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Methionine Decreases the Embryotoxicity of Sodium Valproate in the Rat: In Vivo and In Vitro Observations

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ABSTRACT Methionine provided in the drinking water of pregnant rats injected with sodium valproate reduced the frequency of resorptions but did not improve embryo growth. Rats drinking methionine supplemented water had approximately twice the level of serum-free methionine and consumed only one-half the volume of water of controls. Using whole rat embryo cultures, the simultaneous addition of methionine and sodium valproate to the medium provided no protection from neural tube defects, nor did the addition of methionine to a medium of serum obtained from rats previously dosed with sodium valproate. However, protection from the teratogenic effects of sodium valproate was afforded by methionine when the culture medium was sera from rats consuming methionine and was particularly striking when embryos for culture were taken from pregnant rats that had been consuming methionine. These observations along with those of others indicated the importance of dietary and culture media methionine levels in evaluating experimental and regulatory teratology studies and suggested the possibility that methionine may play an important role in human teratology where multifactorial causes have been implicated in problems such as neural tube closure defects.

Neural tube closure defects have been frequently observed in human subjects with estimated incidences ranging from two per thousand live births (Stein et al., '82) in the United States to as many as 6:1,000 in other countries (Stocks, '70). In spontaneously aborted fetuses, the frequency of such defects may occur at a rate as much as 10 times higher than observed in live births (Byrne and Warburton, '86). In spite of these high frequencies, clear etiologies have not been identified, leading to the suggestion that multiple factors such as genetic predisposition, maternal illness and fetal drug exposure could all be involved (Campbell et al., '86).

Poor nutrition also has been implicated as an important factor in defects of neural tube closure. For example, women receiving adequate diets had fewer children with neural tube defects than women on inadequate diets (Laurence et al., '80) and, if the diet was improved, the risk of having another child with a neural tube defect was reduced from 18% to 3% (Laurence et al., '81). In a recent multinational double-blind trial, women who had a previous child with a neural tube defect were given folic acid supplements before and during pregnancy. This treatment lowered the chance of producing a second child with a neural tube defect by 72% compared to those not given the folic acid supplement (MRC Vitamin Study Research Group, '91). In addition, deficiencies of zinc and copper also have been associated with an increase in neural tube defects in humans (Hurley, '81; Zimmerman, '84; Mulinare et al., '88; Milunsky et al., '89) and in animals (Warkany and Petering, '73; Hurley, '81).

Women taking anticonvulsant medications have a two to three times greater risk

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of having a baby with a birth defect than do women not taking such drugs (Center for Disease Control, '82), with the predominant defects associated with the skeleton and neural tube. Exposure to valproic acid or its sodium salt have been found in human epidemiological studies (Robert and Rosa, '83; Martinez-Frias, '90) and experiments with rodents both in vivo (Turner et al., '90; Dencker et al., '90) and in vitro (Kao et al., '81; Chatot et al., '84; Hanson and Grafton, '91) to cause a higher incidence of embryos with neural tube defects than those not exposed. In addition, sodium valproate was found to concentrate in the neuroepithelium of embryos when injected into pregnant rodents (Dencker et al., '90) causing these cells to become severely disorganized and irregular (Turner et al., '90).

Studies with cultured whole rat embryos have shown that nutritional deficiencies, as well as the presence of teratogens, could cause neural tube defects. When rat embryos were cultured on sera from women taking anticonvulsant drugs, 72% of the sera samples were considered teratogenic (Chatot et al., '84), and the embryos showed both neural tube defects as well as limited growth. When these same serum samples were supplemented with vitamins and amino acids, 60% of the teratogenic samples now supported normal rat embryo development.

Other studies from this laboratory using whole rat embryo cultures have shown that the amino acid methionine was specifically required for neural tube closure when sera from cows were used as culture media (Coelho et al., '89). In addition, sera from some women with histories of spontaneous abortions when used as culture media also required additional methionine to support normal rat embryo development (Ferrari et al., '86). With dog sera as the culture media methionine was needed in conjunction with glucose and a lipophilic iron chelate (Flynn et al., '87) to support normal embryo growth and development. In all these studies, it was not clear if methionine played a protective role in the presence of a toxic substance or if it simply overcame a deficiency of the amino acid. Recently, sera from both monkeys and rats fed a methionine deficient synthetic diet were found to be teratogenic to cultured rat embryos unless methionine was added to the culture medium (unpublished data, Dr. Norman Klein). In the present study, we examined the ability of methionine to protect embryos against the teratogenicity of sodium valproate, a drug known to interfere with neural tube closure.

