FUNDAMENTAL AND APPLIED TOXICOLOGY 34, 36–46 (1996)
ARTICLE NO. 0173

Perinatal Methanol Exposure in the Rat

I. Blood Methanol Concentration and Neural Cell Adhesion Molecules

SANDER STERN, KENNETH REUHL,* SIDNEY SODERHOLM,[1] CHRISTOPHER COX,† ARCHANA SHARMA,[2] MARLENE BALYS, ROBERT GELEIN, CHUN YIN,* AND BERNARD WEISS

*Department of Environmental Medicine and †Department of Biostatistics, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642; and *Department of Pharmacology and Toxicology, College of Pharmacy, Rutgers University, Piscataway, New Jersey 08854*

Received January 18, 1996; accepted August 14, 1996

Perinatal Methanol Exposure in the Rat. I. Blood Methanol Concentration and Neural Cell Adhesion Molecules. STERN, S., REUHL, K., SODERHOLM, S., COX, C., SHARMA, A., BALYS, M., GELEIN, R., YIN, C., AND WEISS, B. (1996). *Fundam. Appl. Toxicol.* **34, 36–46.**

Although the acute toxicity of methanol is well documented, few studies have addressed the consequences of perinatal exposures to the low concentrations that are expected to arise from its proposed use as a component of automobile fuel. This report describes the general research design of a series of studies, the effects of methanol exposures on blood concentrations in dams and neonates, and indices of brain development. Four cohorts of Long–Evans pregnant rats, each cohort consisting of an exposure (n = 12) and a control (n = 12) group, were exposed whole-body to 4500 ppm methanol vapor or air for 6 hr daily beginning on Gestation Day 6. Both dams and pups were then exposed through Postnatal Day 21 (PND 21). Blood methanol concentrations determined by gas chromatography from samples obtained immediately following a 6-hr exposure reached approximately 500–800 μg/ml in the dams during gestation and lactation. Average concentrations for pups attained levels about twice those of the dams. Selected offspring from Cohort 4 were exposed for one additional 6-hr session at ages that extended out to PND 52. Regression analyses showed that the blood methanol concentrations of the pups declined until about PND 48, at which time their levels approximated those of their dams. Such pharmacokinetic differences might increase the risks posed to developing organisms. Light-microscopic analysis showed no significant abnormalities in the brains of the methanol-treated animals. However, assays of neural cell adhesion molecules (NCAMs) in brains of pups sacrificed on PND 4 showed staining for both the 140 and the 180 kDa isoforms to be less intense in the cerebellum of exposed animals. NCAM differences were not apparent in animals sacrificed 15 months after their final exposure.

Concerns about methanol toxicity have grown in parallel with the possibility that methanol may be widely adopted as an additive to, or replacement for, gasoline (Kavet and Nauss, 1990). These emanate, in part, from its egregious history, which is filled with episodes of severe, often fatal poisonings.

The consequences of methanol intoxication were recognized soon after its introduction into commerce. Acute poisoning most frequently results from ingestion, typically in the form of adulterated alcoholic beverages. Poisoning episodes in the workplace can occur from inhalation and from dermal exposure due to methanol-soaked clothing or footwear (Hunter, 1975).

The manifestations of severe acute poisoning include optic nerve edema often leading to blindness, and damage to the basal ganglia, particularly the putamen. These manifestations are now recognized to follow from excessive accumulation of formic acid and the lapse of victims into metabolic acidosis, as described in detail by Kavet and Nauss (1990). The optic nerve damage seems due to formate itself rather than to lowered pH (Hayreh *et al.*, 1980).

The major health questions currently provoked by methanol, however, do not arise from acute intoxication. Instead, they stem from the possibility of low-level environmental exposures, primarily by inhalation, that will occur under circumstances where methanol is used as an automotive fuel. Relatively little research has addressed such questions in the past. Many observers agree that a predominant issue would be neurobehavioral impairment. Cook *et al.* (1991) examined a broad range of neuropsychological endpoints in subjects exposed to 200 ppm methanol (the threshold limit value) and observed some hints, especially on subjective measures, of adverse effects.

Potential consequences of methanol exposure during early development arouse particular concerns because developmental neurotoxicity may emerge insidiously (Needleman and Bellinger, 1994) under conditions of prolonged low-level exposures. Developmental neurotoxicity has emerged, in fact, as a major element in evaluating the health risks posed by exposure to environmental chemicals. One such risk stems from the enhanced susceptibility of the fetal brain

[1] Present address: National Institute of Occupational Safety and Health, Morgantown, WV.

[2] Present address: Purdue University, West Lafayette, IN.

0272-0590/96 $18.00

to agents such as lead (e.g., Bellinger and Stiles, 1993) and methylmercury (Cox *et al.*, 1989). Together with analogous data from other agents, such results have led the USEPA to formulate developmental neurotoxicity guidelines. A particularly compelling reason for evaluating methanol is the huge volume of information devoted to fetal alcohol syndrome. Those findings indicate deficits in cognitive and other behavioral functions at levels of maternal consumption much lower than those evoking malformations and mental retardation (Streissguth *et al.*, 1986).

The current project consisted of a set of experiments selected to gauge potential neurobehavioral effects arising from developmental exposure to methanol in the rat. Methanol administration was accomplished by exposing pregnant rats, and then both dams and their litters until weaning, to 4500 ppm methanol vapor. The experiment was also designed to take account of the asynchrony in development of the human and rodent brain. At birth, the rat brain is roughly equivalent in maturity to the second trimester of the human and primate brain; its growth spurt occurs postnatally (Bayer *et al.*, 1993). To accommodate this feature of rat development, methanol exposures continued until weaning.

Selection of the 4500-ppm exposure concentration was guided primarily by the results of Nelson *et al.* (1985) and Stanton *et al.* (1991), which are now reported in more detail in Stanton *et al.* (1995). Nelson *et al.* (1985) found evidence of teratological effects at 10,000 ppm but not 5000 ppm methanol. Stanton *et al.* exposed rats via inhalation, during Gestation Days 7–19, to a concentration of 15,000 ppm 7 hr daily. Maternal blood concentrations measured about 3 mg/ml. An extensive battery of behavioral tests administered to the offspring failed to reveal any persistent adverse effects. We rejected going to a higher concentration than 4500 ppm because, in contrast to the Stanton *et al.* strategy, we deemed it important to expose the neonates. Furthermore, we wanted to study the effects at a nonteratogenic concentration.

This report describes three aspects of the research program. First, it provides a detailed description of the exposure system and its physical and biological calibration. The latter was especially crucial because of the atypical procedure of simultaneously exposing both dams and neonates during the preweaning period. Next, it presents the results of blood methanol assays conducted both during gestation and during the preweaning period. Initial determinations showing blood methanol concentrations in young neonates twice those of their dams led us to undertake additional observations as the young rats grew. Finally, the report also describes the results of an assay of neural cell adhesion molecules (NCAMs), which are developmentally regulated surface glycoproteins that serve critical roles in the formation and maintenance of the nervous system (Edelman, 1985; Lagunowich *et al.*, 1994; Tomasiewicz *et al.*, 1993). The behavioral data will be described in subsequent reports.

