# Effect of ethanol consumption during pregnancy on folate coenzyme distribution in fetal, maternal, and placental tissues

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*Ethanol has been shown to be a teratogen in humans and experimental animals; however, the mechanism of its actions is not known. Because ethanol interferes with the metabolism of folate coenzymes that participate in various biosynthetic pathways of nucleic acids, the effect of gestational ethanol consumption on the distribution of folate coenzymes in tissues was investigated with an eye to the possibility that such effects might shed light on the mechanism by which ethanol produces its teratogenic effect. Sprague-Dawley rats were fed a 35% ethanol-calorie liquid diet from gestation days 7–21; controls were pair-fed with isocaloric sucrose substituted for ethanol. Rats were killed on day 21. Food intake, maternal weight gain, litter size, and conceptus weight were similar between ethanol and control rats. However, fetal weight was decreased and placental weight increased in ethanol group as compared with the control. Total folates and folate coenzymes were determined in fetal liver and brain, placenta, and maternal liver. Ethanol consumption decreased 5-methyltetrahydrofolate in the placenta, which was compensated by increasing tetrahydrofolate and formyltetrahydrofolates. In fetal liver, formyltetrahydrofolates and formiminotetrahydrofolate were decreased and tetrahydrofolate increased with ethanol. In fetal brain, ethanol not only decreased total folates but also altered the coenzyme pattern by decreasing 5-methyltetrahydrofolate and increasing tetrahydrofolate and formyltetrahydrofolates. These changes in folate coenzyme pattern indicate ethanol-induced alteration in folate metabolism. The possible physiologic significance of these changes in relation to Fetal Alcohol Syndrome is discussed.*

**Keywords:** ethanol; gestation; fetus; total folates; folate coenzymes

## Introduction

Ethanol intereferes with the metabolism of folate coenzymes.[1,2] The major folate coenzymes found in animal tissues are 5-methyltetrahydrofolate (5-methyl-THF), 5-formyl-THF, 10-formyl-THF, 5-formimino-THF, and THF.[3] These folate derivatives participate in various biochemical reactions that involve purine and pyrimidine biosynthesis and amino acid metabolism. Embryonic growth is highly dependent on these reactions for normal development. Alterations in the distribution of these folate coenzymes in fetal tissues may cause functional folate deficiency and lead to fetal malformations. To understand the cellular metabolism of folate, quantitation of these individual folate derivatives is essential. To date, no studies have reported on the effect of gestational ethanol exposure on the distribution of individual folate coenzymes. In this study we examined the effect of ethanol on the distribution of the major folate coenzymes in maternal liver, placenta, fetal liver, and fetal brain, as well as on the total folate content in these tissues.

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## Materials and methods

### Chemicals

2-mercaptoethanol, folic acid, THF, 5-methyl-THF, and 5-formyl-THF were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 10-formyl-THF was synthesized by the procedure of Rabinowitz.[4] 5-formimino-THF was prepared by the method of Tabor and Wyngarden.[5] Standard solutions of these folates were purified and prepared as previously described.[6] Tetrabutyl-ammonium phosphate was obtained from Waters Associates, Inc. (Milford, MA, USA). Methanol (high-pressure liquid chromatography [HPLC] grade) was obtained from Fisher Scientific (Fairlawn, NJ, USA). *Lactobacillus casei* (7469) was obtained from American Type Culture Collection (Rockville, MD, USA). Folic acid casei medium and Lactobacillus broth AOAC were from Difco Laboratories, Inc. (Detroit, MI, USA). All other chemicals were reagent grade.

### Animals

Twenty female Sprague-Dawley rats, weighing 220–250 g (about 80 days old) were obtained from Charles River Breeding Labs, Inc. (Wilmington, MA, USA). They were housed individually in plastic cages with stainless steel wire bottoms and maintained on a stock diet of Purina Lab Chow (Ralston Purina Co., St. Louis, MO, USA) and water ad libitum in our animal quarters (22–23°C, 12-hour light:dark cycle) for 1 week before mating. During mating, female rats were exposed overnight to potent males of the same age and strain, which were also fed Purina Lab chow. The presence of sperm in the vaginal smear the next morning denoted day 1 of pregnancy.