MATERIALS AND METHODS

In vivo procedures

CD strain rats (Charles River Breeding Laboratories, Wilmington, MA) were housed in steel mesh suspension cages in a room with controlled temperature (20–22°C) and a 12-h light–dark cycle. They were given ad libitum food and water. Female rats (one or two) were placed in a cage with a male rat overnight, and the presence of sperm in the vaginal tract was considered day 0.5 of pregnancy. Individual weights and average water consumptions (one to four rats per cage) were monitored thrice a week. Rats treated with methionine received 10 mg/ml L-Methionine (pharmaceutical grade, Degussa Corporation; Allendale, NJ) in their drinking water for a period of at least 2 weeks before mating and throughout pregnancy. Following a recommendation by Dr. Richard Finnell, i.p. injections were made twice a day six hours apart with 325 mg/kg body weight of sodium valproate (Sigma Chemical Co., St. Louis, MO) dissolved in water (650 mg/kg/day) on days 7, 8, and 9 of gestation. The animals were divided into four groups. Group 1 received neither methionine nor sodium valproate, group 2 received methionine alone, group 3 received sodium valproate alone, and group 4 received both methionine and sodium valproate. On day 18, rats were etherized, blood was drawn from the descending aorta, centrifuged immediately (Steele and New, '74); the serum was stored at −20°C for later amino acid analyses by gas chromatography. The uterine horns were removed, the viable and resorbed implantation sites counted and viable embryos were removed from the yolk sacs. The embryos were rinsed in phosphate buffered saline, blotted, weighed and placed in Bouin's fixative for subsequent examination by Wilson sectioning (Wilson and Warkany, '65).

In vitro procedures

Rat embryos were removed from CD strain rats at 9.5 days of gestation, according to the procedures developed by New and associates ('76). Two embryos were placed in a culture tube in a total volume of 1.33 ml (Klein et al., '78) which consisted of 0.9 ml

TABLE 1. Water consumption by pregnant rats receiving methionine# and sodium valproate##

Days of gestation	Treatment			
	None (ml/rat/d)	Methionine (ml/rat/d)	Sodium valproate (ml/rat/d)	Methionine and sodium valproate (ml rat d)
0–6	43 ± 2 (5)[1]*	17 ± 1 (9)[2] (620)**	38 ± 2 (15)[1]	21 ± 1 (11)[2] (750)
7–12	47 ± 1 (6)[1]	24 ± 1 (9)[2] (822)	52 ± 1 (18)[1]	23 ± 1 (19)[2] (764)
13–18	49 ± 1 (6)[1]	30 ± 1 (9)[2] (888)	53 ± 1 (18)[1]	25 ± 1 (19)[2] (762)

#Methionine was provided in the drinking water continuously at a concentration of 10 mg/ml of L-methionine.
##Sodium valproate was injected at a concentration of 325 mg/kg twice a day on days 7, 8, and 9 of gestation.
*Estimated average water consumption in ml/day ±SE (number of rats); between columns on a single row, differing superscripts indicate significance at $P < 0.05$ by t-test.
**Estimated average mg methionine consumed/kg body weight/day.

rat serum and 0.3 ml Tyrode's solution (v/v). The remaining volume was water which also contained 0.66 mg/ml streptomycin sulfate (Sigma Chemical, St. Louis, MO) and 0.006 mg/ml penicillin–G-potassium (Sigma Chemical) per ml of medium, as well as the methionine and sodium valproate as indicated in the Results. The culture tubes were kept at 37.5°C and rotated at 30 rpm. They were gassed at a constant 5% CO_2 with varying ratios of N_2 and O_2 as follows: pregas and after 3 h 90% N_2, 5% O_2; after 20 h 80% N_2, 15% O_2; after 27 h 65% N_2, 30% O_2; after 44 h 55% N_2, 40% O_2.

