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Alcohol Exposure Before Pregnancy: Biochemical and Behavioral Effects on the Offspring of Rats

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LEDIG, M., R. MISSLIN, P. KOPP, E. VOGEL, G. THOLEY AND P. MANDEL. *Alcohol exposure before pregnancy: Biochemical and behavioral effects on the offspring of rats.* PHARMACOL BIOCHEM BEHAV **36**(2) 279–285, 1990.—The effect of maternal alcohol exposure before mating was investigated in the offspring over a period of 6 months concerning some specific aspects of energy metabolism in the brain and the liver. The following biochemical parameters were analyzed: superoxide dismutase (involved in elimination of free radicals produced during ethanol oxidation), enolase isoenzymes (markers of nerve cell maturation), and alcohol and aldehyde dehydrogenase (the main alcohol degradating enzymes). These enzymatic activities were measured at their subcellular level. In these animals never directly exposed to alcohol, superoxide dismutase activity was decreased mainly in the liver cytosol. Only the nonneuronal form of enolase activity was modified. Alcohol dehydrogenase was decreased in the liver as well as in the brain. Aldehyde dehydrogenase was also decreased in the liver and in the brain, mainly in the mitochondria. Behavioral observations showed decreased emotional reactivity as well as an increase in locomotor activity. Our results suggest that long-lasting biochemical and behavioral effects of alcohol may occur in the offspring starting at the earliest stage of development.

Fetal alcohol Brain Liver Enzymes Behavior

A vast body of clinical observations suggest that prenatal alcohol exposure is associated with morphologically, biochemically, physiologically and behaviorally adverse effects (5,27). In order to simulate the human condition, animal models for the study of the Fetal Alcohol Syndrome (FAS) were called upon to determine whether ethanol given prenatally was deleterious to the offspring (1). The results tend to show that ethanol, or its metabolites, acts throughout pregnancy on various developing systems of the blastocyst, the embryo and the fetus (4). While these studies were based on a maternal drinking history during pregnancy, the present report concerns some biological and behavioral effects induced in the offspring of female rats exposed to alcohol only prior to mating. Indeed, some authors suggested that FAS risk may be removed if the mother stops drinking alcohol during pregnancy (21). On the other hand, it has been shown that modifications may occur even after an acute alcohol exposure during specific periods of embryogenesis (19). Also, paternal alcoholism may be a part of the factors involved in the etiology of FAS (2). As in our proposal, the consequences of paternal alcoholism result from premating alcohol effects.

Liver and brain from the offspring were analyzed up to 6 months for some enzymatic activities involved in specific aspects of energy metabolism and which appear during development: cytosolic and mitochondrial superoxide dismutase (SOD) (involved in the elimination of free radicals produced during ethanol oxidation) (6); nonneuronal (NNE) and neuron-specific (NSE) enolase (two forms of the glycolytic pathway enzyme producing phosphoenolpyruvate and used as markers of cell maturation in the nervous tissue) (15); alcohol dehydrogenase (ADH) as well as cytosolic and mitochondrial aldehyde dehydrogenase (ALDH) (the main alcohol degradating enzymes). The behavioral parameters were: open-field activity and novelty-seeking behavior (both reflecting nerve cell functions).

METHOD

Mother's Treatment

Six two-month-old female rats of a Wistar strain, well adapted for alcohol consumption (kindly supplied by Dr. F. Feo, Instituto di Patologia Generale, Universita di Sassari, Italia), were maintained under controlled temperature (20 ± 1°C) and 12/12 hours light-dark cycles in sawdust-covered plastic cages. They were acclimatized to alcohol (ethanol officinalis 95°) by giving first a 10% (v/v) alcohol solution for one week then they were given a 20% (v/v) alcohol solution as sole liquid source for one month before mating. Longer alcohol treatment would induce some tolerance phenomena. Six control animals received tap water. Another group of six animals received 15% (w/v) sucrose. All the groups were fed ad lib with laboratory chow.

At mating, the alcohol was withdrawn from the drinking water, pregnancy started within one week maximum after mating. The number of pups per litter was similar in the control and alcohol-

treated group. At birth, litters were reduced to 8 pups. That method of alcohol treatment seems to be well adapted for our experimental investigations since no apparent signs of undernutrition (weight loss, dull hair) nor withdrawal symptoms were observed over the treatment period.

