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Interaction of Ethanol and Nutrition during Gestation: Influence on Maternal and Offspring Development in the Rat[1]

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ABSTRACT

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Although liquid diets containing ethanol have been widely used in rodent models of the fetal alcohol syndrome, the nutritional adequacy of these diets has not been verified for pregnant rats. Beginning on day 7 of gestation, maternal diet consumption and body weight were monitored in rats receiving Bio-Mix 711-PR with ethanol (B/A), pair-fed diet without ethanol (B/P), *ad libitum* liquid diet without ethanol and *ad libitum* solid diet. The B/P and B/A animals did not gain weight normally during pregnancy, reflecting a significant decrease in caloric and protein intake compared to *ad libitum* liquid diet without ethanol and *ad libitum* solid diet mothers. Offspring of B/A mothers were severely growth-stunted in body and brain weight at birth, but B/P pups were also stunted compared to *ad libitum* controls. Thus, it was concluded that Bio-Mix 711-PR did not provide sufficient nutrients to pregnant rats when intake of diet was reduced by ethanol. Therefore, the diet was reformulated to increase the protein content and caloric value. A comparison of the revised diet containing ethanol and pair-fed revised diet without ethanol with B/A, B/P and *ad libitum* solid diet animals was made. The maternal body weight gain and offspring body and brain weights of revised diet containing ethanol and pair-fed revised diet without ethanol mothers did not differ from controls. However, blood alcohol levels of mothers consuming the revised diet containing ethanol were significantly less than B/A rats despite equivalent ethanol intake. The results of this study indicate the importance of proper nutritional control in rodent models of the fetal alcohol syndrome. The revised diet will permit the investigation of prenatal ethanol exposure without the confounding variable of undernutrition.

Ethyl alcohol, unlike many other pharmacological agents that have abuse potential, must be ingested in gram amounts rather than milligram or less doses in order to exert its effects. Furthermore, ethanol can contribute significant caloric value to the diet of an individual when taken in large quantities. These factors, coupled with the almost universal aversion to voluntary intake by laboratory animals, makes the method of chronic alcohol administration a significant aspect of the design of studies investigating the influence of this drug. This proves to be especially important in studies which attempt to assess the teratogenic potential of alcohol.

The recognition in human infants of a set of signs and symptoms associated with high maternal alcohol intake during pregnancy, termed the fetal alcohol syndrome (FAS) (Jones *et al.*, 1973; Palmer *et al.*, 1974), has raised the need for carefully controlled animal models of this phenomenon. Such models could produce data on how much, how long and when, if ever, ethanol exposure during gestation is detrimental. An essential element of any rodent model of FAS is the method of exposing pregnant rats to alcohol. Criteria for an ideal procedure include the ability to modify the amount of alcohol administered, production of intoxicating blood alcohol levels, provision of adequate nutrition and control for environmental factors which might interact with the alcohol and nonalcohol groups differentially. Although many methods of alcohol administration have been employed with pregnant rats, most do not meet these criteria. Chronic i.p. (Kronick, 1976), s.c. (Morra, 1969) and i.v. (Demers and Kirouac, 1978; Sandor and Amels, 1971) injections are inherently stressful procedures which require daily handling and restraint of the pregnant subject. Intubation procedures (Abel, 1978, 1979; Abel and Dintcheff, 1978; Martin *et al.*, 1978b) suffer similar problems. Self-administration of alcohol through the drinking water (Borgman and Wardlaw, 1977; Øisund *et al.*, 1978; Schwetz *et al.*, 1978; Tze and Lee, 1975) results in only moderate blood alcohol levels but also decreases food intake, disrupting the nutrition of the mother.

Administering ethanol in a liquid diet has proven to be a more efficient procedure (Chernoff, 1977; Detering *et al.*, 1979; Druse and Hofteig, 1977; Henderson and Schenker, 1977; Randall *et al.*, 1977). Substantial blood alcohol levels are achieved and, with proper manipulation, nutrition can theoretically be controlled. Many of these liquid diet studies have used commercial human food product diets, but this approach is not suitable for rigorous experimental analysis because of a number of factors: lack of a long-range supply (*e.g.*, Metrecal, a widely

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used diet, is no longer marketed); variability of diet composition between shipments (*e.g.*, Nutrament, the diet used as a substitute for Metrecal, was reformulated to decrease protein content in 1979); presence of flavorings and preservatives which might have pharmacological or toxicological effects; undocumented applicability of these human formulations to meet rodent nutritional requirements; and inability to modify the commercial formula.

A diet composed of specified components has been proposed by DeCarli and Lieber (1967). Although the nutritional adequacy of this diet was established for adult male rats, its adequacy has not been verified for pregnant or lactating female rats. The nutritional requirements of the pre- and postpartum periods are clearly greater than those of adult maintenance levels (National Academy of Sciences, 1978). Thus, the following studies were undertaken to develop a nutritionally adequate liquid diet,containing ethanol for use in a rodent FAS model.

