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Behavioral and Neurochemical Alterations in the Offspring of Rats After Maternal or Paternal Inhalation Exposure to the Industrial Solvent 2-Methoxyethanol

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NELSON, B. K., W. S. BRIGHTWELL, J. R. BURG AND V. J. MASSARI. *Behavioral and neurochemical alterations in the offspring of rats after maternal or paternal inhalation exposure to the industrial solvent 2-methoxyethanol.* PHARMACOL BIOCHEM BEHAV **20**(2) 269–279, 1984.—The industrial solvent 2-methoxyethanol (2ME) has antifertility effects in male rats at 300 ppm and is teratogenic in rats and rabbits at 50 ppm. The present research investigated if exposure of paternal or maternal animals to 25 ppm 2ME, the current U. S. permissible occupational exposure limit, would produce detectable effects in the offspring. Eighteen male young-adult Sprague-Dawley rats were exposed to 25 ppm 2ME 7 hr/day, 7 days/week for 6 weeks; they were then mated with untreated females which were allowed to deliver and rear their young. In addition, groups of 15 pregnant rats were exposed 7 hr/day on gestation days 7–13 or 14–20 and allowed to deliver and rear their young. At birth, litters were culled to 4 females and 4 males for behavioral testing of neuromotor function, activity, and simple learning ability on days 10 through 90. In addition, brains from newborn and 21-day-old offspring were analyzed for neurochemical deviations from controls. No effects on paternal or maternal animals, nor on the number or weight of live offspring, were noted. Behavioral testing revealed significant differences from controls only in avoidance conditioning of offspring of mothers exposed on days 7–13. In contrast, neurochemical deviations were observed in brains from 21-day-old offspring from the paternally exposed group as well as from both maternally exposed groups; changes were numerous in the brainstem and cerebrum but were fewer in the cerebellum and midbrain. Thus it appears that both paternal and maternal inhalation of 25 ppm 2ME produces some effect which is reflected in neurochemical deviations in the offspring.

Teratology Reproduction Reproductive hazard Industrial solvents Occupational exposure
Neurochemical deviation Behavioral teratology Methoxyethanol

ETHYLENE glycol monomethyl ether (2-methoxyethanol, 2ME, CAS No. 109-86-4) is widely used as an industrial solvent, primarily in varnishes, stains, enamels, and in varnish and paint removers. It is also used as an anti-icing agent in aviation fuels, and in some consumer products such as nail polish and polish removers. Based upon 1972–1974 surveys, the National Institute for Occupational Safety and Health (NIOSH) estimates that approximately 100,000 workers are exposed to 2ME.

2ME is relatively low in toxicity, with an LD_{50} in rodents of about 3 g/kg, and an LC_{50} of approximately 1500 ppm [38]. The Occupational Safety and Health Administration (OSHA) Permissible Exposure Limit is 25 ppm. The Threshold Limit Value recommended by the American Conference of Governmental Industrial Hygienists (ACGIH) was also 25 ppm until 1982, when a reduction to 5 ppm was recommended [1]. The general toxicity of 2ME has recently been reviewed but reproductive factors were not discussed [42]. However, as will be reviewed shortly, experimental animal research indicates that the reproductive system is particularly vulnerable to 2ME toxicity. The only human study to date gave essentially negative results [10]. Employees at a large manufacturing and packaging plant were examined to determine whether employees potentially exposed to 2ME (N=40) had a higher prevalence of anemia, leukopenia, or sterility than an in-plant comparison group (N=25); only 6 potentially exposed and 9 controls were used for the fertility indices. Industrial hygiene information available since 1976 showed that ambient concentrations of 2ME ranged from nondetectable levels to 20 ppm, though there were exposures to

[1]Mention of company or product names does not constitute endorsement by NIOSH.

other chemicals as well. The results indicated that there were no gross abnormalities or clinically meaningful differences in hematological or fertility indices, with the possible exception of smaller testicular size ($p=0.06$) in the potentially-exposed employees. Other such studies are needed to determine if these testicular effects are indeed real, and, if so, the functional significance of this observation, before defining the risk of reproductive effects in humans.

These possible effects in humans are consistent with effects reported in experimental animals. The effects of ethylene glycol and six ethylene glycol mono alkyl ethers on testicular atrophy have been reported in mice [33]. Groups of 5 male mice (6 weeks old) were given oral doses of 62.5, 125, 250, 500, 1000, 2000, or 4000 mg/kg 2ME 5 days/week for 5 weeks. Upon sacrifice, blood was collected for clinical chemistry, and testes were weighed and subsequently examined for histopathology. Ethylene glycol did not produce measurable effects. Phenoxyethanol and butoxyethanol produced mortality in the mice at 2000 mg/kg, but there were no detectable effects at lower doses. However, 2ME and its acetate, and 2-ethoxyethanol and its acetate all produced marked testicular atrophy and leucopenia at the higher doses used; alterations were still detectable at 250 mg/kg 2ME, 500 mg/kg of its acetate, and at 1000 mg/kg of 2-ethoxyethanol and its acetate. At twice these levels (respectively), white blood cell counts were significantly decreased.

Inhalation of 2ME vapors has also produced reproductive disorders in male experimental animals. 2ME was not mutagenic in a test screen [25]; however, at 50 ppm 2ME (7 hr/day for 5 consecutive days), serial matings showed a dramatic effect on implantation and pregnancy in post-exposure weeks 3–8, although normal values were evident by week 10. In another study [28], male and female Sprague-Dawley rats and New Zealand white rabbits were exposed to 0, 30, 100, or 300 ppm 2ME for 6 hr/day, 5 days/week for 13 weeks. The highest concentration induced toxicity in both species, but particularly in rabbits. At 30 ppm, the only effects were slight microscopic alterations in the testes of 1 of the 5 male rabbits. A follow-up study [29,41] found that 300 ppm 2ME on a similar exposure regime completely suppressed fertility in male rats, but the effect on fertility was partially reversed at 13 weeks. Dominant lethality could not be assessed at this concentration due to infertility, but no treatment-related toxicity was observed at 100 ppm or 50 ppm.

