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ACUTE EFFECTS OF ETHANOL ON BRAIN, PLASMA AND ADRENAL MONOAMINE CONCENTRATIONS IN VIRGIN AND PREGNANT RATS AND THEIR FETUSES

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Abstract—The dose-response relationship in brain, plasma, and adrenal monoamine changes after acute oral ethanol administration (1, 2, 4 g/kg body wt) was studied in virgin rats to determine whether the response to the highest dose differed in 21-day pregnant animals, and to assess the potential consequences of ethanol on the neurotransmitter systems of their fetuses. Blood ethanol and acetaldehyde concentrations in blood increased progressively with the ethanol dose in virgin rats, and values in pregnant animals were very similar. Ethanol concentration in fetal blood and amniotic fluid did not differ from that in mother's blood whereas fetal acetaldehyde concentrations were negligible. In a dose-related manner, ethanol decreased brain DA, DOPAC and 5HT concentrations did not affect those of NA and 5HIAA, or adrenal A and NA concentrations, whereas it enhanced plasma NA levels. Basal levels of monoamines and their changes after ethanol intake did not differ in pregnant and virgin rats. Monoamine and metabolite concentrations were much lower in fetal than in maternal brains whereas plasma and adrenal catecholamine concentrations were very similar and maternal ethanol intake did not modify these fetal parameters in the fetus. Results are in agreement with the known similar metabolic response to ethanol in fed pregnant and virgin rats. The lack of fetal monoamine response to maternal ethanol intake may be a consequence of the incapacity of fetal liver to form acetaldehyde and the ability of the placenta to oxidize maternal acetaldehyde which protects the fetus from maternal alcohol intake at late gestation.

Ethanol intake is known to interfere with different aspects of the central and peripheral nervous systems at different levels. Its spectrum of consequences are very wide, ranging from depressant to excitatory effects on central nervous system functions (Pohorecky and Newman, 1977; Tabokoff and Kijanma, 1982), depending on various factos including dosage and the physiological conditions of the recipient. Ethanol administration affects several neurotransmitter systems (Rahwan, 1974; Bacopoulus *et al.*, 1978; Liljequist and Carlsson, 1978; Hunt and Majchrowicz, 1979; Mena and Herrera, 1980; Ferko *et al.*, 1982; Frye and Breese, 1982; Edwards *et al.*, 1983), and we have previously reported that chronic treatment with moderate doses in the rat enhances monoamine concentrations in specific brain regions (Mena and Herrera, 1980). Alcohol ingested by the mother during pregnancy crosses the placenta freely (Bissonnette, 1981), attaining similar levels in fetal and maternal blood (Kesaniemi and Sippel, 1975; Kaufman and Woolam, 1981). The negative effects of maternal alcohol intake on intrauteral and postnatal offspring development are well established in humans (Jones and Smith, 1973; Streissguth *et al.*, 1980) as well as in experimental animals (Streissguth *et al.*, 1980; Abel and Dintcheff, 1978; Lee and Leichter, 1980; Herrera and Llobera, 1981; Ludeña *et al.*, 1983), the brain being one of the most affected sites (Branchey and Friedhoff, 1973; Rawat, 1975; Barnes and Walker, 1981; Borges and Lewis, 1981; Mena *et al.*, 1982, 1984). Chronic maternal alcohol ingestion modifies brain monoamine concentration although the direction of the change may vary according to the dose and duration of treatment (Mena *et al.*, 1982, 1984; Rawat, 1975; Detering *et al.*, 1980, 1981). It has been recently reported that maternal ethanol intake may produce different effects or have no effect on dopaminergic function in the fetal nervous system of the rat, according the stage of pregnancy which the mother receives the treatment (Lucchi *et al.*, 1984). It is not known, however, whether acute alcohol treat-

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ment affects monoamine metabolism in a different manner in pregnant and non-pregnant subjects. This question is of interest as it has been shown that other nervous system stimuli such as fasting, increase urinary catecholamine excretion in pregnant versus non-pregnant rats (Herrera *et al.*, 1969) and peripheral nervous terminals and adrenal catecholamine pools are depleted in pregnant rats (Young and Landsberg, 1979). The aims of the present study were to determine the dose-response relationship in brain, plasma, and adrenal monoamine changes after acute ethanol administration in virgin rats in order to determine whether the response differed in 21-day pregnant animals and to assess the potential consequences of ethanol in fetal neurotransmitter systems.