Methods

Subjects. Primiparous Sprague-Dawley rats weighing 240 ± 10 g were time-mated with Sprague-Dawley males. Vaginal smears were taken and presence of sperm marked day 1 of gestation. Animals were obtained from Zivic-Miller (Allison Park, PA) on day 5 of gestation. The females were housed one per cage (45 × 24 × 20 cm, polypropylene with wire lid). Wood shavings were provided as bedding and water was available *ad libitum*. The animal room was maintained at 23°C and 70% relative humidity with lights-on at 7 A.M. and lights-off at 7 P.M.

Diet composition and administration. Five diets (4 liquid and 1 solid) were used in these studies. Bio-Mix 711-PR (B) was obtained from Bio-Serv, Inc. (Frenchtown, NJ). This formula is a vitamin- and mineral-enriched modification of the original diet designed by DeCarli and Lieber (1967) for adult male rats. On the basis of the results of experiment 1, this liquid diet was reformulated to increase the protein content and was designated as revised diet 1226 (R). Alcohol-containing diets were formulated to contain 5% (w/v) ethanol (Rossville 190 proof pure ethyl alcohol; IMC Chemical Group, Agnew, CA) and the maltose-dextrin component was reduced accordingly so that the two forms of the diet were isocaloric. Table 1 summarizes the composition of the four liquid diets (with and without ethanol). A casein solid diet, diet 170590 (S), was obtained from Teklad Mills (Madison, WI) for comparison to the liquid diets (see table 2). This solid diet was chosen over standard rat chows because its exact composition is known and it contains the same source of protein, casein, as the liquid diets. Bio-Mix 711-PR, revised diet 1226 and solid diet 170590 provide 0.99 kcal/ml, 1.19 kcal/ml and 4.21 kcal/g, respectively.

TABLE 1
Composition of liquid diets with and without alcohol

	Bio-Mix 711-PR		Revised Diet 1226	
	Control	Ethanol	Control	Ethanol
	g/l	g/l	g/l	g/l
Casein, vitamin free	41.40	41.40	90.00	90.00
L-cystine	0.50	0.50	0.50	0.50
DL-methionine	0.30	0.30	0.30	0.30
Maltose-dextrins	114.04	24.40	114.04	25.30
Ethanol		50.00		50.00
Corn oil	8.50	8.50	8.50	8.50
Olive oil	28.40	28.40	28.40	28.40
Ethyl linoleate	2.70	2.70	2.70	2.70
Vitamin Mix 711-PR[a]	5.00	5.00	5.00	5.00
Salt Mix 711-PR[b]	13.43	13.43	13.43	13.43

[a] Vitamin Mix 711-PR (5.0 g) contains: *p*-aminobenzoic acid, 50 mg; vitamin B_{12}, 0.1 mg; calcium pantothenate, 20 mg; choline chloride, 750 mg; folic acid, 1.0 mg; inositol, 100 mg; menadione, 1.0 mg; niacin, 15 mg; pyridoxine HCl, 2.9 mg; riboflavin, 5.0 mg; thiamine HCl, 503 mg; vitamin A acetate (500,000 I.U./g), 8.0 mg; vitamin D_3 (500,000 I.U./g), 0.4 mg; and vitamin E acetate (500 I.U./g), 1.12 g.

[b] Salt Mix 711-PR (13.43 g) provides: Ca, 1.3 g, Cl, 1.02 g; Cu, 0.7 mg; I, 6.0 mg; Fe, 45.8 mg; Mg, 100.5 mg; Mn, 38.3 mg; P, 708 mg; K, 2.93 g; Na, 658 mg; S, 768 mg; and Zn, 11.4 mg.

TABLE 2
Composition of solid diet 170590

	g/kg
Casein, vitamin free	260.06
Sucrose	448.24
Corn starch	150.00
Vegetable oil	80.00
Cod liver oil[a]	20.00
Vitamin Mix[b]	1.70
Mineral Mix[c], USP XIV	40.00

[a] Cod liver oil (20.0 g) provides: vitamin A, 17,000 I.U.; vitamin D, 1,700 I.U.

[b] Vitamin Mix (1.70 g) contains: *p*-aminobenzoic acid, 10 mg; biotin, 0.3 mg; vitamin B_{12}, 15 mg; calcium pantothenate, 20 mg; choline chloride, 1.0 g; folic acid, 5.5 mg; inositol, 400 mg; menadione, 5 mg; niacin, 20 mg; pyridoxine HCl, 5 mg; riboflavin, 10 mg; thiamine, 5 mg; and dry vitamin E acetate (500 I.U./g), 199.7 mg.

[c] Mineral Mix (40.0 g) provides: Al, 0.2 mg; Ca, 4.4 g; Cl, 4.2 g; Cu, 0.8 mg; F, 9.2 mg; I, 1.2 mg; Fe, 107 mg; Mg, 657.7 mg; Mn, 2.6 mg; P, 2.67 g; K, 6.55 g; Na, 1.22 g; and S, 410.5 mg.

All diets were refrigerated until used. Fresh liquid diets were presented daily to the animals in 180-ml polyethylene bottles with rubber stoppers containing ball-point drinking tubes. Preliminary studies indicated that spillage and evaporation during the 24-hr period was less than 0.5 ml.