At the end of the 48-h culture periods, embryos were examined for viability (all were alive as judged by the presence of a beating heart) and abnormalities, photographed immediately (Polaroid 667 film, Polaroid Corp., Cambridge, MA) and then used either for protein determinations by the Lowry method ('51) or placed in Carnoy's solution for histological sectioning. Paraffin sections were cut at 7 μm and stained with hematoxylin.

Sera samples used as media for whole embryo cultures were collected from the descending aortae of etherized CD strain rats. The blood was immediately centrifuged and fibrin clots removed. Sera were pooled and heat inactivated at 56°C for 30 min, passed through a 0.45-μm HA filter (Millipore Corp., Bedford MA), and frozen at −20°C.

Amino acid determination

The procedure of Desgres et al. ('79) as modified by Coelho et al. ('89) was used for free amino acid determinations. Duplicate 200 μl aliquots of sera containing 50 μl of adipic acid (internal standard) were precipitated with 5-sulfosalicylic acid to remove proteins and centrifuged. Supernatants were placed in tubes containing Dowex 50W-8X beads (Bio-Rad Laboratories, Richmond, CA) incubated with 250 μl of 4N acetic acid for 1 h. The Dowex was washed with 1N acetic acid and distilled water and eluted with ammonium hydroxide (6N). The eluates were placed in glass reaction vials (4 ml; Kontes, Vineland, NJ) and evaporated under N_2 gas. The vials were cooled to room temperature; the remaining amino acids were incubated with 250 μl of acidified isobutanol (4N) at 130°C and dried, and 30 μl of heptafluorobutyric anhydride (Aldrich Chemical Co., Milwaukee, WI) was added. The vials were cooled to room temperature and dried under N_2. Thirty μl of ethyl acetate (Aldrich Chemical Co., Milwaukee, WI) was added and the vials placed at −20°C overnight.

A Hewlett Packard 5890A gas chromatograph (Hewlett Packard, Avondale, PA) with a flame ionization detector and a Hewlett Packard 3392A integrator (Hewlett Packard, Avondale, PA) were used for amino acid analysis. One μl of sample was injected onto a GB-1 capillary column (Foxboro Analabs, North Haven, CT). The carrier gas was helium at a flow rate of 1 ml/min. A step gradient increased the temperature from 90°C to 270°C at 7°C/min. The injector port temperature was 280°C, and the detector temperature was 300°C.

RESULTS

In vivo injections

Water consumption by pregnant rats receiving methionine in their drinking water (10 mg/ml) was approximately one-half the volume consumed by those not receiving methionine ($P<0.05$, t-test) (Table 1). Administration of sodium valproate did not appear to influence water consumption. In

TABLE 2. Weight of pregnant rats receiving methionine# and sodium valproate##

Days of gestation	Treatment			
	None (g/rat)	Methionine (g/rat)	Sodium valproate (g/rat)	Methionine and sodium valproate (g/rat)
0–6	292 ± 18 (5)*	274 ± 9 (9)	278 ± 5 (18)	280 ± 9 (13)
7–12	316 ± 13 (6)	292 ± 10 (9)	297 ± 4 (18)	301 ± 8 (19)
13–18	360 ± 13 (6)	338 ± 10 (9)	318 ± 3 (18)	328 ± 7 (19)

#Methionine was provided in the drinking water continuously at a concentration of 10 mg/ml of L-methionine.
##Sodium valproate was injected at a concentration of 325 mg/kg twice a day on days 7, 8, and 9 of gestation.
*Average weight (in g/rat) during time period ±SE (number of rats). Between columns on a single row, there was no significance ($P > 0.05$) between treatment groups.

spite of this large difference in water consumption, weight gains during pregnancy did not differ appreciably between the controls (no treatment) and those receiving either methionine alone, sodium valproate alone or methionine plus sodium valproate ($P > 0.05$, t-test) (Table 2). It should be noted that, on the basis of water consumption, estimates of methionine intake ranged from 620 to 888 mg/kg/day (Table 1).