2ME has also recently been shown to be teratogenic in experimental animals. 2ME was administered by gastric intubation to mice on gestation days 7 through 14 at doses of 31.25, 62.5, 125, 250, 500, or 1000 mg/kg, and animals were sacrificed on day 18 [34]. No maternal deaths were observed, but maternal weight gain was reduced at the highest 3 levels, and leukocyte count was depressed at the highest dose. All fetuses in the 1000 mg/kg group were dead at the time of observation, and only one fetus was alive at 500 mg/kg. Fetal weights were reduced at 125 and 150 mg/kg. The authors reported that skeletal anomalies were apparent even at the lowest dose of 31.25 mg/kg.

Inhalation of 2ME vapors is also teratogenic in rats and rabbits. Fischer 344 rats and New Zealand white rabbits were exposed to 0, 3, 10, or 50 ppm 2ME for 6 hr/day on gestation days 6–15 (rats) or 6–18 (rabbits), and fetuses were evaluated near term [8] (in a previous probe study, these investigators had observed maternal toxicity and complete resorption of litters in both rats and rabbits at 200 and 400 ppm 2ME). At 50 ppm, significant increases in resorptions and malformations were observed in rabbits, but there were indications of only a slight fetotoxic effect in rats. There was no evidence of maternal or fetal effects at the lower concentrations. In another study [36], groups of pregnant Sprague-Dawley rats were exposed to 50, 100, or 200 ppm 2ME for 7 hr/day on gestation days 7–15. At 200 ppm, all litters were resorbed. At 100 ppm, about one half of the litters were completely resorbed, and all litters had some resorptions; surviving fetuses had depressed weights and an increased incidence of congenital malformations. At 50 ppm, resorptions were still increased, fetal weights were decreased, and congenital malformations were observed.

Thus, significant testicular effects have been reported after exposure of male rabbits, and teratogenic effects have been reported in rats and rabbits after exposure to 50 ppm 2ME. Consequently the present research sought to determine if exposure of male or female rats to 25 ppm 2ME, the current OSHA Permissible Exposure Limit, would induce effects which were detectable in the offspring. As other investigators have reported decreased survivability [18,23] and altered behavior [3,4] in offspring after exposure of males to toxic agents, our testing included the functional evaluation of offspring of rats after paternal exposure throughout one sperm cycle. Also, offspring were functionally evaluated after prenatal administration of 2ME, as such testing often detects alterations at concentrations below those which produce frank malformations [2].

METHOD

Subjects

Virgin female and male Sprague-Dawley rats specified to be free of Mycoplasma, Sendai virus, and of internal and external parasites (Charles River Breeding Laboratories; Wilmington, MA) were acclimated to a 12 hr light/dark cycle (lights on at 6 a.m.) and to a temperature of 24±2C for 2 weeks. The humidity was not controlled but typically averaged 40±20 percent. Breeder males weighed over 300 g and were individually housed in 32×41×18 cm suspended stainless steel wire mesh cages equipped with automatic water dispensers (Hoeltge Inc.; Cincinnati, OH). Virgin females were housed 3/cage in similar cages. Males to be exposed to the test chemical (see below) were individually housed in 18×32×18 cm suspended stainless steel cages. Purina Lab Chow and tap water were available ad lib except when animals were in exposure chambers. Bedding for the litters was cleaned, heat-treated sawdust (Absorb-Dri; Tasty Foods; Cincinnati, OH).

Apparatus and Procedures

For mating, virgin females weighing 200–300 g were placed individually with the breeder males. Unless sperm plugs were detected, vaginal smears were taken each morning. Females with sperm (day 0 of gestation) were placed singly in 30×34×17 cm polycarbonate cages having autoclavable polyester filter covers. Feed and water intake, along with maternal weights were measured over weekly periods (i.e., on days 7, 14, and 21). On the appropriate days of gestation, these females were transported from the animal quarters to the exposure chambers in their home cages (water bottle removed) with filter tops in place. Males to be exposed to methoxyethanol weighed 300–400 g at the initiation of exposures (N=18; X=355 g, SD=48). They were exposed 7 hr/day, 7 days/week for 6 weeks, and were weighed biweekly. After two non-exposure days, these males were mated (1:1) with unexposed virgin females for a maximum of

5 days. The males were then discarded, and the females were handled in the same manner as the controls for gestation days 7–13 for comparison purposes.

The vapor generation equipment was housed above the inhalation chambers (0.5 m^3; Charles Spengler and Associates; Cincinnati, OH) in a glove box which was vented to the building exhaust air. Technical grade 2-methoxyethanol (≥98% purity; Matheson Coleman and Bell Manufacturing Chemists; Cincinnati, OH) was placed into a flask. A micrometering pump (RP model lab pump; Fluid Metering Inc.; Oyster Bay, NY) circulated liquid from the reservoir flask into a 10 cc syringe contained within the flask such that the syringe was constantly overflowing. This assured that the syringe had a constant head of chemical for a second pump which injected the specified amount of liquid into a three way valve which was attached to a Greensmith impinger. Compressed air was introduced through the second inlet of the three way valve. The impinger increased contact time between the air and the liquid to assure total evaporation. This vapor and air mixture was introduced into the chamber air flow prior to the orifice plate. Due to the turbulence resulting from the pressure drop created by the orifice plate, there was uniform mixing of the vapor and air before the mixture entered the chamber. Air flow through the chambers provided approximately one air change per minute.

The concentration within the chamber was monitored continuously by a Miran IA infrared analyzer (calibrated within the range to be tested) and recorded on a stripchart recorder. The chamber concentration, temperature, and room humidity were recorded on an hourly basis onto a daily concentration data sheet. At the end of each day, the chart was attached to the data sheet and the daily mean, range, and CT (concentration × time) were calculated. At the conclusion of the study, these values were averaged for an overall study mean.

In addition, charcoal tube samples were taken from the chamber atmosphere on a regular basis. (Typically, a sample was taken once hourly on alternate days throughout the study; verification samples had been obtained previously.) These charcoal tubes were analyzed by gas chromatography for 2ME by a slight modification of NIOSH method S-79 [39] (performed by the UBTL division of the University of Utah Research Institute; Salt Lake City, UT).

