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Neurochemical and Behavioral Effects of Diet Related Perinatal Folic Acid Restriction[1]

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MIDDAUGH, L. D., T. A. GROVER, L. A. BLACKWELL, AND J. W. ZEMP. *Neurochemical and behavioral effects of diet related perinatal folic acid restriction.* PHARMAC. BIOCHEM. BEHAV. 5(2) 129–134, 1976. Some effects of restricting dietary folic acid during the perinatal period on tissue folate and S-adenosyl-L-methionine (SAM) concentrations and on behavior were examined in 35-day-old DBA/2J mice. In one study dams were started on diets containing no folate (FO), 1.1 mg of folic acid/kg diet (F1.1) or 9.9 mg of folic acid/kg diet (F9.9) on the day of parturition. In a second study some mice were started on the FO or F9.9 diets 6–7 days prior to parturition and some remained on lab chow (LC). The dams and their pups remained on their assigned diets until offspring were killed for biochemical assays. The major findings of the 2 studies are: (1) that eliminating folic acid from diets of dams and developing pups from birth or 1 week prior to birth causes a reduction of folate in brain tissue; (2) that reduction in brain tissue is not as severe as that of liver; (3) that initiating the folate free diet 1 week prior to birth causes reductions in body, liver, and brain weights and in activity levels of surviving offspring; and (4) that offspring of dams started on either the FO or F9.9 diet one week prior to parturition are less viable and have lower levels of SAM in brain tissue than animals reared on the LC diets.

Folic acid SAM Perinatal period Dietary restrictions Folate free diet

FOLIC acid and its derivatives are required for a number of important neurochemical reactions including the synthesis of nucleic acids and S-adenosyl-L-methionine (SAM). SAM, in turn, is important in the synthesis of choline, a component of the neurotransmitter, acetylcholine, and of the neurochemically important lipids, lecithin and sphingomyelin. In addition, SAM serves a methyl donor in the catechol-0-methyl transferase (COMT) catalyzed inactivations of dopamine and norepinephrine. Thus, a deficiency in folic acid might be expected to exert a wide influence on brain development and on brain function. Since mammals are unable to synthesize folic acid, diet is an important source of this vitamin [4].

The effect of reduced maternal folate intake upon the developing fetus and neonate has not been studied extensively. Nelson and coworkers [15,16] reported that a reduction in folic acid levels during critical stages of gestation was teratogenic. Feeding a folate free diet containing succinylsulfathiazole and methyl pteroylglutamic acid during days 8–9½ of gestation produced malformations in 80% of the animals examined. In another study, Briggs [5] reported reduced growth for neonatal mice of several strains reared on a folate free diet. Evidence for reduced tissue stores of folate in these mice was suggested by the presence of formiminoglutamic acid in urine. Klipstein and Lipton [10] reported that growth was more severely retarded if the folate free diet was initiated 1 week prior to birth and that liver folate concentration was reduced in offspring reared on the diet.

In our laboratory we have been studying alterations in plasma, liver, and brain folate concentrations produced by dietary restriction of folic acid. We have also been examining the effects of reduced tissue folate levels on brain development. In a series of early studies [18, 19, 20] employing a diet with low folic acid content (1.1 mg per kg diet) we found that: (1) folate restriction one week prior to parturition lowered liver folate concentrations in offspring of C57BL/6J or DBA/2J; (2) folate restriction started one week prior to parturition increased mortality and reduced growth rate in mice of both strains; (3) folate restriction started at birth produced increased mortality and a transient reduction in body weight in DBA/2J mice but not in C57BL/6J mice; (4) folate restriction started before or at birth produced some rather complex and as yet unclarified alterations in the brain, as evidence by correlations between the amount of folate present in liver and the nucleic acid concentration and serotonin concentration in brain; and (5) folic acid restriction started before or at birth increased the duration of susceptibility of DBA/2J mice to audiogenic seizures.