Offspring's Treatments

Animals. After birth the pups remained with their mothers until weaning at 4 weeks, then they were housed in twos until examination.

The brains and the livers of the offspring provided from the alcohol-treated and the control mothers were analyzed at 2, 4, 8 and 24 weeks of age after birth.

Biochemical determinations. The animals were killed by decapitation; brain and liver were excised and weighed. About 1 g of tissue was homogenized, using a tissue grinder, in 4 ml of ice-cold 0.9% NaCl. The homogenate was centrifuged for 10 min at $1000 \times g$ in order to remove nuclei and tissue fragments. The supernatant was centrifuged for 1 hr at $100,000 \times g$ in order to separate the cytosolic fraction. The pellet was disrupted by 3 liquid nitrogen freezing-thawing cycles in order to liberate the mitochondrial enzymes and recentrifuged for 1 hr at $100,000 \times g$ in order to separate the soluble fraction of the mitochondria. Both supernatants were filtered on small (1 cm high, 0.5 cm diam.) G50 Sephadex columns equilibrated with 0.9% NaCl in order to eliminate low molecular weight compounds interfering in the enzymatic measurements. The filtrates from both extracts (cytosol and mitochondria) were used for biochemical determinations. The following enzymatic activities were measured: SOD by a colorimetric method (10), enolase (nonneuronal and neuron-specific form after separation on small DEAE cellulose columns) (12), ADH (28) and ALDH (20) by measuring initial velocity of the reaction. Proteins were determined by the Lowry method (14).

SOD was given as μg/mg protein (in our conditions, one unit corresponds to 2 μg). The other biochemical results were expressed as units or milliunits (micromoles or nanomoles/min/mg protein ± SD). All values are means ± SD of 12 animals (6 males and 6 females from 3 different litters) were compared by the Student's *t*-test to determine the statistical significance of the differences between controls and animals born to mothers consuming alcohol up to the beginning of pregnancy. No significant sex differences were observed for the biochemical parameters.

Behavioral effects. For the behavioral effects performed in a different laboratory, the animals (only the 8-week-old) were divided into two groups, those born to alcohol-consuming mothers and those born to water drinking mothers. Fifty-three animals from 4 different litters in each group were used. Prior to testing (about 2 weeks), they were housed in groups of 3–5 in a standard sawdust-covered plastic cage containing a constant supply of food pellets and water, and kept on a 12/12-hr light-dark cycle, with lights on at 1 a.m. to observe animals in their high activity period. Behavioral testings were conducted at first in an open-field and five days later in a novelty-seeking behavior test.

For statistical procedures comparison between the performances of controls vs. treated rats, ascertained by the Student's *t*-test, was followed by an analysis of variance (ANOVA) with sex and treatment as factors.

Open-field activity. The apparatus consisted of polyvinyl chloride square open-field (50×50 cm) surrounded by vertical walls 40 cm high. The floor was divided into 25 squares (10×10 cm). A light from a 100-W desk lamp above the center of the apparatus provided the only room illumination. During the observation, the experimenter sat always at the same place, next to the apparatus.

The subjects were individually tested in 10-min sessions. Animals were always placed into the open-field, starting in the same corner. Activity was recorded as the number of squares crossed (locomotor activity) as well as the number of rearing incidents. The number of animals presenting defecation was also recorded.

TABLE 1

MATERNAL WEIGHTS AND CALORIE CONSUMPTION

Maternal Treatment (n = 6)	Initial Weight (g)	Liquids (kcal/kg/day)	Laboratory Chow (kcal/kg/day)	Total (kcal/kg/day)	Final Weight (g)
Control (water)	382	—	172	172	390
Alcohol 20% (v/v)	383	65	98	163	387
Sucrose 15% (w/v)	387	55	122	177	428

Novelty-seeking behavior. The apparatus consisted of a grey plastic box ($60 \times 40 \times 30$ cm) covered with Plexiglas and subdivided into six equal square exploratory units which were all interconnected by small holes (6×7 cm). It could be divided in half lengthwise by closing three temporary partitions. The apparatus was kept in the rat house. The experimenter stood next to the box.