In the exposure chambers, animals were placed in individual compartments (13×25×18 cm) of stainless steel wire mesh cages. In order to maximize use of the inhalation chambers, females were assigned to exposure groups as they became pregnant (i.e., pregnancies were clustered with respect to time). Thus, the assignment was not truly random but was without bias. The following groups were utilized; the first number in parentheses refers to the number of animals exposed to 2ME and the second number refers to the number of litters used for behavioral testing. Litters were discarded if there were fewer than six pups per litter or fewer than three pups of either sex per litter; this explains differences in N within groups. (1) C 7–13—sham exposed controls, days 7–13 (N=18; 15); (2) C 14–20—sham exposed controls, days 14–20 (N=15; 15); (3) 2ME 7–13—25 ppm methoxyethanol, days 7–13 (N=16; 15); (4) 2ME 14–20—25 ppm methoxyethanol, days 14–20 (N=18; 15); (5) 2ME Males—25 ppm methoxyethanol, for 6 weeks (N=15; 14); (Females handled the same as C 7–13); (6) In addition, five litters were not handled at all, and their brains collected for use in neurochemistry (to determine if merely handling the dam early in gestation would result in alterations in neurochemistry).

Exposures were conducted 7 hr/day, and the animals were left in the chamber for an additional hour blow-off time after vapor generation terminated. They were then removed and returned to the animal quarters each night.

On day 21 of gestation, each animal was placed in clean bedding, given a paper towel for nest construction, and left undisturbed until after parturition. Expectant females were examined around 7:30 a.m. and 3:30 p.m. for offspring. Within 16 hr after delivery, the dam was weighed, as was her entire live litter. Generally, four male and four female pups (arbitrarily selected from those that had nursed) were left with their biological mother. Weaned at 25 days of age, individual pups were weighed on postpartum days 7, 14, 21, 28, and 35 and observed for abnormalities. Separate mean weights were determined for female and male pups per litter.

BEHAVIORAL TESTING

Six behavioral tests (see Table 1) were selected to evaluate various CNS functions (neuromuscular, activity, and learning) at several stages of development. The rationale for their selection is outlined in previous publications [35,37]. Behavioral testing encompassed days 10 through approximately 90 as outlined in Table 1. Male and female pups were selected randomly on postpartum day 10, ear marked, and assigned to test groups. For each test, one female and one male were used from each litter; following each group of tests, the participating animals were sacrificed. Where animals were used on more than one test, there was no reason to expect interaction among the tests in the order administered (e.g., see [17]). Since various treatment and control groups were run simultaneously, those involved with animal testing were not aware of the treatment groups to which specific subjects belonged.

Ascent

The apparatus consisted of a 6 mm wire mesh screen attached to a wooden frame (45 cm high and 15 cm wide) inclined at an angle of 70° from horizontal. Attached to the bottom of the screen was a cardboard box filled with approximately 3 cm of sawdust (to cushion the animal's fall).

One male and 1 female, randomly selected from each litter, were tested on days 10, 12, and 14 days of age. The front feet of the test animals were placed on the inclined screen 20 cm from the top, with the hind feet lower on the screen. The time each pup held to the wire, the maximum distance climbed in a 60 sec trial, and the time to reach the top were recorded for each animal tested.

Rotorod

A 9 cm diameter and 10 cm-long rod (made rough by applying sand while the paint on the rod was wet), was rotated by a variable speed motor (3 to 78 rpm) controlled by a digital lock adjustable speed drive (Minarik, from Thal-mor Associates; Dayton, OH). There was approximately a 48 cm fall from the bottom of the rotorod into 3–4 cm of sawdust.

The same male and female rats used for the ascent test were used for the rotorod testing. The test animal was placed on the rod rotating at 6 rpm (day 21), 9 rpm (day 25), or 12 rpm (day 29). If the animal maintained a walking (or running) motion for 15 sec at a given rate of rotation, the velocity was progressively increased by 3 rpm at 15 sec intervals. If the rat fell off during any trial, it was allowed to remain in the sawdust-filled box for 30 sec, and then placed back on the

TABLE 1

BEHAVIORAL AND NEUROCHEMICAL TESTS AND DAYS OF TESTING IN BEHAVIORAL TERATOLOGY STUDY (SEE TEXT FOR EXPLANATION)

Behavioral Tests	Group*	Function Tested	Days of Age
1. Ascent on wire mesh	1	Neuromuscular	10, 12, 14
2. Rotorod	1	Neuromuscular	21, 25, 29
3. Open field	2	Exploratory activity	16, 17, 18, 30, 31, 32, 44, 45, 46, 58, 59, 60
4. Activity wheel	2	Circadian activity	32–33
5. Avoidance conditioning	1, 2	Aversive learning	Begun days 34, 60
6. Operant conditioning	3	Appetitive learning	Begun day 40
Neurochemical Assays			
1. Whole brain	4	Protein, acetylcholine, dopamine, norepinephrine, 5-hydroxytryptamine	Newborn
2. Cerebrum, cerebellum, brainstem, midbrain	4	same chemicals	21

*Rats in the same group were administered the tests in the order shown.

rod rotating at 3 rpm higher than the last rate of speed on which it had been successful. Each rat was given 5 trials (falls) per test day. The score given each animal/day was the highest rpm it maintained walking (or running) for that day. These rats were the same ones used beginning on day 34 of age for the avoidance conditioning test.

Open Field

The open field test apparatus consisted of a 1.0 m diameter circular plexiglass field, subdivided into sections, and enclosed by a wall 0.5 m high. The field was marked into four concentric circles, and divided by segments of radii such that sections were of similar area (approximately 250 cm^2).