### Diet preparation and feeding procedure

The liquid diets were prepared in this laboratory from chemically pure ingredients. The liquid diet composition and preparation procedures were described elsewhere.[7] The liquid diets were given from gestation day 7 through gestation day 21. Impregnated rats were paired according to their body weights. The ethanol-containing diet was given ad libitum to one of the animal pair while its control was pair-fed with liquid diet containing sucrose substituted isocalorically for ethanol. To allow for adjustment to the ethanol diet rats in the ethanol group were introduced to the ethanol-containing diet in progressively increasing concentrations from 20%–35% ethanol-calorie in 1 week's time.

### Autopsy of animals

On gestation day 21, rats were anesthetized with ether, conceptuses were removed immediately by caesarean section, and the number of implantations and resorptions, the size of litters, and fetal and placental weights were recorded. Fetuses were examined for external abnormalities. Maternal liver, fetal liver, and fetal brain were dissected and the weights recorded. Tissues for folate determination were immediately placed on petri dishes on ice, sliced with a razor blade, and prepared for folate assay.

### Tissue preparation for folate assay

Tissues were heated in three volumes of hot 1.5 M 2-mercaptoethanol in a boiling water bath for 5 minutes. After cooling, the samples were homogenized and the homogenates were centrifuged at 40,000*g* for 15 minutes. The supernatants were kept frozen until assayed. Before assay, aliquots were treated with a hog kidney polyglutamate hydrolase (conjugase) preparation as described previously[6] to convert tissue folates to the monoglutamyl forms.

### Determination of folates by microbiological method

Determination of folate content in the conjugase treated tissue extracts (total folates) and in HPLC fractions (individual folate coenzymes) was achieved by a modified *L. casei* microbiologic analysis using 96 well plates and a 96 well plate reader.[8] 5-formyl-THF was the standard folate in this assay.

### Separation of folate coenzymes by HPLC

After determination of total folate levels, the conjugase treated samples were filtered with a 0.2μm membrane filter and individual folate coenzymes were separated by ion pair chromatography.[6,9] Basically, folates were separated by gradient elution (2 mL/min of 27%–32% methanol-water containing 5 mmol/L tetrabutylammonium phosphate) from a 4 mm × 30 cm Varian Micropak (Varian Associates, Palo Alto, CA, USA) MCH-10 column (10 μm particle size) into tubes containing 2-mercaptoethanol (0.4 mL, 1.5 M). Levels of individual folate derivatives in each sample were computed by summing the amount in each peak and dividing by the amount of total folate. Data were expressed as percent of total folate. Because 10-formyl-THF is partly converted to 5-formyl-THF by these procedures,[6] these data have been combined as 5/10-formyl-THF.

### Statistical analysis

Litter means were used as the unit of analysis. The data, presented as means with their standard errors (SEM), were analyzed by Student's $t$ test or Fisher's exact test. Results were considered statistically significant at $P < 0.05$.

## Results

Food and ethanol intakes and pregnancy outcome parameters are presented in *Table 1*. Calorie intakes were comparable to reported values for pregnant rats fed conventional powder diets ad libitum.[10] No significant differences between ethanol-fed rats and their pair-fed controls were found in food intakes, maternal weight gain, maternal liver weight, implant, litter size, conceptus weight, fetal liver, and brain weights. However, the fetal weight was significantly decreased by 10% and the placental weight significantly increased by 22% in the ethanol group as compared with the control. These findings were consistent with the reports in the literature.[11,12] No external anomalies were observed in either group.

*Table 2* presents the levels of total folates in the tissues. Ethanol consumption during pregnancy affected total folate levels in some of the tissues. Maternal liver folates were increased by 22% and fetal brain folates were decreased by 15%. Among the four tissues examined, maternal liver had the highest concentration of folates followed by fetal liver, which had a concentration of 55%–75% of maternal liver, then placenta, 30%–40% of maternal liver. Fetal brain had the lowest concentration, which amounted to only 15%–20% of maternal liver.