Approximately 24 hr before testing, each subject was placed in one half of the apparatus with the temporary partitions in place, in order to be familiarized with it. The floor of this half was covered with sawdust and the animal was given unlimited access to food and water. Next day, the subject was exposed to both familiar and novel environments by the removal of the temporary partitions. The subject was then observed in red light for 10 min. The amount of time spent in the novel compartment (novelty preference) and the number of novel and familiar units entered by the subject were recorded and defined as locomotor activity.

Alcohol consumption. Following these behavioral tests, eight animals (4 males, 4 females from different litters) were fed a 20% (v/v) alcohol solution as sole liquid for 4 weeks, and their alcohol consumption was compared to that of control animals whose mothers received only water prior to conception.

RESULTS

Maternal Parameters

Maternal weight and calorie consumption are shown in Table 1. The calorie consumption was similar in all groups. In the alcohol-treated group alcohol represented about 40% of the calories compared to the controls, their laboratory chow consumption was reduced. The alcohol-fed group had no weight loss during the alcohol treatment, their blood alcohol level measured by the Boehringer test No. 176 290 was: 0.56 ± 0.22 g/l at 9 p.m. For the sucrose-treated animals the total calorie consumption was similar to the water- and alcohol-treated animals, sucrose represented about 35% of the total calories. Their final weight was about 10% higher than the initial one. That increase was not observed with the water-consuming animals. Indeed, sucrose is not metabolized like alcohol. As is also suggested by recent publications (19,26), it is more correct to compare alcohol-treated animals to water controls than to sucrose per fed animals. Our experimental animals were also compared to water-consuming controls and not to the offspring of the sucrose per fed group.

Biochemical Effects in the Offspring

Weights. The body, liver and brain weights are shown in Table

TABLE 2

EFFECT OF MATERNAL PREPREGNANCY ALCOHOL CONSUMPTION ON THE OFFSPRING

	Age (W)	Sex	Body Weight (g)	Liver Weight (g)	Brain Weight (g)
Control	2	m + f	39 ± 7	1.25 ± 0.22	1.17 ± 0.20
Alcohol	2		30 ± 5	1.01 ± 0.31	1.05 ± 0.17
Control	4	m + f	119 ± 6	5.25 ± 0.69	1.33 ± 0.19
Alcohol	4		107 ± 26	4.25 ± 0.66	1.41 ± 0.11
Control	8	m	335 ± 26	14.5 ± 1.6	1.60 ± 0.81
		f	219 ± 14	9.7 ± 0.9	1.47 ± 0.82
Alcohol	8	m	310 ± 12	14.0 ± 0.8	1.40 ± 0.81
		f	237 ± 7	10.8 ± 0.8	1.47 ± 0.13
Control	24	m	523 ± 39	16.6 ± 3.0	1.72 ± 0.21
		f	313 ± 17	10.6 ± 1.9	1.66 ± 0.13
Alcohol	24	m	473 ± 17	13.1 ± 0.9	1.72 ± 0.16
		f	301 ± 13	10.4 ± 0.6	1.54 ± 0.14

Means ± SD, *t*-test (n = 12).

2. We found no significant modification in body, liver and brain weight between the controls and the animals born to alcohol-consuming mothers.

Superoxide dismutase. Liver SOD activity is shown in Figs. 1A and 2A. In the controls both cytosolic and mitochondrial SOD activities increased progressively with age. In the offspring of alcohol-exposed animals, the cytosolic SOD activity was at a lower level in the 8- and 24-week-old animals (respectively −30% and −20%). The mitochondrial SOD level was only significantly lower at 8 weeks (−30%).

Brain SOD activity is shown in Fig. 1B and 2B. In the controls the cytosolic SOD reached a plateau at 8 weeks, while the mitochondrial SOD increased up to 24 weeks. In the alcohol-exposed animals the cytosolic SOD activity was significantly lower at 2 weeks (−40%), while the mitochondrial SOD activity was lower at 4 weeks (−35%). At 24 weeks both activities were close to control values.

