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THE ROLE OF THE TETRAHYDROISOQUINOLINE, SALSOLINOL, IN THE MECHANISM OF ETHANOL TERATOGENICITY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

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To Mom, Dad and Al
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  Pharmacology of Ethanol
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CHAPTER I

INTRODUCTION

TIQ Theory of Alcoholism

Recent evidence in both laboratory animals and in humans suggests that tetrahydroisoquinoline (TIQ) and tetrahydro-β-carboline (TBC) alkaloidal metabolites, formed endogenously from interactions between acetaldehyde and catecholamines or serotonin during alcohol consumption, may play a role in the development of physical dependence to ethanol (for reviews, see Rahwan, 1974; Rahwan, 1975). The simple TIQs were proposed to be formed by the direct condensation of acetaldehyde with endogenous catecholamines to form substituted 6,7-dihydroxy TIQs (Fig. 1) (Cohen and Collins, 1970). The similarity in structure between these TIQs and the catecholamines suggested that the TIQs might interact with mechanisms which normally regulate the physiologic properties of catecholamines.

Indeed, Heikkila et al. (1971) have shown that TIQs may act as false transmitters in that they are taken up and accumulated in rat brain synaptosomes, and interfere with the uptake and release of catecholamines by the synaptosomes. In addition, TIQ alkaloids are formed in acetaldehyde and
Figure 1. Pictet-Spengler condensation of acetaldehyde and a catecholamine to form first an unstable Schiff base and finally one of the simple tetrahydroisoquinolines (TIQs). Shown here is the formation of salsolinol, EPI-TIQ, and NE-TIQ from the condensation of acetaldehyde and dopamine, epinephrine and norepinephrine, respectively.
Figure 1
formaldehyde-perfused adrenal glands, bind within the adrenal chromaffin granules (Greenberg and Cohen, 1972) and are released from the adrenal medulla upon stimulation (Greenberg and Cohen, 1973; Rahwan et al., 1974). Norepinephrine and epinephrine TIQs have been shown to act as α-adrenergic agonists, weak β-agonists, and in higher concentrations have β-receptor blocking properties in in vivo and in vitro preparations.

Dopamine-derived TIQs and norepinephrine-derived TIQs have also been reported as substrates for catechol-O-methyltransferase (COMT) (Rubenstein and Collins, 1973; Collins et al., 1973), and the competitive inhibition of monoamine oxidase (MAO) by dopamine-derived TIQs has been demonstrated (Collins et al., 1973). Collins et al. (1973) demonstrated that salsolinol, the condensation product of dopamine and acetaldehyde, was a competitive inhibitor of the O-methylation of dopamine and a weak competitive inhibitor of the oxidation of serotonin by rat brain MAO. Azevedo and Osswald (1977) recently reported that chronic administration of 2 epinephrine-derived TIQs induced selective degeneration of the adrenergic nerve terminals of adult rats and of the whole adrenergic neurone of newborn rats.

Another hypothesis proposes the formation of more complex TIQs. Davis et al. (1970) suggested that since
Figure 2. Catechol-O-methyltransferase (COMT) catalyzed O-methylation of salsolinol in the presence of S-adenosylmethionine (SAM). The resulting product isosalsoline and salsoline are inseparable by chromatographic means.
Figure 2
acetaldehyde can competitively inhibit the aromatic aldehyde dehydrogenase, the aldehyde product of dopamine, produced normally in the brain by monoamine oxidase, would be prevented from further oxidation to dopacetic acid. This aldehyde, 3,4-dihydroxyphenylacetaldehyde (dopaldehyde), would then condense with dopamine to form the TIQ derivative tetrahydropapaveroline (THP, see Fig. 3). Therefore, a heavy consumption of alcohol could result in a diversion of dopamine metabolism in the central nervous system to produce THP.

Interest developed in the possibility that THP, formed in mammalian cells, could undergo further biotransformation into more complex multi-ring morphine-like alkaloids, and that these compounds are responsible for the development of alcohol addiction. THP biosynthesis has been demonstrated in vitro in the brain stem (Davis et al., 1970; Davis and Walsh, 1970), a tissue with low aldehyde oxidizing capacity, and this biosynthesis is enhanced significantly by incubation with ethanol and acetaldehyde.