MATERIALS AND METHODS

Animals. Long–Evans hooded rats, obtained from Charles River Breeding Laboratories (Wilmington, MA), were housed individually, in a room assigned exclusively to this experiment, in the University of Rochester Medical Center Vivarium, an AAALAC-certified facility. The room was maintained at a temperature of 24 ± 2°C, humidity of approximately 50%, and a 12/12 light/dark cycle beginning at 0600. All experimental procedures had been approved by the University Committee on Animal Research. Virgin females (170–200 g) were maintained in polycarbonate breeder cages (45 × 22 × 20 cm) with a wire cover and filter top. Fresh bedding, provided weekly, consisted of aspen chips (Northeastern Products Corp., Warrensburg, NY) selected for their methanol absorbance properties (see below). Cage locations of methanol-exposed and control group subjects were randomly assigned within the room. Males (250–300 g) were housed in wire-mesh cages (36 × 17.5 × 17 cm), located in the same room, until breeding was completed. Both males and females were allowed free access to Purina RMH 2000 Lab Chow and tap water except when pregnant animals were in the exposure chambers. The rats were adapted to these conditions for approximately 2 weeks. A separate group of test dams was bred at the same time as the other groups. These females were used in one of the neonatal behavioral tests. Since exposure and control group subjects were adapted to the chambers prior to breeding, rats were randomly assigned to the test dam group prior to other assignments.

Females not assigned to the test dam group were adapted to the exposure chambers over the next 2 weeks. The handling procedure duplicated the procedure initiated on Day 6 of gestation except that no exposures to methanol occurred during this adaptation period. For this purpose, the females were removed from the vivarium daily, taken to the inhalation chamber room, and then placed into the chambers, where they remained for 6 hr. Afterward, they were returned to the vivarium.

Breeding. Females were placed individually with males in the hanging wire cages at approximately 3–4 PM. Vaginal smears obtained at 8:30 AM were examined microscopically. A sperm-positive smear determined Day 0 (GD 0) of gestation, at which time the female was assigned to one of the treatment conditions. Assignments were randomized within blocks of two females, with the first of the two dams randomly assigned to one of the treatments and the second specifically assigned to the other. This ensured that the date of conception was counterbalanced across treatment conditions. Litter number within a treatment group was also designated at this time as the ordinal position to which the subject was assigned in that group. The female was then returned to a polycarbonate breeding cage and maintained under standard conditions.

A total of four cohorts was bred. When a litter was discovered in the morning prior to transport from the vivarium to the exposure chambers, that date was designated Postnatal Day 1 (PND 1). All litters greater than eight were culled to eight offspring on PND 4 using a random assignment procedure. Whenever possible, four male and four female pups were selected. An India ink injection of the paw identified pups, who were randomly assigned identification numbers. These numbers determined subsequent treatments of the pups.

From GD 6 through PND 21, pregnant and lactating dams and litters were transported from the vivarium to the inhalation chambers in their home cages. The number of rats in the chamber varied, depending on gestation and postnatal dates, with a maximum of 12 cages per exposure chamber. Cage location within chambers was rotated systematically across days of exposure Exposures to 4500 ppm methanol vapor or control air were conducted 6 hr daily. Following a 30-min degassing period after vapor generation was terminated, the rats were returned to the vivarium.

INHALATION FACILITY AND PROCEDURES

Chambers. Exposures were conducted in hexagonal "Rochester" chambers (Cheng and Moss, 1989; Leach *et*

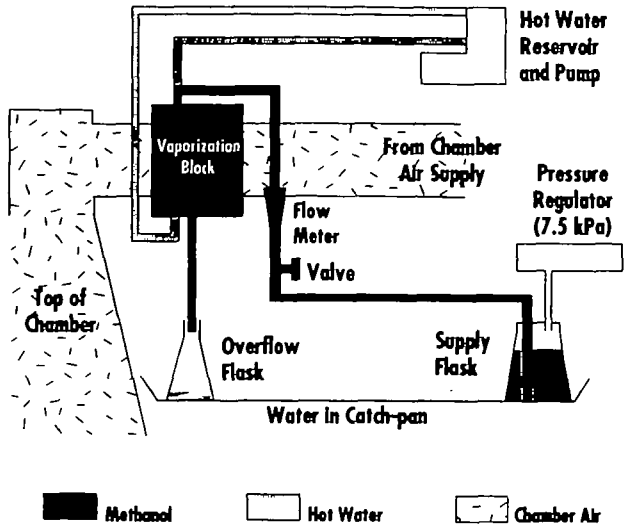

FIG. 1. Diagram depicting the system for delivering methanol vapor to the exposure chamber. Inside the vaporization block, the methanol evaporated from the outside of metal tubes containing the hot water.

al., 1959) with a volume of 2 m^3. Each chamber was supplied with filtered and warmed outside air pumped into the chamber through an intake duct at the top and exhausted at the bottom. Humidity was not regulated. HPLC-grade methanol (J. T. Baker, Phillipsburg, NJ, catalog no. 9093-03) was introduced into the air stream by passing liquid methanol through a heated aluminum block mounted on the intake duct adjacent to each chamber (Fig. 1) from which the vaporized portion then flowed into the chamber. The rat cages were placed on two large mesh shelves located in the midregion of the chamber. Methanol concentration within the chamber was monitored continuously by a Miran 1A (Foxboro Corporation, Foxboro, MA) gas infrared analyzer calibrated from gas chromatographic results and connected to a chart recorder. A turnover of 12 chamber volumes per hour was the nominal air flow rate. Chamber illumination was provided by fluorescent lighting in the room housing the exposure chambers.

Calibration. The Miran 1A infrared spectrophotometer used to monitor chamber concentration was calibrated against duplicate silica gel (silica gel tubes; SKC 226-10, SKC, Eighty Four, Pennsylvania) samples obtained at 5-min intervals, simultaneously with the Miran 1A readings at three different chamber concentrations of methanol (500, 1500, and 4500 ppm). The silica gel samples were analyzed for methanol content with a gas chromatograph using the NIOSH Analytic Method 2000 (NIOSH, 1984). Passive dosimeters, consisting of a distilled-water-filled SKC Developing Vial (226-02A) with the Teflon lining of the septum cap replaced by a 5.0-μm pore, LS-type filter (LSWP 01300) (Millipore Corp., Bedford, MA), were positioned at various chamber locations and subsequently analyzed for methanol content with a gas chromatograph. These samples were obtained simultaneously with the silica gel samples used to calibrate the Miran 1A. The dosimeters provided a means for determining methanol concentrations in quality control experiments in which use of the silica gel sampling method was not feasible or appropriate.

Once the system for generating methanol vapor was turned on, about 15 min were required to reach steady state corresponding to 4500 ppm. An equivalent amount of time was required for the chamber concentration to fall to baseline values when the generating system was turned off. During an exposure, the strip chart and other instruments were monitored frequently to ensure that no systematic deviations were taking place.

Table 1 summarizes the methanol exposure concentrations attained for each cohort. Generally, the exposure concentrations closely approximated the nominal value of 4500 ppm. The differences in number of exposure sessions across cohorts reflect the outcome of the breeding program, since not all females became pregnant on the same day.