From the results of these studies we recognized that variability in the data obtained for brain concentrations of nucleic acids, protein, and serotonin might be due to a variability in the concentration of folate present in the brains of some experimental animals. It has been well documented that the brain is frequently spared when other organs show changes as a result of dietary manipulations [7]. Studies recently completed on adult DBA/2J mice in

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our laboratory demonstrated that plasma and liver folate levels can be reduced extensively before reductions in brain tissue folate concentrations are observed [21]. Although McClain and Bridgers [14] have reported changes in brain tissue folate concentration in developing Swiss-Webster mice, no one has yet demonstrated that brain folate concentration can be affected by dietary manipulation during development. The following experiments were undertaken to determine if brain tissue folate concentration could be lowered in developing DBA/2J mice by restricting dietary folate starting at birth or 1 week prior to birth. In the groups of mice exposed prenatally to dietary manipulation, additional experiments were designed to determine if lowered concentration of folate in the brain would produce changes in SAM concentration in stressed and unstressed mice. Stress was used to increase the turnover of catecholamines thus increasing the utilization of SAM in the COMT catalyzed metabolism of these amines. If reduced folate levels limit the supply of SAM, the effect should be magnified by increasing the demand for SAM by stress. In addition, possible behavioral malfunctions produced by lowered folate concentrations were assessed by observing open field activity and conditioned escape behavior.

METHOD AND PROCEDURES

Animals and Procedures

DBA/2J mice of the Jackson Laboratories stock were used in both experiments. They were maintained in a temperature regulated colony room (23° ± 2°C) on a 12 hr light:12 hr dark cycle with lights on at 0700 hr. Dietary manipulation was begun either at birth or 1 week prior to birth. Litters were culled to 6 upon delivery and litters with less than 4 pups were eliminated from the study. For the postnatal study, dams and their pups were started on 1 of 3 diets differing only in concentration of folic acid on the day of delivery. The diets were obtained from Nutritional Biochemical Company, Cleveland, Ohio, and contained no folate (FO), 1.1 mg folic acid per kg food (F1.1) or 9.9 m folic acid per kg food (F9.9). The F9.9 diet containe approximately 5 times more folic acid than recommende requirements (Nutritional Biochemical Company) and was used as a control to assess the effects of restricted folate intake. The dams and pups remained on their assigned diet until the pups were 35 days of age at which time the pups were weighed and killed for assays. In this and the following experiment, food was removed at 1700 hr and the animals were killed by decapitation between 0900 hr and 1300 hr the following day. The order of killing was counter-balanced among the 3 groups.

For the prenatal study, pregnant DBA/2J mice were obtained from the Jackson Laboratories (Bar Harbor, Maine). One week after their arrival (*i.e.*, on Day 15 of pregnancy), they were started on the FO diet, the F9.9 diet or remained on Wayne Breeder Blox Lab Chow (LC). The dams and pups were maintained on their assigned diet until the pups were 35 days of age at which time the pups were tested behaviorally or received shock stress as described below and then killed for biochemical assays.

Folic Acid Assays

In the postnatal study, folic acid was determined by the microbiological assay of Bennet *et al.* [3]. The brains and livers were quickly removed, weighed, and homogenized with a Brinkman polytron with power set at 4 for 5 sec at 4°C in 5.0 volumes of freshly prepared ascorbic acid (10 mg/ml, pH 6.0). The samples were heated in 75°C water bath for 30 min and centrifuged for 10 min at 10,000 RPM in a Sorval RC2B (SS 34 rotor) centrifuge. The liver supernatants were diluted 1:50 with the ascorbic acid and stored along with brain supernatants at −75°C until time of assay. Assays were done in the laboratories of the Communicable Disease Center in Atlanta, Georgia.

For the prenatal studies, folate levels were determined with a competitive protein binding radioassay supplied by the Schwarz/Mann Co. [8]. We have substituted this method for the microbiological assay due to frequent difficulties we have experienced in obtaining reproducible results with the microbiological assay.