When rats were injected with sodium valproate on days 7, 8 and 9 of gestation, 66% of their embryos were resorbed by day 18 (183 resorptions out of 276 implants; Table 3). In comparison, rats treated in the same manner with sodium valproate but given methionine in their drinking water showed a resorption frequency of 42% (102 resorptions of 242 implants). Using the arcsine transformation (Steel and Torrie, '80) this difference was found to be significant when the outcome of each litter was calculated ($P < 0.05$, one-tailed t-test). For the no-treatment group, no resorptions were found in 88 implants; for the methionine alone group, only one resorption in 128 implants was observed in spite of the large amount of methionine consumed.

For the sodium valproate treated animals, whether given methionine or not, abnormalities were not observed on day 18 of gestation in surviving embryos either at the gross level or when examined by Wilson sectioning. In an effort to determine whether embryos were abnormal before being resorbed, four rats previously injected with sodium valproate were sacrificed on day 12 of gestation. Abnormalities were not observed, although resorptions could already be detected at this time.

The average wet weight of the viable embryos for the sodium valproate-treated alone group at gestational day 18 was 0.98 g, which did not differ from those receiving sodium valproate plus methionine (1.00 g). However, the groups lacking valproate (methionine alone and no treatment) had average wet weights that were comparable to each other and were significantly higher (1.37 g and 1.47 g, respectively; $P < 0.05$, t-test) than the sodium valproate-treated experimental groups. This indicated that sodium valproate rather than methionine adversely affected the growth of embryos in utero.

The serum methionine level of the dams in the sodium valproate alone group at day 18 was approximately one-half that of the group treated with sodium valproate plus methionine (8.6 vs. 15.0 μg/ml; see Table 3) ($P < 0.05$ by t-test). The methionine levels of the control groups (those not receiving sodium valproate injections) did not differ from their respective sodium valproate-injected experimental groups. For example, the no treatment group did not differ from the sodium valproate alone group (9.0 vs. 8.6 μg/ml, respectively), and the methionine-alone group did not differ from the sodium valproate plus methionine-treated group (17.0 vs. 15.0 μg/ml, respectively). This indicated that sodium valproate did not adversely affect methionine uptake and/or absorption by the pregnant dam.

In vitro experiments

Sodium valproate at 0.9 μmoles (0.15 mg/ml final concentration in the medium or 0.68 mM) was used for all the whole rat embryo cultures as higher concentrations (0.98–1.05 μmoles) were embryo lethal; lower levels (0.45–0.82 μmoles) caused abnormalities in only 25% or less of the embryos. At 0.9 μmoles with rat sera as the culture medium, approximately 78% of the embryos were abnormal, showing either open neural tubes, collapsed neural tubes (observed in whole embryos as an irregular

TABLE 3. Effects of methionine[§] on sodium valproate[§§]-induced resorption in the 18-day pregnant rat

Treatment	Rats (n)	Resorbed embryos/ total implants (%)	Embryo wet weight (g/embryo)	Serum methionine (μg ml)
None	6	0/88 (0)	1.47 ± 0.02[1]* (88)**	9.0 ± 1.1[1,2]# (6)##
Methionine	9	1/128 (1)	1.37 ± 0.02[1] (127)	17.0 ± 5.1[2] (9)
Valproate	19	183/276 (66)[1]	0.98 ± 0.02[2] (92)	8.6 ± 0.5[1] (18)
Methionine and valproate	18	102/242 (42)[2]	1.00 ± 0.02[2] (139)	15.0 ± 3.4[2] (18)

[§]Methionine was provided in the drinking water continuously at a concentration of 10 mg/ml of L-methionine.
[§§]Sodium valproate was injected at a concentration of 325 mg/kg twice a day on days 7, 8, and 9 of gestation.
*Average weight in grams ±SE. Within columns, differing superscripts indicate significance at $P < 0.05$ by t-test.
**Number of embryos examined.
#Average serum methionine level (in μg/ml) serum ± SE.
##Number of rats examined.

inward folding of the neural epithelium), or a combination of both abnormalities, although all embryos were alive (Fig. 1; Table 4). When methionine was added to the medium with sodium valproate, the frequency of abnormal embryos remained at approximately 75%. The methionine level used in these studies was 0.44 μmoles (0.049 mg/ml final concentration in the media or 0.33 mM). However, both higher (0.66 μmoles, 0.88 μmoles and 1.10 μmoles) and lower (0.22 μmoles) concentrations did not provide greater protection against the adverse effects of sodium valproate.