Characterization of methanol exposure. Different strategies have been employed to maintain well-defined, experimental control of the agent in inhalation studies (Cheng and Moss, 1989). Head/nose-only exposures ensure not only a well-defined concentration in the flowing air stream but preclude the potential of additional exposure via whole-body contamination, which may lead to excessive dermal uptake or exposure due to ingestion resulting from grooming. From the perspective of the current study, such a design was rejected because (1) the subjects are stressed during the procedure (e.g., Paré and Glavin, 1986), an outcome which can directly alter fetal, and, especially, brain development; and (2) because normal maternal care of the neonates would be prevented during exposures. Whole-body exposures in systems in which the subject is bathed in a nearly unrestricted free-flowing air stream by open mesh housing (wire or plastic) eliminate the stress factor produced by restraint in a head/nose-only system, but they are not adequate for construction of the nest, which ensures normal development in the neonatal rat. To address this difficulty, we exam-

TABLE 1
Methanol Exposure Concentration Summary

	Cohort 1	Cohort 2	Cohort 3	Cohort 4
Exposure sessions	40[a]	42	44	41
Mean concentration (ppm)	4352	4382	4582	4471
SEM (SE of mean) (ppm)	63	26	46	17
Maximum (ppm)	5363	4880	5209	4776
Minimum (ppm)	3558	4033	3552	4197

[a] 38 sessions included in the summary statistics due to system failures that resulted in two sessions that were less than 6 hr.

ined the feasibility of using standard plastic cages with wire tops used in rat breeding facilities. One concern with this type of caging was that the bedding would absorb methanol, which would alter the exposure conditions, especially because the degassing of the methanol would extend the exposure beyond the period when methanol was being actively administered in the exposure chamber. Although we could not totally eliminate this factor, we attempted to minimize and document such an outcome.

In addition, we were concerned about the consequences of handling the dams and pups when transferring them on a daily basis between the polycarbonate home cages and wire mesh enclosures. An extensive literature in psychobiology indicates that the early environment, including the process of handling pregnant dams and neonates, leads to significant modifications of subsequent behavioral and neuroendocrine responses (e.g., Smythe *et al.,* 1994). Our concern about introducing such variables into the experiment led us to ascertain the possibility of exposing the animals in their home cages.

We first compared methanol concentrations from passive dosimeters placed in the breeder cages containing aspen-chip bedding with dosimeters placed on the chamber racks. The ratio of methanol concentration in the cage to the concentration outside was 0.77. Next, blood methanol concentrations (see "Determination of Blood Concentrations" below) were compared between four female rats housed in breeder cages and four housed in standard wire mesh cages following exposure to methanol at 4500 ppm for 6 hr. Blood methanol concentrations between the two groups did not differ, yielding concentrations of 1.26 mg/ml (SD, 0.15) for the breeder cages and 1.28 (SD, 0.08) mg/ml for the wire mesh cages. These concentrations were higher, probably because of the novel environment, than those obtained later, from animals acclimated to the chambers. Perkins *et al.* (1995) note that activity levels and breathing patterns may account for some observed differences in attained blood methanol concentrations; such factors would be expected to differ between nonacclimated and acclimated subjects. Cooper *et al.* (1992) found that, following a single 6-hr exposure to 5000 ppm methanol, blood methanol concentrations were higher in unacclimated than acclimated rats. This experiment, therefore, confirmed the validity of using breeder cages in the inhalation chambers.

Additional independent experiments evaluating blood methanol concentration were conducted as part of our characterization of exposure to methanol. Twelve female rats were exposed to 4500 ppm methanol for 6 hr daily over 3 consecutive days. Blood samples were obtained by tail nick from groups of three rats at the end of the first hour of exposure, at the termination of the 6-hr exposure, at 1 hr postexposure, and at 17 hr postexposure. (Note that the 17-hr postexposure samples were obtained the following morning; for Days 1 and 2, therefore, they were obtained just prior to the exposures on Days 2 and 3, respectively.) Gas chromatographic analyses were used to determine the concentration of methanol in blood. The results appear in Fig. 2. They show higher elevations on the first exposure day, perhaps because of greater exploratory activity in a novel setting. Within our limits of detection at 10 ng/μl, we did not detect any more than trace amounts of methanol in the blood at the 17-hr postexposure time (data not shown). We also did not detect methanol in the blood immediately prior to the first exposure session (data not shown).

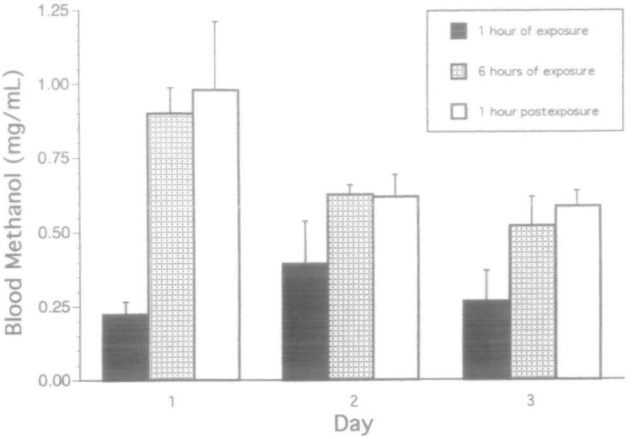

FIG. 2. Mean (±SD) blood methanol levels attained by a group of 12 female rats exposed for 3 successive days to 4500 ppm of methanol for 6 hr daily. Blood samples were drawn at 1 and 6 hr during exposure, and at 1 hr following cessation of exposure. Methanol was not detected in the blood 30 min prior to the first day of exposure, nor at 17 hr in the morning following each exposure (data not shown).

Degassing of methanol. Because any bedding material absorbs some quantity of methanol, degassing of methanol from the bedding must be assumed to occur following termination of methanol vapor generation in the exposure system. The magnitude of the degassing was evaluated in the following experiments. In the first, in the absence of rats, cages containing aspen chips were exposed to 4500 ppm methanol for 6 hr. Thirty minutes after methanol vapor generation ceased, passive dosimeters were placed on top of the bedding in each cage for a 1-hr sample period. Passive dosimeters again were placed into three cages 17 hr after termination of the exposure. In the first hour following exposure, methanol concentrations averaged 17% of the 4500-ppm exposure; 17 hr later the concentration was about 1% for the cages that matched the conditions for housing the rats.

A second experiment was conducted to determine whether the presence of animals would modify the results above. Two rats were placed in the inhalation chamber and exposed to 4500 ppm methanol for 6 hr. After 1 hr of exposure, the rats were removed and the dosimeters then placed on the bedding in each cage while the cages remained in the inhala-

tion chamber. The dosimeters were removed 1 hr later and the rats returned to their respective cages, which still remained in the inhalation chamber. One hour after termination of exposure, the rats were returned to the vivarium and then removed from those cages for 1 hr during which dosimeters were again placed in the cages. Upon removal of the dosimeters, the rats were returned to their cages. The next morning, 16 hr after termination of exposure, the procedure was repeated to obtain another 1 hr sample with the dosimeters. These 1- and 16-hr postexposure assessments were also conducted following the second and third consecutive days of exposure to 4500 ppm methanol for 6 hr. Fresh bedding was used at the start of the first day of exposure only. Cage concentrations during the first hour of exposure averaged 4400 ppm. Across all 3 days, during the 1-hr postexposure period, they fell to 224 ppm and 16 hr later the values had fallen to a range of 0.3 to 2.6 ppm. The results showed that, although the bedding did absorb some methanol, degassing occurs rapidly. These results confirmed that the nominal specification of a 6-hr exposure to methanol corresponded closely to our measurements. In addition, the results indicated that by the time the rats were removed from the inhalation chambers for return to the vivarium, potential exposure of the control group to methanol degassing from the other group would be insignificant. GC analyses were unable to detect methanol in the blood of control rat samples obtained 1 hr after the cages of exposed rats were returned to the vivarium and placed adjacent to theirs.

DETERMINATION OF BLOOD CONCENTRATIONS

For samples from Cohorts 1 and 2 blood methanol was analyzed on a Packard 2000 GC equipped with a flame ionization detector, chart recorder, and reporting integrator. Apparatus difficulties left us without a GC during Cohort 3 exposures. Analyses of the TCA blood extracts from Cohort 4 samples were performed with a Hewlett-Packard Model 5890 Series-II gas chromatograph. A detailed description of the procedures is presented in Weiss *et al.* (1996).