S-Adenosyl Methionine (SAM)

SAM levels were measured by the double isotopic dilution method of Baldessarini and Kopin [2]. The tissue was homogenized in 4 volumes of ice cold 10% trichloracetic acid in 0.05N HCl and the homogenate centrifuged at 1,500 × G at 4°C for 30 min. $^{14}CH_3$ S-adenosylmethionine (0.1 ml, 15 nCi, 14 nmole, New England Nuclear) was added to 1.0 ml of the supernatant, and the mixture washed 3 times with ice-cold ether saturated with 0.05N HCl. Phosphate buffer (1.0 ml of 0.067M, pH 6.7 to which 4.2 mg/ml of $NaHCO_3$ has been added just prior to use) and ^{3}H-acetyl-N-acetylserotonin (approximately 21 nmoles, 400 nCi) synthesized according to Kopin *et al.* [12] were added to the washed supernatant. After a pre-incubation of 5 min, 0.1 ml of hydroxyindole-0-methyl-transferase (0.03 units of crude enzyme prepared according to Kopin and Baldessarini [11]), was added and the mixture incubated at 37°C with gentle stirring.

The reaction was quenched by the addition of 2 ml of ice cold 1N NaOH, transferred to a scintillation vial, and evaporated to dryness. The residue, taken up into 10 ml of a toluene:Liquifluor (New England Nuclear) scintillation solution, was assayed for ^{3}H and ^{14}C by liquid scintillation spectrometry.

Identification of melatonin was made by thin layer chromatography of the chloroform extract on cellulose with isopropanol:5% NH_4OH (2:1) solvent. Routinely, there was one radioactive sport with R_f identical to that of melatonin.

Stress Procedure

Some of the animals assayed for SAM in the prenatal study were subjected to shock stress prior to killing. Animals were stressed in groups of 3 by scrambled shock through the grid floor of a 16 × 18 × 21 cm Plexiglas box. Shocks of 4 mA and one sec duration were delivered at a rate of 6 per min for 15 min. Immediately after stress, animals were killed for SAM assay.

Behavioral Tests

Activity of individual animals was assessed over 30 min intervals in 1 of 3 Polycarbonate cages (32 × 21 × 13 cm). The cages were enclosed in sound attenuated chambers and were divided into quadrants by 2 photobeams. Activity was indexed by the activation of photocells produced by interruption of their respective light sources. All testing was

completed between 0800 and 1200 hrs when the pups were 35 ± 2 days of age.

Conditioned escape behavior was assessed using a procedure developed and described by Calhoun and Murphy [6]. The apparatus for this test, previously described by Jarvik [9], was a trough-shaped box divided into a start and a shock compartment by a guillotine door. During training, the animal was placed in the start compartment facing away from the door. Two sec later, the door was raised and the animal was allowed to enter the shock compartment. Animal contact with sensors located 8 cm from the door initiated delivery of 1 sec of 1.0 mA shock. Five sec after shock, the animal was removed from this compartment and returned to its home cage. Testing occurred 24 hrs later and involved placing the animal in the compartment where it had previously been shocked and recording the latency to leave the compartment.

Statistical Analysis

Data were analyzed with one-way analyses of variance followed by t tests for comparisons between groups unless otherwise specified. Differences with probabilities of 0.05 or less were considered significant.

RESULTS

Postnatal Folate Deficiency

The effects of rearing mice on reduced folate diets from birth to 35 days of age on body, brain, and liver weight; and on folate concentration in liver and brain tissue are summarized in Table 1. Although body weights of 35-day-old offspring started on the three diets at birth were not affected by folic acid intake, brain and liver weights were significantly altered. Brain weight of animals reared on the FO diet was 9% lower than that of animals reared on the F9.9 diet and significantly lower than animals reared on either the F1.1, or F9.9 diets. Liver weights for the FO animals were elevated compared to the other 2 groups.