Early studies (Halushka and Hoffman, 1968) found \(^{14}\text{C}\)-THP formation in vivo in guinea pig liver 2 and 5 min after intravenous administration of \(^{14}\text{C}\)-dopamine. In vivo studies (Cohen and Barrett, 1969; Collins and Cohen, 1970) also confirmed the formation of tetrahydroisoquinoline
Figure 3. The formation of tetrahydropapaveroline (THP) from the condensation of dopamine and dopaldehyde.
Figure 3

DOPAMINE $\xrightarrow{\text{MAO}}$ DOPALDEHYDE $\rightarrow$ TETRAHYDROPAPAVEROLINE
condensation products of formaldehyde and epinephrine and norepinephrine in rats treated with methanol.

The in vivo biosynthesis of salsolinol in brain tissue was first demonstrated by Collins and Bigdeli (1975) in rats following acute treatment of alcohol. However, these animals had been pretreated with pyrogallol (COMT inhibitor) and pargyline (MAO inhibitor) which increased salsolinol formation by 7-fold following ethanol administration. Pyrogallol had previously been shown to increase ethanol-derived acetaldehyde blood concentrations in rats (Collins et al., 1974). THP formation however was not detected with their gas chromatography/electron-capture assay. After administration of ethanol and levodopa with a peripheral decarboxylase inhibitor in the drinking water of rats, 2 to 15 ng of THP per g of brain tissue were detected in these animals (Turner et al., 1974). However, those animals receiving ethanol alone showed no significant THP formation.

The presence of significant amounts of salsolinol (700 mg daily) and THP in the urine of parkinsonian patients on L-dopa treatment with concomitant ethanol consumption was detected by a gas chromatographic assay (Sandler et al., 1973). Formation of salsolinol in parkinsonian patients who had not received ethanol was also reported but later discounted as an extracorporeal formation resulting from reagent contamination with acetaldehyde (Cohen, 1976).
Recently Nijm et al. (1977) detected the presence of salsolinol in the urine of levodopa-treated patients, ruling out an extracorporeal source. However, the authors suggested diet as a possible source of the TIQ.

Cashaw et al. (1974) reported the in vivo formation of four tetrahydroprotoberberines found in the urine of rats treated with tetrahydropapaveroline. These tetrahydroprotoberberines are plant alkaloids produced by a series of reactions similar to those occurring in the opium poppy, Papaver somniferum (Shamma, 1972).

One might expect that the administration of levodopa would be capable of producing addiction since the TIQs and tetrahydroprotoberberines are formed in humans treated with L-dopa. However, this has not been reported. In fact, D-dopa (not a substrate for dopa decarboxylase) was more effective than L-dopa in increasing the preference for ethanol in the rat. The reason for this finding is still a mystery (Sprince et al., 1972).

All of the investigations discussed above, however, required pharmacological manipulations which increased aldehyde precursors or inhibited degradation before demonstrating in vivo tetrahydroisoquinoline biosynthesis. O'Neill and Rahwan (1977a) investigated the formation of salsolinol in brain tissue under conditions of physical dependence to ethanol without intervening pharmacological
manipulations. Using a gas chromatographic/electron-capture assay (O'Neill and Rahwan, 1977b), capable of detecting as little as 8 ng of salsolinol per g of mouse brain tissue, they were unable to detect the in vivo formation of salsolinol in individual whole brains, pooled whole brains, or dopamine-rich areas of pooled brains obtained from ethanol-dependent mice with blood ethanol levels as high as 700 mg% (O'Neill and Rahwan, 1977a).