EXPERIMENTAL PROCEDURES

Animals

Virgin female Wistar rats weighing 180–200 g and age-matched 21-day pregnant animals were used (gestational time determined by the presence of spermatozoids in vaginal smears). Animals were fed purina chow diet and maintained under automatically controlled temperature (25 ± 1°C) and 12 h light–dark cycles (light from 9:00 to 21:00 h). Treatments were given at 11:00 h after a 3 h fasting period to minimize differences in alcohol absorption produced by the possible presence of food in the stomach. Ethanol dissolved in saline (0.9% NaCl) or plain saline was given in a total volume of 1 ml/100 g body wt by gastric sonda without anesthesia after which animals were maintained without access to food until sacrifice which was performed by decapitation 3 h after treatment. Just before sacrifice, rectal body temperature was measured with a lubricated thermometer pb 0331 (Panlab, Spain) inserted 2.2 cm into the rectum. In pregnant rats the conceptus was immediately dissected and amniotic fluid was collected with a syringe before the sacs were opened and placed in tubes containing 300 μl of 100 mM chloral hydrate. Fetuses were decapitated and blood samples were collected from the neck wound into two separate ice-cold recipients, one containing 300 μl of 100 mM chloral hydrate to be used for ethanol and acetaldehyde determinations and the other containing heparin and used for plasma separation to which 10% EDTA (25 μl per 0.9 ml plasma) and 5% $Na_2S_2O_5$ (10 μl per 0.9 ml plasma) were added before being frozen at −80°C until monamine analysis. Brain and adrenals were dissected, frozen on dry ice, weighed, and stored at −80°C until processed.

Ethanol and acetaldehyde determinations

These determinations were always done in fresh blood samples the same day of their collection. The method of Von Wartburg and Ris (1979) was followed with minor modifications. Immediately after placing the amniotic fluid or plasma aliquots in chloral hydrate, proportions were adjusted to 1:1 (vol/vol) by weighing the tubes and adding the appropriate amount of chloral hydrate. After being thoroughly mixed at 4°C and centrifuged at 1000 *g* for 15 min, 150 μl of supernatant aliquots were placed in 1 ml glass vials containing 300 μl saline, 500 μl of 1.5 mM 1-propanol (internal standard) and 50 μl of 60% of $HClO_4$ after which the vials were hermetically sealed. External standard vials, containing plasma from untreated animals supplemented with chloral hydrate and saline or different amounts of ethanol or acetaldehyde, were always run in parallel with blank vials. All vials were subjected to head space gas chromatography performed with a Perkin–Elmer Sigma-15 apparatus and a column of Carbowax 1540. Temperatures were 60°C for the sample thermostat and 150°C for the injector and detector block. With this procedure, the amounts of ethanol and acetaldehyde recovered from fresh plasma samples were 106.5 ± 7.5 and 98.4 ± 10.1 respectively.

Monoamine analysis

Monoamines were determined in tissues and plasma by high performance liquid chromatography with electrochemical detection (HLPC/ED).

Standards and reagents. Noradrenaline (NA), adrenaline (A), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), 5-hydroxy-tryptamine (5-HT) and 5-hydroxy-indolacetic acid (5HIAA) and alumina, all of the highest purity, were obtained from Sigma (Saint Louis, Mo., U.S.A.) and methanol for HPLC was from Scharlau (Ag Duren, Germany). Other reagents were obtained from Merck (Darmstadt, Germany).