Liver folate concentration was reduced in animals reared on either of the reduced folate diets (FO or F1.1) compared to that of animals reared on the F9.9 diet. Brain folate concentration for animals reared on the FO diet was 20% lower than that of animals reared on the F9.9 diet. In a separate assay, brain folate concentration of animals reared on the F1.1 diet was not different from that of animals reared on the F9.9 diet, however, values for the F9.9 group were approximately 6–7 fold lower than those reported in Table 1. Hence, we cannot at this time compare the effects of the F1.1 diet with the FO on brain folate.

Perinatal Folate Deficiency

Starting dams on either the FO or F9.9 diets prior to parturition drastically reduced the survival of offspring. By 21 days of age, mortality for animals reared on either the FO or F9.9 diets was significantly (χ^2 test, $p<0.01$) higher than that for animals reared on the LC diet. By 35 days of age mortality was 15% and 26% higher for animals reared on F9.9 and FO diets than for LC controls.

The effects of restricted dietary folate on body weight, organ weights, and folate concentration in offspring surviving until 35 days of age are summarized in Table 2.

Initiating the FO diet 6–7 days prior to birth produced significant reductions in body, brain, and liver weights of animals surviving to 35 days of age compared to animals reared on either the high folate, F9.9, or the LC diets. Folate concentrations in liver and brain tissue of animals reared on the FO diet were 78% and 38% lower than values obtained for animals reared on the F9.9 diet and were also significantly lower than values obtained for the LC animals.

The results of SAM assays are summarized in Table 3. A one-way analysis of variance of these data revealed that liver SAM concentration was altered as a function of dietary condition $F(2,30) = 3.333$; $p<0.05$. As noted in Table 3, mean liver SAM values for animals reared on either the FO or the F9.9 diets were lower than the mean value for animals reared on lab chow. The only significant group difference, however, is between the FO and LC groups ($t(16) = 2.247$, $p<0.05$).

Data for brain SAM were analyzed with a 2 (Stress) x 3 (Diet) analysis of variance. This analysis revealed significant effects of Stress ($F(1,57) = 24.4$, $p<0.01$) and Diet condition $F(2,57) = 4.63$, $p<0.05$). The diet effect was due to lower SAM levels in animals reared on FO or F9.9 diets with no difference between these 2 groups. That the stress procedure did not differentially affect animals reared under the three diet conditions is indicated by the lack of significance ($F(2,57) = 1.49$, $p<0.1$) of the Stress x Diet interaction. As noted in Table 3, the stress procedure lowered concentration of SAM in brain tissue for all 3 groups.

TABLE 1

EFFECTS OF REDUCING DIETARY FOLIC ACID BEGINNING AT BIRTH ON FOLATE CONCENTRATION AND BODY AND ORGAN WEIGHTS OF 35-DAY-OLD DBA MICE

	FO $\bar{X}$ ± SEM (N)	F1.1 $\bar{X}$ ± SEM (N)	F9.9 $\bar{X}$ ± SEM (N)
Body Weight (g)	13.2 ± 0.8 (12)	14.6 ± 0.7 (12)	14.9 ± 0.5 (32)
Brain Weight (mg)*	311 ± 0.8 (10)	330 ± 4 (35)	337 ± 3 (43)
Liver Weight (mg)†	832 ± 62 (10)	565 ± 21 (35)	592 ± 26 (43)
Liver Folate (μg/g)‡	4.6 ± 0.6 (10)	3.1 ± 0.4 (26)	11.3 ± 0.6 (32)
Brain Folate (μg/g)§	0.59 ± .02 (10)	—	0.73 ± .08 (8)

*FO<F1.1, F9.9; $p<0.01$.
†FO>F1.1, F9.9; $p<0.01$.
‡FO, F1.1<F9.9; $p<0.01$.
§FO<F9.9; $p<0.01$.