On days 16, 17, 18; 30, 31, 32; 44, 45, 46; 58, 59, 60 postpartum, each preselected rat (1 male and female per litter) was placed individually in the central circle, and the number of sections entered and number of fecal boluses excreted during a 3-min trial were recorded. All such testing was performed at approximately the same time each morning; on days 16, 17, and 18, the latency to leave the central circle was also recorded. Following each trial, the field was cleaned with a detergent swab and dried with paper towels. The same rats were used in the activity wheel testing and, commencing at 60 days of age, for avoidance conditioning.

Activity Wheel

Activity wheels (Lafayette Instrument Co., Lafayette, IN) were modified slightly to allow a programmed digital recorder (Digitex Model 6150, United Systems Corp.; Dayton, OH) to give a printout of revolutions every hour. The activity wheels were placed in a 1.0×0.7×1.9-m sound-attenuated chamber (Industrial Acoustics Co., Inc.; Bronx, NY) equipped with its own exhaust fan and a 15-watt fluorescent light set for 12 hr light and 12 hr dark. Each rat was allowed individual access to the activity wheel for approximately 24 hr on days 32–33 of age.

Avoidance Conditioning

Commercially available shuttle boxes, housed in sound attenuated chambers each having a baffled air intake and exhaust system (BRS/LVE Beltsville, MD; 45.5×20×19.5-cm), were divided in the center by a partition 4 cm high. Scrambled electric shock (0.7 mA) could be delivered to either side of the grid floor. A trial consisted of placing the rat on one side of the box and presenting a warning tone for 5 sec before the onset of shock. If the animal crossed to the opposite side of the box during the tone presentation, the rat avoided any shock and that trial ended. If the animal did not cross during the tone, it received shock until it did cross. The times between trials (generated from a table of random numbers) varied between 15 and 45, with a mean of 30 sec.

As previously noted, animals used for the ascent test and rotorod testing were used beginning on day 34 of age for the avoidance conditioning, and those previously used in the open field and activity wheel were tested in the avoidance trials beginning on day 60 of age. On the 1st day of testing, the male and female rats were placed individually into either side of the shuttle box. After a 5 min adaptation period during which the number of times the animal crossed the partition was recorded, each rat received 20 trials in the shuttle box. On succeeding days the rats received 20 trials (without adaptation period) per day for a maximum of 14 days until they reached a criterion of ≤ 4 shocks/day for two consecutive days. For those that reached this criterion, testing for extinction was continued for a maximum of 10 days until a criterion of ≥ 16 "shocks"/day was met for two consecutive days (i.e., the shock was turned off to see how long the animals persisted in crossing in response to the warning tone). The data analyzed included: number of crosses in a 5

min adaptation period, mean number of crosses in 20 trials, mean number of shocks received in 20 trials, mean duration of shock delivery in 20 trials, number of trials required to reach criterion, and number of trials required for extinction.

Operant Conditioning

The operant chambers were standard modular test cages (Coulbourn Instruments, Inc., Lehigh Valley, PA) having a 0.635 cm stainless steel grid floor. The animal area was 24×30×29 cm, with the cage top and ends of 0.635 cm metal, and sides of 0.635 cm clear acrylic plastic. The hardware included a response lever on both the left and right side of the water dipper (2 cm into cage; required 13 g force to activate), the water dipper and trough, a house lamp, and a relay (clicker). Each chamber was housed in a Coulbourn sound attenuated isolation cubicle which was equipped with a ventilation fan and baffled air intake and exhaust system.

In the operant chamber, the house light and ventilation fan remained on throughout testing. The water dipper remained in the water trough unless activated by the animal's pressing the response lever, at which time it rapidly elevated so that the animal could lick it for 3 sec, thus delivering the reinforcement for this response. The magazine light over the dipper was off when the dipper was in the down position. The clicker sounded when reinforcement was delivered.

Subjects for the operant tests were drawn from the remaining untested rats. Specifically, the preselected female and male rats from each litter were weighed at 35 days of age, and water was made available in their home cages for only 0.5 hr/day. On days 40 and 41 of age, these rats were magazine trained (water delivered at 60-sec intervals) for 0.5 hr/day. Water availability was then further restricted to 5 min/day in the late afternoon. On day 41, the animals were left overnight in the chambers with water delivered each 10 min. Generally (91 percent of cases), this procedure was sufficient for the rats to learn the lever-press response. In the remaining cases, the experimenter manually trained the rat to press the lever for water reinforcement on day 42. On day 43, the rats were put on a fixed ratio 5 (FR5) schedule of reinforcement for 1.5 hr. On day 44, the rats were put on an FR10 schedule, and on subsequent days the rats were placed on an FR schedule twice that of the previous day (i.e., FR20, 40, 80, 160, 320, and 640). This continued until the rat did not make a sufficient number of responses to receive reinforcement on a given day, at which time the test for that rat terminated.

NEUROCHEMISTRY

At least 10 pups (no more than 2 per litter) per treatment group, either on the day of birth or postpartum day 21, were used for the neurochemical analyses. Newborn pups were selected from the "extra" pups at the time of culling litters to eight pups. Having been so designated during the random selection process on day 10, one male and one female pup which had received no prior testing (except weighing) were used on day 21. Individual pups were placed into a small animal holder, inserted into a focused microwave oven (Model 4104 Metabostat; Gerling Moore Inc.; Santa Clara, CA) and irradiated at 3.5 kW and a frequency of 2.45 GHz. Newborn pups were irradiated for 0.75 sec and 21-day old pups were irradiated for 1.0 sec between 1300 and 1400 hr of the day in order to preserve neurotransmitters by inactivating brain enzymes [8,26]. The animals were then removed from the microwave unit and the entire brain was excised. For newborn animals, chemical analyses were performed on whole-brain samples. For 21-day old animals, the brains were separated into the following operationally-defined regions: cerebrum, cerebellum, brainstem, and midbrain. The cerebrum included the cerebral cortex, hippocampus, and striatum. The cerebellum included only the cerebellum. The brainstem sample was taken from the spino-medullary junction to a transverse section from the caudal boundary of the inferior colliculus to the posterior boundary of the mammillary body. The midbrain sample began at the rostral boundary of the brainstem and included all tissue ventral to the cerebrum and caudal to a section approximately through the middle of the olfactory tubercle. All samples were frozen at −80°C until assays were made.