Using a similar experimental design, Hamilton et al. (1978) were unable to detect the formation of salsolinol in dopamine-rich striatal tissue of mice which had been exposed to a 10-day treatment of ethanol vapor. However, they were able to detect the formation of methoxysalsolinol (the 6-methoxy or 7-methoxy derivatives) which could result from the COMT-catalyzed O-methylation of the parent compound, indicating at least a brief presence of salsolinol. An alternative, proposed by the authors, to this explanation of the generation of methoxysalsolinol may be the direct condensation of 3-methoxytyramine, a dopamine metabolite in the striata, with acetaldehyde (Hamilton et al., 1978).

Riggin and Kissinger (1977) fed rats a 10% alcohol liquid diet for 25 days (8-13 g ethanol per kg per day) without additional pharmacological manipulation. Neither salsolinol nor tetrahydropapaveroline was detectable in the rat brains using their high performance liquid chromatography
assay with a lower limit of sensitivity of 2 ng of either TIQ per g of rat brain tissue.

McIsaac (1961a) demonstrated in vivo formation of tetrahydro-β-carbolines (TBCs) in the rat during ethanol, disulfiram (an aldehyde dehydrogenase inhibitor) and iproniazid (an MAO inhibitor) administration. The formation of the β-carbolines proceeds by a similar reaction as that for the TIQs, as the TBCs are the condensation products of ethanol-derived acetaldehyde with indoles. A general biochemical hypothesis of mental disease was proposed by McIsaac (1961b) suggesting that the TBCs were further metabolized to the hallucinogenic harmala alkaloids. Dajani and Saheb (1973) confirmed the findings of McIsaac (1961a) and suggested that the TBCs may play a role in the development of ethanol dependence.

The recent demonstration that intracerebral administration of TIQs or TBCs in minute amounts to rats mimics ethanol dependence and increases free-choice ethanol consumption over water (Myers and Melchior, 1977a, 1977b; Myers and Oblinger, 1977; Melchior and Myers, 1977a, 1977b) has created a renewed interest in the role of these alcohol-derived alkaloids in the central effects of ethanol. THP was infused directly into the cerebral ventricle of rats automatically every 15 minutes for 12 days. The rats were allowed free access to both water and alcohol. The animals,
which normally were aversive to alcohol, drank alcohol solutions in increasing amounts within 3 to 6 days of the start of the infusion of THP. These animals exhibited symptoms which were similar to those of withdrawal and intoxication (Myers and Melchior, 1977a). In another similar study, Melchior and Myers (1977a) demonstrated that chronic infusion of both the racemic mixture of THP and the S-(−) THP isomer exerted this alcohol-drinking effect and that the excessive intake of alcohol during the intraventricular infusions of THP continued long after the cessation of the infusion regimen. Withdrawal-like symptoms were also observed in the THP-infused rats at the termination of the 12-day experiment. Myers and Melchior (1977b) also studied the effects of four TIQs and a β-carboline, tryptoline, when infused directly into rat ventricles in a dose ranging from 0.0004 - 4.0 μg per 4.0 μl. Each alkaloid was dissolved in a cerebrospinal fluid (CSF) vehicle and delivered over a 58 sec interval every 30 min throughout a 12-day period. The chronic intraventricular infusion of 1-methyl-3-carboxy-6,7-dihydroxy-TIQ or 4,6,7-tri hydroxy-TIQ produced a moderate increase in voluntary alcohol intake. However, salsolinol, 6-methoxy-4,7-dihydroxy-TIQ and tryptoline all produced a significant enhancement in the rats' preference for alcohol within 2 to 6 days of the start of the infusions. Myers and Oblinger (1977) reported
that a single infusion of THP once a day into the lateral ventricle in doses of 0.1 - 1.0 μg produced an increase in alcohol drinking over a period of 12 days in rats normally aversive to alcohol. Several rats consumed as much as 8 - 10 g per kg per day of alcohol, with some animals exhibiting withdrawal signs at the end of the experiment. Alcohol consumption was also enhanced significantly with infusion of mixtures of tryptoline and THP, or salsolinol and THP. Finally, Melchior and Myers (1977b) showed that intracerebral infusion of THP, salsolinol, or noreleagnine in rats in doses ranging from 0.4 ng to 4.0 μg every 30 min for 12 days produced an enhanced alcohol preference over water. In addition, withdrawal symptoms were manifest in certain animals independent of whether or not alcohol was ingested. Therefore, these results offer direct support for the theory that these alkaloids are involved in the central actions of alcohol and may be mediators of the pharmacological or toxicological effects of alcohol.

