Neurotoxicology and Teratology, Vol. 11, pp. 273–284. Pergamon Press plc, 1989. Printed in the U.S.A. 0892-0362/89 $3.00 + .00

Effects of 2-Methoxyethanol on Fetal Development, Postnatal Behavior, and Embryonic Intracellular pH of Rats

B. K. NELSON,*[1] C. V. VORHEES,† W. J. SCOTT, JR.† AND L. HASTINGS*

**Department of Environmental Health, University of Cincinnati Medical School, Cincinnati, OH 45267*
†Institute for Developmental Research, Children's Hospital Research Foundation
Elland and Bethesda Avenues, Cincinnati, OH 45229

Received 20 April 1988

NELSON, B. K., C. V. VORHEES, W. J. SCOTT, JR. AND L. HASTINGS. *Effects of 2-methoxyethanol on fetal development, postnatal behavior, and embryonic intracellular pH of rats.* NEUROTOXICOL TERATOL **11**(3) 273–284, 1989.—The industrial solvent 2-methoxyethanol (2ME) is a reproductive and developmental toxicant when administered by inhalation, gavage, and IP injection. The present research established that this solvent can produce teratogenicity in rats when administered in liquid diet. Groups of 10 Sprague-Dawley rats were given various percentages of 2ME in liquid diet on gestation days 7–18. Day 20 fetuses were examined for visceral or skeletal malformations. Concentrations above 0.025% 2ME (approximately 73 mg/kg/day) produced total embryomortality. Cardiovascular malformations were produced at lower levels. The teratogenic no-effect level was 0.006% 2ME (16 mg/kg). In a second experiment, groups of 12 Sprague-Dawley rats were given 0, 0.006 and 0.012% of 2ME as above. Litters were culled to 8 pups, and tested for auditory and tactile startle and conditioned lick suppression, and for performance in figure-8 activity and the Cincinnati water maze on postnatal days 48–65. The high dose of 2ME produced approximately 50% mortality in the offspring and increased the number of errors in the Cincinnati maze. No other behavioral effects were observed at either dose. An interaction study was conducted to determine if simultaneous exposure to 2ME and ethanol would reduce the teratogenicity of 2ME, but no reduction was observed. The hypothesis that 2ME acts by altering embryonic intracellular pH was tested by injecting 0.33 ml/kg of 2ME into rats on gestation day 13, and determining embryonic intracellular pH at 2, 4, 8, and 24 hours thereafter. There was an increase in pH at 4 hours, but not at later time points. Another group of rats was given 2ME along with amiloride, which blocks the sodium/hydrogen antiporter. The combined 2ME-amiloride exposure produced an incidence of cardiovascular malformations in fetuses twice that of 2ME alone. These studies confirmed the structural teratogenicity of 2ME even when given in liquid diet, as it was given for the first time in the present study. At nonteratogenic doses, developmental toxicity (e.g., postnatal deaths) persisted, but only limited evidence of behavioral teratogenicity was observed. The pH data are consistent with the concept that 2ME may alter embryonic intracellular pH at critical stages of organogenesis.

2-Methoxyethanol Glycol ethers Teratology Behavioral teratology Intracellular pH

ETHYLENE GLYCOL monomethyl ether (2-methoxyethanol, 2ME; CAS No. 109-86-4) is one of a class of alkyl ethers of ethylene glycol which are widely used as industrial solvents. It has been used in numerous surface coatings and removers, as an anti-icing additive in aviation fuels, and in some consumer products such as nail polish and its remover.

The general toxicity of 2ME has been reviewed (44). It is relatively low in acute toxicity, having an LD_{50} in rodents of approximately 3 g/kg and an LC_{50} of approximately 1500 ppm for a 7-hr exposure. Until recently, the guidelines for human exposure included the Permissible Exposure Limit [29 CFR 1910.1000] of the Occupational Safety and Health Administration (OSHA) and the Threshold Limit Value (51) of the American Conference of Governmental Industrial Hygienists (ACGIH) of 25 ppm (80 mg/m^3). Based upon more recent reports of reproductive toxicity in animals, the ACGIH recommended lowering the TLV from 25 to 5 ppm in 1982, and the change was adopted in 1984. In 1983, the National Institute for Occupational Safety and Health (NIOSH) recommended that 2ME be regarded as a reproductive toxicant in the workplace for both males and females (30). Concurrently, many manufacturers adopted exposure maxima below 10 ppm (14,30).

Reproductive and Developmental Toxicology

Male reproductive effects were reported for 2ME as early as 1938 (60), but were largely overlooked until the late 1970's when testicular effects (e.g., histopathology, biochemical alterations,

[1]Requests for reprints should be addressed to B. K. Nelson, Division of Biomedical and Behavioral Science, National Institute for Occupational Safety and Health, 4676 Columbia Parkway, Cincinnati, OH 45226.

infertility) were reported for 2ME and other glycol ethers (27). Shortly thereafter, reports of teratogenic effects (e.g., skeletal and cardiovascular malformations) induced by 2-ethoxyethanol (2EE) appeared (1,37). Subsequent studies reported the teratogenicity of 2ME and other glycol ethers following administration by gavage to mice (28,29), and by inhalation to rats (33, 38, 39). Teratogenesis of 2ME has been reported for rats, mice, and rabbits (12,13), as have studies of gestational stage, dose-response, and target organ characteristics (18, 49, 52–54). The route of exposure to 2ME does not appear to be important, as cardiovascular and skeletal malformations have been reported following most common routes, including inhalation (12, 13, 39), IP injection (7,42), and gavage (18,29). It is likely that 2ME is teratogenic when absorbed through the skin, as 2EE and 2-ethoxyethyl acetate are teratogenic after either inhalation or cutaneous exposure (15, 16, 39). Recently, Scott *et al.* (47) found that monkeys (*Macaca fascicularis*) gavaged with 0, 12, 24, or 36 mg/kg 2ME from gestation days 20–45 had dose-related increases in embryonic death, but few malformations. (By comparison, workers exposed at the current OSHA standard of 25 ppm would experience a daily dose of 5–10 mg/kg.)

Behavioral Teratology

It has previously been shown that at nonmalforming doses, 2EE is behaviorally teratogenic to rats (37). Because of its structural similarity to 2EE, 2ME was hypothesized to produce similar effects, and tested in a behavioral teratology investigation administered by inhalation (33). Groups of 15 pregnant Sprague-Dawley rats were exposed to 25 ppm 2ME for 7 hours/day on gestation days 7–13 or 14–20. Offspring were tested from days 10–90 on a series of behavioral tasks and neurochemical tests that had identified 2EE-induced differences from controls. No significant differences from controls were detected on the behavioral measures, but several neurochemical differences were noted after prenatal exposure to 2ME (greater concentrations of brainstem acetylcholine, norepinephrine, and 5-hydroxytryptamine, and cerebral dopamine; less brainstem and cerebellar dopamine). Although functional changes were expected to be produced by 2ME, the dose may have been insufficient. Alternatively, it is possible that the particular tests used in this experiment did not assess these functions that are affected by 2ME exposure.

Biotransformation

As with ethanol and other glycol ethers (8, 17, 20, 23), 2ME is metabolized via alcohol and aldehyde dehydrogenase to methoxyacetic acid (MAA). MAA administered to male rats produced testicular effects comparable to those of 2ME, leading to speculation that MAA may be the proximate toxin (25). That MAA is also the likely proximate teratogen is indicated from in vitro research in which MAA but not 2ME produced malformations (48,62), thus suggesting that metabolism of 2ME is necessary to produce malformations. In addition, MAA produced teratogenic effects similar to those of 2ME (cardiovascular and skeletal malformations) in rats (5,42) and mice (49). Coadministration of the alcohol dehydrogenase inhibitor 4-methylpyrazole reduced the embryotoxicity of 2ME from 100% to 17% (42). Similar results have been reported in mice using coadministration of ethanol as a competitor for alcohol dehydrogenase (49,50). It should be noted that 2ME is a much more potent teratogen than is ethanol, as at least 20 times more ethanol is required to produce defects than is the case with 2ME. Extending the concern for potential malformations produced by 2ME is the observation that some phthalate esters undergo biotransformation to yield 2ME or MAA (2, 7, 40).

The study with ethanol (49,50) is interesting in light of two other observations. First, there is evidence that ethanol affects the behavioral teratogenicity produced by 2EE (34–36). Ethanol was administered in drinking water to rats concomitantly exposed via inhalation to 100 ppm 2EE on gestation days 7–13 or 14–20. Testing was essentially identical to that of the previous behavioral teratology study of 2ME (33). Compared with 2EE administration alone, addition of ethanol resulted in fewer behavioral and neurochemical effects when administered early in gestation, but it resulted in more effects when administered later in gestation.

Second, IP coadministration of ethanol and 2ME significantly reduces the rate of excretion of 2ME in nonpregnant animals (43). Blood levels of 2ME remained nearly constant as long as ethanol was also in the blood. The complementary effect, reduction in the rate of ethanol excretion, was only slightly affected after 2ME administraton (43).