TABLE 2

EFFECTS OF REDUCED DIETARY FOLATE BEGINNING THE LAST THIRD OF GESTATION ON FOLATE CONCENTRATION AND ON BODY AND ORGAN WEIGHTS OF 35-DAY-OLD DBA/2J MICE

	FO $\bar{X}$ ± SEM (N)	F9.9 $\bar{X}$ ± SEM (N)	LC $\bar{X}$ ± SEM (N)
Body Weight (g)*	8.8 ± 0.4 (13)	13.3 ± 0.6 (15)	14.4 ± 0.5 (28)
Brain Weight (mg)†	300 ± 4 (13)	327 ± 5 (15)	345 ± 5 (15)
Liver Weight (mg)	508 ± 52 (10)	655 ± 32 (15)	726 ± 21 (12)
Liver Folate (μg/g)§	9.0 ± 1.4 (13)	40.8 ± 4.6 (14)	35.2 ± 2.7 (15)
Brain Folate (μg/g)	0.8 ± 0.1 (13)	1.3 ± 0.1 (15)	1.2 ± 0.1 (15)

*FO<F9.9, LC; $p<0.01$.
†FO<F9.9<LC; $p<0.05$.
‡FO<F9.9, LC; $p<0.05$.
§FO<F9.9, LC; $p<0.001$.
FO<F9.9, LC; $p<0.001$.

TABLE 3

EFFECTS OF REDUCED DIETARY FOLATE ON CONCENTRATION OF S-ADENOSYL-L-METHIONINE (μg/g) IN LIVER AND BRAIN TISSUE OF 35-DAY-OLD DBA/2J MICE

	NONSTRESS		STRESS
	Liver* $\bar{X}$ ± SEM (N)	Brain† $\bar{X}$ ± SEM (N)	Brain $\bar{X}$ ± SEM (N)
LC	40.3 ± 3.1 (9)	21.1 ± 1.6 (14)	12.8 ± 1.1 (11)
F9.9	35.8 ± 1.5 (15)	14.1 ± 0.9 (13)	10.8 ± 0.7 (5)
FO	32.3 ± 1.3 (9)	16.3 ± 1.3 (15)	10.0 ± 0.3 (5)

*FO<LC; $p<0.05$.
†FO, F9.9<LC; $p<0.05$.

The results of the behavioral tests are summarized in Table 4 which shows that animals reared on the FO diet had lower activity scores than those reared on the F9.9 or LC diets and that animals from the three groups did not differ in escape latencies on the conditioned escape task.

TABLE 4

EFFECTS OF REDUCED DIETARY FOLATE ON LOCOMOTOR ACTIVITY AND ON CONDITIONED ESCAPE BEHAVIOR OF 35-DAY-OLD DBA/2J MICE.

Dietary Condition	Activity Counts* $\bar{X}$ + SEM (N)	Escape Latency Sec. $\bar{X}$ + SEM (N)
LC	261 ± 15 (20)	11.6 ± 1.8 (18)
F9.9	282 + 27 (20)	13.6 + 1.8 (11)
FO	191 ± 19 (17)	17.3 + 3.3 (12)

*FO<F9.9, LC, $p<0.02$.

DISCUSSION

From the results obtained with animals started at birth on the diets, it is evident that either of the restricted folate diets (FO or F1.1) initiated at birth is capable of reducing liver stores of folic acid in 35-day-old DBA mice. Brain folate stores were reduced in mice reared on a folate free diet (FO), however not to the extent as liver folate. This finding is in agreement with a previous study on adult DBA mice [21] and is of importance for studies assessing the effects of folate deficiency on brain function. Body weight was not altered by initiating folate restrictions at birth. This finding is in agreement with previous findings reported by Schreiber and Zemp [18] for 35-day-old DBA mice reared on the F1.1 diet. Brain weights were significantly reduced in mice reared on the FO diet which may suggest retarded brain growth. However, the reduction is slight (~9%) and in the absence of other indices of growth (e.g., DNA, RNA, protein concentration) should not be emphasized. Liver weights in animals reared on the FO diet were greater than controls. We have noted this on occasion in folate deprived animals of other studies and hypothesized that the increase may indicate fatty infiltration of liver. Histology on livers in the current study, however, provided no support for this hypothesis. It should also be pointed out, that increased liver weight as a result of folate restriction has not been a consistent finding.

