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Neonatal Behavioral Toxicity in Rats Following Prenatal Exposure to Methanol

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ABSTRACT Although methanol (MEOH) may assume a significant role as a fuel, which implies wide availability, little is known of its toxicity apart from acute poisoning episodes in human adults. Even less is known about its toxicity in developing organisms. This experiment studied the early behavioral development of rats whose mothers had consumed MEOH during gestation by measuring the responses of suckling (postnatal day 1) and nest-seeking (postnatal day 10). Primigravida Long-Evans rats were divided into three groups (N = 10). Two of the groups consumed drinking solutions of 2% MEOH instead of distilled water either on gestational days 15–17 (MEOH 1) or 17–19 (MEOH 2). No maternal toxicity was apparent as measured by weight gain, gestational duration, and daily fluid intake. Daily MEOH consumption averaged 2.5 gm/kg over the 3-day period in both MEOH groups. Litter size, birth weight, and infant mortality did not differ among the three groups. Postnatal growth and date of eye opening were unaffected. MEOH pups required longer than controls to begin suckling on postnatal day 1. On postnatal day 10, they required more time to locate nesting material from their home cages. These data suggest that prenatal MEOH exposure induces behavioral abnormalities early in life that are unaccompanied by overt toxicity.

Several national energy programs, including that of the United States (Environmental Protection Agency, '83), are considering methanol (CH_3OH) as an alternative to gasoline. It already is a significant fuel or fuel additive in Europe and elsewhere. Since methanol is a potent neuropoison (Aquilonius et al., '78), an increase in its availability and likelihood of exposure makes it imperative that we fully understand the health hazards it may pose.

Reports of methanol consumption and its toxic consequences abound (Posner, '75) because it is erroneously perceived by many lay persons as a substitute for ethyl alcohol. It also could be abused by inhalation. Some groups in this country, as well as others (e.g., India and Mexico), deliberately inhale gasoline and glue products (Sharp and Carroll, '78) for their intoxicating properties and might abuse methanol in the same way. Furthermore, widespread distribution carries an increasing risk of inadvertent exposure, a risk that is amplified by the ease of methanol's absorption through intact skin (NIOSH, '76). Hunter ('75) describes several cases of poisoning attributable to this property.

In high doses, methanol induces blindness and motor disturbances in adult humans (Posner, '75). Blindness appears to result from a methanol metabolite (formate) that damages the optic nerve and that produces optic disk edema (Hayreh et al., '77; Martin-Amat et al., '77). Survivors of methanol intoxication also exhibit signs resembling Parkinsonism, and histopathology and computerized axial tomography (C.A.T.) scans reveal damage to the basal ganglia (Aquilonius et al., '78).

Animal models based upon adult organisms reveal wide species differences in susceptibility to methanol toxicity (Roe, '82). Old World primates resemble humans in their

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sensitivity to the toxic consequences of methanol exposure (Hayreh et al., '80). Nonprimate species such as the rat and mouse are much more resistant (Smith and Taylor, '82). These differences in susceptibility arise from species differences in the metabolism of methanol and formate accumulation in plasma (Tephly et al., '74).

Little is known about the developmental toxicology of methanol. One report showed a lower LD_{50} of methanol in 2-week-old (7.4 ml/kg) than in young adult (13.0 ml/kg) rats (Kimura et al., '70). A recent examination of the teratogenic potential of methanol showed it to be a teratogen only at relatively high inhalation concentrations and corresponding blood levels (Nelson et al., '85). The current investigation was designed to examine the early behavioral development of rats exposed prenatally to methanol. Although rodents would not be among the most preferred species, our interest was directed toward the consequences of mild or moderate exposures too low to produce toxic acidosis in primates (Hayreh et al., '80). The criteria consisted of two behavioral paradigms that permit the assessment of neural function early in life among rodents and other altricial mammalian species. The first is suckling behavior, which is characteristic of all mammals. The integrity of this behavior is essential for normal growth and development. Suckling behavior is a well investigated phenomenon in neonates of a large variety of mammalian species (Blass and Teicher, '80). Suckling behavior aberrations have been reported in newborns whose mothers drank alcohol and smoked cigarettes during pregnancy (Martin et al., '77), in the offspring of narcotic-addicted women (Kaplan et al., '75), and in rat pups exposed prenatally to ethanol (Chen et al., '82). The second behavioral endpoint was nest-seeking (homing) behavior, which assesses the tendency for neonates to return to their nesting site after removal (Rosenblatt, '79). This behavior is biologically significant because delay in or failure to return to the nest may decrease suckling time and maternal care. Homing behavior is sensitive to both the effects of amphetamine and the modulating influences of circadian rhythms (Infurna, '81).