The tissue distributions of individual folate coenzymes, expressed as percentage of total folates, are presented in *Figure 1*. The major folate coenzymes found in maternal liver, placenta, and fetal brain were

**Table 1** Food and ethanol intake and pregnancy outcome

| | Ethanol | Control |
|---|---|---|
| Food intake (Cal/kg/day) | | |
| Day 7 to 14 | 302.9 ± 8.6 | 305.7 ± 9.0 |
| Day 14 to 21 | 249.5 ± 7.5 | 250.5 ± 8.5 |
| Ethanol intake (g/kg/day) | | |
| Day 7 to 14 | 10.42 ± 0.78 | — |
| Day 14 to 21 | 12.19 ± 0.27 | — |
| Maternal weight gain (g) | | |
| Day 7 to 21 | 79.5 ± 20.4 | 85.9 ± 15.3 |
| Maternal liver weight (g) | 13.92 ± 0.33 | 12.90 ± 0.44 |
| Implant | 13.80 ± 0.55 | 14.40 ± 0.61 |
| Litter size | 12.80 ± 0.55 | 14.00 ± 0.65 |
| Conceptus weight (g) | 62.5 ± 3.9 | 68.9 ± 3.00 |
| Fetal weight (g) | 3.32 ± 0.08* | 3.67 ± 0.04 |
| Placental weight (g) | 0.509 ± 0.014† | 0.417 ± 0.013 |
| Fetal liver weight (g) | 0.259 ± 0.008 | 0.275 ± 0.006 |
| Fetal brain weight (g) | 0.146 ± 0.003 | 0.154 ± 0.002 |

Values are given as mean ± SEM of ten dams or litters.
* Significantly different from control at $P = 0.0006$.
† Significantly different from control at $P = 0.0001$.

**Table 2** The effect of ethanol on total tissue folates

| | Ethanol | Control |
|---|---|---|
| | (nmole/g) | |
| Maternal liver | 12.85 ± 0.79* | 10.50 ± 0.55 |
| Placenta | 3.58 ± 0.17 | 4.10 ± 0.21 |
| Fetal liver | 6.92 ± 0.43 | 7.83 ± 0.35 |
| Fetal brain | 1.66 ± 0.06† | 1.96 ± 0.07 |

Values are given as mean ± SEM of ten dams or litters
* Significantly different from control at $P = 0.025$.
† Significantly different from control at $P = 0.005$.

5-methyl-THF, which comprised 70%–80% of total folates, followed by THF (10%–20%) and 5/10-formyl-THF (5%–10%). 5-formimino-THF was not detected in placenta and fetal brain, and was found only as minor component (1%–12.5%) in maternal liver.

The distribution of individual folate coenzymes in fetal liver was different from that in the other tissues examined. In fetal liver, 5/10-formyl-THF were the major forms (about 40% of the total), whereas 5-methyl-THF and THF existed in somewhat smaller proportions (25%–30% of total folates). 5-formimino-THF comprised about 6% of total folates, a greater proportion than that in the other tissues.

The effects of ethanol on individual folate coenzymes differed from tissue to tissue. In the maternal liver, no difference was observed between the ethanol-fed and the control groups in the distribution of individual folate coenzymes expressed as percentage of total folates. Thus, the concentrations of each individual folate coenzyme were increased in parallel with the increase in the total folates by ethanol treatment.