Enolase. Nonneuronal enolase activity is shown in Fig. 3A and 3B. The control activity increased slightly in the liver but decreased in the brain mainly between 2 and 4 weeks. In the liver no significant modification of nonneuronal enolase activity was found for the alcohol-exposed animals.

In the brain, the nonneuronal enolase, mainly localized in immature neurons and in the glial cells, was lower by about 20–30% up to 8 weeks in the alcohol-exposed animals. The neuron-specific enolase form localized in the mature neurons was not significantly modified (results not shown).

Alcohol dehydrogenase. Liver ADH activity is shown in Fig. 4A. In the controls, ADH activity reached a maximum at 8 weeks, then a decrease occurred. Alcohol-exposed animals exhibit a significant (35–80%) reduction up to 24 weeks.

Brain ADH activity is shown in Fig. 4B. The control ADH activity behaved as in the liver. In the alcohol-exposed animals we observed also a reduction at 24 weeks (−40%).

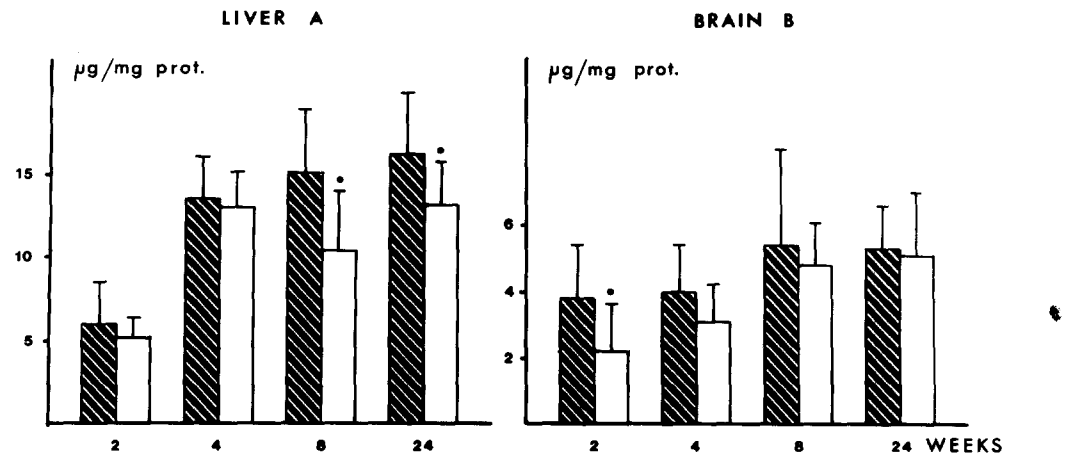

FIG. 1. Superoxide dismutase activity of liver (A) and brain (B) cytosol: control (hatched bars), alcohol withdrawal at mating (open bars), ●$p<0.05$.

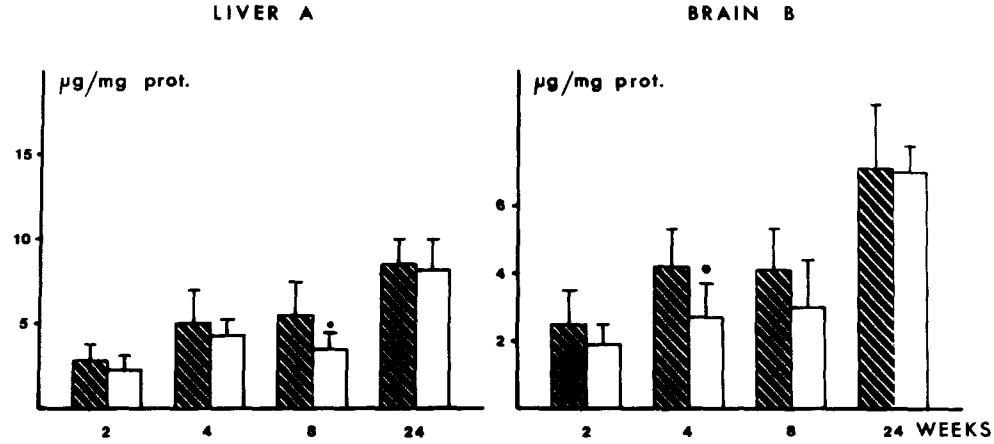

FIG. 2. Superoxide dismutase activity of liver (A) and brain (B) mitochondria: control (hatched bars), alcohol withdrawal at mating (open bars), ●$p<0.05$.