Blood samples during the experimental phase were collected immediately following cessation of the 6-hr exposure from selected exposed and control dams on GDs 7, 13, and 19, and from both dams and pups on PNDs 7, 14, and 21.

Cohort 4: Postweaning blood samples. When initial results showed that the blood methanol concentrations of the pups exceeded those of the dams, we decided to more closely examine the relationship between blood methanol concentration and age. At the time of weaning, we had already designated subjects for the adult phase of the behavioral testing. From the remaining, extra nonbehavioral subjects, we constructed the following design.

On 6 different days, pups selected from those remaining subjects were exposed once to 4500 ppm methanol for 6 hr. Across all 6 days, the same two dams, one from the exposure group and one from the control group, were exposed. On any 1 day, male–female littermates from both the originally exposed and control groups were exposed; i.e., in this experiment only, "control" designates the history of such subjects. All subjects were exposed to 4500 ppm methanol in this procedure. A total of 9 pairs from six different exposure-group litters, and 13 pairs from eight different control-group litters provided our sample. No litter contributed more than 1 pair to an individual exposure day. No litter contributed more than 2 pairs to the protocol. Because pups were not all born on the same day, on any given exposure day during this procedure, ages spanned a 3-to 5-day range. Dates of exposure and litters for that date were selected to provide postweaning ages that extended out to 52 days. This procedure, therefore, allowed us to examine how blood methanol concentration changed as age increased. It also provided an opportunity to evaluate the potential roles of gender and prior exposure history. In addition, since weight of the subjects was known, possible relationships between blood methanol concentration, weight, and an estimate of surface area based on weight could be examined.

Interlaboratory quality control assessments. As part of the quality control procedures of the research program, this laboratory participated in three HEI-sponsored interlaboratory studies designed to confirm the overall adequacy of the blood methanol determinations, and to provide an estimate of the variation among the four participating laboratories. Both gas chromatographs in our laboratory were used, the Packard 2000 in the first two assessments, the HP 5890 in the third. Methanol concentrations ranging from 1 to 100 μg/ml in both blood and water samples were produced by direct mixing by an experimenter from one of the laboratories, or blood methanol concentrations were produced by exposing rats for 6 hr to either 1500 ppm or 4500 ppm. Coded samples were analyzed by investigators. At values greater than 10 μg/ml, the coefficient of variation fell below 25%. No systematic attempt was made to identify interlaboratory sources of variation.

MORPHOLOGICAL ASSESSMENT

Neuropathology. Selected pups from Cohorts 2 and 3 were sacrificed on either PND 1 or PND 21. Samples were immersion-fixed in Bouin's solution for a minimum of 1 week prior to processing. Bouin's solution is a rapid fixative well-suited to brain and to tissues of juvenile animals. All samples were received at Rutgers with the codes assigned at Rochester. Upon receipt, the samples were assigned accession numbers by the pathologist, who broke the code only after the slides had been read. Full brains were cut down the sagittal midline. The left hemisphere was then cut into three sagittal sections with the aid of a Plexiglas template

to yield reproducible sections. The right half was cut into four sections along coronal landmarks. The samples were then embedded to provide one paraplast block each of right and left hemispheres. In addition, selected adults from Cohort 3 were euthanized following completion of the long-term behavioral testing with matched half-brains submitted to morphology and NCAM assessments. Every 4th section was stained by hematoxylin/eosin/phloxine, yielding 10 H& E slides per animal. Two slides were stained with LFB/PAS, and other techniques were used as suggested by preliminary study. All slides for each animal were read without knowledge of treatment assignment. The absence of significant anatomic differences obviated the need for detailed damage assessment scales.

NCAM determinations. For SDS–PAGE and Western blotting, frozen samples of rat brain were divided into cerebellum and cerebrum and homogenized in 1× sample buffer (0.0625 M Tris–Cl, pH 6.8, 2% SDS, 10% glycerol, and 5% mercaptoethanol) as described by Laemmli (1970). Total protein concentration of each sample was measured on a COBAS FARA II (Roche Instruments, Nutley, NJ), using the Bradford dye-binding assay (Bradford, 1976). Split samples were run with or without boiling for 5 min. Boiling cleaves the sialic acids from NCAM, giving more compact bands on electrophoresis and permitting better assessment of sialic acid status. Samples were run on a 7.5% polyacrylamide gel with a Bio-Rad mini protein II system. After protein separation, the gels were transferred onto nitrocellulose membranes (Towbin *et al.*, 1979) prior to overnight incubation in primary antibody. Antibodies 5B8 (University of Iowa Developmental Studies Hybridoma Registry) and 0B11 (Sigma, St. Louis, MO), recognizing different portions of the intracellular domain, were employed. Blots were then incubated with alkaline phosphate-linked goat anti-mouse IgG (Fisher Scientific), with cross-reaction to rat NCAM. Visualization of the bands was obtained using the 5-bromo-4-chloro-3-indole phosphate/nitro blue tetrazolium substrate system (Bio-Rad, Melville, NY).

Immunoblots were digitized using a Presage CV-6 Image Analysis System (Advanced Imaging Concepts, Princeton, NJ) into black and white images encoded as a sequence of integer values between 0 and 255. Images were acquired with a NEC TI-23 EX CCD camera and numerical values placed on the gray-scale calculation by in-house algorithms.

RESULTS

Litter indices. Within cohorts, control and methanol-exposed litters showed virtually identical litter sizes, sex distributions, and dam and pup weight gains. Across all four cohorts, weights of the methanol-exposed vs control pups on PND 18, for example, were 37.13 g (1.94 SEM) vs 39.65 g (1.23) for males and 37.16 g (1.22) vs 38.05 g (1.37) for females. Although litter sizes differed among cohorts, the variability was independent of exposure history. (Additional details are found in Weiss *et al.*, 1996.)

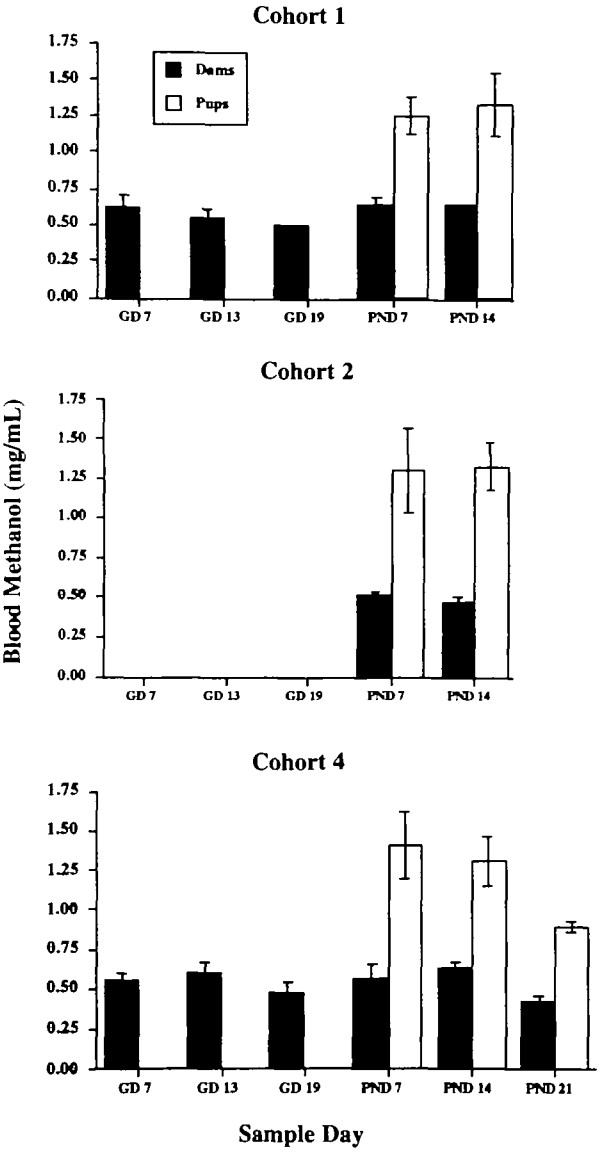

FIG. 3. Mean (±SD) blood methanol levels achieved at the end of the 6-hr exposure period during gestation and lactation, on the days indicated, for Cohorts 1, 2, and 4.