Blood alcohol determination. Mixed arterial and venous blood samples were obtained from the tails of unanesthetized animals and blood alcohol levels were assayed by a micromodification of the alcohol dehydrogenase method (Bonnichsen and Theorell, 1951). In experiment 1, blood samples were taken on days 14 and 18 at 1 P.M. and 11 P.M., respectively. In experiment 2, blood samples were taken on days 13 and 19 of gestation at 11 P.M. and 5 P.M., respectively.

Experimental design. The subjects of experiment 1 were 28 time-mated Sprague-Dawley females divided into four (n = 7) weight-matched groups: 1) *ad libitum* access to Bio-Mix 711-PR with 5% (w/v) ethanol (B/A); 2) limited access (pair-fed the amount consumed by B/A animals) to Bio-Mix 711-PR without ethanol (B/P); 3) *ad libitum* access to Bio-Mix 711-PR without ethanol (B/C); and 4) *ad libitum* access to solid diet 170590 (S/C). Diet was presented daily at 1 P.M., beginning on day 7 of gestation.

The subjects of experiment 2 were 65 time-mated Sprague-Dawley females divided into the following five (n = 13) weight-matched groups: 1) *ad libitum* access to Bio-Mix 711-PR with 5% (w/v) ethanol (B/A); 2) limited access (pair-fed to B/A animals) to Bio-Mix 711-PR without ethanol (B/P); 3) *ad libitum* access to revised diet 1226 with 5% (w/v) ethanol (R/A); 4) limited access (pair-fed to R/A animals) to revised diet 1226 without ethanol (R/P); 5) *ad libitum* access to solid diet 170590 (S/C). Diet was presented daily at 7 P.M. beginning on day 7 of gestation. On day 13 of gestation, the circadian rhythm of food intake was determined by measuring the consumption during 4-hr segments of the 24-hr period.

Beginning on day 20 of gestation, the animals were routinely checked for births in both experiments. When pups were noted, they were removed from the home cage within 6 hr of birth. The sex and body weights of individual pups were recorded. Pups were inspected for external anomalies (*e.g.*, head, digits and limbs). From each litter a random sample of six to eight pups, equally distributed between males and females, were decapitated. The brains were removed and weighed.

In experiment 2, six litters in each diet condition were examined on day 19 of gestation. The pregnant rat was anesthetized with chloral hydrate (0.35 g/kg i.p.) and the fetuses were removed from the uterus in a distal to proximal direction. The placenta was dissected from the fetus and weighed. After examination for sex and external anomalies, fetal body weight was recorded. The brain and heart were then removed and weighed.

Data analysis. Maternal body weight was analyzed by a two-way

(maternal diet × days of gestation) analysis of variance (ANOVA) with a repeated measure design for the second factor. Daily food intake was averaged over the 1st and 2nd 7 days of diet-administration and analyzed by a two-way (maternal diet × week of gestation) ANOVA for protein and caloric intake. Offspring data were expressed as litter averages and subjected to one-way ANOVA. Where indicated, significant interactions were further analyzed for simple main effects and subsequent *post hoc* analysis by the Newman-Keuls method (Winer, 1971).

Results

Experiment 1. The analysis of the maternal growth curves during gestation (F = 15.89; dF = 15,120; P < .001; see fig. 1) indicated that by day 14 of gestation the rats consuming the B/A and B/P diets weighed significantly less than S/C mothers (P < .05). On the other hand, the B/C mothers with *ad libitum* access to the liquid diet without ethanol were able to maintain normal growth throughout pregnancy. The failure of B/A and B/P mothers to gain weight normally during pregnancy may be directly attributable to the 40% reduction in food intake compared to the level consumed by the B/C animals. This reduced food intake resulted in a 40% deficit in caloric intake and a 50% deficit in protein intake when compared to the females on the solid casein diet (see table 3).

Blood alcohol levels of the B/A animals on day 14 of gestation at 1 P.M. were 60.00 ± 12.63 mg/dl and on day 18 at 11 P.M. were 185.83 ± 15.24 mg/dl. This difference in blood alcohol level likely reflects a circadian rhythm of food intake rather than increased ethanol intake since the intake was relatively constant over the 2 weeks of diet presentation (week 2 of gestation: 2.98 ± 0.08 g of ethanol per day; week 3 of gestation: 3.38 ± 0.11 g of ethanol per day).

Parturition was delayed (F = 5.59; dF = 3,24; P < .01) in the B/A mothers by approximately 1 day (21.9 ± 0.1 days) as compared to the other mothers (B/P: 21.3 ± 0.2 days; B/C: 21.0 ± 0.2 days; and S/C: 21.0 ± 0.2 days). However, the number of live births and the sex ratio per litter did not differ among the groups (see table 4). Although no external malformations were noted in any pups, analysis of offspring body weight at birth indicated a significant effect of maternal diet (F = 15.83; dF = 3,21; P < .001). The B/A pups weighed significantly less than B/C or S/C pups (P < .01) by approximately 1.5 g and B/P pups (P < .05) by approximately 1.0 g. Analysis of the offspring brain weights revealed a significant effect of maternal diet (F = 3.43; dF = 3,21; P < .05) which indicated that whereas S/C and B/C pups did not differ in brain weight, the B/P and B/A pup brains weighed significantly less than S/C pups (P < .05).