METHODS

Subjects, experimental design, and dosing

The Long-Evans rats used in this investigation were obtained from Blue Spruce Farms in Altamont, NY. The females were 90–120 days of age and the males were 150–180 days of age at the beginning of the experiment. All animals were maintained under a 16-hour light, 8-hour dark illumination cycle, to mimic the breeding season, and were provided with both Charles River Rodent Chow and tap water at all times unless specified otherwise. Mating was conducted by placing two or three females overnight in the same cage with two males. Day 1 of presumed gestation was designated as the day on which spermatazoa were detected in the vaginal smear. After parturition, all offspring remained with their biological dams.

Thirty primigravida Long-Evans rats were divided randomly into three groups. Two of the groups were given drinking solutions, containing 2% v/v methanol (MEOH) instead of water, during gestational days 15–17 (prenatal methanol group 1) or during gestational days 17–19 (prenatal methanol group 2). These periods lie beyond those during which major organ formation occurs and were selected because of our focus on functional endpoints rather than morphological ones. The selection of a 2% (v/v) methanol solution was based on preliminary studies of preference indicating that, at this concentration, animals did not distinguish between methanol and tap water, consuming about equal amounts of each.

Several variables were recorded during gestation and throughout the preweaning period to evaluate both maternal and fetal toxicity. These included (1) weight gained during the 3rd week of pregnancy, (2) daily fluid intake during this period, (3) duration of gestation, (4) birth weight and body weight on postnatal days 0, 7, 14, and 20, recorded to the nearest 0.1 g, (5) day of eye opening (as an easily scored developmental landmark), (6) maternal behavior following parturition, and (7) litter size. On the day of parturition, the nesting material was changed, and the pups were removed for weighing and gross inspection. After refilling the maternity cage with new bedding material, the pups were placed at the end of the cage furthest from the original nesting site. The dam was then returned to the cage, and the time taken to retrieve the pups to that site was recorded. This measure was used to assess whether methanol treatments produced any lasting perturbations of maternal behavior that would be critical for the normal development of the young. The nesting material was not

changed again until after the homing behavior tests on postnatal day 10.

Behavioral testing

Postnatal behavioral evaluations began the day after birth by testing the suckling behavior of rat pups from all three groups. The assessment of a pup's suckling capabilities requires that movement and the milk ejection reflex be suppressed in the test dam. An anesthetic dose (65 mg/kg) of pentobarbital is sufficient for these purposes (Lincoln et al., '73). A separate group of control dams was used specifically for the suckling behavior test and was not involved in any other aspect of this study. Three to five pups from each litter were randomly selected for the suckling behavior evaluations. The pups were discarded following the suckling test, which reduced litter size to eight pups, containing four males and four females whenever possible. All litters contained at least 10 offspring. These pups served as the subjects for the homing behavior tests conducted on postnatal day 10.

The suckling behavior test was conducted as follows: Each pup was removed from its nest and placed on the ventral surface of the anesthetized dam in a standardized position facing a nipple (Fig. 1a). The environment around the test dam was warmed (31–33°C) and illuminated by two 100-W incandescent light bulbs suspended approximately 40 cm above the ventrum. The dependent variable was the latency of attachment to the nipple during a 2-minute test. The observer was not aware of the pup's prior treatment.

The suckling behavior sequence is composed of three distinct phases: (1) Pups search for the nipple using their forepaws and snout. (2) The mouth closes around the nipple and mouth movements can be observed. (3) The activity of the pup ceases when the nipple has been tugged into the mouth and clasped. This last phase is the criterion for attachment and can be verified by lifting the pup by its hind quarters (Fig. 1b).