Blood methanol determinations. Samples were drawn from dams during gestation and from both dams and pups after parturition on the occasions shown in Fig. 3. (Operational difficulties with the GC left us with no data for the Cohort 2 dams during gestation; a replacement instrument became available only after the Cohort 3 exposures had ceased.) Maternal blood concentrations remained fairly constant during gestation (mean, 0.55 mg/ml; SD, 0.07) and lactation (mean, 0.56 mg/ml; SD, 0.09), although, for both

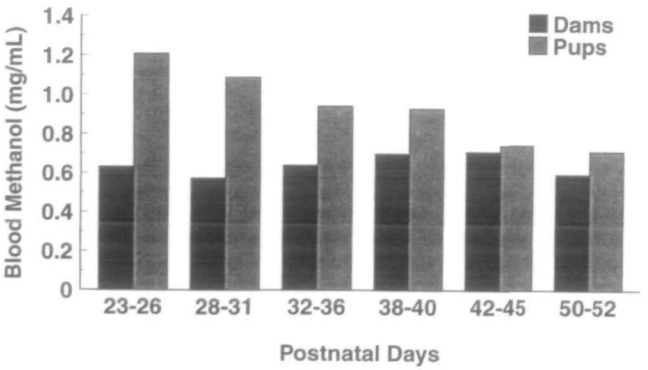

FIG. 4. Mean blood methanol levels achieved at the end of the 6-hr exposure period in offspring from Cohort 4 on the days indicated. Offspring had been maintained in individual cages since PND 21 (weaning).

Cohorts 1 and 4, values based on GD 19 appeared lower than those secured earlier during gestation.

For all three cohorts analyzed, the pups, before weaning, exhibited blood concentrations approximately twice those attained by their dams (mean, 1.26 mg/ml; SD, 0.23). Upon confirming this result across cohorts, subjects from Cohort 4 not used in the adult-phase behavioral testing were used to evaluate how blood methanol concentration, following a 6-hr exposure to 4500 ppm, varies with age up to PND 52. Even after weaning and assignment of pups to individual cages, the dam–pup differences persisted until about PND 48.

These data induced us to pursue additional analyses of the relationship between blood methanol concentration and age. The data from both the preweaning and postweaning blood methanol determinations in Cohort 4 are shown in Fig. 5. (Data from PND 21 were not included because values for the dams fell substantially below those recorded for all the other days, and those for the pups also showed a major departure from the trend across time that was observed graphically.) For the postweaning assays, subjects from both the 4500-ppm and control groups that were not designated for later behavioral testing were exposed for one 6-hr session to 4500 ppm methanol at a designated age. Data were available for age, weight, an estimate of surface area ($SA^{9.85} \times BW^2$), sex, and exposure history. We initially examined, by BMDP multiple linear regression procedures (BMDP2V), whether any of these variables would predict the concentration of methanol. A series of regressions indicated that age, weight, and surface area were highly correlated (Pearson $r > 0.90$), so that the individual contributions of these three variables could not be determined. No indication of any effect of sex or exposure history appeared.

Two regression models were then applied to the relationship between methanol and age. (In the absence of a known mechanism for the decline in blood methanol concentration, developmental changes based on age seem likely candidates.) The first model was a straight line fit, i.e., the data were analyzed by ordinary linear regression. The second, a four-parameter nonlinear regression model, using the BMDP statistical software BMDP2V, hypothesized an initial plateau in the concentration of methanol, followed by a linear decline, which in turn is followed by a plateau at older ages. The four parameters consisted of the initial concentration, the onset of the decline, the slope of the linear decline, and the level for the second plateau.

The initial plateau was estimated to be 1.4 mg/ml (SD, 0.05), the onset of the linear decline was estimated to occur at 11.22 days (SD, 3.49), the estimated slope was −19.26 (SD, 2.08), and the second inflection in the function was estimated to occur at 47.67 days. The final plateau was estimated to be 0.71 mg/ml, as determined by the second change point. From a statistical point of view, the four-parameter nonlinear model provides only a small improvement in fit to the data over the two-parameter linear regression which yielded a slope of −17.18 (SD, 1.27) and an intercept of 1.56 (SD, 0.04).

Mean blood methanol concentrations for the two dams exposed during all six of the postweaning exposures were 0.58 (SD, 0.06) and 0.69 (SD, 0.06) mg/ml, respectively. These values showed no trends across the six exposures, which occurred from 2 to 31 days following weaning. The final plateau of the offspring falls above these values.

Morphology. Light-microscopic surveys of the brains of methanol-exposed pups revealed no detectable evidence of neuropathology according to the criteria listed earlier.

NCAM indices, in contrast, revealed a significant treatment difference in the brains of neonates sacrificed on PND 4. The brains of offspring sacrificed at 15 months of age showed no NCAM differences. Figure 6 plots the results of densitometer readings based on Western blots. NCAM 140 and 180 assays were performed on the same brains. Each plot is based on determinations from six control brains and eight methanol-group brains. Four of the methanol-group brains represent the average of two separate determinations performed to check assay reliability. t tests performed on each set of assays yielded p values of 0.01 (NCAM 140) and 0.001 (NCAM 180).

DISCUSSION

Four findings are described in this report: (1) Blood methanol concentrations in adult rats corresponded to those reported in the literature. (2) Neonatal blood methanol concentrations exceeded those of the dams; the neonatal levels declined gradually to approach those of the dams at about PND 48. (3) NCAM differences due to methanol were observed in neonatal cerebellum. (4) No evidence of neuropathology was evident.