The microwaved brain samples were homogenized by sonication in 8 ml of 0.1 N HCl. Aliquots were then removed for determination of (A) protein [22] (0.5–5.0 μl), (B) acetylcholine (ACh, 25 μl) [13] as modified [16,24]; (C) 5-hydroxytryptamine [43] (5HT, 10 μl); (D) norepinephrine [11] (NE), and (E) dopamine [11] (DA, 25 μl each). Samples were randomized for processing sequence and the individual performing the assay was unaware of the group to which the sample belonged.

STATISTICAL ANALYSES

Data were analyzed using multivariate analysis of variance (MANOVA) [32] or, in cases where the data did not fit normal distributional assumptions required for parametric analyses, the Wilcoxon 2-sample nonparametric test [9] was used with $p=0.05$ (though p values between 0.05 and 0.1 are also reported). When the same group of animals was used for multiple comparisons in the MANOVA, Bonferroni corrections [27] were made to eliminate the multiple comparisons problem.

RESULTS

The concentration of 2ME in the exposure chambers remained very near to the desired concentration of 25 ppm. The mean chamber concentration over 99 exposure days based upon hourly readings from the infrared analyzer was 24.9 ppm (SD=1.6). The mean concentration from 45 charcoal tubes collected throughout this period was 25.6 ppm (SD=3.9).

Parental Data

No treatment related differences were observed in maternal data, including weight gain, feed consumption, water intake, number or proportion of live offspring, or pregnancy duration. Feed and water intake were not monitored in the exposed males, but weight gain was monitored and appeared to be normal. Sixteen of the 18 males copulated within 5 days after placing them with females (one male was observed to have slight nasal bleeding about mid-way through the exposure period, and he continued to lose weight from that point in time. After termination of exposures, he did not mate with a female; however this was judged not to be treatment related). One of the 16 litters had only female pups, and was discarded due to the lack of males. Thus there was likely no treatment-related general toxicity in either the female or male exposed animals.

Offspring Data

No treatment-related differences in weight gain were evi-

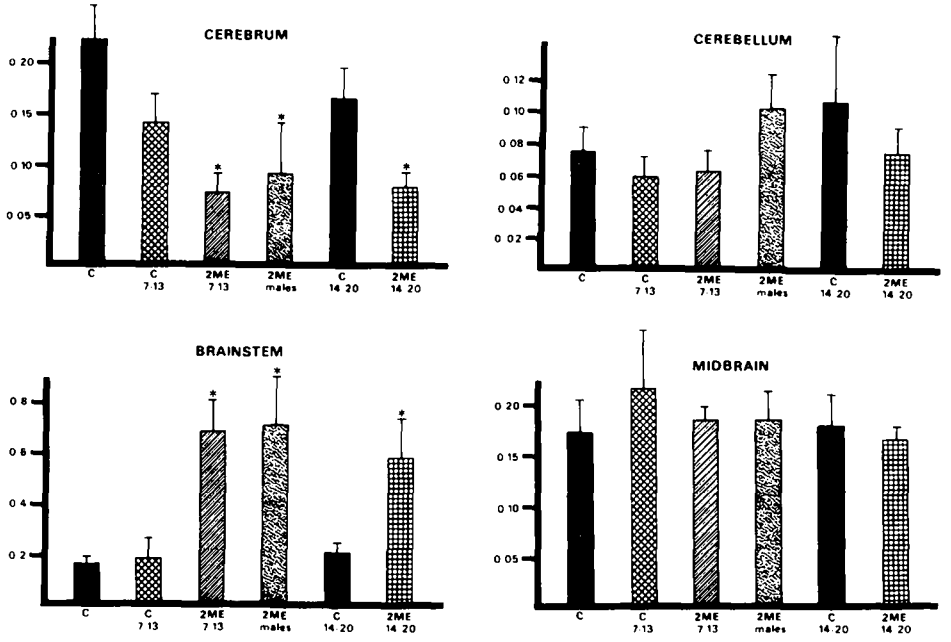

FIG. 1. Concentration of acetylcholine (pmole/μg protein) by brain region in 21-day old rats (C=unhandled controls; c 7–13=sham exposed controls on gestation days 7–13; 2ME 7–13=exposed to 25 ppm 2ME on gestation days 7–13; 2ME males=offspring of males exposed to 25 ppm 2ME for six weeks; c 14–20=sham exposed controls on gestation days 14–20; 2ME 14–20=exposed to 25 ppm 2ME on gestation days 14–20). (*=$p<0.05$).

dent. Based on a multivariate analysis with weight as repeated measures and birth weight as the covariate, the group 2ME 14–20 males gained more weight than their controls (approximately 5%) but this was not likely related to the experimental treatment. As expected, the males gained significantly more weight than the females ($p<0.001$).

Behavioral Testing

Very few behavioral tests revealed differences from controls. For the ascent test, a χ^2 analysis to examine contingency table distributions for each of the test days revealed no differences among groups. On the rotorod, a Wilcoxon 2-sample test for group comparisons of each of the three test days found only one difference: a performance deficit (mean rpms of 10.5 vs. 16.0 and 11.2 vs. 12.4 for females and males respectively) in the 2ME 7–13 group relative to controls, but only on day 21. As this was the only day on which performance was affected, this difference may be of questionable biological significance.

The activity measures also showed no differences among the relevant comparisons. In the open field data, Wilcoxon 2-sample testing revealed no differences among groups on the latency scores. The sum of 3-day boluses were categorized by intervals of five, and a contingency table analysis revealed no differences among groups; sex differences were apparent, with males excreting more boluses than females. A MANOVA using repeated measures at the different ages tested for activity found no differences among groups; females were significantly more active than males, with the differences increasing with age. Likewise in the activity wheel females were more active than males. The scores from the activity wheel were divided into day and night scores, and the activity at night was greater than during the day. However, no differences among groups were detected.