Potential Mechanism of Action

The majority of proven human teratogens are weak acids and none are weak bases, thus suggesting that acidity may be important in determining teratogenic potency. The intracellular pH (pHi) of early mouse and rat embryos is higher than that of maternal plasma (31). It was shown (31,46) that acids accumulated in the embryo, reaching a greater concentration than in maternal plasma—in some cases over twice as much. Amide forms of the two acids initially examined did not accumulate in the embryo and were much less potent teratogens than the acid forms (31). The authors hypothesized that some human teratogens may cause developmental abnormalities because they are weak acids and thus accumulate in the embryo and exert an unspecified effect. Conversely, weak bases are not teratogenic because they are largely excluded from the embryo during critical phases of organogenesis.

A corollary of this hypothesis is that a mechanism of action for some teratogens may be the alteration of embryonic pH at critical stages of development (46). After fertilization, there are rapid and dramatic pH changes in the zygote (6). It was stated: "Clearly many (perhaps all?) pronounced metabolic activations or inactivations involve modest-to-large pHi changes . . . and such changes have been convincingly shown to play a regulatory role" [(6) pp. R432]. Since the rate of cellular proliferation decreases dramatically during embryogenesis, Scott and collegues hypothesized—and found—that pHi followed the same course. That is, higher pH was associated with higher proliferative rates during development. The pHi of day 11 rat embryos was 7.61 and decreased to 7.07 by day 14, compared with maternal blood pH of 7.46 (46). Maintenance of the appropriate intracellular pH is known to be at least partially under the control of a growth-factor-regulated Na+/H+ antiporter. Antiporter activation is associated with cellular proliferation (26), although a causative role is not yet established. Thus, teratogens may alter the normal changes in pHi, possibly through changing the activity of the antiporter, during critical stages of development.

The purpose of the present research was to investigate the teratogenic and behavioral teratogenic effects of 2ME alone and in combination with ethanol when administered in liquid diet [this route of exposure was selected since it is frequently used to administer ethanol and has several advantages (59)], as well as to determine 2ME's effect on embryonic intracellular pH (pHi). Current evidence supports MAA as the proximate teratogen of 2ME [in contrast to ethanol, where the parent compound has been implicated (3,4)]. Since MAA is acidic (pKa estimated to be 3.5), pHi changes were determined after 2ME administration. The role of the Na+/H+ antiporter in mediating effects on pHi involved coadministration of 2ME along with the diuretic amiloride, which

is known to block the Na+/H+ antiporter. Amiloride exacerbates the teratogenicity of acetazolamide (24) and valproic acid (46). We hypothesized that 2ME's structural and behavioral teratogenicity would be reduced by coadministration of ethanol. A second hypothesis was that administration of 2ME would reduce embryonic pHi.

METHOD

The subjects were female Sprague-Dawley rats (VAF plus, Charles River Breeding Laboratories, Inc., Portage, MI) weighing between 200 and 300 g and mated to males of the same stock and supplier (presence of sperm plug was designated day 0). Females were housed individually in 18 × 24 × 18 cm hanging metal cages with ad lib access to water and Purina Lab Chow except on gestation days 7–18, when their only nutrition source was a liquid diet commonly used for administration of ethanol (41,56); the diet was administered in calibrated 100 ml glass bottles with double ball bearings designed to minimize evaporation and spillage (Lab Products Inc., Maywood, NJ). The diet consisted of 355 ml Sustacal, 40 ml distilled water (except as reduced for addition of 2ME), 48.4 g sucrose, 2.13 g mineral salts, and 1.3 g vitamin mixture, with fresh diet (mixed every 2–3 days and refrigerated) given daily at 1500 hr. The various concentrations of 2ME diet were made by replacing 1 ml of distilled water with 1 ml of 2ME for the 0.25% diet and as appropriate for the other percentages. Consumption was recorded daily from days 7–18, and maternal weights were recorded on days 0, 7, 14, and 20. The room temperature was maintained at 22 ± 2°C, 50 ± 10% relative humidity, on a 0700/1900 hr light/dark cycle.

Morphological Examinations

Groups of approximately 10 rats were assigned sequentially to the groups described below. On day 20, the rats were sacrificed by CO_2 overdose. Fetuses were removed, blotted of excess fluids, weighed, examined for external malformations, and sexed. Two-thirds were fixed in Bouin's solution and subsequently sliced and examined for internal malformations using the Wilson technique (61). The remaining one-third were placed in 95% ethanol and prepared using a modified [(21), Fradkin, personal communication] double staining technique (19) for skeletal examinations.

2ME. Investigation of the teratogenicity of 2ME when administered in a liquid diet began at 0.5% 2ME, based upon research in which 2ME was administered to mice in drinking water (10,11). Doses were systematically decreased (0.25, 0.1, 0.05, 0.025, 0.012, and 0.006% 2ME) until a no-observable effect level was found (i.e., no malformations were detected).

MAA. To verify the teratogenicity of molar equivalents of 2ME and MAA, rats were given 0.03 and 0.014% MAA in the diet.

2ME plus ethanol. Based upon the dose-finding results with 2ME, two levels (0.05 and 0.025% 2ME) were selected to investigate the possible interactive effects of 2ME and ethanol. The diet was prepared as described, except distilled water and sucrose were replaced with ethanol to provide 35% ethanol-derived-calories (EDC). In order to provide an equivalent basis for direct comparison of 2ME alone and 2ME + ethanol, a third concentration was selected. For this group, 0.035% 2ME + 35% EDC was administered to one group of animals, and another group was given 0.035% 2ME and pair-fed to the 2ME + ethanol group.

2ME plus amiloride. Another group of animals was given amiloride along with 0.035% 2ME. As we had previously observed significant diet rejection when amiloride was added to the liquid diet, the 0.035% 2ME group was pair-fed to this group. For these animals, 2.75 mg amiloride chloride was added per 100 ml diet, which was predicted to provide 4.0 mg/kg/day. This dose, when given SC, has been shown to be effective in increasing the teratogenicity of other acidic compounds (46). As there were no data in which a comparable dose of amiloride was administered to pregnant rats, a group of 5 animals was included in which amiloride alone was added to the liquid diet, and these were pair-fed to those receiving amiloride + 0.035% 2ME.

Behavioral Procedures

Additional groups of 10–12 pregnant Sprague-Dawley rats were administered 0.014, 0.012, 0.006, or 0.000% 2ME in liquid diet from gestation days 7–18 (the control group was weight-matched and pair-fed to the 0.014% 2ME group). Upon delivery (day 0), the pups were weighed and randomly culled to 8 (with a goal of 4 of each sex). Offspring were weighed at weekly intervals. They were weaned at day 25, and randomly assigned to test groups on day 42, with one female and one male per litter assigned to each test listed below.

Figure-8 activity. Measurement of figure-8 activity involved testing on days 48–50 (± 1) for 60 min/day for 3 successive days. The data were analyzed in blocks of 5 min, both with and without transformation of the data.

Cincinnati maze. The Cincinnati maze, a modification of Biel's original design, is used to detect changes in complex problem solving after early brain insult (55). The apparatus consisted of a 150 cm straight alley for initial testing of swimming ability, and a 9-unit asymmetrical multiple T-maze filled with water maintained at room temperature (23 ± 1°C). Beginning at approximately day 50, rats received 4 trials in the straight alley on the first day, 2 trials per day for 3 days in the maze in path A (forward), followed by 2 trials per day for 3 days in the maze in path B (reverse) (55).

Startle response. Rats were tested at 63 ± 1 days of age in a facilitated (background noise) acoustic startle test, and, on the next day, for tactile startle (San Diego Instruments, SR apparatus). The acoustic signal was 115 dB and the tactile stimulus was a 12-psi air puff presented against a fixed background of 70 dB. Each session consisted of 100 trials, with stimulus presentation for 20 msec and an intertrial interval of 8 sec. Responses were recorded as maximum voltage (V_{max}) and mean voltage (V_{mean}) outputs and latency to the maximum response (T_{max}).

Conditioned lick suppression. The test was modified from (45) and utilized a standard operant chamber into which a sipper tube was inserted, with the tube attached to a drinkometer circuit. Water-deprived (24 hr) rats at 63 ± 1 days of age were allowed to drink freely, with the number of licks on the sipper tube monitored. The following day, in the absence of the sipper tube, a tone sounded and a brief electric shock (1 sec, 1 mA) was delivered through the floor grid. On the third day, the time it took the animals to lick 100 times was recorded; this was followed by tone presentation (no shock), and time to resume drinking was recorded as a measure of response suppression.