Maternal blood levels averaged 0.55 mg/ml, although a

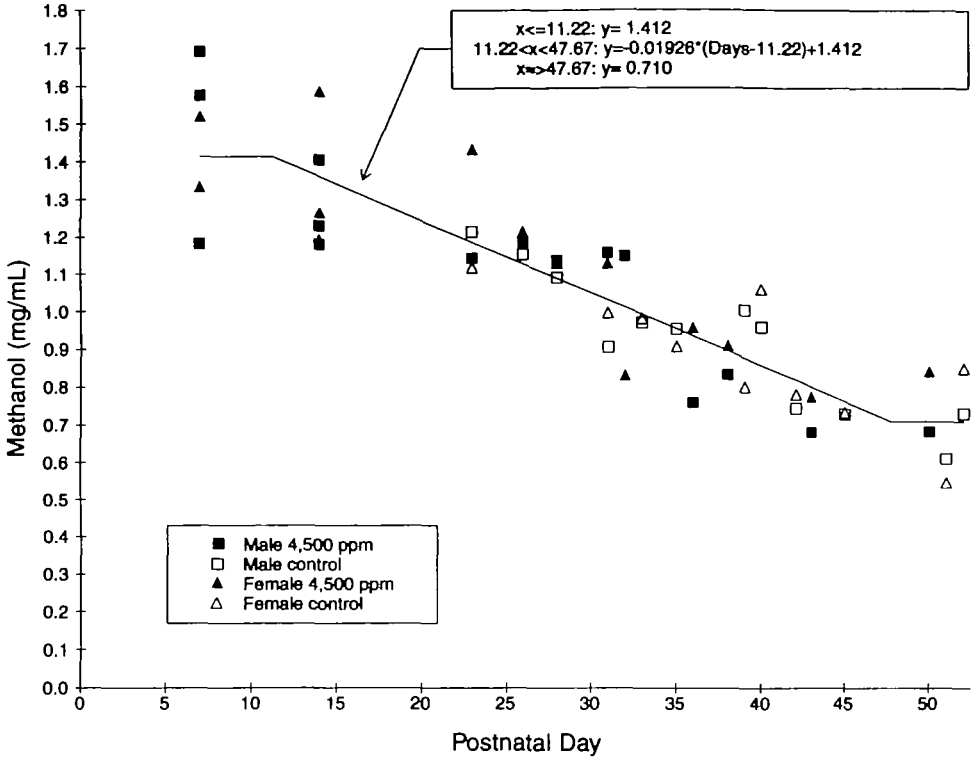

FIG. 5. Nonlinear regression function fitted to offspring blood values between PND 7 and PND 52. Exposures to 4500 ppm lasted 6 hr.

minor decrease occurred on GD 19. Nelson *et al.* (1985) reported a similar decline. The concentrations themselves are not substantially different from those reported by Nelson *et al.* for exposures of 5000 ppm.

One unexpected pharmacokinetic finding is the difference between methanol blood concentrations attained by dams and pups. A possible explanation for such differences might be the position of the pups in the breeding cages and their more intimate contact with the bedding chips. Such an explanation seems unlikely because differences in pup size and activity between PND 7 and PND 21 are quite substantial. In addition to a marked growth in size, the processes of sensory and motor development equip the neonates with rapidly expanding visual, auditory, and locomotor capacities.

Blood methanol concentrations in offspring declined from about PND 11 to PND 48. Because the two- and four-parameter regression models differed only slightly, there is no compelling reason for choosing one over the other. Also, even as late as PND 48–52, offspring levels remained slightly above those of the dams. More data with a greater range of ages are needed provide a definitive profile of how blood methanol levels vary with age. In addition, other studies are required for evaluating how variables such as body weight, surface area, activity, ventilation rates, metabolic processes, etc., which may be highly correlated with age, contribute to the changes in blood-methanol levels.

The observed differences might depend on maturation of metabolic processes during development. According to Card *et al.* (1989), due to low alcohol dehydrogenase activity in fetal and neonatal liver of the guinea pig, the fetus has no capacity to oxidize ethanol during gestation. In rodents, catalase is the predominant enzyme for the conversion of methanol to formaldehyde (Tephly and McMartin, 1984). At least in the rat lung, catalase activity increases progressively during late prenatal development and postnatally as well (Chen and Frank, 1993). A more extended longitudinal assessment of catalase levels in various tissues during development might help explain the differences between dams and pups observed in the current project. Interspecies predictions and comparisons of exposure effects depend on such information.

Although neonatal blood methanol concentrations before weaning were determined from PND 4 through PND 21 (PNDs 1–14 are held to correspond to the third trimester of human development), we made no direct assessments of *in utero* methanol concentrations. Studies of ethanol elimination raise special concerns here. Brien *et al.* (1983), for example, showed in humans that the clearance rate of ethanol from amniotic fluid fell below that from maternal blood. In addition, Hill *et al.* (1983), in monkeys, and Idanpaan-Heikkila *et al.* (1972), in humans, showed that ethanol elimination from neonatal blood proceeds more slowly than that from

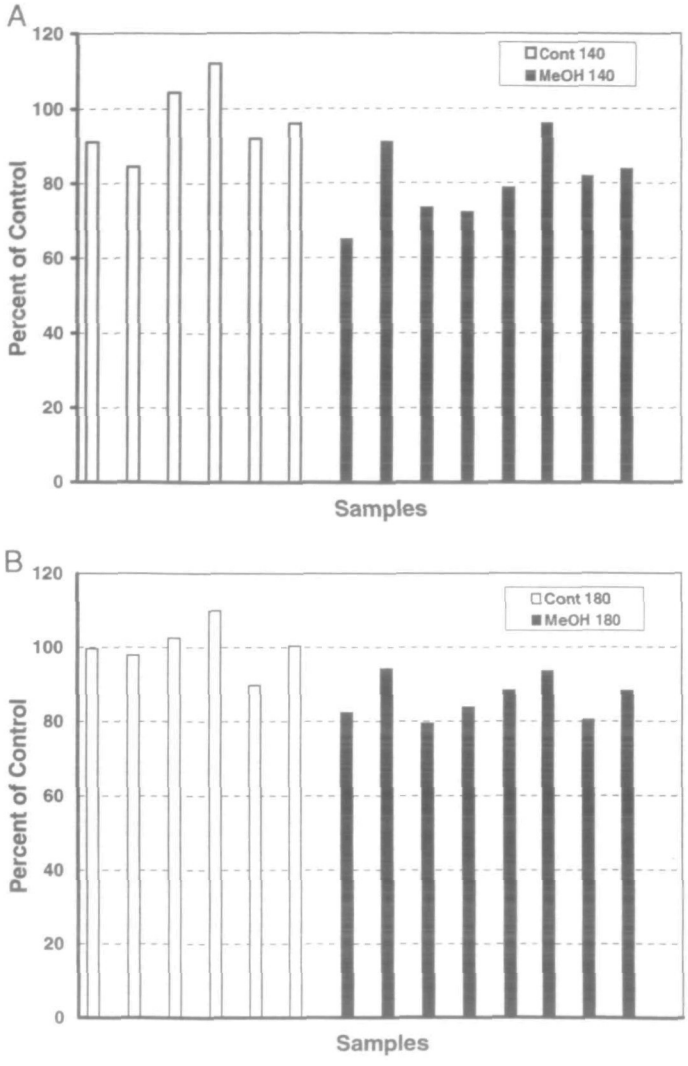

FIG. 6. Neural cell adhesion molecule (NCAM) assays: results of densitometer readings based on Western blots. NCAM 140 (A) and 180 assays (B) were performed on the same brains. Each plot is based on determinations from 6 control brains and 8 methanol-group brains.

maternal blood. This lengthened persistence may pose additional risks for the developing organism in addition to the enhanced vulnerability of the fetal brain.

Perkins *et al.* (1995) recently raised concerns about extrapolation of dose across species when attempting to assess the risk to humans. They demonstrated that the same concentrations of methanol-vapor exposures produced two- to threefold higher concentrations in the blood of mice than rats. While noting differences between species in pharmacokinetic parameters, perhaps more significantly, they note the current lack of understanding of the underlying mechanisms. The present findings amplify such concerns, especially in attempting to assess risk to the developing human.

