant growth and physical development. The most concerning result to emerge from this study was the severe wasting syndrome that began at approximately one year of age in two of the seven female offspring in the 1,800 ppm–exposure group. Such events are rare in U.S. primate colonies, and the occurrence of two cases in one exposure group should be considered a serious event that warrants follow-up. At the same time, it is unlikely that pregnant women would experience prolonged exposures to such high concentrations of methanol.

In this study, no systematic pattern of adverse effects from prenatal methanol exposure was evident on most of the measures of neurobehavioral development (neonatal behavior, reflex responses, infant gross motor development, spatial memory, and social behavior). Burbacher and colleagues reported two possible methanol-related exposure effects, one on visually directed reaching in male infants and one on novelty preference. In the case of the Visually Directed Reaching Test, these effects were small and emerged after extensive statistical analyses. The novelty preference results were inconsistent and may have resulted, in part, from cohort effects. However, both outcomes warrant further investigation because they are complex perceptual functions that take time to develop and may be subject to latent neurotoxic effects.

The findings of this study increase our understanding of methanol metabolism, toxicity, and reproductive effects in a methanol-sensitive animal model. Nevertheless, uncertainties remain. The consequences of prolonged methanol exposure on specific target organs, particularly the eye, need to be addressed. Although formate did not accumulate in maternal serum of adult monkeys chronically exposed to methanol vapors, the possible accumulation of formate in the placenta and in fetal tissues cannot be ruled out. Additional studies on sex-specific differences in methanol metabolism and toxicity may be useful. Also, the effect of methanol exposure on folate status during pregnancy has not been completely resolved. The animals in this study were well nourished and the serum folate results suggest that, for these animals, pregnancy in combination with long-term methanol exposure did not produce overt folate deficiency. However, serum folate measurements would not identify folate deficiency localized in fetal or other tissues. Moreover, the results of this study cannot be extrapolated to those pregnant women who are folate-deficient. Prenatal methanol exposure did not affect most measures of early behavioral development; however, the suggestive effects on visually directed reaching and novelty preference need to be evaluated further using more sophisticated tests of cognitive performance at later stages of development. Also, further testing is warranted to determine whether latent effects emerge later in life.

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