Intracellular pH

Intracellular pH (pHi) was determined in whole-animal preparations of rat embryos using the distribution of a weak acid as described (31,46) and by Duggan *et al.* (in preparation). Approximately 6 pregnant rats/group were administered 330 μl/kg of 2ME on the morning of gestation day 13 (this day was selected because of the ease in handling the embryos at this age). A group of pooled controls was used for 2-, 4-, and 8-hr comparisons, and a separate group was used for the 24-hr comparisons. At 2, 4, 8, and 24 hr postinjection, they were injected IP with 10 μCi/kg of

TABLE 1

EFFECT OF 2-METHOXYETHANOL, METHOXYACETIC ACID, 2-METHOXYETHANOL PLUS ETHANOL, 2-METHOXYETHANOL PLUS AMILORIDE, OR AMILORIDE ADMINISTERED IN LIQUID DIET TO RATS

% in Diet	No. Pregnant/ No. Bred	No. Resorbed/ No. Pregnant	Mean Weight Gain (g)	Mean (ml) Diet Consumed	Estimated 2ME Dose (mg/kg/day)
2-Methoxyethanol					
0.5	9/9	9/9	(−)25*	34	620
0.25	9/9	9/9	16*	36	290
0.1	11/12	11/11	45*	60	198
0.05	10/10	10/10	97*	83	140
0.025	9/10	4/9	137	89	73
0.012	11/12	0/11	156*	91	31
0.006	9/9	0/9	133	85	16
0.000	10/11	0/10	122	85	0
Methoxyacetic acid					
0.03	4/4	4/4	65*	63	79
0.014	8/9	0/8	121	73	39
2-Methoxyethanol plus ethanol					
0.05	10/10	7/10	77*	53	81
0.025	8/10	0/8	98*	48	44
2-Methoxyethanol plus amiloride					
0.025	3/3	0/3	49	55	41
Pair-feeding Study					
0.035% 2ME plus ethanol	7/9	0/7	81	44	59
0.035% 2ME†	9/9	0/9	77	44	59
0.035% 2ME plus amiloride‡	9/9	0/9	58	44	54
amiloride only§	5/5	0/5	32	43	—

*Significantly different from controls at $p<0.05$.
†Pair-fed to the group given 0.035% 2ME plus ethanol.
‡Pair-fed to the group given 0.035% 2ME.
§Pair-fed to the group given 0.035% 2ME plus amiloride.

^{14}C-labelled 5,5-dimethyloxazolidine-2,4-dione (DMO) with a specific activity of 50 mCi/mM. After 1 hr for transplacental equilibration, the pregnant animal was anesthetized with ether. Five embryos with yolk sacs intact were removed from the uterus, dissected free of their membranes, and blotted dry; each embryo was placed in a scintillation vial. A pooled sample of approximately 0.1 g exocoelomic fluid was placed into a 6th vial. Yolk sacs from 3 embryos were placed into a 7th vial. A maternal aortic blood sample (3–5 ml) was taken for determination of pH, using a Corning pH/Blood Gas Analyzer (model 165). After centrifugation, two samples of maternal plasma from each dam weighing approximately 25 mg were placed into additional vials. Finally, a sample weighing approximately 50 mg was collected from the maternal thigh muscle. To each vial was added 400 μl of distilled water. Samples were vortexed to disperse the tissues (muscle samples were also sonicated). Biofluor was added to the vials, bringing the volume to approximately 20 ml, and this mixture was counted in a Beckman scintillation counter, after remaining in the dark for at least 24 hr.

From a weighed plasma aliquot, the DMO concentration was determined as dpm of ^{14}C DMO/μg of protein-free plasma. The dpm of ^{14}C labelled DMO/μg wet weight was determined for all other tissue samples. Determination of pHi was made using a formula derived from the Henderson-Hasselbalch equation (57). This calculation requires information about the intracellular water content, which was not measured. Consistent with assumptions by others [e.g., (46)], values of 30% for intracellular water and 60% extracellular water, were used in the present studies.

Statistical Analyses

Data were analyzed using parametric methods with the litter as the N, and with a probability level of ≤0.05 required for significance. Post hoc comparison utilized Duncan's Multiple Range Test. When repeated measures analysis of variance was used and significant interaction terms were present, the Geisser-Greenhouse F-ratio was used as a correction.

RESULTS

Table 1 summarizes the effects of various percentages of test chemicals administered in liquid diet on pregnancies carried to term, resorptions, weight gain, diet consumption, and dose. For litters which survived to term, Table 2 presents the percentage of conceptuses resorbed, mean survivor weights, and percentage of survivors malformed. Table 3 shows the number of litters (fetuses) examined for malformations along with the number of malformations observed.

Morphological Observations

2ME. The highest level of 2ME (0.5%) was toxic to the dams and produced 100% resorption of all litters (Table 1). Initially, the mean body weights were 248 ± 21 g (mean ± SD) with day 7

TABLE 2

EMBRYOTOXIC EFFECTS OF 2-METHOXYETHANOL, METHOXYACETIC ACID, 2-METHOXYETHANOL PLUS ETHANOL, 2-METHOXYETHANOL PLUS AMILORIDE, OR AMILORIDE ADMINISTERED IN LIQUID DIET TO RATS

Dose	No. Implants (No. Litters)	% Dead/ Resorbed	Mean Survivor Weights (g;F,M)	% Survivors Malformed
2-Methoxyethanol				
0.025	136 (9)	92	2.5*; 2.3*	40
0.012	156 (9)	14	2.7*; 2.8*	4
0.006	135 (9)	7	2.8*; 2.9*	0
0.000	131 (10)	11	3.3; 3.3	0
Methoxyacetic acid				
0.014	109 (8)	58	2.1*; 2.4*	15
2-Methoxyethanol plus ethanol				
0.05	133 (10)	89	1.9*; 2.2*	25
0.025	124 (9)	8	2.3*; 2.4*	1
2-Methoxyethanol plus amiloride				
0.025	46 (3)	35	1.6*; 1.7*	10
Pair-feeding study				
0.035% 2ME plus ethanol	100 (7)	15	2.1*; 2.3*	14
0.035% 2ME†	111 (9)	31	2.2*; 2.3*	9
0.035% 2ME plus amiloride‡	115 (9)	23	2.0*; 2.1*	19
amiloride only§	65 (5)	8	2.7*; 2.9*	0

*Significantly different from controls at $p<0.05$.
†Pair-fed to the group given 0.035% 2ME plus ethanol.
‡Pair-fed to the group given 0.035% 2ME.
§Pair-fed to the group given 0.035% 2ME plus amiloride.

weights averaging 277 ± 24 g. However, the day 14 and day 20 weights were reduced: 271 ± 21 and 223 ± 20 g, respectively. There was a mean of 16.3 resorptions per litter. Dams in this group exhibited symptoms of diarrhea, respiratory difficulties, eye and nose exudate, hair loss, lack of grooming, and malaise. One female died on day 19. Consumption of diet was only 40% of that of controls, for a mean consumption of 170 mg 2ME/rat/day. Using a weight of 274 g (the average of the day 7 and day 14 means), this represented a dose of 620 mg/kg/day of 2ME.

The 0.25% level of 2ME was not as toxic to the dams, although malaise and ungroomed appearance were noted; one dam died. This dose produced resorption of all litters (Table 1). Body weights for days 0, 7, 14, and 20 were 294 ± 26, 316 ± 27, 304 ± 29, and 310 ± 28 g, respectively. The estimated dose of 2ME was 290 mg/kg/day.

The 0.1% level of 2ME did not produce overt maternal toxicity, but all litters were resorbed (Table 1). Body weights were 271 ± 20, 294 ± 22, 312 ± 25, and 317 ± 16 g, for days 0, 7, 14, and 20, respectively. The estimated dose of 2ME was 198 mg/kg/day. Similarly, all litters were resorbed at 0.05% 2ME, and no maternal toxicity was observed. Body weights were 242 ± 11, 278 ± 14, 322 ± 17, and 340 ± 19 g for days 0, 7, 14, and 20 respectively. Consumption of diet was similar to that of controls, and provided a dose of 140 mg/kg/day.

At 0.025% 2ME, 4 of 9 litters were totally resorbed (Table 1). Of the remaining 5 litters, only 10 fetuses survived (Tables 2 and 3). Maternal body weights were 243 ± 11, 275 ± 18, 328 ± 19, and 381 ± 22 g for days 0, 7, 14, and 20, respectively. The estimated dose of 2ME was 73 mg/kg/day. No external malformations were observed in the 10 surviving fetuses. Fetal weights averaged 2.5 and 2.3 g for females and males, respectively. No malformations were detected in the three fetuses examined for skeletal defects. Four of the seven fetuses examined for soft-tissue malformations were abnormal: two (in different litters) had esophogeal and tracheal stenosis, misplaced ductus arteriosus, and double and/or misplaced aortic arch; one had a ventricular-septal defect. Two other fetuses in the same litters as above had double and/or misplaced aortic arches.

At 0.012% 2ME, a shift occurred. No litters were totally resorbed, and the incidence of resorptions was not increased significantly from controls (Table 2). Maternal body weights were 297 ± 19, 322 ± 18, 381 ± 25, and 453 ± 31 g on days 0, 7, 14, and 20, respectively. This gain was significantly larger than for any other group. Dietary consumption provided a dose of approximately 31 mg/kg/day of 2ME. No external malformations were apparent, but fetal weights were significantly lower (Table 2) than in controls, $F(2,25)=21.39$, $p<0.001$, for females and, $F(2,25)=11.17$, $p<0.001$, for males: 2.7 and 2.8 g for females and males, respectively. One litter had two pups (Table 3) with double/misplaced aortic arches, and a low frequency of wavy, rudimentary, and fused ribs.

At 0.006% 2ME, no adverse effects were observed in the dams, and no malformations were seen among the fetuses (Table 3). Fetal weights were still significantly less than controls (Table 2): 2.8 and 2.9 g, for females and males, respectively. Maternal weights were 275 ± 15, 305 ± 16, 346 ± 20, and 409 ± 30 g for days 0, 7, 14, and 20, respectively. Diet provided a dose of 16 mg of 2ME/kg/day. Control maternal weights were 265 ± 29, 287 ± 36, 327 ± 36, and 387 ± 47 g, respectively.