Experiment 2. The results of experiment 2 not only replicated the findings of experiment 1, but also verified the nutritional adequacy of the revised diet. The analysis of the maternal growth curves during gestation (F = 13.02; dF = 16,120; P < .001; see figure 2) indicated the B/A and B/P mothers weighed significantly less (P < .05) than S/C mothers from day 14 to 20 of gestation, whereas the R/A and R/P mothers did not differ from S/C animals throughout gestation. Analysis of caloric intake (see table 5) indicated that during the 2nd week of gestation S/C mothers consumed more calories than R/A or R/P mothers (P < .05); however, during the last week of gestation the S/C, R/A and R/P mothers consumed the same number of calories. As in experiment 1, the B/A and B/P mothers took in fewer calories than S/C animals during both weeks (P < .01). Analysis of protein intake indicated that the S/C mothers received more protein during the 2nd week of gestation than R/A or R/P mothers (P < .01); however, during the 3rd week of gestation, R/A and R/P subjects consumed more protein than S/C mothers (P < .01). Replicating the results of experiment 1, the B/A and B/P mothers took in less protein than S/C mothers (P < .01) during both weeks.

The analysis of maternal blood alcohol levels indicated that the B/A mothers displayed higher levels than R/A mothers at

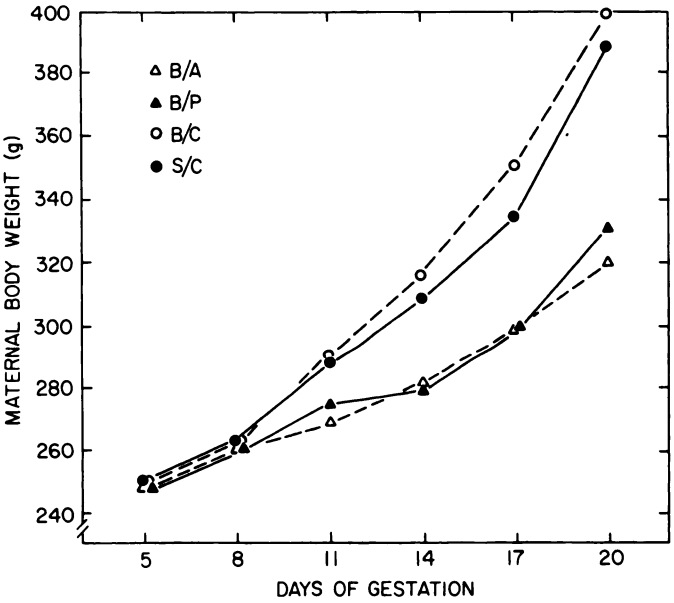

Fig. 1. Experiment 1: Mean maternal body weight (grams) during gestation: B/A (△), B/P (▲), B/C (○), S/C (●); n = 7 per group.

TABLE 3
Comparison of caloric and protein intake of experiment 1

Diet	Calories[a]		Protein[b]	
	Week 1	Week 2	Week 1	Week 2
	kcal/day		*g/day*	
B/A	59.00 ± 1.58[c]	66.88 ± 2.14	2.26 ± 0.06	2.57 ± 0.08
B/P	65.00 ± 1.83	71.22 ± 2.72	2.50 ± 0.07	2.73 ± 0.10
B/C	98.41 ± 3.11	116.23 ± 3.03	3.78 ± 0.12	4.46 ± 0.12
S/C	92.94 ± 4.71	96.42 ± 3.74	5.22 ± 0.26	5.41 ± 0.21
	[S/C = B/C] > [B/A = B/P][d]	B/C > S/C > [B/A = B/P]	S/C > B/C > [B/A = B/P]	S/C > B/C > [B/A = B/P]

[a] Diet × week interaction: P < .01; F = 7.38; dF = 3,24.
[b] Diet × week interaction: P < .05; F = 3.74; dF = 3,24.
[c] Mean ± S.E., n = 7 mothers.
[d] Newman-Keuls *post hoc* analysis, P < .05.

both testing times (see table 6). This large difference could not be explained on the basis of either the absolute intake of ethanol (R/A mothers consumed more ethanol than B/A mothers during the last week of gestation) or on the basis of the dose of ethanol per kilogram of body weight since the R/A animals, in fact, received slightly greater amounts of ethanol (see table 6).

The results of the measurement of circadian rhythm of food intake are illustrated in figure 3. Whereas animals fed a solid diet ate 85% of their food during the lights-off period, the liquid diet animals displayed different patterns of eating depending on whether their diet contained ethanol or not. The B/A and R/A mothers spaced out their consumption over the day in what appeared to be eating spurts. The low blood alcohol levels observed at 1 P.M. in experiment 1 thus correspond to the end of a period of apparent abstinence during the early morning. The two pair-fed groups, B/P and R/P, consumed their entire food allotment during the lights-off period and thus were effectively food deprived during the lights-on period. Thus, none of the animals fed a liquid diet showed the normal rodent circadian pattern of food consumption.