Sample preparation. Whole brain samples were homogenized in the proportions of 1/10 (wt/vol) in a glass homogenizer with ice-cold 0.1 N $HClO_4$ containing EDTA (0.05%) and $Na_2S_2O_5$ (0.05%). Whole adrenal glands from each animal (30–50 mg) were homogenized by sonication at 0–4°C in 3 ml 0.1 N $HClO_4$ containing EDTA (0.05%) and $Na_2S_2O_5$ (0.05%), and 1 ml aliquots of the tissue homogenate supernatants or 0.9 ml plasma were placed in vials containing 400 μl of 1 M Tris–HCl buffer pH 8.6 and 25 mg aluminum oxide pretreated according to Anton and Sayre (1962). After shaking for 15 min and centrifugation at 4000 *g* for 10 min, supernatants were discarded and the vials were washed three times with 1 ml distilled water. Catecholamines were eluted by adding 100 μl of 0.1 N $HClO_4$ containing 0.1 mM $Na_2S_2O_5$ and supernatants were injected in the HPLC/ED system as described below. At this stage, recovery of purified standards added to the initial samples was 75.2 ± 3.0% (n = 11) for NA, 77.1 ± 7.6% (n = 11) for A, and 72.0 ± 3.8 (n = 11) for DA. Other aliquots of the supernatants from the centrifuged tissue homogenates were filtered through Millex HA 0.4 μm (Millipore Co. Bedford, Mass., U.S.A.) and directly injected into the HPLC/ED system for indolamine and DOPAC determinations.

HPLC/ED apparatus and analytical conditions. A model 6000A solvent delivery system and a U6K injector from Waters (Waters Ass. Inc., Milford, Mass., U.S.A.) were coupled with a BAS LC-4B amperometric detector and a LC-16 transducer equipped with a glassy carbon electrode (Bioanalytical Systems, La Fayette, Ind., U.S.A.). Two reverse phase columns of Nucleosil $5C_{18}$ (Scharlau Ag Duren, Germany) (12.5 cm × 4 mm i.d. and 20 cm × 4 mm i.d.) were used, protected by a guard column containing Bondapak C_{18}/Corasil (Waters Ass.). Except for minor modifications, chromatographic conditions were similar to those in previous studies (Maruyama *et al.*, 1980; Reinhard *et al.*, 1980; Goldstein *et al.*, 1981; Westerink and Mulder, 1981). The column was maintained at room temperature, flow rate was 0.8 ml/min, and the potential was set at +0.5 V vs the Ag/AgCl reference electrode for catecholamines and at +0.75 V for indolamines and DOPAC.

Table 1. Ethanol and acetaldehyde concentrations in blood and amniotic fluid 3 h after oral treatment with ethanol of virgin and 21-day pregnant rats

	Ethanol (mmol/l)	Acetaldehyde (μmol/l)
Virgin rats		
treated with 1 g EtOH/kg	0.26 ± 0.05 (5)	7.65 ± 3.73 (6)
treated with 2 g EtOH/kg	13.96 ± 1.09 (5)	19.27 ± 6.36 (6)
treated with 4 g EtOH/kg	37.10 ± 4.45 (5)	33.19 ± 12.60 (6)
21-day pregnant rats		
treated with 4 g EtOH/kg		
Mother's blood	41.24 ± 8.60 (5)	45.51 ± 21.64 (5)
Fetus's blood	41.84 ± 9.75 (5)	7.38 ± 4.85 (8)
Amniotic fluid	41.09 ± 0.25 (5)	Not detectable (8)

Results are means ± SEM. ( ) = number of animals/group.

The injection volume was 20 μl for all determinations. Separation of NA, A and DA was achieved by an isocratic mobile phase containing a McIlwain buffer 0.1 M citrate–phosphate pH 6.5 and elution time was less than 7 min. Separation of 5HT, 5HIAA and DOPAC was performed with a mobile phase containing 0.1 M citrate–phosphate buffer pH 3.5 plus 8% methanol and elution time was less than 15 min.

Expression of the results

Results were expressed as mean ± SEM and statistical comparison among the groups was done by the Student's *t*-test.

RESULTS

Ethanol and acetaldehyde blood levels and body temperature

As shown in Table 1, 3 h after oral ethanol administration to virgin rats their blood concentration of ethanol began to increase progressively with dosage. Blood acetaldehyde concentrations were negligible using 1 g ethanol/kg body wt but they increased with greater doses (Table 1). When the highest amount of ethanol was given to virgin and to 21-day pregnant rats, as shown in Table 1, their blood ethanol and acetaldehyde concentrations were similar. Ethanol concentrations in both fetal blood and amniotic fluid were almost the same as in maternal blood whereas acetaldehyde concentrations were negligible in fetal blood and undetectable in amniotic fluid (Table 1). Body temperature was only measured in virgin animals, values being 36.18 ± 0.16°C for controls and 35.37 ± 0.24, 35.35 ± 0.24 and 35.18 ± 0.24°C for those treated with 1, 2 and 4 g ethanol/kg respectively (P vs controls were <0.05 for those receiving the 1 and 2 g dose and <0.01 for those of 4 g).