MAA. At a dose of 0.03% of MAA, maternal body weights were 208 ± 13, 244 ± 14, 263 ± 15, and 272 ± 20 g on days 0, 7, 14, and 20, respectively. Consumption provided a dose of 79

TABLE 3

SUMMARY OF DEFECTS OBSERVED AFTER EXPOSURE TO 2-METHOXYETHANOL, METHOXYACETIC ACID, 2-METHOXYETHANOL PLUS ETHANOL, 2-METHOXYETHANOL PLUS AMILORIDE, OR AMILORIDE TO RATS IN LIQUID DIET

% in Diet (Approx. mg/kg)	Litters (fetuses) Examined			Malformations Observed	
	Total	Visceral	Skeletal	Visceral*	Skeletal†
2-Methoxyethanol					
0.025 (73)	4(10)	4(7)	2(3)	2(4)	0
0.012 (31)	9(145)	9(96)	9(49)	1(2)	3(4)
0.006 (16)	9(126)	9(84)	9(42)	0	0
0.000	10(118)	10(77)	10(41)	0	0
Methoxyacetic acid					
0.014 (39)	8(46)	8(32)	6(14)	3(6)	1(1)
2-Methoxyethanol plus ethanol					
0.05 (81)	3(12)	3(9)	1(3)	2(3)	0
2-Methoxyethanol plus amiloride					
0.025 (41)	3(30)	3(22)	2(8)	2(3)	0
Pair-feeding study					
0.035% 2ME plus ethanol (59)	7(85)	7(56)	7(29)	3(4)	2(2)
0.035% 2ME‡ (59)	9(79)	9(54)	7(25)	4(5)	0
0.035% 2ME plus amiloride§	9(54)	9(87)	9(29)	5(11)	0
amiloride¶	5(61)	5(41)	5(20)	0	0

*Most defects were double and/or misplaced aortic arches ("ringed aorta") and/or ventricular septal defects, but cases of esophogeal or tracheal stenosis were also observed.

†Skeletal defects included fused ribs, rudimentary 14th ribs, missing vertebrae.

‡Pair-fed to the group given 0.035% 2ME plus ethanol.

§Pair-fed to the group given 0.035% 2ME.

¶Pair-fed to the group given 0.035% 2ME plus amiloride.

mg/kg/day, and all litters (4) were resorbed (Table 1).

At 0.014% MAA, maternal weights were 217 ± 18, 247 ± 21, 285 ± 30, and 338 ± 36 g on days 0, 7, 14, and 20, respectively. This dose (39 mg/kg/day) produced death or resorption of 58% of the fetuses (Table 1), and 5/8 of the litters had at least one fetus with cardiovascular defects typical of 2ME (Table 3; primarily double/misplaced aortic arch). Another litter had a fetus with fused ribs.

2ME plus ethanol. The addition of ethanol significantly reduced diet consumption, with intake approximately one-half that of 2ME alone (Table 1). Thus, on a mg/kg basis, intake of 0.05% 2ME together with ethanol provided a similar dose of 2ME as the 0.025% 2ME alone (81 vs. 73 mg/kg/day, respectively). With 0.025% 2ME combined with ethanol, the resulting intake of 2ME was not much greater than 0.012% 2ME when given alone (44 mg/kg vs. 31 mg/kg/day). Hence another dose level as administered in which the 2ME intake was determined by the intake of the same level of 2ME (0.035%) + ethanol (35% EDC). By design, then, these two diets provided identical exposure to 2ME.

As only the 0.035% 2ME and the 0.035% 2ME + ethanol groups had comparable exposure to 2ME (both providing an estimated dose of 59 mg/kg/day of 2ME; the mean consumption was approximately 4.6 ml ethanol, or 16,000 mg/kg/day), only their results are presented here. Maternal body weights for the 2ME group were 238 ± 14, 262 ± 14, 261 ± 19, and 315 ± 16 g for days 0, 7, 14, and 20, respectively. For the 2ME + ethanol group, maternal weights were 246 ± 30, 280 ± 26, 279 ± 28, and 327 ± 42 g for days 0, 7, 14, and 20, respectively. Resorptions were increased after 2ME alone (31%) and slightly after 2ME + ethanol (15%). No external malformations were observed in either group, but fetal weights were depressed both for females (2.1, 2.2 g) and males (2.3, 2.3 g) for 2ME and for 2ME + ethanol. The incidence of malformations (Table 3) was similar between groups, with 4/9 litters affected (44%) by 2ME and 3/7 litters affected (43%) by 2ME + ethanol. In the 2ME group, 5 of 54 (9%) fetuses examined viscerally had cardiovascular malformations (misplaced aortic arches and/or ventricular septal defects); no skeletal malformations were detected. In the 2ME + ethanol group, 4 of 56 fetuses (7%) examined for visceral malformations had cardiovascular defects, and 2 of 29 (7%) of those examined for skeletal malformations had defects (missing sacral vertebrae and/or tail).

2ME plus amiloride. No increase in resorptions or defects was noted in the animals given amiloride (Table 1), although fetal weights were slightly decreased from controls (Table 2). The maternal body weights for days 0, 7, 14, and 20 were 275 ± 27, 313 ± 27, 271 ± 11, and 307 ± 14 g, respectively. Diet provided an estimated dose of 4.1 mg/kg/day of amiloride. The group given 2ME + amiloride had intake providing a dose of 54 mg/kg/day of 2ME and 4.2 mg/kg/day of amiloride. Maternal body weights were 265 ± 14, 288 ± 14, 279 ± 18, and 323 ± 22 g. There was an increase in resorptions (23%), and a reduction in fetal weights (2.0 and 2.1 g for females and males, respectively); by comparison, there were 11% resorptions and fetal weights of 3.3 g for females and males in controls. In the group given 2ME + amiloride, 5 of 9 litters (56%) had affected fetuses, within 19% of the fetuses having cardiovascular defects (primarily septal defects). None of the 29 fetuses examined for skeletal defects had malformations.

Postnatal Observations

Each of four groups (0.014, 0.012, 0.006, and 0.000% 2ME) was assigned 12 sperm-positive females. There were no signs of

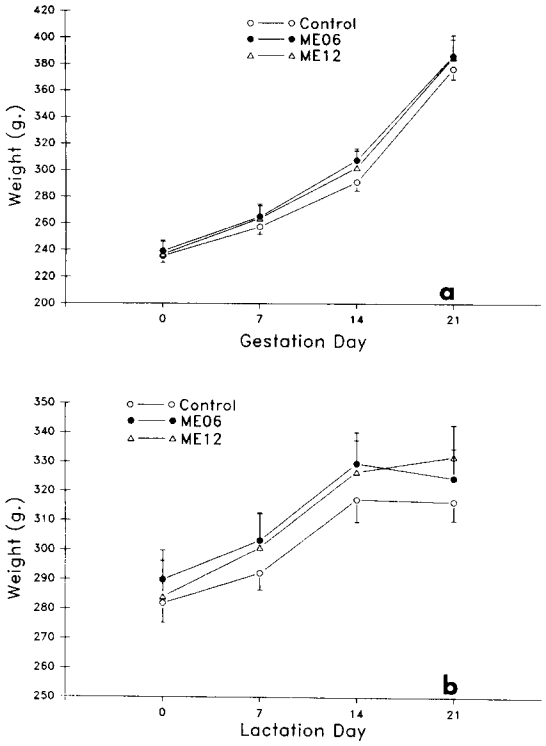

FIG. 1. (a) Maternal weight gain during gestation, with 2ME treatment on days 7–18. (b) Maternal weight gain during lactation following 2ME treatment on gestation days 7–18 (means with standard error).

toxicity, although 1 dam died in the 0.014% 2ME group. No cause of death was ascertained, but the death did not appear to be treatment-related. Mean liquid consumption values for the groups were 74, 79, 78, and 80 ml/rat/day, yielding doses of approximately 40, 33, 17, and 0 mg/kg/day of 2ME. Using analysis of variance and covariance with repeated measures, no significant effects were noted on maternal weight gain during gestation, $F(2,26)=0.36$, $p=0.70$, or lactation, $F(2,26)=0.43$, $p=0.65$; Fig. 1. As expected for gestational weight gain, the Days variable was significant, but the Group by Days interaction was not significant.

Observations at birth. No statistically significant effects were observed on the total number of pups born, $F(2,32)=0.05$, $p=0.95$, although the mean litter size from the highest dose group was lower than for the other groups (11 vs. 13 for each of the other groups). There was no effect on sex distribution, $F(1,3)=0.24$, $p=0.63$. Gestation length was significantly increased, $F(2,33)=15.65$, $p<0.001$, by 2ME treatment (Table 4), with the highest dose producing a delay of approximately one day. At birth, females were significantly lighter than males, $F(1,13)=13.34$, $p<0.001$. Birth weights were significantly reduced in the pair-fed controls (0.00%, Table 4) compared with the low dose of 2ME, $F(7,84)=5.38$, $p<0.001$; the 0.014% 2ME group was significantly lighter than the 0.006% group, but the Group by Sex interaction was not significant, $F(1,3)=0.22$, $p=0.88$.