TABLE 4

Offspring data of experiment 1

Diet	Total Live Births	Pup Body Weight	Brain Weight
		g	*mg*
B/A	9.0 ± 0.9[a]	4.81 ± 0.24	217.8 ± 8.3
B/P	11.6 ± 0.9	5.90 ± 0.13	223.8 ± 5.3
B/C	11.0 ± 0.6	6.41 ± 0.15	239.7 ± 5.4
S/C	11.0 ± 0.6	6.42 ± 0.21	246.4 ± 8.7
	No difference	S/C > B/P > B/A[b]	S/C > [B/A = B/P]
		S/C = B/C	S/C = B/C
		B/C > B/A	B/C > B/A

[a] Mean ± S.E.; n = 7 litters.
[b] Newman-Keuls *post hoc* analysis, P < .05.

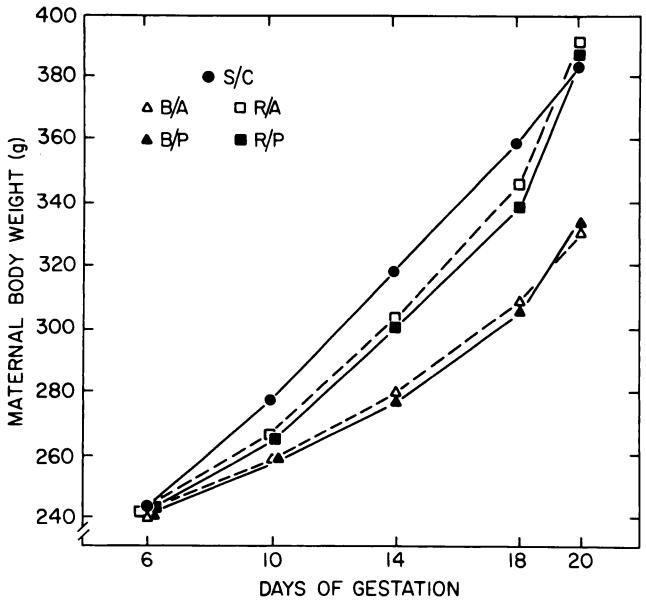

Fig. 2. Experiment 2: Mean maternal body weight (grams) during gestation: B/A (△), B/P (▲), R/A (□), R/P (■), S/C (●); n = 7 per group.

Parturition was delayed by approximately 1 day for the B/A and R/A mothers when compared to the S/C, B/P and R/P animals (F = 5.53; dF = 4,28; P < .01, see table 7). Neither the number of live births nor their sex ratio were significantly different among the groups. However, there were more litters in the B/A and R/A groups which had one or more pups found dead in the cage. As in experiment 1, none of the pups exhibited any gross malformations. Analysis of newborn pup body weight revealed a significant effect of maternal diet (F = 11.87; dF = 4,24; P < .001, see table 8). Whereas the body weight of R/A and R/P offspring did not differ from S/C pups, the B/A pups were again significantly growth-stunted (P < .01). Although the B/P pups were also lower in body weight than S/C pups (P < .05), they weighed significantly more than B/A pups (P < .05). Analysis of brain weight at birth indicated a significant effect of maternal diet (F = 4.84; dF = 4,24; P < .01). In this case, the R/A and R/P pup brain weights did not differ from the brain weights of S/C offspring. Replicating the findings of experiment 1, the B/A and B/P brain weights were significantly less than S/C brain weights (P < .01), but did not differ from each other.

On day 19 of gestation, neither the number of live fetuses nor the number of resorption sites were different among the groups. In addition, no external malformations were observed in the examined fetuses. Analysis of fetal body weight demonstrated a significant effect of maternal diet (F = 5.79; dF = 4,25; P < .01; see table 9). Whereas the fetal body weight of S/C, R/A and R/P mothers did not differ from each other, the B/A and B/P fetuses weighed less than the others (P < .05). However, unlike the newborn body weight results, the B/A and B/P fetuses did not differ from each other in body weight on day 19 of gestation. A pattern of differences between groups similar to those observed in body weight were present in fetal brain weights (F = 4.59; dF = 4,25; P < .01). However, fetal heart weights did not differ among the groups. There was a significant

TABLE 5

Comparison of caloric and protein intake of experiment 2

Diet	Calories[a]		Protein[b]	
	Week 1	Week 2	Week 1	Week 2
	kcal/day		*g/day*	
B/A	58.87 ± 2.77[c]	71.91 ± 3.41	2.26 ± 0.11	2.76 ± 0.13
B/P	63.56 ± 2.05	85.08 ± 3.93	2.44 ± 0.08	3.27 ± 0.15
R/A	80.70 ± 3.69	97.43 ± 4.87	5.53 ± 0.25	6.68 ± 0.33
R/P	78.39 ± 3.34	102.31 ± 5.77	5.38 ± 0.23	7.02 ± 0.40
S/C	90.20 ± 3.92	100.07 ± 4.10	5.06 ± 0.22	5.62 ± 0.23
	[S/C = R/A = R/P] > [B/A = B/P][d]	[S/C = R/A = R/P] > [B/A = B/P]	[S/C = R/A = R/P] > [B/A = B/P]	[R/A = R/P] > S/C > [B/A = B/P]

[a] Diet × week interaction: P < .01; F = 15.12; dF = 4,29.
[b] Diet × week interaction: P < .01; F = 9.17; dF = 4,29.
[c] Mean ± S.E.; n = 7 mothers.
[d] Newman-Keuls *post hoc* analysis, P < .05.