The most significant effect of initiating the FO diet prior to birth rather than at birth was the large reduction in body weight. In addition, brain weight and folate concentration were more extensively lowered in these animals. Compared to animals reared on the F9.9 diet brain weight was reduced 13% for animals started on the FO diet prior to birth and 9% for animals started on the diet at birth. Brain folate concentration was reduced 38% and 19% for animals started on the diet prior to birth and at birth respectively.

The folate assays establish that dietary folate restriction during ontogenesis can reduce tissue folate concentration in brain and liver. Initiating a folate free diet prior to birth produced a more severe reduction in brain stores of folate than initiation at birth and also clearly retarded develop-

ment as evidenced by reduced body, brain, and liver weights. It is important to note that the reduced folate levels observed in these experiments were produced by restricting dietary folate alone. It has been reported that folic acid can be synthesized by intestinal bacteria of rodents [10] and addition of sulfa drug to either the diet or drinking water is commonly used to eliminate this source of folic acid [5, 15, 16, 17, 21]. We have observed that addition of sulfasuxidine to drinking water of adult DBA mice maintained on the F1.1 diet causes a more extensive reduction of liver folate than that produced by the F1.1 diet alone [21]. This procedure was not utilized in the current study however, because our primary interest was to induce a folate deficient state that is compatible with survival and our previous work [20] suggests that virtually none of the neonates maintained on the F1.1 diet plus sulfamethazine survive.

The results of the SAM assays were unexpected. SAM was reduced in liver and brains of animals reared on the folate free diet compared to those reared on lab chow. This finding agrees with Ordonez and Wurtman's report of reduced SAM concentration in liver and brain tissue of adult rats maintained on a diet regimen which reduced serum folate to about 14% of control values [17]. Animals maintained on a diet containing 5 mg folic acid per kg diet had SAM levels comparable to controls. In the present study, however, we observed reduced SAM concentration in animals reared on a high folic acid diet (F9.9) even though brain a liver folate concentrations for the group were comparable to those of the LC group. Thus, the lower SAM concentration observed in this study cannot be attributed exclusively to lower tissue folate stores. We currently have no satisfactory explanation for the different results obtained in the 2 studies. Procedural differences in using different species, age groups, and length of time on the diet make a direct comparison impossible.

Shock stress lowered brain concentration of SAM, however, it did not differentiate folate deficient animals from those with adequate folate stores. Reduced SAM concentration in brain following environmental stress is a new finding. Although various drugs known to increase catecholamine metabolism have been reported to lower SAM levels in brain [1, 2, 17], environmental stressors such as saline injections and cold stress were reported to be ineffective in altering SAM levels [1]. Data from the current study, however, suggest that a rather severe stress procedure which has previously been shown to increase catecholamine release [13] produces the anticipated decreases in SAM concentration.

The results of the behavioral measurements show that animals with low brain levels of folate are less active than those with adequate folate stores. The relationship between the folate depletion and activity is not clear and may be only a reflection of the overall state of health of the mice. It should be noted, however, that they perform as well as control animals on a simple conditioned escape task.

In summary, it is evident from these data that liver and brain tissue concentrations of folic acid can be reduced by dietary manipulations of this vitamin during ontogenesis. Reduction of folate concentration in brain is not as extensive as that for liver for the 35 day period studied. Initiation of a folate free diet during gestation produces a more severe reduction in brain folate concentration than starting the diet at birth. Prenatal reduction of dietary folate also clearly retards body growth as evidence by reductions in body, brain, and liver weights. Initiating folate restriction at birth does not produce the extensive retardation of growth, but can still cause a slight reduction in brain weight. Behaviorally, animals deprived prenatally were less active than those maintained on diets containing higher folate concentrations F9.9 and LC; however, they performed as well as controls on a shock motivated escape task. The additional findings of increased mortality and reduced SAM concentrations cannot be attributed exclusively to lowered folate intake since animals reared on either high or low folate concentrations in special diets had reduced survival and reduced concentrations of SAM relative to those reared on LC.

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