Offspring weights. Preweaning weights (days 7, 14, and 21; Fig. 2) did not differ, either by treatment group, $F(2,52)=1.45$, $p=0.24$, or sex, and the Group by Sex interaction was nonsignificant. Postweaning weights (days 28, 35, 42, 49, 56, and 63; Fig. 2) differed by sex, but not by Group, $F(2,51)=2.86$, $p=0.07$; the Group by Sex interaction was not significant. The Days variable was significant, but the Day by Group interaction was nonsignificant. The Day by Sex interaction was significant, and the Day by

TABLE 4

GESTATION LENGTH, NEWBORN WEIGHTS, AND POSTNATAL DEATHS IN OFFSPRING OF ANIMALS EXPOSED TO 0.000, 0.006, 0.012, OR 0.014% 2ME

	0.00	0.006	0.012	0.014%
Number of litters	12	12	12	11
Day of delivery	21.9	22.4*	22.8*	22.9*
% Pups dead				
birth	2	1	7	14
d 1–25	9	8	51*	83*
d 26–63	0	0	3	—
Birth weights (g)	5.6	6.2	6.1	5.8
Litters tested	11	12	6	0

*Significantly different from controls at $p<0.05$.

Group by Sex interaction was significant, $F(10,255)=3.53$, $p=0.02$. Simple effect tests to determine what was responsible for the significance revealed that the only significant effect was on day 63, when the control females were lighter than the 0.006% 2ME females, $F(2,25)=4.32$, $p=0.024$; 180 vs. 201 g.

Mortality. Significant mortality was observed in both of the two higher 2ME groups (Table 4). Of pups dead on the day of birth, control and low level 2ME had low mortality compared with higher rates in the 0.012% and 0.014% groups. During the preweaning period (days 0–25), however, even larger differences

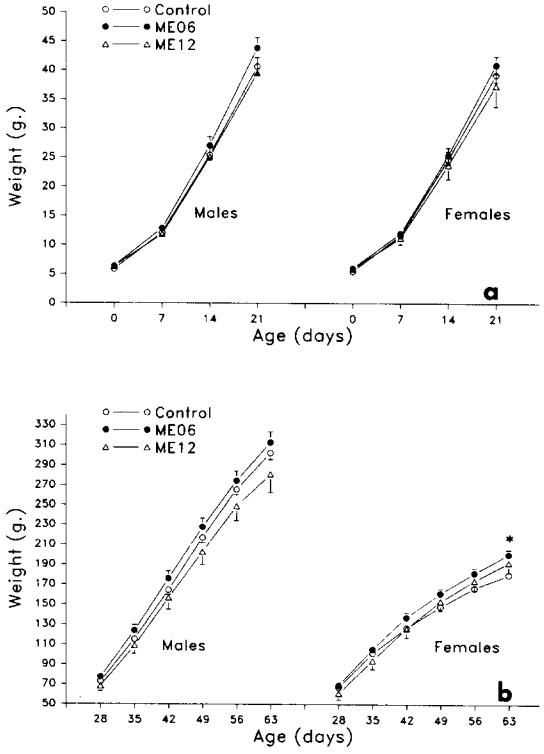

FIG. 2. (a) Mean (with standard error) offspring weight during the nursing period following maternal 2ME treatment on gestation days 7–18. (b) Offspring weight from the time of weaning (day 25) to termination of testing (means with standard error). *$p<0.05$ from control.

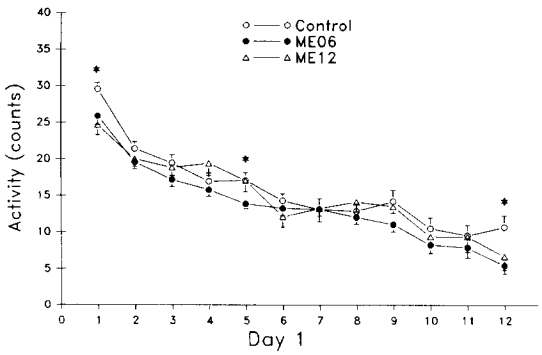

FIG. 3. Figure-8 activity on the first day of testing (day 48) of offspring following prenatal treatment with 2ME (nontransformed data collapsed by sex). *Indicates significantly different from control ($p<0.05$) in nontransformed data (significance disappeared in transformed data).

were apparent. From days 25–63, one of 38 animals in the 0.012% group died, whereas 0 of 84 in the control group and 0 of 88 in the lower dose group died. By day 7, all pups had died in 5 of the 12 litters at 0.012%, and 8 of the 12 litters at 0.014% 2ME. An additional litter in this group had too few pups to test. Only 13 pups survived to weaning at 0.014%, too few to test. One control litter had only 1 male pup, and was discarded. Thus, there were 11, 12, and 6 litters (control, 0.006%, and 0.012% 2ME, respectively) which were assessed behaviorally.

Figure-8 activity. Figure 3 shows the pattern of activity in the figure-8 test on the first day (other days were similar). The main effect of Group was nonsignificant, $F(2,45)=2.39$, $p=0.10$, as was the Group by Sex interaction. Sex and Days were significant, as was the Days by Sex interaction. Neither the Day by Group nor the Day by Group by Sex interaction was significant. The Blocks effect was significant, as was the Blocks by Sex interaction. Neither the Blocks by Group interaction nor the Blocks by Group by Sex interaction was significant. The Days by Blocks interaction was significant. The Days by Blocks by Group interaction was also significant, $F(44,990)=1.61$, $p=0.03$, as was the Days by Blocks by Sex interaction. However, the Days by Blocks by Group by Sex interaction was not significant, $F(44,990)=0.94$, $p=0.55$.

Concern over normality in these data led us to do a reciprocal transformation. When this was done, no significant Group main effect occurred, $F(2,45)=2.87$, $p=0.07$, and the interaction term involving Group also became nonsignificant, $F(44,990)=0.68$, $p=0.28$.

Cincinnati maze. There were no significant group differences, $F(2,47)=1.42$, $p=0.25$; Table 5, in mean swimming speed in the straight alley, but males had significantly faster times than females [45 vs. 64 sec; $F(1,47)=12.77$, $p<0.001$]; there was no significant Group by Sex interaction, $F(2,47)=0.98$, $p=0.38$. There were no differences in fastest trial time for Group, $F(2,47)=0.67$, $p=0.52$, Sex, or the Group by Sex interaction. Similarly, there were no last trial differences for Group, $F(2,47)=0.22$, $p=0.80$, Sex, or the interaction. In times to swim path A of the maze, no Group, $F(2,47)=1.57$, $p=0.22$, Sex, or Group by Sex interaction differences were apparent. The number of errors committed in path A did not differ by sex, but there was a significant treatment effect in which the 0.012% 2ME group committed more errors than the other groups, $F(2,47)=5.23$, $p=0.01$; the Group by Sex interaction was nonsignificant. In path B of the maze, the times in the maze did not differ by Group, $F(2,46)=0.15$, $p=0.87$, Sex, or the Group by Sex interaction. Errors in path B did not differ by Group, $F(2,46)=0.51$, $p=0.60$, Sex, or the interaction of Group and Sex. The total number of errors (errors in path A + B) also did not differ by Group, $F(2,46)=2.13$, $p=0.13$, Sex, or the interaction of Group and Sex.

TABLE 5

SUMMARY OF OBSERVATIONS FROM CINCINNATI MAZE ON OFFSPRING OF ANIMALS EXPOSED TO 0.000, 0.006, OR 0.012% 2ME

	0.000	0.006	0.012
Number of litters	11	12	6
Number of offspring	20	22	11
Speed (sum in sec)	49 ± 4	53 ± 5	61 ± 8
Errors, forward	58 ± 4	64 ± 3	81 ± 8*
reverse	56 ± 6	65 ± 6	58 ± 8
total	114 ± 8	129 ± 6	139 ± 11
time, forward	517 ± 52	523 ± 30	647 ± 89
reverse	736 ± 107	796 ± 98	821 ± 128
total	1253 ± 123	1319 ± 102	1468 ± 162

*Significantly different form controls at $p<0.05$.

Startle response. The first trial of auditory and tactile startle were analyzed separately, then the remaining trials were analyzed in 10-trial blocks. Neither auditory nor tactile startle revealed significant differences between the control and treatment groups for V_{max}, V_{mean}, or T_{max} [data not shown, but are available upon request, see (32)].

Conditioned lick suppression. On the first day of testing on the lick suppression test, the number of licks emitted and the latency to 100 licks were unaffected by Group, $F(2,40)=0.43$, $p=0.65$, and $F(2,41)=0.37$, $p=0.69$, Sex, or the Group by Sex interaction. On the third day of testing, the latency to tone and the latency to criterion were unaffected by Group [two subjects did not reach criterion; $F(2,41)=0.08$, $p=0.94$ and $F(2,39)=0.18$, $p=0.84$], Sex, and the interaction. Transforming the latency data for normality by taking the reciprocal of each latency did not affect the outcome. A suppression index (defined as the latency to emit 10 licks following the tone, minus one tenth the latency to the first 100 licks on the third day divided by the latter term, and multiplied by 100) also did not show significant effects by Group, $F(2,39)=1.10$, $p=0.34$, Sex, or the Group by Sex interaction.

Intracellular pH

Table 6 presents the results of the 0-, 2-, 4-, and 8-hour data which were analyzed by analysis of variance, and the 24-hour data which were analyzed by *t*-test. The main effect of 2ME treatment on all variables examined was nonsignificant [for the primary test of interest, pHi, $F(3,24)=2.25$, $p=0.11$; for maternal plasma pH, $F(3,24)=0.07$, $p=0.98$; for, muscle pH, $F(3,24)=1.73$, $p=0.19$; and for embryo weight, $F(3,24)=2.79$, $p=0.06$]. The pHi data were also analyzed by analysis of covariance using maternal plasma pH as the covariate. The effects of Group remained nonsignificant, $F(3,23)=2.49$, $p=0.09$. Despite this, the trend toward increased pHi is suggestive, particularly at 4 hours.