Support for the hypothesis implicating acetaldehyde in alcohol dependence stems from the observation that after chronic exposure to either acetaldehyde or ethanol vapor, mice exhibit similar changes in behavior upon withdrawal (Ortiz et al., 1974). Ortiz et al. (1974) demonstrated that ethanol inhalation during acetaldehyde withdrawal was able to prevent the withdrawal syndrome in the same way
acetaldehyde inhalation during ethanol withdrawal prevented the behavioral changes induced by ethanol withdrawal.

However, Wood and Laverty (1979) have criticized this hypothesis. They have fed rats liquid diets containing ethanol or tert-butanol, and have observed withdrawal reactions upon removal of either alcohol from the diet after 4 to 20 days (Wood and Laverty, 1979). Only the time course of the withdrawal reactions differed. Tert-butanol (2-methyl-propan-2-ol) is a tertiary alcohol which, unlike ethanol, cannot be metabolized to an aldehyde. In addition, depletion of brain norepinephrine and dopamine by 6-hydroxydopamine pretreatment did not prevent the development of physical dependence on either alcohol, suggesting that the development of physical dependence on these alcohols was not dependent on aldehyde formation or brain catecholamines.

It has been shown, however, that agents capable of complexing acetaldehyde in vitro can protect animals in vivo against the toxic effects of ethanol or acetaldehyde (Sprince et al., 1974; O'Neill and Rahwan, 1976; Blum et al., 1974). Furthermore, Hupka and Yoon (1978) presented evidence that the administration of D-penicillamine (which forms stable complexes with acetaldehyde in vivo) during ethanol consumption, prevents the development of physical dependence to ethanol.
If the morphine-like metabolites of ethanol are responsible for ethanol dependence (Davis and Walsh, 1970), then a known opiate antagonist such as naloxone should induce the withdrawal syndrome in ethanol-dependent animals. Indeed, Blum et al. (1977) demonstrated that naloxone inhibits the development of ethanol dependence in mice, and it was also shown in this same species that both morphine and dopamine produced a pronounced suppression of alcohol-induced withdrawal convulsions (Blum et al., 1976). In addition, Jones and Spratto (1977) reported that chronic ethanol administration during the development of dependence on morphine significantly suppressed the symptoms of morphine withdrawal without altering brain morphine levels.

The TIQs, salsolinol and 3-carboxysalsolinol (the condensation product of levodopa and acetaldehyde) were found to prolong ethanol-induced narcosis in mice (Marshall and Hirst, 1976). It has also been reported that salsolinol behaves as a partial opiate agonist in the guinea pig ileum (Hamilton et al., 1976). Recently, Mackenzie (1979) reported the reversal of alcohol-induced comas in 2 patients admitted to the hospital with blood alcohol levels of 210 mg% and 350 mg%, suggesting a relationship between alcohol and opiate effects in humans.
The Fetal Alcohol Syndrome

Human Observations

A contemporary aspect of ethanol toxicity concerns its teratogenic action. However, the relationship between maternal alcohol consumption and serious problems in the offspring was alluded to as early as Biblical times (Judges 13,4). In 1834 a Select Committee of the British House of Commons, established to investigate "drunkenness," presented evidence that infants born to alcoholic mothers sometimes had a "starved, shriveled and imperfect look." In 1900, an increased frequency of early fetal death and early infant mortality in the offspring of female alcoholics was reported by Sullivan. Since that time intermittent reports have been published indicating an association between chronic alcoholic mothers and serious anomalies in their offspring (Ladraque, 1901; Roe, 1944; Lecomte, 1950; Christiaens et al., 1960). But it wasn't until 1967 (Lemoine et al., 1967) that the first detailed scientific report on ethanol teratogenesis appeared in the French literature. This report detailed 127 children born to alcoholic mothers who suffered from abnormalities including growth deficiencies, cranio-facial anomalies and psychomotor retardation.
In 1972, Ulleland reported a study of 10 children of alcoholic mothers with intrauterine growth failure, where no catch-up growth was observed. This condition is in contradistinction to malnutrition where catch-up growth and development can be achieved (Brock, 1961). In 1973, Jones et al. described the characteristic pattern of anomalies in 8 children of 3 different ethnic groups born to chronic alcoholic mothers. This was followed that same year by a second report (Jones and Smith, 1973) describing an additional 3 children suffering from this disorder which was termed the "fetal alcohol syndrome."