Aldehyde dehydrogenase. Liver ALDH activity is shown in Figs. 5A and 6A. In the controls, cytosolic ALDH activity was optimal at 4 weeks, but the mitochondrial ALDH activity only at 24 weeks.

In the cytosol of the alcohol-exposed animals the activity was significantly lower up to 8 weeks (40–60%). In the mitochondria, the activity was reduced at 24 weeks (−30%).

Brain ALDH activity is shown in Figs. 5B and 6B. In the controls the activity varied as in the liver. Cytosolic ALDH of alcohol-exposed animals exhibited no significant modification of the activity. In the mitochondria, the activity was significantly reduced up to 24 weeks (20–40%).

Behavioral Effects in the Offspring

Open-field activity. When the males and females were taken together (Fig. 7), there was a significant increase in locomotion ($p<0.02$) as well as in incidents of rearing (t-test: $p<0.001$) for the treated animals. Furthermore, there was a significant decrease in the number of animals presenting defecation between the treated group (11/24) and the controls (22/27) ($\chi^2 = 10.53$, $p<0.001$).

ANOVA analysis (Table 3) also shows significant differences between treated rats and controls as well as between males and females for rearing incidents.

Novelty-seeking behavior (Fig. 8). There was a significant increase in the time spent by the treated rats in the novel compartment (t-test; $p<0.03$) as well as in the locomotion (t-test; $p<0.01$). A sign test also revealed that treated animals were seen in the novel half significantly more often than in the familiar one ($p<0.001$), while controls showed no preference for any compartment. ANOVA analysis (Table 3) showed significant differences between controls and treated animals only for novelty preference.

Alcohol consumption. When the animals whose mothers were alcohol exposed up to the beginning of pregnancy were fed with 20% alcohol, their consumption was reduced considerably (5.51 ± 1.29 g/kg/day) compared to control animals (10.17±0.76 g/kg/day) over a period of 4 weeks (t-test; $p<0.001$).

DISCUSSION

The present experiments indicate that adult rat offspring born to alcohol-consuming mothers before pregnancy did not differ significantly in body, liver and brain weight. But they differ from controls in certain enzymes related to energy metabolism, as well

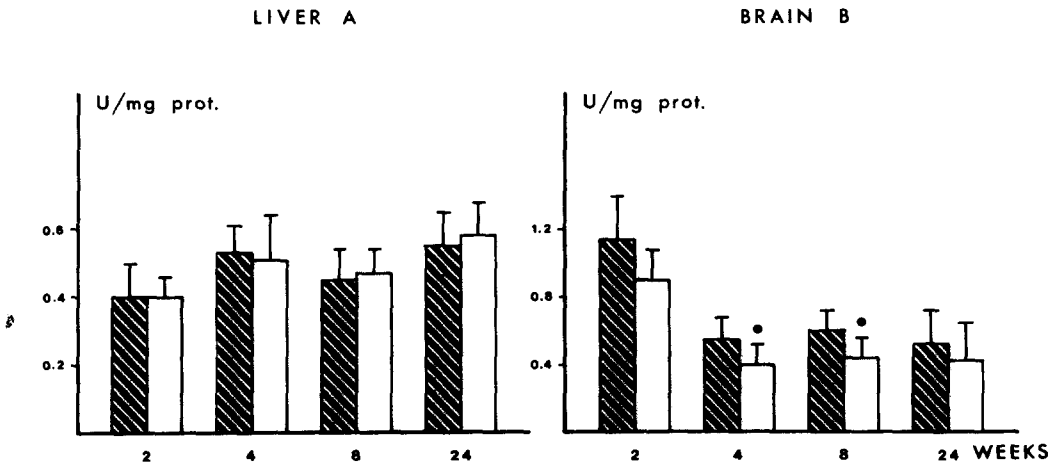

FIG. 3. Nonneural enolase activity of liver (A) and brain (B): control (hatched bars), alcohol withdrawal at mating (open bars), ●$p<0.05$.