The curve of best fit was determined using polynomial regression analysis for linear, quadratic, and cubic functions. The first order function (Fig. 4) did not account for significantly more of the variance than the intercept. Addition of the second order term accounted for significantly more of the variance than the first order term, indicating that the quadratic curve (Fig. 5) constituted a better fit to the data. This reflects an increase in pHi at 4 hours,

TABLE 6

INTRACELLULAR pH IN RAT EMBRYOS AT 2, 4, 8, AND 24 HR FOLLOWING ADMINISTRATION OF 0.33 mg/kg 2ME (MEAN ± S.E.)

Hours after Injection (Number of Litters)	Maternal Plasma pH	Maternal Muscle pH	Embryonic Tissue pH	Embryo Weight (mg)
0 (10)	7.40	6.92	7.32	73
	±0.03	±0.03	±0.03	±3
2 (5)	7.42	6.94	7.40	65
	±0.06	±0.10	±0.07	±2
4 (8)	7.40	6.96	7.43	80
	±0.03	±0.03	±0.04	±4
8 (5)	7.42	6.86	7.29	77
	±0.04	±0.02	±0.03	±4
24 Control (4)	7.41	6.90	7.01	152
	±0.04	±0.04	±0.07	±4
24 2ME (6)	7.39	6.90	7.18	145
	±0.02	±0.07	±0.03	±6

$F(1,27)=6.08$, $p=0.021$, with a return to control levels by 8 hours. Addition of a third order term did not account for significantly more of the variance than the quadratic model. The R-square value for the quadratic model was 21%, compared with 7% for the linear model, and remained at 21% for the cubic model, the latter indicating no gain in variance accounted for by the cubic component. The pHi of the 2ME-treated animals at 24 hours showed an apparent increase at 24 hours, but this increase was not significant [t-test, $F(3,5)=3.62$, $p=0.12$].

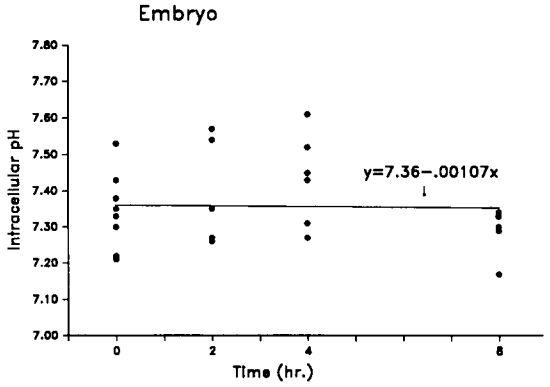

FIG. 4. Linear line of best fit to embryonic intracellular pH data.

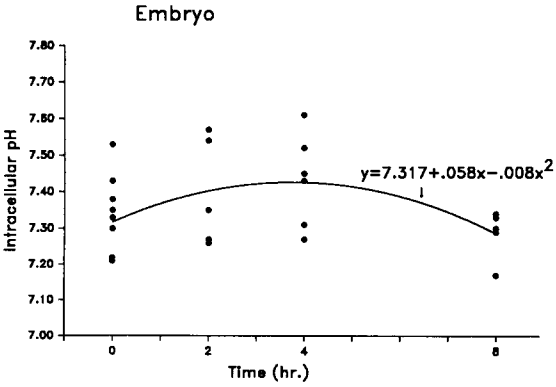

FIG. 5. Quadratic model curve of best fit to embryonic intracellular pH data.

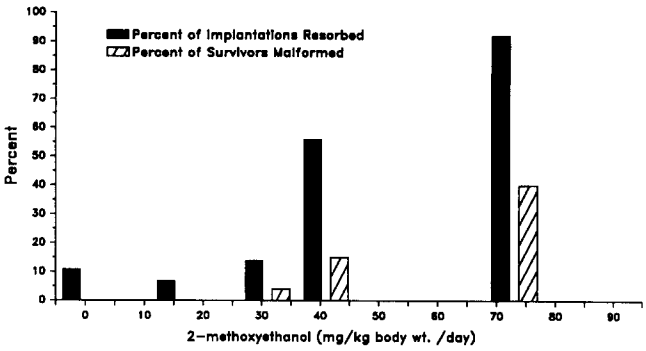

FIG. 6. Percent of implantations resorbed and of survivors malformed, illustrative of the steepness of the dose-effect curve for 2ME.

DISCUSSION

The present study established a dose-effect curve for the teratogenicity of 2ME administered in liquid diet to rats, and investigated a possible mechanism by which it may act. In contrast to the lack of teratogenic effects in rats after administration of 2-ethoxyethanol in drinking water (9), but in support of significant reproductive effects of 2ME in drinking water (9–11), the present study found teratogenicity of 2ME in a liquid diet. The ease with witch 2ME can be added to a liquid diet, along with the advantages of such an exposure route (59), opens new possibilities for future investigations of the reproductive toxicity of 2ME when extended exposures may be desired. However, in studies involving extended exposures, it would be important to consider monitoring possible accumulation of MAA. When 2ME is administered daily by gavage, there is a gradual accumulation of MAA in maternal plasma (46). Implications from these observations for the present study will be discussed below.

2ME

As illustrated by the present results (e.g., see Fig. 6), the dose-effect curve for the embryotoxicity of 2ME is steep, and the threshold for effects in maternal animals was approximately 0.05% 2ME. From this concentration to about one half that concentration (0.025%), a low percentage of fetuses survived to term. From this latter concentration, providing approximately 73 mg/kg/rat/day, there were systematic doses down to 0.006%, providing approximately 16 mg/kg/day 2ME. At this last concentration, the only effect was a minimal weight reduction in the fetuses. It appears that the steepest part of the curve is between 30 and 40 mg/kg/rat/day under the present exposure regimen. At the upper end (0.014% MAA) to the lower (0.012% 2ME), the shift in embryotoxicity went from high resorptions with malformations in survivors, to low or no increase in resorptions and few malformations at the next lower dose—although the postnatal mortalities at this concentration (0.012% 2ME) indicated that the fetuses were still slightly affected.

2ME plus Ethanol

In contrast to the reduction in malformations reported from

simultaneous administration of ethanol with 2ME in mice (49), we saw no indication that ethanol reduced the teratogenicity of 2ME in rats. Since coadministration of 4-methyl pyrazole reduced the teratogenicity of 2ME in rats (42), one would expect a similar reduction with ethanol. However, both of these studies used an acute dose of 2ME, and it may be that our extended dosing period, or the different route of exposure, accounted for the different results. Further, our method of exposure would not be expected to saturate alcohol/aldehyde dehydrogenase, whereas bolus administration would, and this may have contributed to the different outcome. It should also be noted that the consumption patterns were undoubtedly different. The ethanol group consumed diet ad lib, while the pair-fed animals, having restricted intake, consumed their diet in a shorter period of time soon after being fed. The effects of this difference in exposure pattern are unknown.

2ME plus Amiloride

Amiloride reduced consumption of the liquid diet. The 4.1 mg/kg dose, however, was accepted by the animals. Although the small number of litters makes the differences in effects less clear (4/9 litters for 2ME versus 5/9 litters for 2ME + amiloride), the percentage of fetuses affected by 2ME + amiloride was twice that seen with 2ME alone (19% versus 9%). If this is a reliable increase in malformations, it provides an additional area (viz., additional research focussed on the Na+/H+ antiporter) to pursue the mechanism of action of 2ME, particularly when considered along with the intracellular pH data. If, however, malformations and resorptions for these groups are summed, group differences in embryotoxicity are not apparent (for 2ME, 9 + 31=40% affected; for 2ME + amiloride, 19 + 23=42% affected).

Behavioral Testing

The only behavioral task to show significant differences from controls was the Cincinnati maze. In this test, only the number of errors committed in path A (forward path) of the maze differed from controls. Such results have been reported in some early work with the maze, but it is more common for the number of errors committed in the path B (reverse path) to be different from controls. Considering the unusual nature of the maze effect and the lack of significant differences from controls in the other behavioral tests, some uncertainty exists concerning the meaning of this finding. Only replication can reconcile this point.

The weak evidence (if any) of behavioral teratogenicity of 2ME is puzzling in light of the effects reported for 2-ethoxyethanol (37). With 2EE, behavioral effects were seen at levels which produced no detectable maternal toxicity or mortality in the offspring. In contrast, 2ME did not produce reliable behavioral effects even at a dose which produced mortality in one half the offspring. Because of the close structural similarity between these two glycol ethers, this discrepancy is puzzling. However, a recent study provided some potentially relevant information (58). A relatively wide range of levels of both 2ME and MAA in both maternal and embryonic tissues was reported 1 and 6 hours after administration of 280 mg/kg 2ME in a single gavage on gestation day 11 to CD-1 mice. At 1 hour, very little MAA was detectable, but 6-fold differences in values were obtained for both 2ME and MAA in maternal plasma, and there were even larger differences in embryonic levels. At 6 hours, the concentrations of 2ME and MAA were more similar, but a high degree of variability was still observed. We did not measure blood levels in our study, but if similar differences in blood levels of MAA were observed in our rat embryos, this might account for the differences observed. That is, it may be that fetuses with the highest exposure died, and those with lower levels were not affected. However, there were no apparent differences in mean consumption data in litters where the offspring died, as compared with those in which the offspring lived.