The choice of rats in the current research evolved from the prevailing literature. Until the recent reports of Rogers *et al.* (1993) and Bolon *et al.* (1993) based on mice, all of the previous developmental studies had relied on rats, and even the ethanol literature was dominated by rat experiments. At least superficially, rats may seem to be far from an optimal choice. Unlike humans and other primates, they rapidly degrade formate, the metabolite presumed to underlie the most severe effects of methanol poisoning, unless their normal mechanisms, dependent on folate, are impaired (Eells, 1991). At exposure concentrations short of those promoting the accumulation of formic acid, however, rats and monkeys metabolize methanol at comparable rates (cf. Horton *et al.*, 1992). In addition, it appears that methanol-induced exencephaly in mice is dependent on methanol itself, rather than formate (Dorman and Welsch, 1996). The rat, therefore, was deemed a suitable species for addressing questions about potential neurotoxic consequences of low and moderate concentrations of methanol vapor.

One source of evidence that methanol vapor exposure under the conditions of the present experiment could result in modification of brain development is the NCAM assays. These were undertaken because, given the plasticity of the developing nervous system, and the possibility of silent damage (Weiss and Reuhl, 1994) not expressed in behavioral measures, mechanistic indices of brain development also had to be examined.

Development and maintenance of the nervous system depend upon the proper temporal and spatial expression of CAMs, which mediate the complex cell–cell and cell–substrate adhesion systems responsible for migration, axonal outgrowth, and establishment of the complex connective patterning that underlies mature neuronal function. The NCAM is the best characterized of the adhesion molecules.

NCAM assays based on Western immunoblot analysis were selected for several reasons: (1) Temporal disturbance of brain NCAM expression has been documented following developmental exposure to heavy-metal neurotoxicants (Dey *et al.*, 1994; Cookman *et al.*, 1987) and to ethanol. (2) Persistent expression of a juvenile NCAM isoform in the brain of treated animals after its normal down-regulation might serve as a marker of injury even in the absence of neuropathological findings. (3) Morphoregulatory molecules appeared to be a logical area of study given the possibility that methanol might cause alterations in neuronal migration or defective and diminished synaptogenesis, much as other developmental neurotoxicants. Although conventional light microscopy might detect the former, NCAM assays are better equipped to detect the latter condition. (4) One possible mechanism of action of methanol is as a membrane poison, altering either receptors or membrane-associated processes such as adhesion or recognition. NCAMs, and particularly examination of their isoforms, reflect the intactness of these processes.

Methanol treatment decreased NCAM expression in both

NCAM 140 and NCAM 180. Because NCAM 180 concentrations peak at about PND 14, assays based on later developmental ages might be even more revealing. Specific alterations of NCAM 180 levels and sialylation state have been noted in hippocampus following excitotoxic damage (Le Gal La Salle *et al.*, 1992) and toxic metals (Reuhl *et al.*, 1994).

NCAM 140 is the primary isoform expressed during the stages of neuronal migration and NCAM 180 is expressed during synaptogenesis. Decreases in NCAM 140 might be expected to result in abnormal cell movement following methanol treatment. The absence of evidence of migratory disturbance by light microscopy is not readily reconciled with the NCAM data. However, NCAM is not the only cell adhesion molecule involved in cell movement, and it may be that other adhesion molecules, combined with the residual NCAM 140, may have been sufficient to mediate normal migration. Additionally, interrupted movement of single cells, or even small groups of cells, would be difficult to detect by conventional light-microscopic techniques, even when such changes are anticipated and specifically sought. Although no heterotopias or evidence of gross migration arrest were noted in the older animals, such indices require isotopic tagging of neuron populations. The reduction in NCAM 180 is particularly intriguing given the indications of subtle behavioral changes, to be reported elsewhere. NCAM 180 is primarily localized at synapses, where it is critical to neuronal plasticity, learning, and memory (Regan, 1993). Even subtle alterations of this molecule at critical times of life could potentially alter the learning profile of an individual (Doherty *et al.*, 1994; Poltorak *et al.*, 1993; Jorgensen, 1995).

The estimated concentrations of methanol to which the general public would be exposed (cf Kavet and Nauss, 1990) were it to be adopted as an automotive fuel are far below the concentration selected for the research reported here. Consequently, our results might lead to the conclusion that the health risks of methanol fuels, on the basis of developmental neurotoxicity, do not pose a grave concern. Excessive complacency, however, is not justified. Although methanol concentrations in the communal environment should be quite modest, even with widespread adoption of methanol fuels, the delivery chain from producer to consumer may offer opportunities for exposure at much higher concentrations. Furthermore, the marked differences in blood concentrations observed here between dams and pups, together with the human ethanol data noted earlier, suggest possible pharmacokinetic differences in humans that deserve further investigation. In addition, a comprehensive risk analysis requires description of the maturational course of methanol metabolic processes, which currently are not available.

ACKNOWLEDGMENTS

Funding for this work was provided in part by Agreement 90-8 from the Health Effects Institute, B. Weiss, Principal Investigator, and by NIEHS Center Grant ES-01247. Additional support was provided by NIEHS Grant ES-04976, K. Reuhl, Principal Investigator. David Anderson provided valuable technical assistance while the exposure chamber was being established early in this project.

REFERENCES

Bayer, S. A., Altman, J., Russo, R. J., and Zhang, X. (1993). Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. *Neurotoxicology* **14,** 83–114.

Bellinger, D., and Stiles, K. M. (1993). Epidemiologic approaches to assessing the developmental toxicity of lead. *Neurotoxicology* **14,** 151–160

Bolon, B , Dorman, D. C., Janszen, D., Morgan, K. T., and Welsch, F. (1993). Phase-specific developmental toxicity in mice following maternal methanol inhalation. *Fundam. Appl. Toxicol.* **21,** 508–516.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

Brien, J. F., Loomis, C. W., Tranmer, J., and McGrath, M. (1983). Disposition of ethanol in human maternal venous blood and amniotic fluid. *Am. J. Obstet. Gynecol.* **146,** 181–186.

Card, S. E., Tompkins, S. F., and Brien, J. F. (1989). Ontogeny of the activity of alcohol dehydrogenase and aldehyde dehydrogenases in the liver and placenta of the guinea pig. *Biochem. Pharmacol.* **38,** 2535–2541.

Chen, Y., and Frank, L. (1993). Differential gene expression of antioxidant enzymes in the perinatal rat lung. *Pediatr. Res.* **34,** 27–31.

Cheng, Y.-S., and Moss, O. R. (1989). Inhalation exposure systems. In *Concepts in Inhalation Toxicology* (R. O. McClellan and R. F. Henderson, Eds.), pp. 19–62 Hemisphere, New York.

Cook, M. R., Bergman, F. J., Cohen, H. D., Gerkovich, M. M., Graham, C., Harris, R. K., and Siemann, L. G. (1991). *Effects of Methanol on Human Behavioral Measures.* Research Report No. 42. Health Effects Institute, Cambridge, MA

Cookman, G. R., King, W., and Regan, C. M. (1987). Chronic low-level lead exposure impairs embryonic to adult conversion of the neural cell adhesion molecule. *J. Neurochem.* **49,** 399–403.

Cooper, R. L., Mole, M. L., Rehnberg, G. L., Goldman, J. M., McElroy, W. K., Hein, J., and Stoker, T. E. (1992). Effect of inhaled methanol on pituitary and testicular hormones in chamber acclimated and non-acclimated rats. *Toxicology* **71,** 69–81.

Cox, C., Clarkson, T. W., Marsh, D. O., and Amin-Zaki, L. (1989). Dose–response analysis of infants prenatally exposed to methylmercury: An application of a single compartment model to single-strand hair analysis. *Environ. Res.* **49,** 318–332.

Dey, P. M., Graff, R. D., Lagunowich, L. A., and Reuhl, K. R. (1994). Selective loss of the 180-kDa form of the neural cell adhesion molecule in hippocampus and cerebellum of the adult mouse following trimethyltin administration. *Toxicol. Appl. Pharmacol.* **126,** 69–74.