In the placenta the distribution of the individual folate coenzymes was altered by ethanol. 5-methyl-THF, the most abundant constituent, was decreased by 14%, but THF and 5/10-formyl-THF, secondary

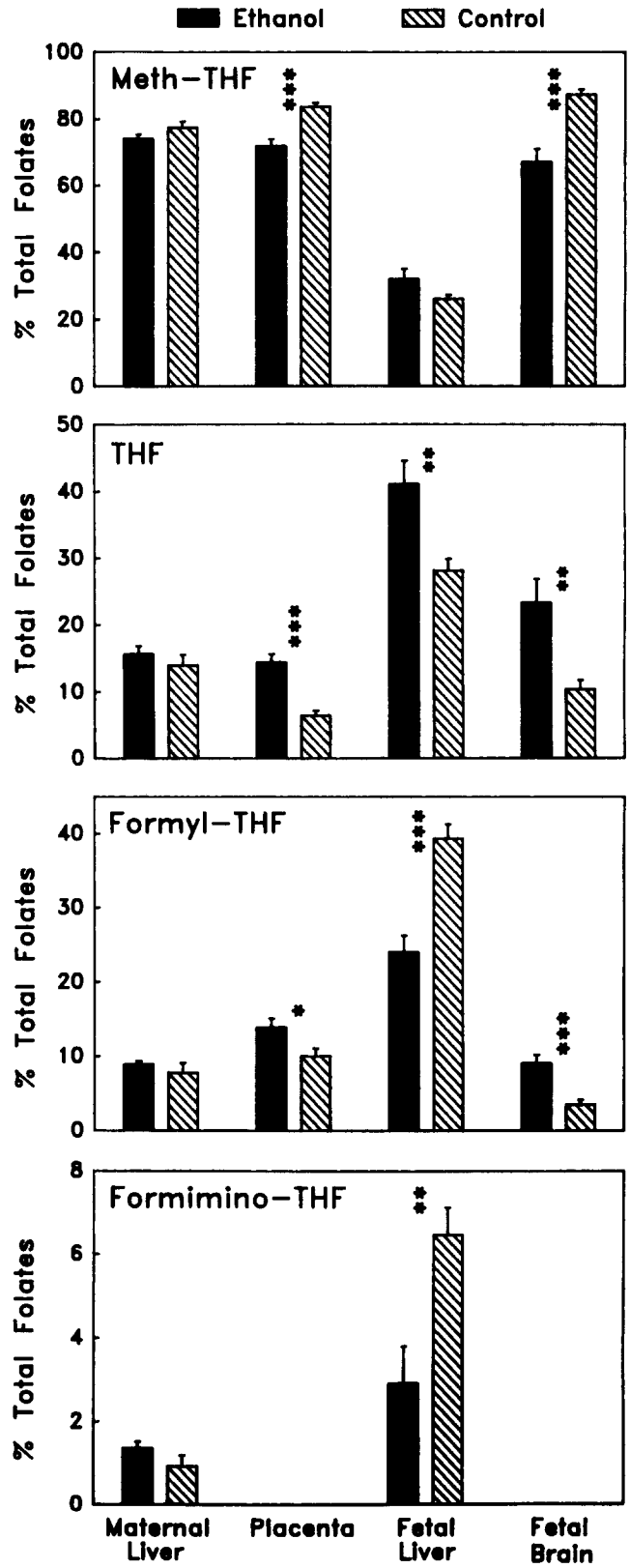


**Figure 1** The effect of ethanol feeding during pregnancy on the distribution of folate coenzymes in maternal, placental, and fetal tissues. Meth-THF, 5-methyltetrahydrofolate; THF, tetrahydrofolate; Formyl-THF, 5/10-formyltetrahydrofolates; Formimino-THF, 5-Formiminotetrahydrofolate; Each bar represents the mean ± SEM; * $0.01 < P < 0.05$, ** $0.001 < P < 0.005$, *** $P < 0.001$.

constituents, were increased by 122% and 38%, respectively. Consequently, total folates remained unchanged.

In the fetal liver, ethanol had no effect on the level of 5-methyl-THF, but the other folate derivatives were greatly affected. THF, one of the major components, was increased by 46%, which was compensated by decreases in 5/10-formyl-THF (39%) and 5-formimino-THF (55%). Therefore, as in the placenta, total folates were not changed.