TABLE 6
Alcohol intake and blood alcohol levels of experiment 2

	B/A	R/A	
Ethanol intake (g/day)			
Week 1	3.06 ± 0.05[a]	3.00 ± 0.09	B/A = R/A[b]
Week 2	3.97 ± 0.08	4.46 ± 0.08	R/A > B/A
Maternal body weight (g)			
Week 1	260.3 ± 4.1	266.1 ± 3.4	B/A = R/A
Week 2	311.6 ± 4.9	337.0 ± 6.2	R/A > B/A
Ethanol dose (g/kg)			
Week 1	11.74 ± 0.14	11.25 ± 0.37	B/A = R/A
Week 2	12.73 ± 0.15	13.23 ± 0.22	B/A = R/A
Blood alcohol level (mg/dl)			
Day 13 11:00 P.M.	200.0 ± 22.5	106.0 ± 20.0	B/A > R/A
Day 19 5:00 P.M.	146.7 ± 64.9	66.0 ± 27.3	B/A > R/A

[a] Mean ± S.E.; n = 7 mothers.
[b] Neuman-Keuls *post hoc* analysis, $P < .05$.

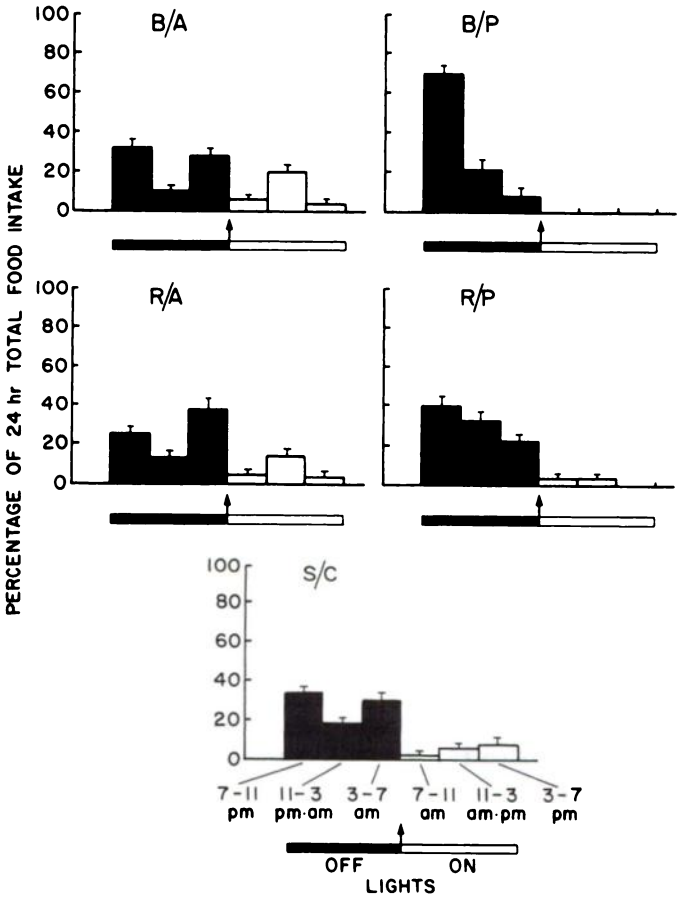

Fig. 3. Experiment 2: Mean percentage of 24-hr food intake (±S.E.M.) for each measurement period; n = 7 per diet condition.

effect of prenatal ethanol exposure on placental weight (F = 7.82; dF = 4,25; $P < .001$) which represents an increase in placental weight in both R/A and B/A mothers ($P < .01$) compared to their pair-fed groups and solid diet mothers.

Discussion

Experiment 1. The results of experiment 1 indicate that pregnant animals maintained from day 7 of gestation on Bio-Mix 711-PR with ethanol or pair-fed the same diet without ethanol do not grow normally during pregnancy. At the depressed level of dietary intake produced by the presence of ethanol in the diet, this diet does not provide sufficient nutrients when compared to rats fed the solid casein diet or the liquid diet without ethanol fed *ad libitum*. Thus, the alleged nutritional control group, mothers pair-fed the nonalcohol diet, are themselves malnourished when compared to the animals on the standard solid diet and their offspring are likewise affected. Furthermore, the effects of ethanol and malnutrition appear to be additive on the measure of pup body weight since the B/A pups were even more growth-stunted than B/P pups. However, on the basis of the gross measurement of brain weight, the B/A and B/P pups appear to be equally affected. Until more detailed analyses of these brains can be carried out, a specific effect of ethanol on central nervous system development in the offspring to B/A mothers cannot be excluded, but no visibly detectable brain or somatic abnormalities were observed.

Experiment 2. The results of experiment 2 not only demonstrated the nutritional adequacy of the revised diet but also replicated the results of experiment 1 on the original formula. It appears that pregnant females consuming the revised diet, regardless of the presence or absence of ethanol, gain weight normally throughout gestation and give birth to pups which are not different in body or brain weight from offspring of mothers fed the solid diet. However, mothers receiving the revised diet with ethanol did display a delay in parturition and an increased number of litters with more than one dead pup at birth similar to rats consuming the original diet with ethanol.