Monoamine and metabolite concentrations in brain

As shown in Table 2, acute ethanol administration of 1, 2 or 4 g/kg to virgin rats did not affect brain NA and 5-HIAA concentrations while DA, DOPAC and 5-HT concentrations decreased in a dose-related manner. In comparison with virgin rats receiving saline (controls), brain DA values decreased significantly in those receiving 2 and 4 g ethanol whereas DOPAC and 5-HT changes were only significant with the 4 g dose (Table 2). In 21-day pregnant rats receiving saline (controls), brain monoamine and metabolite concentrations did not differ from values in virgin controls (Table 2). When compared with their respective controls, pregnant and virgin rats given 4 g/kg ethanol had similarly decreased brain DA, DOPAC and 5-HT concentrations. After ethanol treatment, brain NA concentration was unaffected in pregnant rats and in virgins (Table 2) while 5-HIAA concentration decreased significantly (Table 2). In fetuses from control mothers, brain monoamine concentrations were similar to those previously reported (Mena *et al.*, 1982; Rawat, 1975) and values were much lower than those found in virgin rats and in their respective mothers (Table 2). In contrast with the effects found in adults, acute maternal alcohol intake did not modify any fetal brain monoamine or metabolite concentrations (Table 2).

Catecholamines in plasma

Ethanol treatment did not modify plasma A levels in virgin and pregnant rats nor in their fetuses (data not shown). Values of plasma NA levels are summarized in Table 3. In virgin animals, acute ethanol intake of 2 and 4 g/kg produced significant increments in plasma NA levels whereas 1 g/kg appeared ineffective when compared with controls receiving saline. Plasma NA values in pregnant rats were significantly greater than in virgins and ethanol at 4 g/kg produced a similar effect in pregnant and in virgin rats, consisting of a significant increment in NA (Table 3). In contrast with the change in brain monoamines, plasma catecholamine levels were similar in fetuses and in their mothers and plasma NA

Table 2. Effect of ethanol on brain monoamine and metabolite concentrations in virgin and 21-day pregnant rats, and their fetuses

	NA (ng/g)	DA (ng/g)	DOPAC (ng/g)	5HT (ng/g)	5HIAA (ng/g)
Virgin rats					
treated with saline (controls)	498.8 ± 21.4	1047.8 ± 20.3	154.9 ± 6.7	378 ± 16.6	241.1 ± 17.3
treated with 1 g EtOH/kg	409.6 ± 18.8	960.7 ± 45.5	152.8 ± 14.5	361.6 ± 24.2	258.0 ± 10.3
P	N.S.	N.S.	N.S.	N.S.	N.S.
treated with 2 g EtOH/kg	430.4 ± 25.5	875.0 ± 33.2	145.5 ± 9.8	343.5 ± 27.2	235.0 ± 27.3
P	N.S.	≤0.01	N.S.	N.S.	N.S.
treated with 4 g EtOH/kg	513.2 ± 14.6	850.8 ± 43.0	126.4 ± 9.2	318.8 ± 19.8	234.4 ± 14.9
P	N.S.	≤0.01	≤0.01	≤0.05	N.S.
Pregnant rats					
treated with saline (controls)	523.8 ± 14.0	941.6 ± 44.9	182.2 ± 23.5	329.2 ± 18.8	281.0 ± 22.5
treated with 4 g EtOH/kg	523.7 ± 46.9	735.2 ± 39.9	124.7 ± 5.1	276.2 ± 14.9	208.8 ± 13.5
P	N.S.	≤0.01	≤0.05	≤0.05	≤0.05
Fetuses					
mother treated with saline	145.3 ± 10.9	99.8 ± 7.2	30.1 ± 6.3	116.9 ± 20.6	172.8 ± 15.4
mother treated with 4 g EtOH/kg	153.8 ± 17.2	104.0 ± 10.1	42.5 ± 6.2	116.2 ± 24.2	143.3 ± 5.1
P	N.S.	N.S.	N.S.	N.S.	N.S.