The avoidance conditioning test was the only one in which differences between groups were consistent. At both ages tested, the following data were analyzed: number of crosses in the 5-min adaptation period, mean number of shuttles in 20 trials/day during the learning trials, mean number of shocks received during these trials, mean duration of shocks received, number of trials required to reach criterion, and for those that did reach criterion, the number of trials to reach extinction. A Wilcoxon 2-sample test showed that group 2ME 7–13 had significantly lower ($p\leq0.01$) values for both the number (13 vs. 15 and 13 vs. 14 in younger; and 12 vs. 18 and 15 vs. 17 trials in older rats for females and males, respectively) and duration of shocks (43 vs. 52 and 33 vs. 35 in younger; 34 vs. 48 and 25 vs. 42 seconds in older rats for females and males, respectively). There was also a reduced number of shuttles during the adaptation period and the conditioning trials, but these differences were not significant. In the same group, the number of trials to reach criterion was fewer than in controls 263 vs. 275 and 225 vs. 269 in younger; 233 vs. 296 and 271 vs. 292 trials in older rats for females and males, respectively).

In the 2ME males group, univariate analyses indicated that in the older group of animals tested, the duration of shock was shorter (29 vs. 48 and 33 vs. 42 seconds, females and males respectively) than in controls. While this effect was significant, groups similarly tested at a younger age showed no differences from control. The absence of more-robust differences here raises doubt regarding the biological significance of the difference in this group, as compared with those seen in the 2ME 7–13 group. Once again, sex differences were observed. Females at both ages had more shuttles than the males and were shocked less time than were the

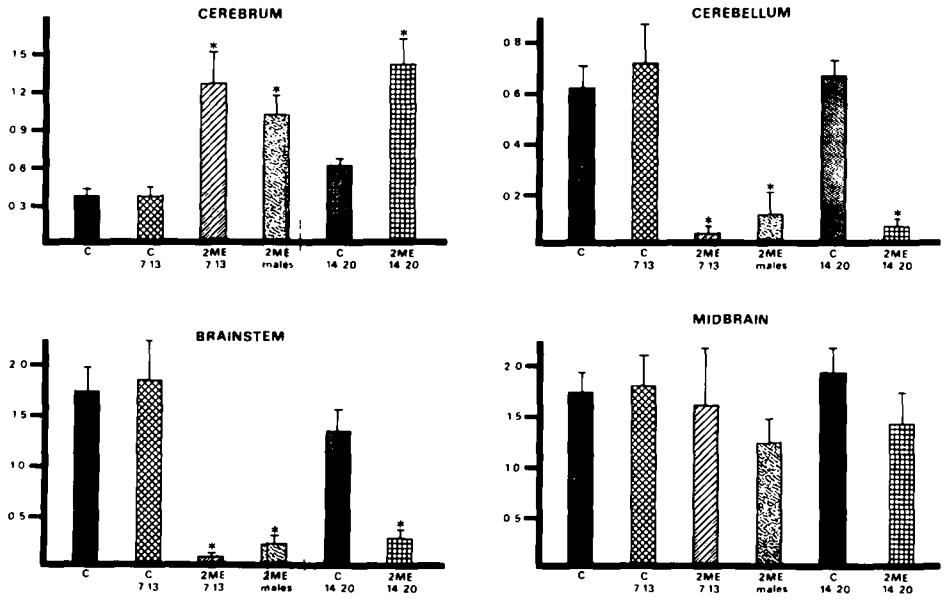

FIG. 2. Concentration of dopamine (pg/μg protein) by brain region in 21-day old rats (see legend to Fig. 1 for explanation of groups).

males; also more females than males reached the learning criterion.

In operant conditioning, 91% of the animals autoshaped with our procedure, but no differences were observed among groups on this variable. Using the Wilcoxon 2-sample test, no differences were observed among groups in the number of responses emitted during the conditioning trials nor on any of the fixed ratio schedules of reinforcement.

Neurochemical Analyses

The Wilcoxon 2-sample test indicated few differences among the control groups. When comparing the non-handled control group with C 7–13, the former had lower levels of cerebellar 5HT and NE; when comparing the non-handled control group with C 14–20, the former had significantly ($p \leq 0.05$) lower cerebral DA and possibly ($p \leq 0.06$) lower cerebral protein. Comparing C 7–13 and C 14–20, one sees that C 7–13 had elevated midbrain 5HT but lower cerebral DA than C 14–20.

In contrast, numerous alterations are observed when comparing the groups exposed to 2ME with the appropriate control group. Differences were apparent in ACh (Fig. 1), DA (Fig. 2), NE (Fig. 3) and 5HT (Fig. 4), with only sporadic deviations in protein (Fig. 5). In whole brain samples from newborn animals, there were increases in ACh (0.134 vs. 0.099 pmole/μg protein) and 5HT (1.054 vs. 0.367 pg/μg protein) in the 2ME males group relative to controls, but no other differences were significant.

Of particular interest in the complex pattern of neurochemical results seen in the 21 day old offspring was a consistent increase in NE and ACh in the brainstem of all three treatment groups (compared with their respective controls), accompanied by a decrease in NE and ACh in the cerebrum. A similar increase in brainstem 5HT was seen in all three treatment groups; however, 5HT in the cerebrum was significantly decreased only in the 2ME 14–20 group. In contrast to this pattern, DA was decreased in the brainstem and cerebellum of all three treatment groups, but was elevated in the cerebrum.

DISCUSSION

The neurochemical results of this study, and to a far lesser extent the behavioral results, indicate that inhalation of 25 ppm 2ME during pregnancy can exert a teratogenic effect that is reflected in alterations of the offspring. Perhaps even more surprising, paternal inhalation of 25 ppm 2ME results in some effect that is reflected in neurochemical deviations in the offspring. In fact, the deviations observed in offspring after paternal exposure to 2ME were very similar to those induced by maternal exposure to 2ME. A similar observation has been previously reported for lead [7].