On day 19 of gestation, the pattern of offspring body and brain weight changes is similar to that observed at birth. The only exception is that fetuses of mothers fed the original ethanol diet did not weigh less than their pair-fed control group. The last 2 to 3 days of gestation before parturition are a time of rapid body weight gain for the fetuses since they approximately double their weight during this period. It appears that the rate of body weight gain of B/A fetuses is severely retarded since they weigh less at birth than their pair-fed counterparts even with an extra day *in utero*. On the other hand, fetuses of R/A mothers might be considered to be only mildly retarded in growth rate since with the additional day *in utero* they are born at a body weight similar to their pair-fed group.

The apparent lack of effect on body and brain weight of offspring of mothers receiving the revised ethanol diet is somewhat surprising, since they consumed as much ethanol as the original ethanol diet. However, the significantly lower blood alcohol levels displayed by the revised diet mothers might

TABLE 7
Parturition data of experiment 2

Diet	Gestation Length	No. of Live-Born Pups	No. of Litters with > 1 Pup Dead
	days		
B/A	22.0 ± 0.0[a]	9.0 ± 1.8	5
B/P	21.3 ± 0.2	9.9 ± 0.7	1
R/A	22.0 ± 0.0	10.4 ± 0.7	5
R/P	21.4 ± 0.2	10.1 ± 1.0	2
S/C	21.3 ± 0.2	10.5 ± 0.7	1
	[R/A = B/A] > [S/C = B/P = R/P][b]	No difference	[R/A = B/A] > [S/C = B/P = R/P]

[a] Mean ± S.E., n = 7 litters.
[b] Neuman-Keuls *post-hoc* analysis, $P < .05$.

TABLE 8
Offspring body and brain weights at birth of experiment 2

Diet	Body Weight	Brain Weight
	g	*mg*
B/A	5.14 ± 0.15[a]	228.6 ± 5.3
B/P	5.98 ± 0.17	229.0 ± 4.3
R/A	6.68 ± 0.25	255.3 ± 7.4
R/P	6.73 ± 0.23	259.7 ± 8.3
S/C	6.76 ± 0.08	258.8 ± 7.7
	[S/C = R/A = R/P] > B/P > B/A[b]	[S/C = R/A = R/P] > [B/A = B/P]

[a] Mean ± S.E., n = 7 litters.
[b] Neuman-Keuls *post hoc* analysis, $P < .05$.

TABLE 9
Fetal data at day 19 of gestation of experiment 2

Diet	Fetuses per Mother	Fetal Body Weight	Fetal Brain Weight	Fetal Heart Weight	Placental Weight
		g	*mg*	*mg*	*g*
B/A	9.7 ± 1.1[a]	2.29 ± 1.3	133.7 ± 5.3	12.2 ± 0.9	0.74 ± 0.06
B/P	11.2 ± 1.3	2.46 ± 0.11	138.2 ± 2.7	13.0 ± 0.4	0.53 ± 0.03
R/A	9.4 ± 1.3	2.62 ± 0.07	148.2 ± 2.1	14.0 ± 0.4	0.73 ± 0.03
R/P	9.2 ± 1.2	2.64 ± 0.08	147.2 ± 3.4	13.9 ± 0.6	0.55 ± 0.02
S/C	11.2 ± 0.9	2.80 ± 0.18	155.9 ± 5.8	14.1 ± 1.0	0.59 ± 0.03
	No difference	[S/C = R/A = R/P] > (B/A = B/P][b]	[S/C = R/A = R/P] > [B/A = B/P]	No difference	[B/A = R/A] > [S/C = B/P = R/P]

[a] Mean ± S.E., n = 6 mothers.
[b] Neuman-Keuls *post hoc* analysis, $P < .05$.

account for the lack of growth-stunting of the pups. One possible explanation for the difference in blood alcohol levels might lie with the apparent undernutrition of the original diet mothers. It has been reported that rats fed a low protein diet had slower rates of ethanol metabolism resulting from decreased liver alcohol and aldehyde dehydrogenase activity (Horn and Manthei, 1965; Lindros *et al.*, 1977). If this phenomenon can be generalized to protein-deprived pregnant females, the undernourished animals (B/A) would be expected to have higher blood alcohol levels than adequately nourished females (R/A) receiving the same dose of ethanol.

The alterations in the circadian rhythm of food intake, especially in the pair-fed animals, poses some problem with experimental design. Shifts in the rhythm of food intake might alter the rhythm of other behaviors, as well as hormonal and biochemical phenomenon. The circadian rhythm of maternal behavior has been demonstrated to be altered with shifts in feeding behavior (Stern and Levin, 1976) and might prove to be a confounding variable in experiments continued postnatally. Food-seeking behaviors of the pair-fed subjects during the periods when they have already finished their daily ration might also interfere with normal behaviors. In experiment 2, the animals were presented with their diet at the beginning of the lights-off period and as a result the usual nocturnal feeding pattern was not destroyed. However, if the presentation of fresh food is made in the early morning, as it is often done (Henderson *et al.*, 1979), a more severe alteration in the feeding rhythm might be expected.