On postnatal day 10, all eight pups from each litter were tested for homing behavior. The test was conducted in a box constructed of translucent plastic with the dimensions 37.5 × 20.5 × 10 cm. The floor of the box was a stainless steel wire mesh screen marked into five distinct sections, each 20 × 7.5 cm. Each section was further marked off into 10 subsections, each 4.10 × 3.75 cm. Located beneath the wire mesh floor of the testing arena were five plastic compartments, each containing 500 cm^3 of bedding. Four contained clean pine shavings, while the fifth contained shavings from the home cage. These compartments measured 20 × 7.5 × 4.5 cm and were located directly under and aligned with the five sections outlined on the wire mesh floor. The home nesting material (now 10 days old) was placed in one of the compartments at the end of the test box. Each pup was tested with material from its own maternity cage, and the location of the home cage material alternated from left to right after each test. The floor of the testing arena was surrounded by a translucent plastic wall 10 cm high. A removable translucent plastic top covered the box to minimize extraneous odors (Infurna, '81).

The pups were tested individually by first placing them in the middle of the field. The observer recorded the following details. (1) Initial choice—the (original) direction chosen by pups: Control animals typically choose the

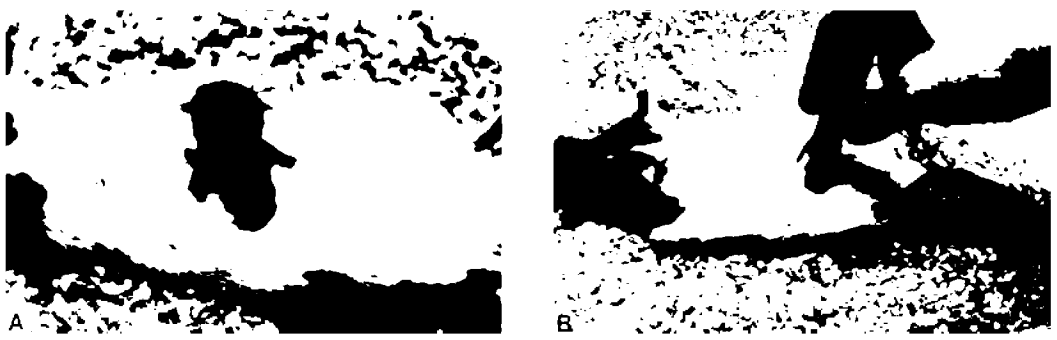

Fig. 1. Components of suckling behavior. a. Pup placement on anesthetized dam. b. Test of attachment to nipple.

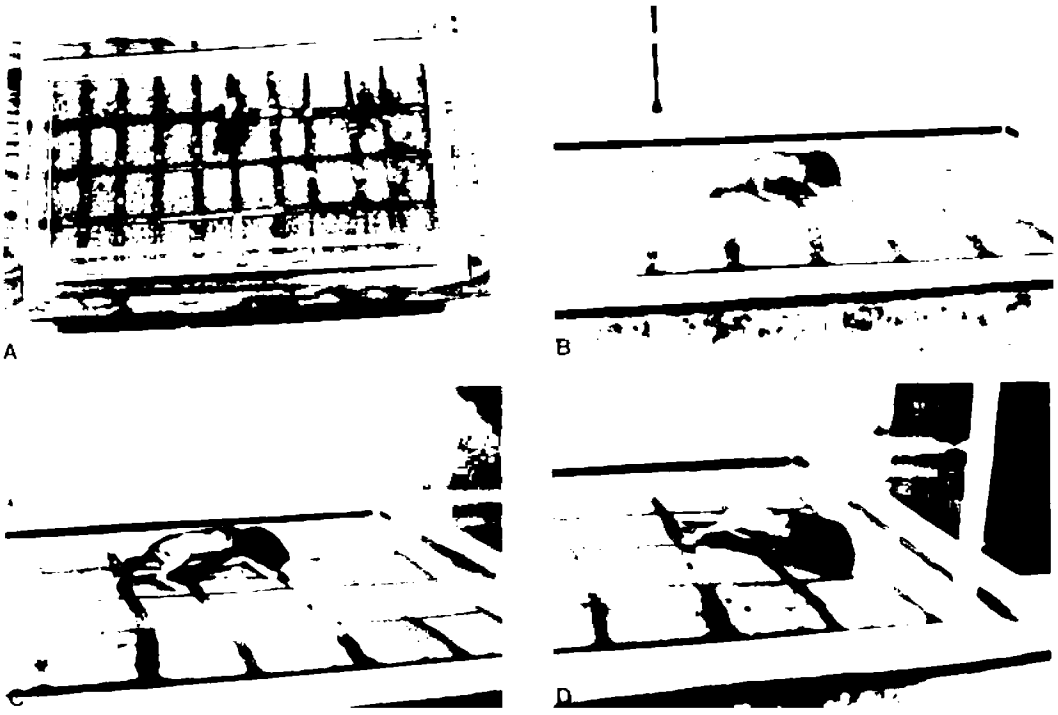

Fig. 2. Components of homing behavior. a. Initial pivoting behavior. b. Orientation toward home cage bedding. c. Entrance into home area. d. Locomotion within home area.