As simply adding methionine to sera containing sodium valproate failed to protect embryos from the teratogenicity of this anticonvulsant, a series of in vitro experiments were then conducted in an attempt to determine the mechanism of protection observed in the intact animal. First, preculturing embryos (starting with 9.5-day mid-headfold stage embryos) with methionine for periods of up to 8 h before adding sodium valproate did not decrease the abnormality frequency (83%) (data not shown). In order to consider the possibility that a metabolite of sodium valproate rather than the parent compound could be responsive to methionine in vivo, sera from rats that had recently finished the 3-day sodium valproate injection protocol (3–6 h after the last injection) were used as the media. It was found that sera drawn approximately 6 h after the last injection was still teratogenic (72%) to cultured embryos and again methionine failed to reduce the frequency of abnormal embryos (data not shown).

In another series of whole embryo culture experiments, the possibility that methionine consumption by the dam altered serum constituents other than simply increasing methionine levels was considered. For this, sera from rats that had been drinking methionine water for at least 2 weeks before collection were used as the culture media and then sodium valproate was added directly to the sera. The results showed a decrease in the frequency of abnormal embryos from 75% to 45% (compare line 3 with line 2; Table 4) ($P<0.05$, chi-square test). As others have shown that methionine could influence the levels of such substances as glutathione in rat liver (Fernandez-Checa et al., '90) or a rat hepatocyte culture system (Henning et al., '89), glutathione was added at concentrations of 0.033–0.65 mg (0.5 and 10 times that of methionine used in the above experiments) to untreated sera with sodium valproate. The abnormality frequency still remained at over 75% (data not shown).

Although we had not seen protection after 8 h of preculturing with methionine, to examine further the value of early exposure to methionine, embryos at the headfold stage were isolated from dams which had been consuming methionine supplemented water continuously starting at least 2 weeks before mating and then cultured on untreated rat sera containing the same 0.9 μmoles of sodium valproate as used in the other experiments. The frequency of abnormal embryos decreased dramatically from 78% when methionine had been added exogenously in previous experiments to only 25% ($P<0.05$; chi-square test).

The growth of the cultured embryos in these studies followed abnormality frequencies (Table 4) as the protein content of em-

TABLE 4. Responses of cultured rat embryos to methionine and sodium valproate

Sera donor treatment	Embryo donor treatment	µmoles added to culture* Met (.44)	µmoles added to culture* NaVP (.9)	Abnormal embryos/total (%)	Somites (pairs/embryo)	Embryo protein (µg/embryo)
None	None	−	+	14/18 (78%)[1]	16 ± 1 (18)[1]**	99 ± 11 (5)[1]#
None	None	+	+	18/24 (75%)[1]	15 ± 1 (24)[1]	115 ± 9 (4)[1,2]
Methionine fed	None		+	10/22 (45%)[2]	17 ± 1 (22)[1]	155 ± 13 (9)[3]
None	Methionine fed	−	+	3/12 (25%)[2]	17 ± 1 (10)[1]	137 ± 5 (8)[2,3]

*NaVP, sodium valproate; Met, methionine.
**Average number of somite pair/embryo ±SE (number of embryos examined). Within columns, differing superscripts indicate significance at $P < 0.05$ by chi-square test.
#Average protein content of embryos ±SE (number of embryos examined).

bryos exposed in vitro to sodium valproate alone or with added methionine had significantly lower protein content ($P<0.05$, t-test) than did those embryos taken from methionine-treated dams. Unlike these protein differences, somite numbers did not differ appreciably between the groups.

DISCUSSION

In the present study, when provided in the drinking water of pregnant rats, methionine was found to reduce the frequency of resorptions caused by the anticonvulsant drug sodium valproate. Although this drug caused reduced weights in surviving embryos as others have noted (Vorhees, '87; Klug et al., '90), unlike the previous reports abnormalities were not detected. As the impetus for this study was to evaluate the ability of methionine to overcome the teratogenicity of a drug known to interfere with neural tube closure (Ehlers et al., '92), it was not clear whether the protection from resorptions afforded by methionine involved neural tube closure. However, in the present study, it was possible that embryos exposed in utero to sodium valproate were not examined at a time sufficiently early after drug exposure to detect such defects, particularly as others have reported neural tube closure defects in the embryos of rats. For example, Klug et al. ('90) treated rats on day 10 of gestation and observed defects when examined on day 11.5.