Clarren and Smith (1978) grouped the abnormalities typically associated with alcohol teratogenicity into 4 categories: central nervous system dysfunctions; growth deficiencies; a characteristic cluster of facial abnormalities; and variable major and minor malformations. Alcohol teratogenicity presents a broad range of abnormalities. However, in general, there has been a reluctance to positively identify a child with the fetal alcohol syndrome without some alteration in brain function, growth, and facial appearance (Clarren and Smith, 1978).

Specific anomalies which have been identified in this condition include: prenatal and postnatal growth deficiency, developmental delay, microcephaly, short palpebral fissures, epicanthal folds, myopia, maxillary
hypoplasia, cleft palate, micrognathia, elbow and joint limitation, hip dislocation, altered palmar crease pattern, cardiac anomalies, anomalous external genitalia, accessory nipple, capillary hemangiomata, abnormal fine-motor function, shortened nose, hirsuitism, narrow upper vermilion, upturned nares, renal insufficiency and liver abnormalities. Jones and Smith (1975) and Clarren et al. (1978) also reported the aberration of neuronal migration and incomplete development of the cerebral cortex in the brain of a child having the fetal alcohol syndrome. They suggest that some of the structural and functional abnormalities in this syndrome, such as microcephaly, developmental delay and motor dysfunction may be secondary to a malorientation of the brain.

Cases documenting the fetal alcohol syndrome have now been reported by a number of investigators (for review, see Clarren and Smith, 1978).

It has been estimated in a collaborative study of the National Institute of Neurological Disease and Stroke (Jones et al., 1974) that maternal alcoholism is associated with a perinatal mortality rate of 17%. In the surviving infants, a 44% incidence of mental deficiency and a 32% incidence of the fetal alcohol syndrome have been reported. Totally safe levels of maternal alcohol consumption are unknown. However, the National Institute on Alcohol Abuse
and Alcoholism has stated that a risk to the fetus is established with the ingestion of 3 oz of absolute alcohol or about 6 drinks per day. Since the risk for lesser amounts is uncertain and because the peak blood alcohol concentration is probably the most critical teratogenic factor, it is recommended that pregnant women not exceed 2 drinks per day (FDA Bulletin, 1977).

Hanson et al. (1976) recently provided evidence of a crude dose-response curve relating maternal alcohol intake to outcome of pregnancy. Their data suggest that the risk of giving birth to an infant with the fetal alcohol syndrome increases proportionately with the average daily alcohol intake, and that as little as 1 to 2 oz of absolute alcohol per day may result in a 10% risk of the syndrome, particularly if drinking occurs during the first 2 months of pregnancy.

**Animal investigations**

To delineate the causes of the fetal alcohol syndrome, animal models have been developed (for review, see Randall, 1977). Combemale (1888) published one of the earliest reports of in utero exposure to alcohol in mammals. Two normal dogs were mated and the pregnant female was exposed to ethanol for the first 23 days of gestation. Three of the 6 pups were stillborn and the remaining 3 were
of "weak intelligence." Stockard (1912) devised a specific apparatus for administering alcohol to pregnant guinea pigs without handling them. The animal was prodded into a chamber filled with vapors from 95% ethanol and left until "intoxicated" for 6 days per week. "Defective" young were often found with hind limb and eye anomalies. Later Stockard (1924) reported that "superior" offspring resulted from ethanol-treated parents and proposed that ethanol was a selective agent allowing only the fittest of the species to survive.