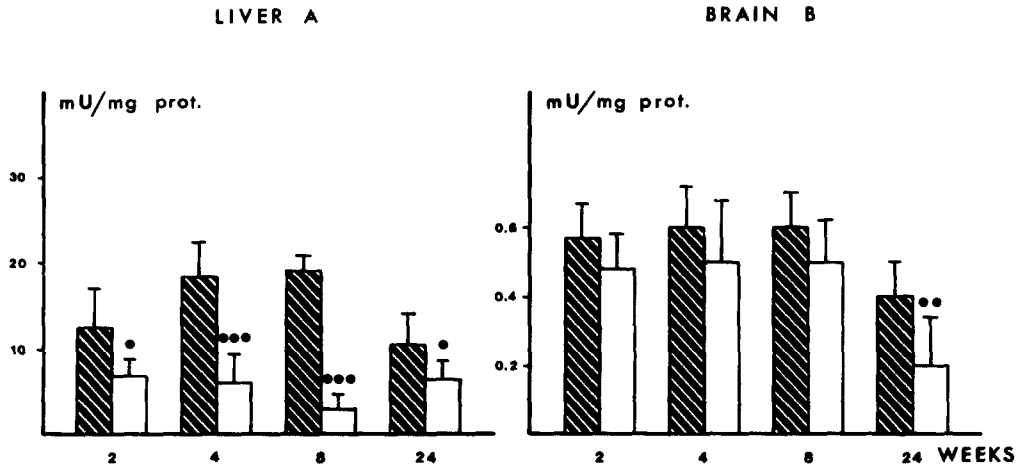

FIG. 4. Alcohol dehydrogenase activity of liver (A) and brain (B): control (hatched bars), alcohol withdrawal at mating (open bars); ●●●$p<0.001$, ●●$p<0.01$, ●$p<0.05$.

as in some behavioral parameters.

There appear to be correlations between biochemical modifications and the subcellular localization of the enzymes. SOD activities were different between the cytosolic and the mitochondrial forms in liver and in brain. In both tissues the activity of mitochondrial SOD was reduced up to 8 weeks and was close to control values at 24 weeks. The cytosolic activity was lowered mainly in the adult animals whereas in the brain the activity was lower in the young animals. The same observation was done in the brain and the liver of pups born to alcohol consuming mothers during the pre- and postnatal period (11). While there was a progressive inhibition in the liver cytosol, an adaptation phenomenon seems to occur in the brain cytosol; it may be related to the difference in SOD appearance during development depending on the tissue (16). Decreased SOD activity allows an increase of free radicals which may be particularly teratogenic during embryogenesis (25).

For liver enolase no modifications by prepregnancy alcohol exposure were found. Also, the neuron-specific enolase form, a marker of neuronal maturation was not modified significantly while for the nonneuronal form localized in immature neurons and in the glial cells a decrease was found up to 8 weeks. The absence of alcohol effect on the neuron-specific enolase may be due to the fact that a switch from the nonneuronal to the neuron-specific form occurs during neuronal maturation (24), as it may also be seen by the decrease of the nonneuronal enolase activity in the control brains between 2 and 4 weeks. The reduced activity of the nonneuronal form in presence of alcohol indicates also an impaired glial cell maturation.

ADH was the enzyme found to be decreased most significantly in the liver, suggesting that these animals may poorly metabolize alcohol. Indeed, when these animals were given 20% alcohol as sole liquid their daily intake was much lower (about 5 g/kg/day) than for the control animals consuming about 10 g/kg/day. In the