Two design limitations are acknowledged. (1) There were no control males for the 2ME-exposed males. Consequently, we cannot be certain that our handling the males while placing them into inhalation chambers, or that perhaps their first copulation, did not exert some effect on the results we observed. However, we do not feel that either of these contributed significantly to our results. (2) The offspring were not fostered at birth. Consequently there is a possibility that some factor(s) other than only the prenatal exposure had some unspecified effect on the offspring. However, since the literature reports over 90% of a single PO dose of 2ME was recovered within 20 minutes after dosing and 95% was recovered within 48 hr after dosing [31], it is virtually impossible that any residual chemical could account for the effects we observed in the 2ME 7–13 group; it is also highly unlikely that significant amounts of 2ME could have been passed through the milk in the 2ME 14–20 group. Thus, the most tenable hypothesis is that the effects we observed were the result of exposure to 2ME.

The cell bodies of most noradrenergic and serotonergic neurons in the CNS of the rat are found in the brainstem [21,44], with axons projecting throughout the neuraxis to innervate terminal regions. Monoaminergic neurons are

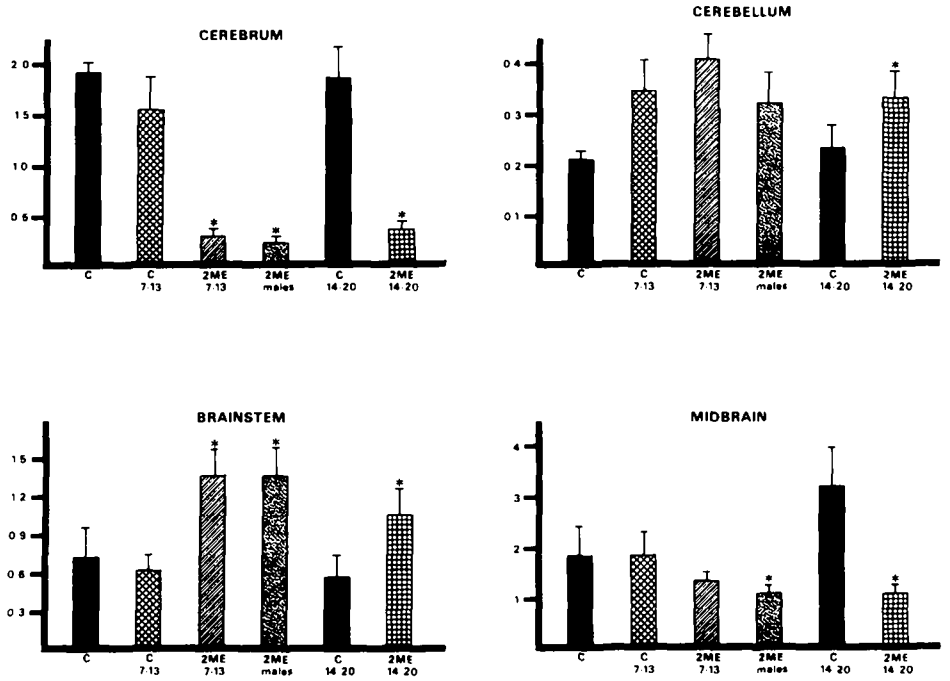

FIG. 3. Concentration of norepinephrine (pg/μg protein) by brain region in 21-day old rats (see legend to Fig. 1 for explanation of groups).

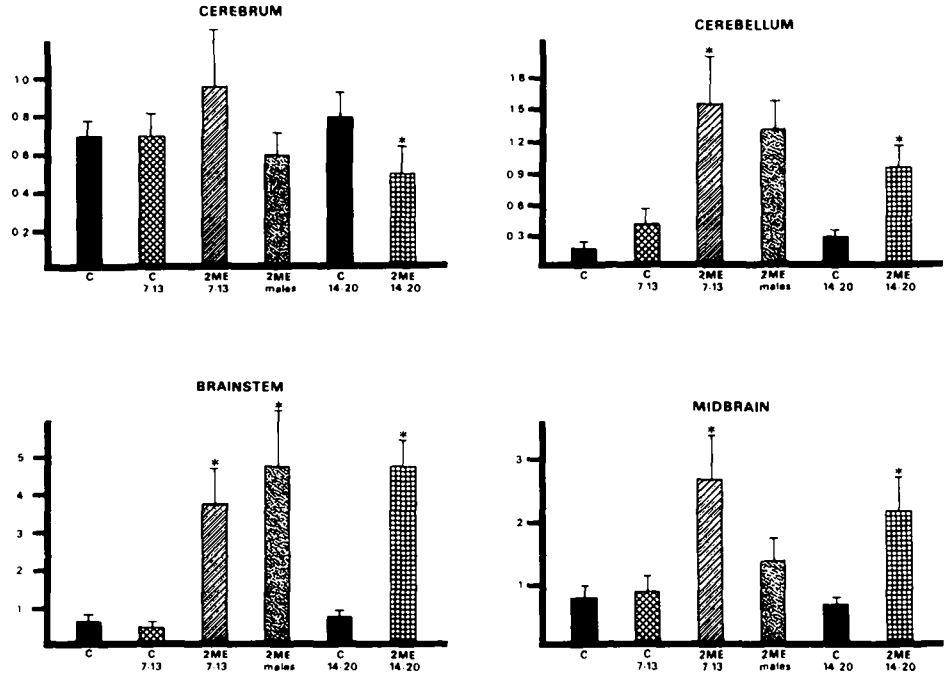

FIG. 4. Concentration of 5-hydroxytryptamine (pg/μg protein) by brain region in 21-day old rats (see legend to Fig. 1 for explanation of groups).