correct direction on more than 80% of the trials (Infurna, '81). (2) The number of rectangles crossed before reaching the home area: This measure reflects the extent to which pups take the most direct route to the home area. (3) Latency in seconds to reach the home shavings area: Maximum duration was set at 120 sec. (4) Preference for the home area: The number of rectangles crossed within the home area compared to the number crossed outside the home area after pups have entered the home area. This measure assesses whether the pup's locomotor activity was random or directed toward the nesting material (i.e., true homing behavior). The photographs in Figure 2a–d depict the apparatus and the typically observed behavioral sequence. When placed in the start area (the middle of the field), pups initially exhibit pivoting behavior (Fig. 2a). Then, they orient (Fig. 2b), move toward, and eventually enter the home area (Fig. 2c). Once a control pup enters the home area, it will move back and forth within the home area and rarely leave it (Fig. 2d).

Statistical analysis

The litter served as the unit of observation for all the behavioral data described below. One-way analyses of variance were performed employing the BMDP Statistical Program P7D (Dixon, '79) as adapted for the PDP-10.

RESULTS

The amount of methanol ingested in g/kg/day did not vary between the two methanol groups ($F(1,18) < 1$). Daily consumption of methanol averaged 2.5 g/kg/day (S.D. = 0.25 gm). The duration of gestation did not differ across treatment conditions ($X = 22.0 \pm 1$ day; $F(2,27) < 1$). Body weight gained each day during the 3rd week of gestation did not differ between treatment groups. Control dams had gained a mean of 10.4 g by day 20. Both methanol groups gained a mean of 11.6 g.

Observation of maternal behavior on the day of parturition revealed no treatment differences in latency to retrieve pups after they had been weighed and replaced in their ma-

ternity cage (F(2,27) < 1). All the pups in this study were retrieved by dams within the 5-minute observation period, indicating that methanol exposure did not disrupt maternal behavior and that cross-fostering was not crucial to the experimental design.

Neither litter size, birthweight, weight gain during the preweaning period, infant mortality, nor day of eye opening differed across groups.

Suckling behavior test

The proportion of pups successfully attaching to nipples did not differ significantly across the three treatment groups (F(2,27) = 2.35; see Table 1). Figure 3 shows the mean latency to nipple attachment for each of the three treatment groups. The individual circles represent mean latencies within each of the ten litters assigned to a treatment condition. The methanol groups differed only slightly from one another, but both differed significantly from control group latencies (F,(2,27) = 7.57, $P < .01$) as is evident from Figure 5. Prenatal exposure to methanol, therefore, produced a significant impairment in suckling behavior that was evident 24 hours after birth.

Homing behavior

Figures 4 and 5 illustrate the results of the homing behavior tests conducted on postnatal day 10. The proportion of pups successfully reaching the home area within 3 minutes did not differ across treatment groups, (F(2,27) = 2.16; see Fig. 4, bottom). On the other measures of homing behavior, the two methanol groups were quite similar, and both differed sharply from the control group (Figs. 4, 5). Of pups that successfully reached the home area, those exposed prenatally to methanol exhibited significantly longer latencies than controls (F(2,27) = 23.01, $P < .001$; see Fig. 4, top). The methanol-exposed animals took about twice as long as control pups. Their increased latencies may have been due, in part, to the tendency for methanol-exposed pups to choose the wrong initial direction more often than controls (Table 2). Further, pups in both methanol groups crossed significantly more rectangles than controls to reach the home area (F(2,27) = 11.34, $P < .01$; see Fig. 5, top). In addition, the total number of rectangles crossed during the entire homing test was significantly elevated over control levels (F(2,27) = 7.19, $P < .01$; see Fig. 5, bottom).