Animals were killed 3 h after treatment. Results are mean ± SEM of 6–8 rats group. Statistical comparisons between the ethanol groups and their respective controls (treated with saline) are shown by the *P* values and those between fetuses and their respective mothers by crosses: + = $P \leq 0.01$, + + = $P \leq 0.001$. Statistical comparisons between pregnant and virgin animals were not significant ($P > 0.05$) for any of the parameters studied.

Table 3. Effect of oral ethanol on plasma NA levels in virgin and 21-day pregnant rats, and their fetuses

	NA (ng/ml)
Virgin rats	
treated with saline (controls)	5.15 ± 0.34
treated with 1 g ethanol/kg	4.64 ± 0.19
P	N.S.
treated with 2 g ethanol/kg	6.74 ± 0.61
P	≤0.05
treated with 4 g ethanol/kg	6.95 ± 0.73
P	≤0.05
Pregnant rats	
treated with saline (control)	7.34 ± 0.51**
treated with 4 g ethanol/kg	9.38 ± 0.98
P	≤0.05
Fetuses	
mother treated with saline	8.96 ± 0.66
mother treated with 4 g ethanol/kg	8.39 ± 0.66
P	N.S.

Animals were killed 3 h after treatment. Results are means ± SEM of 6–8 rats/group. Statistical comparisons between the ethanol groups and their respective controls (treated with saline) are shown by the *P* values and those between pregnant and virgin rats by asterisks: ** = $P \leq 0.01$. Statistical comparisons of values between fetuses and their respective mothers were not significant ($P > 0.05$).

levels did not change in fetuses of mothers receiving alcohol as compared with controls (Table 3).

Catecholamines in adrenals

As expected, A content in adrenals was approximately four times greater than NA (Table 4). Values of both catecholamine concentrations in the adrenals of pregnant rats were very similar to those in virgins (Table 4). In spite of the modifications of plasma and brain monoamine concentrations following ethanol treatment, no given dosage of ethanol modified adrenal catecholamine concentrations in virgin or pregnant rats (Table 4). This parameter was not measured in fetal adrenals due to the difficulties involved in their rapid dissection.

DISCUSSION

Present findings in virgin rats show that acute oral ethanol intake decreased brain DA, DOPAC, and 5HT concentrations in a dose-related manner but did not affect NA and 5HIAA or A and NA concentrations in adrenals, whereas it enhanced plasma NA levels. Changes in brain monoamines differed from our previous findings in the rat in which chronic ethanol treatment produced an enhancement in their brain concentrations (Mena and Herrera, 1980) but this difference may well be due to the known fact that ethanol effects on cerebral neurotransmitters metab-

Table 4. Effect of oral ethanol on catecholamines concentration in adrenals in virgin and 21-day pregnant rats

	NA (μg/g)	A (μg/g)
Virgin rats		
treated with saline (control)	162.11 ± 9.26 (6)	668.31 ± 45.60 (6)
treated with 1 g EtOH/kg	161.78 ± 11.63 (5)	643.67 ± 41.24 (5)
treated with 2 g EtOH/kg	179.25 ± 19.68 (5)	746.60 ± 93.89 (5)
treated with 4 g EtOH/kg	174.76 ± 24.6 (5)	558.59 ± 7.24 (5)
Pregnant rats		
treated with saline (control)	159.54 ± 16.56 (6)	702.38 ± 52.14 (5)
treated with 4 g EtOH/kg	198.37 ± 20.40 (6)	724.74 ± 71.13 (6)

Animals were killed 3 h after treatment. Results are mean ± SEM of 5–6 rats/group. No statistical significance ($P > 0.05$) was found between ethanol and control groups and between pregnant and virgin animals.