As others had found that sodium valproate caused neural tube defects in cultured rat embryos (Klug et al., '90; Bruckner et al., '83; Kao et al., '81; Hanson and Grafton, '91), this technique was used to gain some insights into the interactive mechanisms of the amino acid and drug. First, simply adding methionine and sodium valproate simultaneously to the culture medium failed to reduce the frequency of abnormalities, suggesting that methionine did not block the uptake of sodium valproate. Protection by methionine also was not achieved when sera from sodium valproate dosed rats were used as culture media, which may have excluded the possibility that methionine acted on a metabolite of sodium valproate. Some protection was achieved when sera for culture came from rats receiving methionine. This finding suggested that methionine altered serum constituents as others have proposed (Maree et al., '89; Fernandez-Checa et al., '90), but when one product of methionine metabolism, glutathione, was tested the abnormality frequency was not reduced. The greatest protection from sodium valproate teratogenicity was observed when dams consuming methionine were used as the embryo donors for culture. This suggested the possibility that early molecular events involving methionine such as methylation reactions may have helped the neural tube to close. For example, Coelho and Klein ('90) found that, in the absence of methionine, the methylation of amino acids in neural tube proteins of cultured embryos were decreased. Since the neural folds were elevated, but not turned in, this suggested that the methylation of contractile proteins (e.g., actin and myosin) in microfilaments in the neural folds may be necessary for the neural folds to turn in and close the neural tube. The methylation of these amino acids may have occurred in the present study at an early enough time in the presence of excess methionine to avoid the potential teratogenicity of sodium valproate.