Doherty, P., Fazeli, M. S., and Walsh, F. S. (1994). The neural cell adhesion molecule and synaptic plasticity. *J. Neurobiol.* **26,** 437–446.

Dorman, D. C , and Welsch, F. (1996). Developmental toxicity of methanol in rodents. *CIIT Activities* **16**(2), 1–7.

Edelman, G M. (1985). Cell adhesion and the molecular processes of morphogenesis. *Annu. Rev. Biochem.* **54,** 135–169.

Eells, J. T. (1991). Methanol-induced visual toxicity in the rat. *J. Pharmacol. Exp. Ther.* **257,** 56–63.

Hayreh, M. S., Hayreh, G., Baumbach, P., Cancill, G., Martin-Amat, K., and Tephly, T. (1980). Ocular toxicity of methanol. An experimental

study. In *Neurotoxicity of the Visual System* (W. H. Merigan and B. Weiss, Eds.), pp. 33–52. Raven Press, New York.

Hill, D. E., Slikker, W., Jr., Goad, P. T., Bailey, J. R., Sziszak, T. J., and Hendricks, A. G. (1983). Maternal, fetal, and neonatal elimination of ethanol in non-human primates. *Dev. Pharmacol. Ther.* **6,** 259–268.

Horton, V., Higuchi, M. A., and Rickert, D. E. (1992). Physiologically based pharmacokinetic model for methanol in rats, monkeys, and humans. *Toxicol. Appl. Pharmacol.* **117,** 26–36.

Hunter, D. (1975). *The Diseases of Occupations,* 5th ed. English Univ. Press, London.

Idanpaan-Heikkila, J., Jouppila, P., Akerblom, H. K., Isoaho, R., Kauppila, E., and Koivisto, M. (1972). Elimination and metabolic effects of ethanol in mother, fetus, and newborn. *Am. J. Obstet. Gynecol.* **112,** 387–393.

Jorgensen, O. S. (1995). Neural cell adhesion molecule (NCAM) as a quantitative marker in synaptic remodeling. *Neurochem. Res.* **20,** 533–547.

Kavet, R., and Nauss, K. M. (1990). The toxicity of inhaled methanol vapors. *Crit. Rev. Toxicol.* **21,** 21–50.

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227,** 680–685.

Lagunowich, L. A., Stein, A. P., and Reuhl, K. R. (1994). Cell adhesion molecules in normal and abnormal brain development. *Neurotoxicology* **15,** 123–132.

Leach, L. J., Spiegl, C. J., Wilson, R. H., Sylvester, G. E., and Lauterbach, K. E. (1959). A multiple chamber exposure unit design for chronic inhalation studies. *Am. Ind. Hyg. Assoc. J.* **20,** 13–22

Le Gal La Salle, G., Rougon, G., and Valin, A. (1992). The embryonic form of neural cell surface molecule (E-NCAM) in the rat hippocampus and its reexpression on glial cells following kainic acid induced status epilepticus. *J. Neurosci.* **12,** 872–882.

Needleman, H. L., and Bellinger, D. (1994). *Prenatal Exposure to Toxicants.* Johns Hopkins Press, Baltimore.

Nelson, B. K., Brightwell, W. S., MacKenzie, D. R., Khan, A., Burg, J. R., Weigel, W. W., and Goad, P. T. (1985). Teratological assessment of methanol and ethanol at high inhalation levels in rats. *Fundam. Appl. Toxicol.* **5,** 727–736.

NIOSH (1984). *NIOSH Manual of Analytic Methods,* 3rd ed. U.S. Department of Health and Human Services, National Institute for Occupational Health and Human Services, Publication No. NIOSH 84–100, National Technical Information Service (NTIS), Order No. PB-85-179-018, Springfield, VA.

Paré, W. P., and Glavin, G. B. (1986). Restraint stress in biomedical research: A review. *Neurosci. Biobehav. Rev.* **10,** 339–370.

Perkins, R. A., Ward, K. W., and Pollack, G. M. (1995). Comparative toxicokinetics of inhaled methanol in the female CD-1 mouse and Sprague–Dawley rat. *Fundam. Appl Toxicol.* **28,** 245–254.

Poltorak, M., Herranz, A. S., Williams, J., Lauretti, L., and Freed, W. J. (1993). Effects of frontal cortical lesions on mouse striatum: Reorganization of cell recognition molecule, glial fiber, and synaptic protein expression in the dorsomedial striatum. *J. Neurosci.* **13,** 2217–2229

Regan, C. M. (1993). Neural cell adhesion molecules, neuronal development and lead toxicity. *Neurotoxicology* **14,** 69–74.

Reuhl, K. R., Lagunowich, L. A., and Brown, D. L. (1994). Cytoskeleton and cell adhesion molecules: Critical targets of toxic agents. *Neurotoxicology* **15,** 133–146.

Rogers, J. M., Mole, M. L., Chernoff, N., Barbee, B. D., Turner, C. I., Logsdon, T. R., and Kavlock, R. J. (1993). The developmental toxicity of inhaled methanol in the CD-1 mouse, with quantitative dose–response modeling for estimation of benchmark doses. *Teratology* **47,** 175–188.

Smythe, J. W., McCormick, C. M., Rochford, J., and Meaney, M. J. (1994). The interaction between prenatal stress and neonatal handling on nociceptive response latencies in male and female rats. *Physiol. Behav.* **55,** 971–974.

Stanton, M. E., Crofton, K. M., Gray, L. E., Gordon, C. M., Boyes, W. K., Mole, M. L., Peele, D. B., and Bushnell, R. J. (1991). Assessment of offspring development and behavior following gestational exposure to inhaled methanol in the rat. *Toxicologist* **11,** 118.

Stanton, M. E., Crofton, K. M., Gray, L. E., Gordon, C. M., Bushnell, R. J., Mole, M. L., and Peele, D. B. (1995). Assessment of offspring development and behavior following gestational exposure to inhaled methanol in the rat. *Fundam. Appl. Toxicol.* **28,** 100–110.

Streissguth, A. P., Barr, H. M., Sampson, P. D., Parrish-Johnson, J. C., Kirchner, G. L., and Martin, D. C. (1986). Attention, distraction and reaction time at age 7 years and prenatal alcohol exposure. *Neurobehav. Toxicol. Teratol.* **8,** 717–725.

Tephly, T. R., and McMartin, K. E. (1984). Methanol metabolism and toxicity. In *Aspartame: Physiology and Biochemistry* (D. Stegink and J. R. Filer, Jr., Eds.), pp. 111–140. Dekker, New York.

Tomasiewicz, H., Ono, K., Yee, D., Thompson, C., Goridis, C., Rutishauser, U., and Magnuson, T. (1993). Genetic deletion of a neural cell adhesion molecule variant (N-CAM-180) produces distinct defects in the central nervous system. *Neuron* **11,** 1163–1174

Towbin, M., Staelin, T., and Gorden, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4359.

Weiss, B., and Reuhl, K. (1994). Delayed neurotoxicity A silent toxicity. In *Handbook of Neurotoxicology* (L. Chang, Ed.), pp. 765–784. Dekker, New York.

Weiss, B., Stern, S., Soderholm, S., Cox, C., Sharma, A., Inglis, G., Preston, R., Balys, M., Reuhl, K., and Gelein, R. (1996). *Developmental Neurotoxicity of Methanol Exposure by Inhalation in Rats.* Research Report No. 73, April 1996, pp. 1–64. Health Effects Institute, Cambridge, MA.