In future behavioral teratology studies of 2ME, it would be prudent to reduce the duration of exposure. Since MAA accumulates as exposure continues (31,46), shorter exposure periods might damage the developing CNS and yet allow the conceptuses to survive. Another study which needs to be completed is one in which 2ME and 2EE are directly compared.

Intracellular pH

It was recently noted that ethanol administered concomitantly with MAA reduced the teratogenicity of MAA (49). This observation would not be predicted, since ethanol would be expected to reduce the metabolic conversion of 2ME to the hypothesized active teratogen, MAA. If the mechanism of MAA's effects is on pHi, however, then these results could be explained. In the presence of large doses of ethanol, sufficient MAA may not enter cells to alter pHi. The interaction of 2ME and ethanol may be that of a weak teratogen (ethanol) competitively inhibiting conversion of a potent teratogen (2ME) to its active metabolite and/or inhibiting the metabolite's (MAA) uptake into embryonic cells. Thus, when both compounds are administered simultaneously, the observed embryotoxicity would be critically dependent on the dose of ethanol, and largely independent of the dose of 2ME. At (or below) marginally embryotoxic doses of ethanol, ethanol should prevent the embryotoxic effects of 2ME and no effects would be observed. At embryotoxic doses of ethanol, the same relationship holds; viz., ethanol blocks the effects of 2ME, and only the effects from ethanol are seen.

If the above mechanism is incorrect, then a synergistic, rather than an antagonistic, interaction would occur. While current morphological evidence supports the antagonistic model for 2ME, the behavioral and neurochemical data support a synergistic model in the case of ethanol and 2EE. Since there is strong evidence that 2ME and 2EE are metabolized in the same way, the nonantagonistic interaction of ethanol and 2EE cannot be readily explained by the mechanism described above.

The intracellular pH data of the present study are interesting in light of these hypotheses. The quadratic analysis supports the idea that there is an elevation in pHi at 4 hours, and a return to control levels at 8 hours. The possible changes at 24 hours are equivocal. In the assay, the assumption was made that the pH of the embryo's extracellular fluid (ECF) was equivalent to that of the maternal ECF. Hence, the distribution of a weak acid would be equal in the embryo's extracellular space (ECS) and the maternal ECS, and pH in maternal ECS would be an appropriate referent on which to base the pHi calculations. However, work by Scott *et al.* (personal communication) suggests that this assumption may not always be valid. Using embryo plasma, they found differences between the pH of embryonic ECF versus maternal ECF. When this difference occurs, the assumption that the weak acid distributes evenly between embryonic and maternal ECS is incorrect. In the present study, blood was drawn from two embryos in a single litter at 4 hours after administration of 2ME. These results suggested that use of maternal plasma pH as the referent may have overestimated the apparent pHi increease induced by 2ME. Using embryonic plasma pH as the referent, the embryonic pHi was 7.36, rather than 7.52. Further research will be needed to resolve such questions.

It is not known how 2ME (or MAA) acts to produce teratogenicity or an elevation in pHi. As MAA is acidic (pka estimated to be 3.5), a reduction in pHi had been expected. A potentiation of teratogenicity produced by coadministration of amiloride had been anticipated, similar to that reported for acetazolamide (24). Had a

decreased pHi been observed, then one could speculate that amiloride prevented the hydrogen ions from leaving the cell, and a potentiation in teratogenicity could be explained. Since the pHi was higher after 2ME treatment [similar to that reported for other acidic compounds (46)], the results with amiloride cannot be explained. Future research will be required to determine the mechanism by which 2ME alters pHi and how it exerts its teratogenic effects.

ACKNOWLEDGEMENTS

We thank Cindy Blanton, Mary Moran, Dan Minck, Mike Collins, Carol Duggan and Clare Schreiner for their assistance during the conduct of this research.

REFERENCES

1. Andrew, F. D.; Hardin, B. D. Developmental effects after inhalation exposure of gravid rabbits and rats to ethylene glycol monoethyl ether. Environ. Health Perspect. 57:13–23; 1984.
2. Autian, J. Toxicity and health threats of phthalate esters: review of the literature. Environ. Health Perspect. 4:3–26; 1973.
3. Blakely, P. M.; Scott, W. J., Jr. Determination of the proximate teratogen of the mouse fetal alcohol syndrome. 1. Teratogenicity of ethanol and acetaldehyde. Toxicol. Appl. Pharmacol. 72:355–363; 1984.
4. Blakely, P. M.; Scott, W. J., Jr. Determination of the proximate teratogen of the mouse fetal alcohol syndrome. 2. Pharmacokinetics of the placental transfer of ethanol and acetaldehyde. Toxicol. Appl. Pharmacol. 72:364–371; 1984.
5. Brown, N. A.; Holt, D.; Webb, M. The teratogenicity of methoxyacetic acid in the rat. Toxicol. Lett. 22:93–100; 1984.
6. Busa, W. B.; Nuccitelli, R. Metabolic regulation via intracellular pH. Am. J. Physiol. 246(155):R409–R438; 1984.
7. Campbell, J.; Holt, D.; Webb, M. Dimethoxyethylphthalate metabolism: teratogenicity of the diester and its metabolites in the pregnant rat. J. Appl. Toxicol. 4:35–41; 1984.
8. Cheever, K. L.; Plotick, H. B.; Richards, D. E.; Weigel, W. W. Metabolism and excretion of 2-ethoxyethanol in the adult male rat. Environ. Health Perspect. 57:241–248; 1984.
9. Chester, A.; Hull, J.; Andrew, F. Lack of teratogenic effect after ethylene glycol monoethyl ether (EGEE) in rats via drinking water. Teratology 33(3):57C; 1986(57C abstract P24).
10. Gulati, D. K.; Hommel, L.; Russel, S.; Pooncha, K. B.; Lamb, J. C. IV. Ethylene glycol monomethyl ether: reproduction and fertility assessment in CD-1 mice when administered in drinking water. Final Report of NTP contract 85-214, 1985.
11. Gulati, D. K.; Mounce, R. C.; Shaver, S.; Russell, S.; Pooncha, K. B.; Lamb, J. C., IV. Ethylene glycol monomethyl ether: reproduction and fertility assessment in CD-1 mice when administered in drinking water. Final Report of NTP contract 86-023, 1985.
12. Hanley, T. R., Jr.; Yano, B. L.; Nitschke, K. D.; John, J. A. Comparison of the teratogenic potential of inhaled ethylene glycol monomethyl ether in rats, mice, and rabbits. Toxicol. Appl. Pharmacol. 75:409–422; 1984.
13. Hanley, T. R., Jr.; Young, J. T.; John, J. A.; Rao, K. S. Ethylene glycol monomethyl ether (EGME) and propylene glycol monomethyl ether (PGME): inhalation fertility and teratogenicity studies in rats, mice, and rabbits. Environ. Health Perspect. 57:7–12; 1984.
14. Hardin, B. D. Reproductive toxicity of the glycol ethers. Toxicology 27:91–102; 1983.
15. Hardin, B. D.; Goad, P. T.; Burg, J. R. Developmental toxicity of four glycol ethers applied cutaneously to rats. Environ. Health Perspect. 57:69–74; 1984.
16. Hardin, B. D.; Niemeier, R. W.; Smith, R. W.; Kuczuk, M. H.; Mathinos, P. R.; Weaver, T. E. Teratogenicity of 2-ethoxyethanol by dermal application. Drug Chem. Toxicol. 5:277–294; 1982.
17. Hawkins, R. D.; Kalant, H. The metabolism of ethanol and its metabolic effects. Pharmacol. Rev. 24(1):67–157; 1972.
18. Horton, V. L.; Sleet, R. B.; John-Greene, J. A.; Welsch, F. Developmental phase-specific and dose-related teratogenic effects of ethylene glycol monomethyl ether in CD-1 mice. Toxicol. Appl. Pharmacol. 80:108–118; 1985.
19. Inouye, M. Differential staining of cartilage and bone in fetal mouse skeleton by alcian blue and alizarin red. S. Cong. Anom. 16:171–173; 1976.
20. Jonsson, A. K.; Steen, G. n-Butoxyacetic acid, a urinary metabolite from inhaled n-butoxyethanol (butylcellosolve). Acta Pharmacol. Toxicol. 42:354–356; 1978.
21. Kimmel, C. A.; Trammell, C. A rapid procedure for routine double staining of cartilage and bone in fetal and adult animals. Stain Technol. 56(5):271–273; 1981.
22. L'Allemain, G. L.; Paris, S.; Poussegur, J. Growth factor action and intracellular pH regulation in fibroblasts. J. Biol. Chem. 259(9): 5809–5815; 1984.
23. Laposata, E. A.; Lange, L. G. Presence of nonoxidative ethanol metabolism in human organs commonly damaged by ethanol abuse. Science 231:497–499; 1986.
24. Maren, T. H.; Ellison, A. C. The effects of acetazolamide and amiloride on tissue electrolytes, with reference to the teratogenesis of carbonic anhydrase inhibition. J. Pharmacol. Exp. Ther. 181(2): 212–218; 1972.
25. Miller, R. R.; Carreon, R. E.; Young, J. T.; McKenna, M. J. Toxicity of methoxyacetic acid in rats. Fundam. Appl. Toxicol. 2:158–160; 1982.
26. Mills, G. B.; Cragoe, E. J., Jr.; Gelfand, E. W.; Grinstein, S. Interleukin 2 induces a rapid increase in intracellular pH through activation of a Na+/H+ antiport. J. Biol. Chem. 260(23):12500–12507; 1985.
27. Nagano, K.; Nakayama, E.; Koyano, M.; Oobayashi, H.; Adachi, H.; Yamada, T. Mouse testicular atrophy induced by ethylene glycol monoalkyl ethers. Japn. J. Ind. Health 21:29–35; 1979.
28. Nagano, K.; Nakayama, E.; Oobayashi, H.; Nishizawa, T.; Okuda, H.; Yamazaki, K. Experimental studies on toxicity of ethylene glycol alkyl ethers in Japan. Environ. Health Perspect. 57:75–84; 1984.
29. Nagano, K.; Nakayama, E.; Oobayashi, H.; Yamada, T.; Adachi, H.; Nishizawa, T.; Ozawa, H.; Nakaichi, M.; Okuda, H.; Minami, K.; Yamazaki, K. Embryotoxic effects of ethylene glycol monomethyl ether in mice. Toxicology 20:335–343; 1981.
30. National Institute for Occupational Safety and Health. Current Intelligence Bulletin No. 39. Glycol Ethers, DHHS (NIOSH) Publication No. 83-112, 1983.
31. Nau, H.; Scott, W. J., Jr. Weak acids may act as teratogens by accumulating in the basic milieu of the early mammalian embryo. Nature 323(6085):276–278; 1986.
32. Nelson, B. K. Effects of 2-methoxyethanol on fetal development, postnatal behavior, and embryonic intracellular pH in rats. Dissertation submitted to the University of Cincinnati, August, 1987.
33. Nelson, B. K.; Brightwell, W. S.; Burg, J. R.; Massari, V. J. Behavioral and neurochemical alterations in the offspring of rats after Maternal or paternal inhalation exposure to the industrial solvent 2-methoxyethanol. Pharmacol. Biochem. Behav. 20:269–279; 1984.
34. Nelson, B. K.; Brightwell, W. S.; Setzer, J. V. Prenatal interactions between ethanol and the industrial solvent 2-ethoxyethanol in rats: maternal and behavioral teratogenic effects. Neurobehav. Toxicol. Teratol. 4:387–394; 1982.
35. Nelson, B. K.; Brightwell, W. S.; Setzer, J. V.; O'Donohue, T. L. Prenatal interactions between ethanol and the industrial solvent 2-ethoxyethanol in rats: Neurochemical effects in the offspring. Neurobehav. Toxicol. Teratol. 4:395–401; 1982.
36. Nelson, B. K.; Brightwell, W. S.; Setzer, J. V.; O'Donohue, T. L. Reproductive toxicity of the industrial solvent 2-ethoxyethanol in rats and interactive effects of ethanol. Environ. Health Perspect. 57: 255–259; 1984.
37. Nelson, B. K.; Brightwell, W. S.; Setzer, J. V.; Taylor, B. J.; Hornung, R. W.; O'Donohue, T. L. Ethoxyethanol behavioral teratology in rats. Neurotoxicology 2:231–249; 1981.
38. Nelson, B. K.; Setzer, J. V.; Brightwell, W. S.; Mathinos, P. R.; Kuczuk, M. H.; Weaver, T. E. Comparative inhalation teratogenicity of three industrial glycol ether solvents in rats. Teratology 25:65A; 1982.