In 1957, Papara-Nicholson and Telford reported that ethanol administration during pregnancy in guinea pigs resulted in microscopic changes in the brain of the offspring and affected reproductive parameters in the dams. Greater emotionality and inferior learning ability were reported in neonatal rats exposed to ethanol in utero (Vincent, 1958). St. Sandor and St. Elias (1968) reported on ethanol-induced dysmorphogenesis in the developing chick embryo.

Criticism of these animal experiments initially designed to assess the effect of maternal alcohol consumption on fetal development is based on their lack of nutritional controls (Randall, 1977). However, it can generally be concluded from these reports that maternal ethanol consumption results in decreased birth weight and/or
number, retarded development and impaired learning ability.

More recently, Chernoff (1977) reported a mouse model of the fetal alcohol syndrome attempting to circumvent the nutritional issue. CBA and C3H female mice were maintained on liquid diets of Metrecal and ethanol, containing 15-35% ethanol-derived calories. Control animals received the same diet with isocaloric amounts of sucrose substituting for ethanol. The ethanol diets resulted in alcohol blood levels of 73-398 mg per 100 ml blood in non-pregnant females, and were the sole sustenance for the females for at least 30 days before and throughout gestation. Females were killed on day 18 of gestation and the offspring examined for skeletal and soft tissue anomalies. Prenatal death and maldevelopment increased with the level of alcohol intake. CBA mice were more sensitive to the induction of the syndrome than C3H mice. It was found that day-18 mouse fetuses had a pattern of malformations similar to those seen in children with the fetal alcohol syndrome. The most common skeletal abnormality was an incomplete or apparently missing supraoccipital bone, occurring in both strains even on the lowest alcohol-containing diet. At higher ethanol concentrations apparently missing sternebrae and rib anomalies, including fusion and misalignment were produced. Thirty-six percent of the CBA mice and 78% of the C3H mice suffered from brain anomalies at the lowest
ethanol containing diet, including dilated or immature cerebral ventricles and absence of the corpus callosum. Cardiac anomalies, including ventricular septal defects and hemopericardium were observed at the lowest concentration of ethanol in the diet and in increasing frequency with increasing ethanol doses. Open eyelids occurred in 100% of the CBA mice in the highest treatment diets. This dysmorphology could not be evaluated in the C3H strain since this strain is genetically open lidded at birth. At high ethanol concentrations exencephaly and gastroschisis were observed in both strains. This pattern of malformations exhibited both a dose-response effect and strain differences in susceptibility.

Kronick (1976) also described a mouse model for the fetal alcohol syndrome. Alcohol treatment consisted of intraperitoneal injection of 0.03 ml per gram of body weight of a 25% (v/v) solution of 95% ethanol in saline. The mice used were the progeny (F\textsubscript{1}) of a cross of the C57BL/6J (female) and DBA/2J (male) inbred strains. Following alcohol treatment on both of gestation days 8 and 9 or days 10 and 11, the frequency of fetal death was substantially elevated compared to saline-treated controls. Alcohol treatment on one of gestation days 7, 8, 9, 10, 11 or 12 produced a significant increase in fetal death on days 9, 10, 11, and 12. Congenital anomalies were increased
significantly following alcohol treatment on gestation days 8, 9, or 10 only. The malformations most frequently observed were coloboma of the iris and ectrodactyly involving only the forepaws. These malformations were similar to the eye, digital and palmar abnormalities of the human fetal alcohol syndrome.

In another mouse model, Randall et al. (1977b) used oral ethanol administration in the presence of adequate nutrition to determine if ethanol was a teratogen in inbred mice under controlled laboratory conditions. Pregnant C57BL/6J mice were fed a liquid diet containing 25% of the total calories as ethanol from days 5 to 10 of gestation. A pair-fed control group was treated similarly, except that sucrose was substituted isocalorically for ethanol. The gravid females were sacrificed on gestation day 19. In the alcohol-fed group twice as many fetuses were resorbed as compared to the control groups. The alcohol treated group had both external and internal anomalies not seen in the control fetuses. Limb anomalies observed included syndactyly, adactyly and ectrodactyly of the fore-limbs and occasional distortion of hind limb digits. Cardiovascular anomalies included abnormalities of both the major branches of the aorta and vena caval system, and mitral valve and septal defects. Urogenital abnormalities,
exencephaly, hydrocephalus, anophthalmia and microphthalmia were also observed in the alcohol-exposed group.