TABLE 1. Mean proportion of pups successfully attaching to nipples in suckling behavior test 24 hours after birth[1]

Prenatal treatment	Mean proportion of successful pups	SD
Control	.819	.168
Methanol group 1	.751	.263
Methanol group 2	.668	.217

[1]Each group contained ten litters of three to five pups per litter.

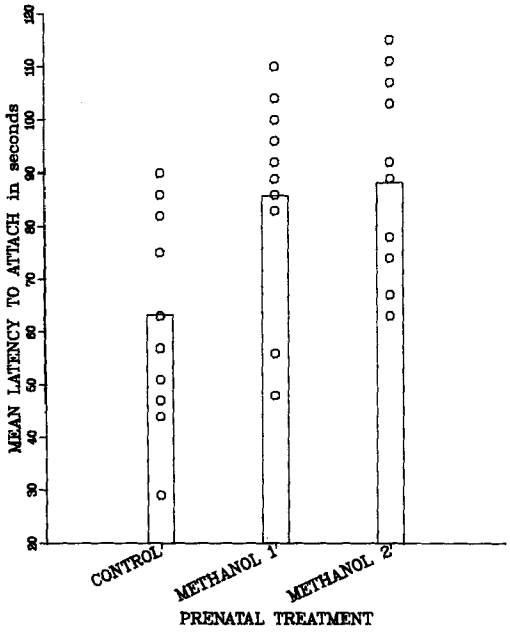

Fig. 3. Latency to nipple attachment in control and methanol treatment groups. Bar height represents mean. Individual litters are represented by circles.

Once the pups reached the home area, more than 90% of their activity (i.e., rectangles crossed) was confined to this section regardless of treatment condition. These latter findings indicate that the pups were not merely exhibiting random locomotor activity.

DISCUSSION

These data indicate that methanol, like ethanol and ethoxyethanol (Abel, '80; Nelson et al., '81) can be defined as a behavioral teratogen in rats, since no other signs of toxicity were apparent either in the mothers or the offspring. They also confirm previous observations in children and animals (Chen et al., '82; Martin et al., '77; Kaplan et al., '75) that altered suckling behavior in mammalian neonates can serve as a useful index of

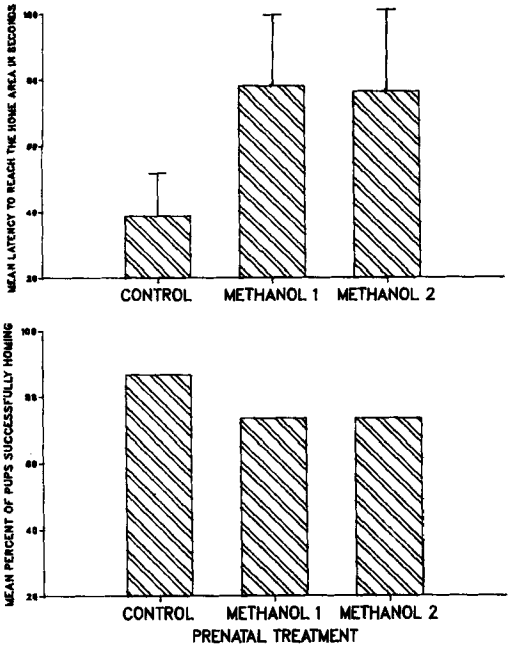

Fig. 4. Homing behavior test results. Bottom: Proportion of pups homing successfully. Top: Mean and standard deviation of latency to reach home area among pups homing successfully.

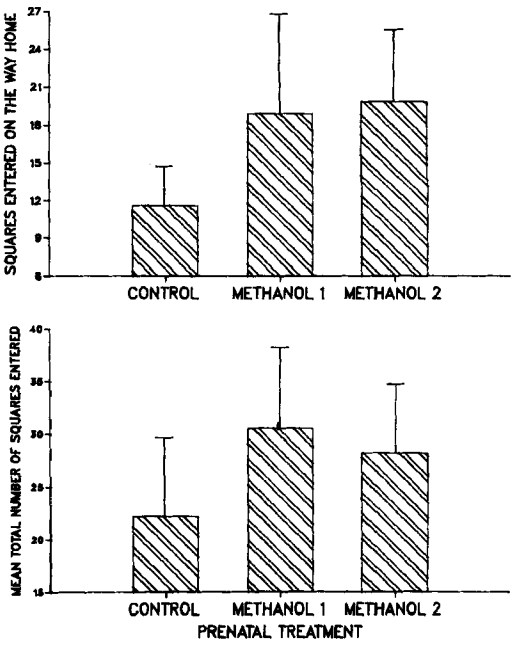

Fig. 5. Homing behavior test results. Top: Means and standard deviations for total squares entered before reaching home area. Bottom: Means and standard deviations for total squares entered during entire homing test.