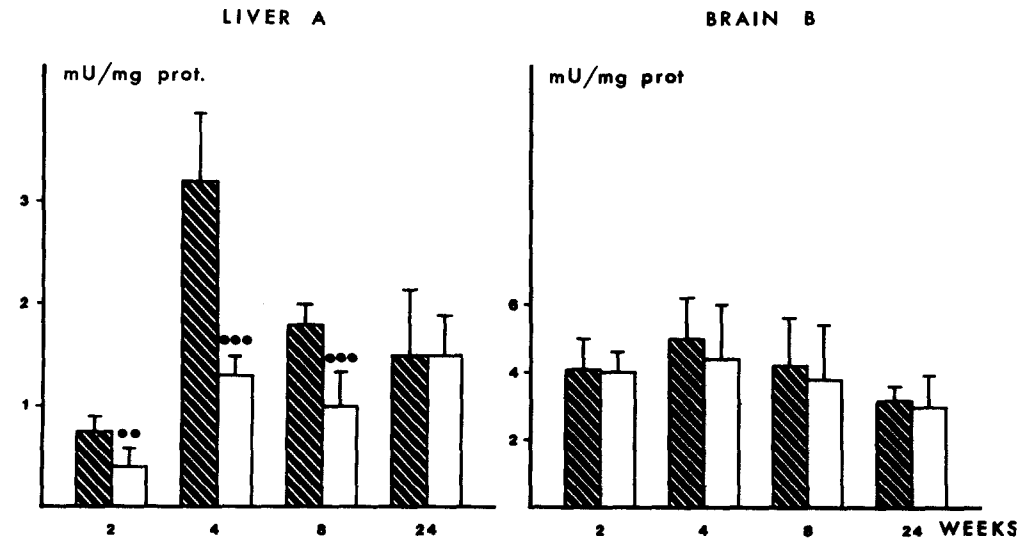

FIG. 5. Aldehyde dehydrogenase activity of liver (A) and brain (B) cytosol: control (hatched bars), alcohol withdrawal at mating (open bars), ●●●$p<0.001$, ●●$p<0.01$.

TABLE 3

EFFECT OF MATERNAL PREPREGNANCY ALCOHOL CONSUMPTION ON THE OFFSPRING

	Open-Field		Exploration	
	Locomotion (No. of squares crossed)	Number of Rearings	Novelty Preference [time (sec) spent]	Locomotion (No. of units entered)
Control	m 38 ± 5 (13) f 43 ± 5 (14)	‡ 20 ± 2 (13) 27 ± 3 (14)	353 ± 48 (14) 306 ± 51 (13)	43 ± 3 (14) 43 ± 5 (13)
Alcohol	m 45 ± 3 (9) * f 53 ± 4 (15)	28 ± 3 (9) § 38 ± 3 (15)	448 ± 32 (9) † 423 ± 23 (16)	45 ± 3 (9) 53 ± 3 (16)

Means ± SEM (ANOVA): *$p<0.05$, †$p<0.01$, ‡$p<0.005$, §$p<0.002$. In parentheses, the number of animals tested.

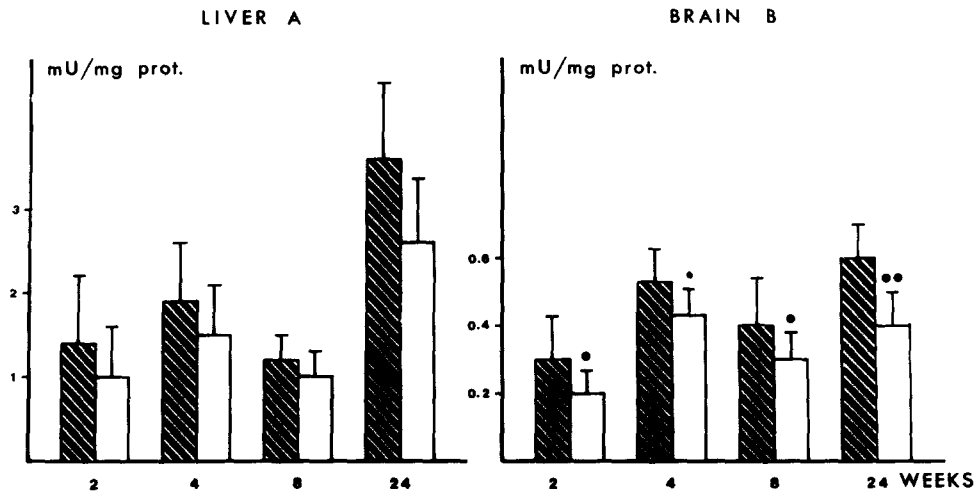

FIG. 6. Aldehyde dehydrogenase activity of liver (A) and brain (B) mitochondria: control (hatched bars), alcohol withdrawal at mating (open bars), ●●$p<0.01$, ●$p<0.05$.