In the fetal brain, ethanol feeding increased THF and 5/10-formyl-THF levels by 125% and 157%, respectively. However, 5-methyl-THF, the major folate coenzyme of fetal brain, was decreased by 22%, resulting in a net decrease in total folate level in ethanol group.

## Discussion

Maternal alcohol consumption has been recognized as a major fetal health hazard—a leading cause of growth deficits, mental retardation, and physical anomalies, the Fetal Alcohol Syndrome (FAS). However, the underlying mechanisms of the various aspects of ethanol teratogenicity are not known. Proposed mechanisms[13] have included direct effects of ethanol or its metabolite, acetaldehyde, on developing cells, fetal hypoxia due to decreased oxygen delivery, altered prostaglandin metabolism,[14] and maternal or fetal malnutrition.[15] Maternal malnutrition could result from decreased food intake, impaired digestion or absorption of nutrients, or altered metabolism of absorbed nutrients. Undernourishment of the fetus could result from maternal malnutrition, inhibition of placental transfer of nutrients,[16] or altered metabolism of nutrients within the fetus. Ethanol-induced changes in fetal metabolism of nutrients could produce a relative or functional deficiency of a key nutrient form, even though the total level of the nutrient is not deficient.

Folic acid is essential for the processes of cell differentiation and tissue growth because of its role in nucleic acid synthesis. Interference in folate metabolism by ethanol may cause deficiency of one of the coenzymic forms of the vitamin that are involved as cofactors in the biosynthetic pathway of nucleic acids. Key folate forms involved in nucleic acid synthesis are 10-formyl-THF, as the one carbon donor in purine synthesis, and THF as the immediate precursor to 5,10-methylene-THF, the folate involved in thymidylate synthesis. 5-methyl-THF, because of its role in methionine synthesis and thus production of S-adenosylmethionine,[17] would influence methylation reactions involving nucleic acids, protein, and lipid biosynthesis.

Results from this experiment showed that gestational ethanol consumption altered total folate levels (*Table 2*), as well as the distribution of folate coenzymes within certain fetal tissues, as summarized in *Figure 1*. The most noticeable changes occurred in the fetal brain, where ethanol significantly decreased total folates and also altered the coenzyme pattern. The brain is a well-protected organ. It is widely accepted that in nutritional deficiency the brain is spared relative to the rest of the body. Thus, a 15% decrease in fetal brain folate by ethanol ($P = 0.005$) could be physiologically significant. In fact, Fehling et al.[18] fed rats a folate-deficient diet for 9.5 months and observed only a 16% reduction in brain folate content, in contrast to a marked hepatic folate depletion. Ethanol decreased the proportion of 5-methyl-THF in the fetal brain and increased that of THF and the formyl-THF. The relevance of these changes is not clear from these studies. Several studies have linked folate deficiency with metabolic changes that might result from decreased methyl group in the brain. Shaw et al.[19] reported that folic acid deficiency decreases brain levels of DNA, RNA, and protein, and could produce changes in the development of the brain. Hirono and Wada[20] observed that gestational folate deficiency significantly decreases the myelin content of fetal brain as well as the brain weight. Arakawa et al.[21] also observed that folic acid deficiency decreases myelin lipid and delays the maturation of brain function. Clinical evidence also exists for a role of folate in the development of brain function. Erbe[22] reviewed the cases of inborn errors of folate metabolism and reported that functional and structural disorders of the brain have been observed in children with congenital malabsorption of folate, defective folate interconversion, or defective folate utilization. The most serious and probably the most sensitive manifestation of maternal alcohol abuse during pregnancy is central nervous system (CNS) dysfunction. As such, the decreased total folate content and the alteration of folate coenzyme pattern in fetal brain by ethanol could contribute to the CNS effects observed in FAS.