General Discussion

Comparing the results of our experiments to those already published is complicated because of the variety of methods of alcohol exposure, the dose of alcohol administered, the length of alcohol exposure, the species and strain of subject and the lack of nutritional control in many studies. Limiting the discussion to rat models, it appears that most researchers have reported, as we do, that the number of live births is unchanged after prenatal alcohol exposure (Abel and Dintcheff, 1978; Detering *et al.*, 1979; Henderson and Schenker, 1977; Martin *et al.*, 1977). Gestation length has been reported to be longer in studies where appreciable blood alcohol levels were also reported (Abel and Dintcheff, 1978; Martin *et al.*, 1977, 1978b).

The body weights at birth of pups born to mothers exposed to alcohol have been reported to be lower than offspring of nonpair-fed control mothers in a number of studies (Auroux and Dehaupas, 1970; Martin *et al.*, 1978a; Pilström and Kies-

sling, 1967; Volk, 1977). Similar to the results of our findings with the original diet formulation, the offspring of alcohol mothers weigh less than pups of pair-fed mothers (Abel and Dintcheff, 1978; Detering *et al.*, 1979; Harris and Case, 1979; Martin *et al.*, 1978b). Finally, the offspring of pair-fed mothers have been reported to weigh less than pups of *ad libitum* control mothers by investigators who have included this comparison (Abel, 1978; Harris and Case, 1979; Martin *et al.*, 1977).

Of the four fetal rat studies in the literature, three have reported an increase in number of resorptions (Henderson *et al.*, 1979; Sandor and Amels, 1971; Skosyreva, 1973) and one reported, as we do, no change in this measure (Schwetz *et al.*, 1978). This discrepancy in results is possibly due to the differences in timing and amount of alcohol exposure used in the various experiments. However, unlike the reported increased incidence of fetal malformations in alcohol-exposed mice (Chernoff, 1977; Kronick, 1976; Randall *et al.*, 1977), none of the researchers using rats have reported any evidence of gross malformations in their alcohol-exposed fetuses. Similar to our results with the fetuses of the original ethanol diet mothers, decreased fetal body weight has been observed by other investigators (Henderson *et al.*, 1979; Schwetz *et al.*, 1978; Skosyreva, 1973). Although placental weight was unchanged in one study (Henderson *et al.*, 1979), Skosyreva (1973) has reported an increase in placental weight in alcohol-exposed subjects similar to our results.

Researchers investigating the influence of prenatal alcohol exposure on offspring development have become increasingly aware of the need to control for the important variable of nutrition. Pregnant animals exposed to alcohol consume less food than *ad libitum* nonalcohol subjects, whether the ethanol is in their drinking fluid (Borgman and Wardlaw, 1977; Martin *et al.*, 1977), in a liquid diet (experiment 1 results; Detering *et al.*, 1979; Martin *et al.*, 1978a) or intubated (Abel, 1978; Abel and Dintcheff, 1978). For the most part, these investigations have used a pair-feeding technique to control for nutrition in their studies. Although only one study using the technique of alcohol in the drinking fluid has attempted to control for the nutrition variable (Tze and Lee, 1975), some form of pair-feeding has been routinely carried out in liquid diet experiments (Detering *et al.*, 1979; Druse and Hofteig, 1977; Henderson and Schenker, 1977; Riley *et al.*, 1979a,b) and intubation studies (Abel,1978; Abel and Dintcheff, 1978; Martin *et al.*, 1978b). However, in studies where a standard lab chow group was also included (Abel and Dintcheff, 1978; Detering *et al.*, 1979; Martin *et al.*, 1977), maternal weight gain during pregnancy was greater in this group than either the alcohol or pair-fed mothers as we report for females receiving the original diet. The only exception to this finding is when ethanol administration was limited to either a low dose (Riley *et al.*, 1979a) or a short period of exposure (Riley *et al.*, 1979b). Thus, undernutrition of the pregnant rat confounds all of these studies. Although the degree of undernutrition might be milder than that used in the investigation of perinatal malnutrition (see reviews of malnutrition methods: Levine and Wiener, 1975; Plaut, 1970), the potential interaction of undernutrition and ethanol cannot be ignored. The use of the revised diet developed here will permit investigation of prenatal ethanol exposure in well nourished pregnant rats.

The results of our experiments indicate that the offspring of ethanol-exposed mothers, which are also protein and calorie malnourished, weigh even less than the growth-stunted offspring of the undernourished pair-fed mothers. Since the offspring of the revised ethanol diet mothers were not growth-stunted compared to either their pair-fed group or solid diet subjects in the present study, it appears that an effect of ethanol on pup body weight occurs only under conditions of malnutrition. Whether this influence of ethanol under conditions of inadequate nutrition can be interpreted as meaning undernourished fetuses are at greater risk for the influence of prenatal alcohol remains to be studied. Consideration of that possible conclusion must recognize that the maternal blood alcohol levels are much higher in the original diet animals than in the nutritionally adequate revised diet mothers. Thus, we are presently attempting to increase the blood alcohol levels observed in well nourished pregnant animals. In doing so, we may be able to investigate the effects of varying blood alcohol levels on the development of offspring under conditions of adequate nutrition. Future studies will also include investigation of the effects of ethanol administration begun earlier in gestation or before conception to further elucidate critical periods of exposure.

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