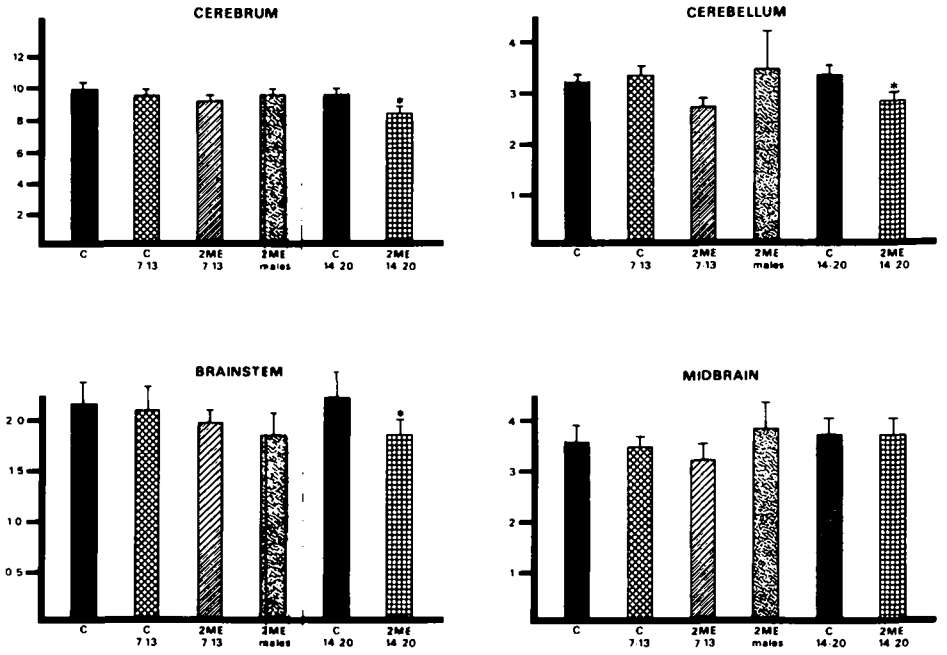

FIG. 5. Concentration of protein (μg/sample) by brain region in 21-day old rats (see legend to Fig. 1 for explanation of groups).

capable of axoplasmic transport of organelles, enzymes, and transmitters [12, 19, 45]. Transection of these axons can lead to an increase in the concentration of a somatofugally transported element proximal to the lesion, and a decrease in its concentration distal to the lesion. In view of this, it is interesting to note in the present study that NE was elevated in its major cell body area (brainstem) and depleted in a terminal region (cerebrum) in all three treatment groups. Similarly, 5HT was elevated in the brainstem and depleted in the cerebrum in the 2ME 14–20 group. 5HT was also increased in the midbrain in this group and some 5HT neurons are found in the midbrain [44] and project to the cerebrum.

Several cholinergic nuclei are also found in the medulla and pons of the rat [5]. Many of these neurons project to peripheral skeletal and smooth muscles, although some may project to the forebrain [20]. 2ME exposure caused an increase in ACh in this area and was associated with a decrease in ACh in the cerebrum. These similar results for three transmitters are compatible with the hypothesis that exposure to 2ME may interfere with the axoplasmic transport of ACh, NE, and 5HT, perhaps by causing lesions which transect some cholinergic, noradrenergic, and serotonergic axon bundles in the brainstem. Lesion of the dorsal NE bundle (which provides the major NE input to the cortex) also results in elevated levels of NE in the cerebellum, probably as a result of compensatory sprouting of NE neurons of the locus coeruleus [40]. The present data also show an increase in NE and 5HT in the cerebellum in the 2ME 14–20 group, which is consistent with the hypothesis that 2ME caused a brainstem lesion.

Dopaminergic nuclei in the rat are found in the midbrain and hypothalamus [21]. Axons from these nuclei project as far rostrally as the forebrain and caudally to the spinal cord [6]. In the present study, no significant changes in DA in cell body areas were noted; however, brainstem and cerebellar DA was consistently depleted by 2ME exposure while cerebral DA was increased. This pattern of results, which is analogous to those seen by Pickel *et al.* [40], is also consistent with the hypothesis that 2ME caused a brainstem lesion, though it is also possible that complex metabolic changes in the activity of DA neurons might have caused these effects.

This study extends the dose-effect curve for 2ME. As is typical in teratology, the curve is quite steep, with no fetuses surviving maternal exposure to 200 ppm, frank malformations at 100 ppm, but effects barely detectable at 25 ppm. As frequently occurs in behavioral teratology, functional alterations were evident at concentrations lower than those required to produce terata.

Based upon the previously reported negative effects at 50 ppm 2ME [14], (6 hr exposure in Fischer 344 rats) and the positive effects at 50 ppm 2ME [36] (7 hr exposure in Sprague-Dawley rats), it is likely that 50 ppm is near the threshold for producing malformations in rats. The paucity of behavioral effects at 25 ppm 2ME reported in this study, in spite of the neurochemical effects at that concentration, suggests that 25 ppm 2ME is or may be near the threshold for producing effects in our behavioral test system. This is the lowest concentration reported to cause significant effects in experimental animals and may lend support to the ACGIH recommendation to reduce the Threshold Limit Value for 2ME from 25 to 5 ppm.

The present study does not elucidate the mechanism by which 2ME induced the effects in either maternal or paternal animals. Nor is it known if 2ME or a biotransformation product is the active moiety. Methoxyacetic acid, a primary urinary metabolite of 2ME [31], was recently reported to produce testicular and other effects very similar to those of 2ME [30]. Thus it is possible that biotransformation products could be responsible for the adverse effects. Because of its (likely) similar biotransformation, it is probable that a comparable molar equivalent of 2-methoxyethyl acetate would produce teratogenic effects very similar to those from 2ME.

This is supported by the fact that similar testicular effects were reported for the two chemicals [30], and by inference from observations with 2-ethoxyethanol and 2-ethoxyethyl acetate in which equivalent teratogenic effects were observed [36]. Further, by inference from 2-ethoxyethanol data, it is likely that 2ME would also be teratogenic when given by cutaneous exposure [15]. Though it is impossible to be certain with only two glycol ethers tested, and with differences in the concentration at which these two were tested, it does appear that there is an inverse relationship between chain length and the concentration of glycol ether required to produce behavioral teratogenic effects. That is, 100 ppm 2-ethoxyethanol produced behavioral teratogenic effects [35], but only 25 ppm 2ME produced such effects. This observation is consistent with the differences in concentrations of these two chemicals required to produce gross teratogenic effects [36].

In summary, this study has reported that maternal or paternal inhalation of 25 ppm 2ME has produced alterations in the behavioral and/or neurochemical development of rat offspring. Whether or not such effects may occur in humans awaits further research.

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