In the placenta, 5-methyl-THF was significantly decreased by ethanol, but THF and the formyl-THF were increased so that total folate levels were not changed. Lin and Lester,[23] using a microbiologic procedure to measure folate derivatives, reported that ethanol consumption during pregnancy altered the ratio of methyl-THF to nonmethyl-THF in the placenta from 50:50 to 70:30. This suggests an increase in 5-methyl-THF at the expense of nonmethyl-THF, although the individual nonmethyl-THF derivatives were not determined. The reason for the discrepancy between studies is not certain, although it may have arisen from differences in the diets used. For example, Case and Steele[24] reported that the distribution of hepatic folates varies between rats fed a casein-based purified diet and those fed a standard commercial diet (the latter decreases the proportion of 5-methyl-THF). In the present study, liquid diets were prepared in this laboratory from chemically pure ingredients with specified composition. In the aforementioned study, liquid diets were prepared from Nutrament (Mead Johnson, Evansville, IN, USA), a commercial liquid food with undefined composition. The different assay procedures may also have led to the differences observed because microbiologic differentiation of folate derivatives is less specific than HPLC separation.[6]

Ethanol did not alter total placental folate levels, but it may affect the transfer of folate from the maternal plasma to fetal plasma, as suggested by others.[25] Ethanol has been shown to decrease the activity of the placental folate binding protein (FBP).[25] Because this protein is probably responsible for the transport of physiologic amounts of folate across the placenta,[26] the inhibitory effect of ethanol would be expected to decrease the transfer of folate to the fetus. Although there was an altered placental distribution of folates, it is not clear how these changes might affect the transfer of folate to the fetus. Because the present study measured the total tissue levels of 5-methyl-THF, including that within the cell (mostly polyglutamate forms) as well as that bound to FBP on the surface (monoglutamate form), we could not determine whether the altered distribution of placental folates reflected changes in the transport pathway to the fetus. Further study is needed.

It is interesting to observe that the folate coenzyme distribution in fetal liver was much different from that of maternal liver, placenta, or fetal brain. In control fetal liver, the formyl forms were the major folates, although 5-methyl-THF and THF were equally represented at 25%–30% of the total folates. In contrast, in maternal liver, placenta, and fetal brain, the major folate was 5-methyl-THF, as generally found in animal tissues.[27] The differences may reflect the fact that the fetal liver is capable of a high degree of turnover, which would require a ready source of substrates for DNA synthesis. A greater proportion of folates in THF and the formyl-THF forms, rather than as 5-methyl-THF, would favor purine and pyrimidine synthesis[28] for DNA production. The effects of ethanol on fetal liver folate coenzymes were substantial. There were significant decreases in the 5/10-formyl and formimino forms. Since the formyl forms are directly involved in purine biosynthesis, the reduction in these folate coenzymes in fetal liver may alter cell differentiation and tissue growth.

Formimino-THF is formed when histidine is metabolized through the formiminoglutamic acid pathway to glutamic acid. Histidine can also be converted to histamine by the action of histidine decarboxylase, a folate independent pathway. In a previous study, we observed that ethanol consumption increases histamine produciton by 173%.[29] The shift of histidine metabolism to histamine pathway should decrease the histidine available for formiminoglutamate production and consequently the formation of formimino-THF in the fetal liver.

Ethanol increased the total folate level in the maternal liver, but did not alter the distribution of folates among coenzyme forms. Previous studies have shown similar results in adult male Sprague-Dawley rats given ethanol chronically[30] and acutely.[9] The reason for the apparent increase in hepatic folate levels is not known, but could be related to a decrease in biliary release of folate.[1] The proportion of 5-methyl-THF in maternal liver folates (70%–80% of total) is higher than what has been found in our other studies of rat liver folates.[6,9,31] As recently reviewed,[32,33] 5-methyl-THF generally comprises about 30%–60% of total rat liver folates. These previous studies of tissue folate distribution were done with male Sprague-Dawley (or Wistar) rats, whereas the present results came from pregnant female rats. The differences could therefore be sex- or condition-related. As discussed above, the distribution of hepatic folates can vary among different diets,[24] so another important difference would be the diets used in the various studies. Since the fetal liver contained less than 30% of the total folate as 5-methyl-THF, the high proportion of this folate in the other tissues did not result from an artifact of the preparation procedures (the same for all tissues).

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