TABLE 2. Mean proportion of pups in each group initially choosing the correct direction on the way home[1]

Prenatal treatment	Mean proportion of pups initially choosing correct direction	SD
Control	.834	.22
Methanol group 1	.704	.29
Methanol group 2	.634	.29

[1]Each group contained ten litters of eight pups per litter.

behavioral function, and indicate the potential of homing behavior as a measure of impaired neonatal development.

The distribution of ^{14}C-methanol and its metabolites in maternal and fetal tissue has been studied in rats (Infurna and Berg, '82). After acute intragastric administration of 2 g/kg, methanol in fetal brain reached maternal tissue levels (about 50 μm/g) within 1 hour. Placental transfer of methanol was also demonstrated in rats allowed to consume a 2% (v/v) methanol solution overnight (a total daily dose of about 2 g/kg). Under these conditions, maternal tissues averaged 20 μm/g, and fetal brain levels reached 30 μm/g. These concentrations are several times lower than those inducing deformities in the teratology study by Nelson et al. ('85). Based on the previously established insensitivity to methanol toxicity among mature rats, a sharp contrast to mature primates, these data probably reflect a conservative estimate of the risk of adverse effects in developing primates.

The present findings suggest yet one more health hazard that might be posed by the widespread distribution of methanol as an automotive fuel. They surely warrant further research on the developmental and reproductive toxicity of methanol, and support concerns expressed earlier about the problems that widespread use of methanol may present (Posner, '75).

sults of both experiments emphasize the need to consider toxic interactions in the evaluation of suspected human teratogens. (Supported in part by a grant from the Appalachia Chapter of the March of Dimes Birth Defects Foundation).

115 **DEVELOPMENTAL TOXICOLOGY OF METHANOL.** Robert Infurna, Wendy Schubin and Bernard Weiss, Div. of Toxicology and Environmental Health Sciences Center, Dept. of Rad. Biology and Biophysics, Univ. of Rochester School of Med. and Dent., Rochester, NY 14642, Sponsor: Frank A. Smith

Methanol (MEOH) may assume a significant role as a fuel, which implies wide availability. Yet, surprisingly little is known of its toxicity apart from acute poisoning in mature humans. Even less is known about its toxicity in developing organisms. We studied the early behavioral development of rats whose mothers had consumed MEOH during gestation. Neonatal responses included suckling (postnatal day 1), and nest-seeking (postnatal day 10). Locomotor activity was assessed on postnatal day 15. Prima gravida Long-Evans rats were divided into 3 groups (N=10). Two of the groups were given drinking solutions of 2% MEOH instead of distilled water either on gestational days 15-17 (MEOH 1) or 17-19 (MEOH 2). No maternal toxicity was apparent by weight gain, gestational duration, and daily fluid intake. Daily MEOH consumption averaged 2.5 gm/kg over the 3-day period in both MEOH groups. Litter size, birth weight, and infant mortality did not differ among the three groups. Postnatal development, such as growth and date of eye opening, were unaffected. Headpace gas chromatography revealed MEOH in placenta and in maternal and fetal brain and blood. MEOH pups took longer than controls to begin suckling on postnatal day 1. On postnatal day 10, they took longer to locate nesting material from their home cages. MEOH 2 rats were significantly more active on day 15 than controls (circular alley). These data suggest that prenatal MEOH exposure induces behavioral abnormalities early in life that are unaccompanied by overt toxicity. (Supported in part by grant MH-11752 from NIMH, grant ES-01247 from NIEHS, and in part by a contract with the US Dept. of Energy).

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LITERATURE CITED

Abel, E. (1980) Fetal alcohol syndrome: Behavioral teratology. Psychol. Bull., *87*:29–50.

Aquilonius, S.M., H. Askmark, P. Enoksson, P. Lundberg, and U. Mostrom (1978) Computerized tomography in severe methanol intoxication. Br. Med. J., *30*:929–930.

Blass, E.M., and M.H. Teicher (1980) Suckling. Science, *210*:15–22.