olism differ substantially when administered either chronically or acutely (Kuriyama *et al.*, 1971), and according to dosage and mode of administration (Pohorecky *et al.*, 1974). An inhibitory or biphasic effect on brain tryptophan and serotoninergic system after acute ethanol treatment in the rat has been previously reported (Stowell and Morland, 1984; Badaway and Evans, 1976) in agreement with present findings. The hypothermia of our ethanol treated virgin animals is in agreement with that reported by Poherecky *et al.* (1974) and Pohorecky *et al.* (1976). Our finding of reduced brain 5HT in ethanol treated virgin rats fits with their hypothesis that hypothermic effects of ethanol may be caused by reduced stimulation of 5HT receptors (Pohorecky *et al.*, 1976). Lack of change in catecholamine adrenal content and plasma A levels after alcohol intake indicates that the doses administered (1–4 k/kg) did not affect adrenal medullary secretion, in agreement with previous reports (Perman, 1961; Deturck and Vogel, 1982). These findings should be also considered together with those of others showing that higher oral ethanol doses (Perman, 1961) or lower doses but given under stressed conditions (Deturck and Vogel, 1982; Perman, 1960) may increase circulating catecholamines following adrenal medulla stimulation. It seems then that adrenal medulla ethanol response is very much dependent on the dose and mode of administration. Changes in plasma NA levels are indirect index of sympathetic activity. The specific rise in plasma NA levels found after acute administration of 2–4 g/kg ethanol in virgin animals therefore indicates an increased sensitivity to alcohol which has a greater effect on peripheral noradrenergic neurons than on adrenal medullary function.

Blood ethanol and acetaldehyde concentrations were similar in pregnant and virgin rats receiving the same ethanol dose per unit of body weight, suggesting that their endogenous distribution and metabolism are similar. Basal levels of monoamines and their changes after ethanol intake did not differ in pregnant and virgin rats, indicating that they have similar monoamine stores and alcohol sensitivity affecting neurotransmitter metabolism. While these findings contrast with reported depletions of adrenal catecholamine content (Young and Landsberg, 1979) and enhanced urinary excretion of catecholamines (Herrera *et al.*, 1969) in the untreated pregnant rat, the changes only occurred in the fasting state (Herrera *et al.*, 1969; Young and Landsberg, 1979), whereas the present study was performed in fed animals. The similar response to alcohol in pregnant and virgin rats is in agreement with our finding that alcohol produces similar hyperglycemia in both types of fed rats (Villarroya *et al.*, 1985), and differences in their nervous system responses to alcohol may occur only in conditions of hypoglycemia such as in the fasted state. This hypothesis is supported by the differing metabolic responses to anesthetics in virgin and pregnant rats when fasted but not when fed (Zorzano and Herrera, 1984), although it must be further tested in studies of alcohol intake.

Monoamine and metabolite concentrations were, as expected, much lower in the brains of 21-day old rat fetuses than in their respective mothers. In contrast with the effects of chronic ethanol maternal intake (Mena *et al.*, 1982; Mena *et al.*, 1984; Rawat (1975); Detering *et al.*, 1980, 1981), there were no changes in these parameters after acute treatment, indicating that at late gestation the fetal rat brain

is less vulnerable to maternal alcohol intake than during earlier fetal stages. The recent report of Lucchi *et al.* (1984) describing permanent changes in brain dopaminergic transmission in rat offspring of mothers given alcohol only on the 4th day of gestation supports this possibility. A similar explanation may be proposed for the lack of change in plasma catecholamine concentrations in the 21-day old fetus after maternal ethanol intake as decreased sensitivity of this parameter to ethanol cannot be explained by an immature peripheral catecholamine metabolism. We found that plasma levels of catecholamines did not differ in 21-day fetuses and their mothers, in agreement with previous findings (Roffi, 1968; Phillippe and Ritzmiller, 1981; Ben-Jonathan, 1978) and it is also known that at late gestation the rat fetus undergoes plasma catecholamine changes as a result of metabolic and/or hormonal stimulus (Ben-Jonathan, 1978).

Lack of fetal monoamine response to acute maternal alcohol intake at late gestation contrasts with the changes reported following chronic alcohol administration from the onset of gestation (Mena *et al.*, 1982, 1984; Rawat, 1975; Detering *et al.*, 1980, 1981) and indicates that negative fetal alcohol effects are not produced by alcohol crossing the placenta and being metabolized by fetal tissues, but occur during early gestation when the placenta is not yet developed. Acetaldehyde production by the fetus appears negligible as evidenced by its low blood levels in fetuses as compared with their mothers, and this agrees with the low alcohol dehydrogenase activity detected in fetal liver even at late gestation (Sjoblom *et al.*, 1978; Horton and Mills, 1979). It is well known that acetaldehyde formed after ethanol ingestion participates actively in the neurological effects of ethanol in adults. The incapacity of fetal liver to form acetaldehyde and the ability of the placenta to oxydize maternal acetaldehyde (Sippel and Kesaniemi, 1975) may protect the fetus against maternal alcohol intake at late gestation.

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