The present observation that the simultaneous addition of methionine and sodium

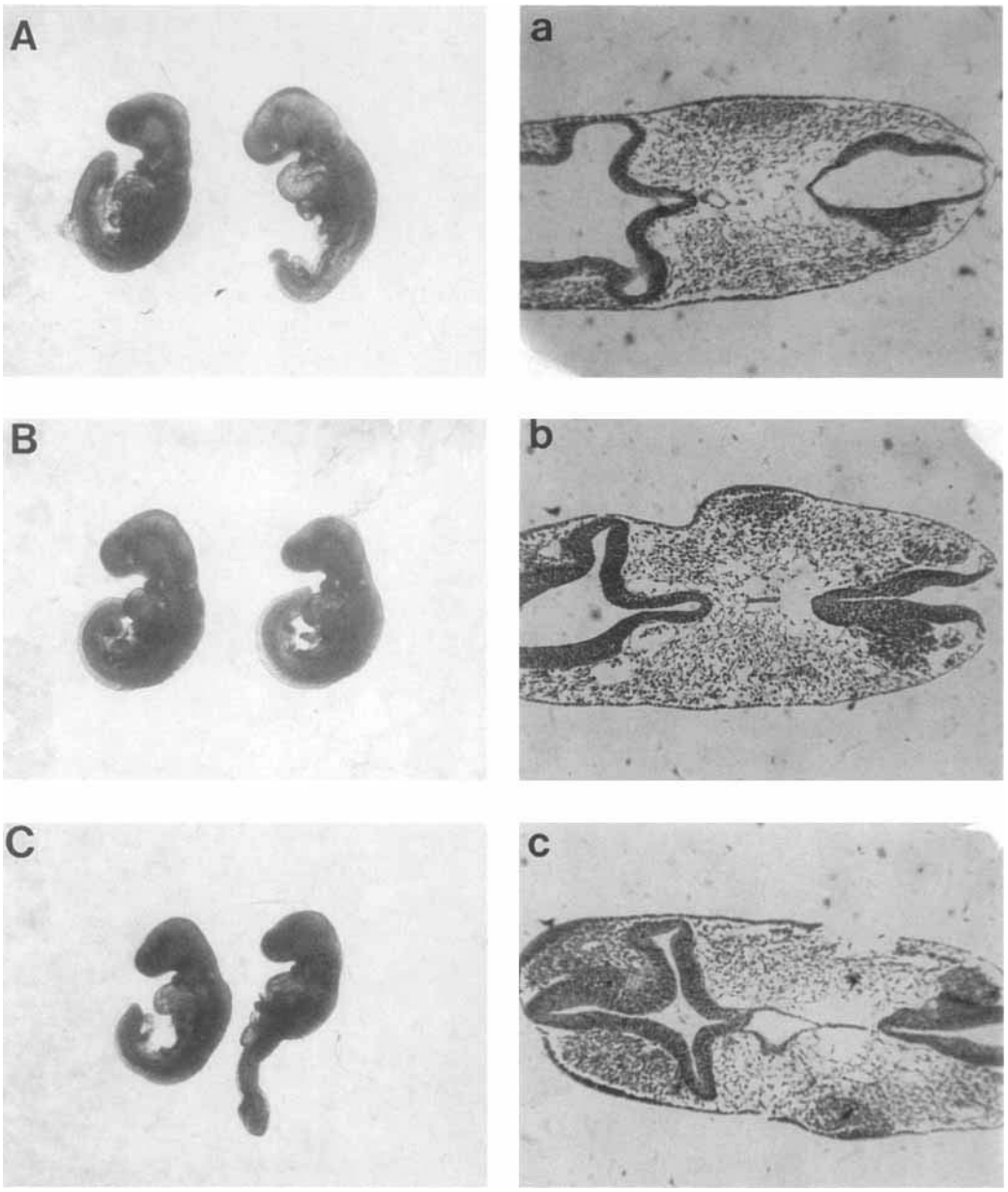

Fig. 1. Rat embryos after 48 h of culture (**A–C**) in serum containing 0.9 μmoles of sodium valproate and sections through the head region (**a–c**). A–C: photographs taken immediately after culture following removal of extraembryionic membranes of A, normal embryos; B, embryos with open neural tubes; and C, embryos with collapsed neural tubes. Note the normal neural tube (a) has an uninterrupted, expanded neural epithelium in the hindbrain, the open neural tube (b) has an opening in the neural epithelium in the hindbrain area and the collapsed neural tube (c) showing an inwardly turned neural epithelium with limited space in the neural tube. A,B,C: ×12; a,b,c: ×100.

valproate to the culture medium failed to protect embryos was consistent with the findings of Hansen and Grafton ('91) that folinic acid (a folic acid derivative) failed to protect rat embryos in culture from valproic acid when both were added to the medium simultaneously. Our observations also may have supported the study of Wegner and Nau ('91), who found in the pregnant mouse that folinic acid reduced the frequency of neural tube defects caused by sodium valproate. However, quite distinct from the present study, these investigators observed that folinic acid actually increased the frequency of resorptions, which may have influenced the apparent decrease in neural tube defects that they reported.

Although we demonstrated here that methionine could protect embryos from the harmful effects of sodium valproate, it should be noted that this action of methionine did not appear specific for this drug. For example, we have observed that methionine protected cultured embryos from the toxicity of a very different agent, antilaminin antibodies (B. Chambers, unpublished data). In addition, it should also be noted that deficiencies in methionine were teratogenic to rat embryos when cultured on cow sera (Coehlo et al., '89), or sera from rats and monkeys that had been fed methionine deficient diets (N. Klein, unpublished data). Finally, Essien ('92) recently reported protection by methionine against a mouse genetic defect leading to posterior open neural tube defects. These experimental observations of the diverse etiologies that could be overcome by methionine may help explain the ability of folinic acid to overcome the multifactorial causes of human neural tube defects (MRC Vitamin Study Research Group, '91), particularly if the role of the folinic acid was to make more methionine available through the pathway involving the conversion of homocysteine to methionine (Finkelstein, '90). In addition to these clinical implications, the present study indicated that consideration must be given to methionine levels in the diet of animals as well as in the culture media for whole embryos for future studies in experimental teratology.

NOTE ADDED IN PROOF

Dr. Victor H. Denenberg and his associates of the Biobehavioral Sciences Graduate Program, University of Connecticut, conducted two behavior tests on two litters each of 19 rats exposed to sodium valproate plus methionine during pregnancy and 19 rats given methionine alone. The tests were given at three to six months after birth and involved the Morris water maze and a test for Swimming Rotation. Although the numbers of animals were small, the animals exposed to sodium valproate (and methionine) in utero did not differ from the controls in these two behavioral tests.

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