39. Nelson, B. K.; Setzer, J. V.; Brightwell, W. S.; Mathinos, P. R.; Kuczuk, M. H.; Weaver, T. E.; Goad, P. T. Comparative inhalation teratogenicity of four glycol ether solvents and an amino derivative in rats. Environ. Health Perspect. 57:261–271; 1984.
40. Peters, J. W.; Cook, R. M. Effect of phthalate esters on reproduction in rats. Environ. Health Perspect. 3:91–94; 1973.
41. Riley, E. P.; Lochry, E. A.; Shapiro, N. R.; Baldwin, J. Response perseveration in rats exposed to alcohol prenatally. Pharmacol. Biochem. Behav. 10:255–259; 1979.
42. Ritter, E. J.; Scott, W. J., Jr.; Randall, J. L.; Ritter, J. M. Teratogenicity of dimethoxethyl phthalate and its metabolites methoxyethanol and methoxyacetic acid in the rat. Teratology 32:25–31; 1985.
43. Romer, K. G.; Balge, F.; Freundt, K. J. Ethanol-induced accumulation of ethylene glycol monoalkyl ethers in rats. Drug Chem. Toxicol. 8(4):255–264; 1985.
44. Rowe, V. K.; Wolf, M. A. Derivatives of glycols. In: Clayton, G. D.; Clayton, F. E., eds. Patty's industrial hygiene and toxicology. 3rd revised ed. vol. 2C. New York: John Wiley & Sons; 1982:3911–3919.
45. Schneider, A. M.; Weinberg, J.; Weissberg, R. Effects of ACTH on conditioned suppression: A time and strength of conditioning analysis. Physiol. Behav. 13:633–636; 1974.
46. Scott, W. J., Jr.; Duggan, C. A.; Schreiner, C. M.; Collins, M. D.; Nau, H. Intracellular pH of rodent embryos and its association with teratogenic response. In: Welsch, F., ed. Approaches to elucidate mechanisms in teratogenesis. Washington, DC: Hemisphere Publishing Corp.; 1987:99–107.
47. Scott, W. J., Jr.; Fradkin, R.; Wittfoht, W.; Nau, H. Teratologic potential of 2-methoxyethanol and transplacental distribution of its metabolite 2-methoxyacetic acid, in non-human primates. Teratology 39:363–373; 1989.
48. Scott, W. J., Jr.; Nau, H.; Wittfoht, W.; Merker, H.-J. Ventral duplication of the autopod: chemical induction by methoxyacetic acid in rat embryos. Development 99:127–136; 1987.
49. Sleet, R. B.; John-Greene, J. A.; Welsch, F. Paw dysmorphogenesis in CD-1 mice treated with 2-methoxyethanol and methoxyacetic acid in combination with ethanol. Teratology 31:48A; 1985.
50. Sleet, R. B.; Greene, J. A.; Welsch, F. Teratogenicity and disposition of the glycol ether 2-methoxyethanol and their relationship in CD-1 mice. In: Welsch, F., ed. Approaches to elucidate mechanisms in teratogenesis. Washington: Hemisphere Publishing Corp.; 1987:33–57.
51. Threshold Limit Values and Biological Exposure Indices for 1986–1987. American Conference of Governmental Industrial Hygienists, Cincinnati, OH, 1987.
52. Toraason, M. B.; Breitenstein, M. J. Ethylene glycol monomethyl ether (EGME) inhibits rat embryo ornithine decarboxylase (ODC) activity. Drug Chem. Toxicol. 9(3&4):191–203; 1986.
53. Toraason, M. B.; Stringer, B.; Smith, R. Ornithine decarboxylase activity in the neonatal rat heart following prenatal exposure to ethylene glycol monomethyl ether. Drug Chem. Toxicol. 9:1–14; 1986.
54. Toraason, M.; Stringer, B.; Stober, P.; Hardin, B. D. Electrocardiographic study of rat fetuses exposed to ethylene glycol monomethyl ether (EGME). Teratology 32:33–39; 1985.
55. Vorhees, C. V. Maze learning in rats: A comparison of performance in two water mazes in progeny prenatally exposed to different doses of phenytoin. Neurotoxicol. Teratol. 9(3):235–241; 1987.
56. Vorhees, C. V. A fostering/crossfostering analysis of the effects of prenatal ethanol exposure in a liquid diet on offspring development and behavior in rats. Neurotoxicol. Teratol. 11(2):115–120; 1989.
57. Waddell, W.; Butler, T. Calculation of intracellular pH from the distribution of 5,5 dimethyl-2,4 oxazolidine (DMO). Application to skeletal muscle of the dog. J. Clin. Invest. 38:720–729; 1959.
58. Welsch, F. Attenuation of 2-methoxyethanol (ME)- induced digit malformations in CD-1 mice by delayed dosing with sodium acetate (NaAc). Teratology 35(2):59A; 1987.
59. Wiener, S. G. Nutritional considerations in the design of animal models of the Fetal Alcohol Syndrome. Neurobehav. Toxicol. 2(3): 175–180; 1980.
60. Wiley, F. H.; Hueper, W. C.; Bergen, D. S.; Blood, F. R. The formation of oxalic acid from ethylene glycol and related solvents. J. Indust. Hygiene Toxicol. 20:269–277; 1938.
61. Wilson, J. G. Methods for administering agents and detecting malformations in experimental animals. In: Wilson, J. G.; Warkany, J., eds. Teratology: Principles and techniques. Chicago: Univ. of Chicago Press; 1965:251–277.
62. Yonemoto, J.; Brown, N. A.; Webb, M. Effects of dimethoxyethyl phthalate, monomethoxyethyl phthalate, 2-methoxyethanol and methoxyacetic acid on post implantation rat embryos in culture. Toxicol. Lett. 21:97–102; 1984.