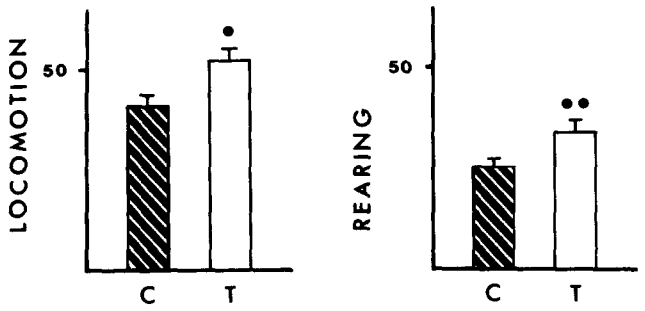

FIG. 7. Open-field activities (means ± SEM, *t*-test) C: controls (n = 27), T: treated rats (n = 25). ●$p<0.02$; ●●$p<0.001$.

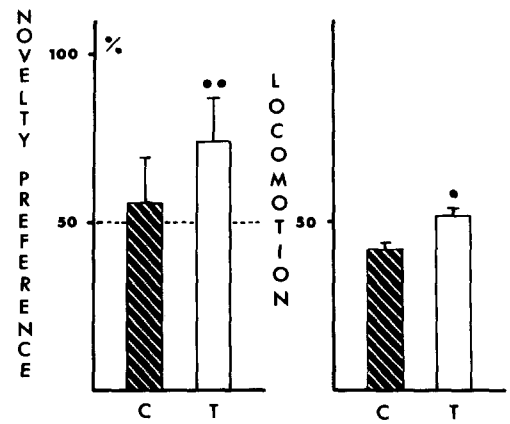

FIG. 8. Novelty-seeking behavior (means ± SEM, *t*-test). C: controls (n = 27), T: treated rats (n = 24). ●$p<0.03$; ●●$p<0.01$.

brain the ADH activity remained also significantly decreased. Lowered ADH activity may be responsible for the initiation of other metabolic pathways of alcohol degradation, i.e., the microsomal ethanol oxidizing system (13) or oxidation by free radicals (6,17). Decreased ADH activity found in the animals born to mothers which were alcohol deprived at the beginning of pregnancy may result from an impaired synthesis of the enzyme during development (22,23).

ALDH activity was mainly decreased in the liver cytosol as well as in the brain mitochondria. That observation correlates also with studies concerning chronic alcohol treatment (7,8). Since acetaldehyde combines with proteins, it may be possible that its accumulation results in an inhibition of ALDH activity (9) as well

as the activity of other enzymes. Decreased ALDH activity may result in an increase of acetaldehyde, known to be one of the most toxic agents in alcoholism.

Moreover, the results from the behavioral observations showed that alcohol exposure of mothers before pregnancy modified in adult offspring the locomotion as well as the emotional reactivity towards novelty. Indeed, compared to controls, treated rats exhibited a significant increase of locomotion in an open field and in a novel/familiar choice situation. This effect resembles the one observed by Abel (1) in adult rat offspring born to pregnant alcohol-consuming mothers. Furthermore, the treated animals were found to be less reactive towards the open-field procedure in so far as they exhibited significantly more rears than controls. The decreased emotional reactivity of treated rats was also illustrated by the fact that a few of them displayed autonomic responses in the open-field, while they exhibited less avoidance responses towards novelty than controls. Taken together, the behavioral data suggest that offspring born to mothers consuming alcohol before mating were hyporeactive to novelty.

The present study indicates that maternal alcohol exposure prior to pregnancy can significantly affect offspring in the very early stages of development. These alcohol effects could be mediated via free radicals known to produce DNA alterations and/or modified alcohol metabolism following an impaired activity of the alcohol-metabolizing enzymes. On the basis of our results, it appears that some effects on the embryo attributed by several authors (3) to prenatal alcohol exposure in utero may in fact be related to prepregnancy alterations.

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