Chen, J.S., C.D. Driscoll, and E.P. Riley (1982) Ontogeny of suckling behavior in rats postnatally exposed to alcohol. Teratology, *26*:145–153.

Dixon, W.J. (1979) BMDP Statistical Software 1979. University of California Press, Berkeley.

Environmental Protection Agency (1983) Methanol/gasoline blends workshop. Fed. Reg., *48*(No. 135, July 13):32075–32076.

Hayreh, M., S. Hayreh, G. Baumbach, P. Cancilla, G. Martin-Amat, T. Tephly, K. McMartin, and A. Makar (1977) Methyl alcohol poisoning. III. Ocular toxicity. Arch. Ophthalmol., *95*:1851–1858.

Hayreh, M., S. Hayreh, G. Baumbach, P. Cancilla, G. Martin-Amat, and T. Tephly (1980) Ocular toxicity of methanol: An experimental study. In: Neurotoxicity of the Visual System. W.H. Merigan and B. Weiss, eds. Raven Press, New York, pp. 35–33.

Hunter, D. (1975) The Diseases of Occupations (5th Edition). English Universities Press, London.

Infurna, R.N. (1981) Daily biorhythmicity influences homing behavior, psychopharmacological responsiveness, learning, and retention of suckling rats. J. Comp. Physiol. Psychol., *95*:896–914.

Infurna, R.N., and G. Berg (1982) Distribution of methanol and metabolites in maternal and fetal tissues. Toxicologist, *2*:73.

Kaplan, S.L., R.E. Kron, M. Litt, L.P. Finnegan, and M.D. Phoenix (1975) Correlations between scores on the Brazelton Neonatal Assessment Scale, measures of newborn sucking behavior, and birthweight in infants born to narcotic addicted mothers. In: Aberrant Development in Infancy. Human and Animal Studies. N.R. Ellis, ed. Erlbaum, Hillsdale, NJ, pp. 139–148.

Kimura, E.T., D.M. Ebert, and P.W. Dodge (1971) Acute toxicity and limits of solvent residue for sixteen organic solvents. Toxicol. Appl. Pharmacol., *19*:699–704.

Lincoln, D.W., A. Hill, and J.B. Wakerley (1973) The milk-ejection reflex of the rat: An intermittent function not abolished by surgical levels of anesthesia. J. Endocrinol., *57*:459–476.

Martin-Amat, G., T. Tephly, K. McMartin, A. Makar, M. Hayreh, S. Hayreh, G. Baumbach, and P. Cancilla (1977) Methyl alcohol poisoning. II. Development of a model for ocular toxicity in methyl alcohol poisoning using the rhesus monkey. Arch. Ophthalmol., *95*:1847–1850.

Martin, J., M. Martin, C. Lund, and A.P. Streissguth (1977) Maternal alcohol ingestion and cigarette smoking and their effects on newborn conditioning. Alcoholism Clin. Exp. Res., *1*:243–247.

Nelson, B.K., W.S. Brightwell, J.V. Setzer, B.J. Taylor, R.W. Hornung, and T.L. O'Donohue (1981) Ethoxyethanol behavioral teratology in rats. Neurotoxicology, (Park Forest Ill.) *2*:231–250.

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

NIOSH (1976) Occupational Exposure to Methyl Alcohol. DHEW Publication No. (NIOSH) 76-148, Washington, DC.

Posner, H.S. (1975) Biohazards of methanol in proposed new uses. J. Toxicol. Environ. Health, *1*:153–171.

Roe, O. (1982) Species differences in methanol poisoning. Crit. Rev. Toxicol., *10*:265–274.

Rosenblatt, J.S. (1979) The sensorimotor and motivational bases of early behavioral development of selected altricial mammals. In: Ontogeny of Learning and Memory. N.E. Spear and B.A. Campbell, eds. Erlbaum, Hillsdale, NJ, pp. 1–38.

Sharp, C.W., and L.T. Carroll, eds. (1978) Voluntary Inhalation of Industrial Solvents. DHEW Publication No. (ADM) 79-779, Washington, DC.

Smith, E.N., and R.T. Taylor (1982) Acute toxicity of methanol in the folate-deficient acatalasemic mouse. Toxicology, *25*:271–287.

Tephly, T., W. Watkins, and T. Goodman (1974) The biochemical toxicology of methanol. Essays Toxicol., *5*:149–177.