Two years later Randall and Taylor (1979) using a similar experimental protocol, as described above, found a dose-dependent increase in the incidence of fetal resorptions and congenital malformations in the offspring of pregnant mice fed a liquid diet with 17, 25 or 30% of the calories derived from ethanol. Prenatal exposure of mice to a diet containing 30% ethanol-derived calories during days 5 to 11 of gestation resulted in delayed sexual maturation as measured by the time of vaginal opening (Boggan et al., 1979) and a high incidence of hydro-nephrosis in the offspring (Boggan and Randall, 1979).

Tze and Lee (1975) described a rat model which may be of value in evaluating the fetal alcohol syndrome. Females received ethanol in 30 g per 100 ml water as their only available fluid and a balanced powdered diet for a 5-week period before mating. A pair-fed group received water and an isocaloric diet. After mating, alcohol administration continued throughout the gestation period. Alcohol treatment before and during pregnancy reduced litter size and average birth weight of the litters as compared to the control group. In addition, some offspring of alcohol-treated females also exhibited microcephaly.
In another rat model, Henderson and Schenker (1977) maintained rats on a liquid diet containing 3-6% alcohol for an average of 21 weeks before pregnancy and then on a 6% alcohol diet for the period of gestation. Such treatment produced a significant increase in neonatal mortality (30%) and a decrease in body weight as compared to the offspring of pair-fed controls. DNA concentration was significantly lower in the liver and total RNA levels were significantly depressed by about 10-30% in the heart, liver, kidney and brain of the alcohol-exposed group. Maternal blood alcohol levels in this study ranged from 67 to over 150 mg%.

Øisund et al. (1978) investigated the teratogenic potential of what they considered moderate ethanol consumption in the rat. Female rats were given ethanol in their drinking fluid to cover 20-25% of the total calories consumed for 3 to 4 weeks prior to and during pregnancy, and in some cases, continuing after parturition. Pair-fed controls were also included in the study. Maternal blood alcohol levels never exceeded 75 mg%. The general trend showed that there were no significant differences in body, brain, liver, kidney and heart muscle weights between control and ethanol offspring between 0 and 24 days of age.

In a study by Abel and Dintcheff (1978) to investigate the effects of alcohol on growth and development in rats, pregnant rats were intubated daily throughout
gestation with 4.0 or 6.0 g of ethanol per kg. Pair-fed vehicle-treated and non-treated rats fed ad libitum served as controls. All pups were removed from their biological mothers at birth and raised by non-drug-treated surrogate mothers. Litter weight was reduced in alcohol-treated groups. Pups exposed to the lower dosage showed postnatal "catch-up" growth relative to ad libitum controls, but pups from the higher dosage group remained significantly lighter in weight than either their pair-fed controls or ad libitum animals. However, both alcohol-treated groups experienced impaired motor activity and greater postnatal mortality than did the controls. Pair-fed animals did not differ from ad libitum controls indicating that malnutrition was not a factor in the effects of alcohol treatment.

Using a similar experimental protocol, Abel (1979) reported that female offspring prenatally exposed to ethanol performed significantly worse than the offspring of vehicle-injected pair-fed control mothers, on a two-way shock-avoidance task. There were no significant group differences, however, in water-escape maze learning.

Schwartz et al. (1978) did not observe the teratogenic effects of ethanol in mice, rats and rabbits in their experimental system. Pregnant CF-1 mice, Sprague-Dawley rats and New Zealand white rabbits were given 15% ethanol
in their drinking water during the period of major organogenesis. Maximum blood alcohol levels, measured in non-pregnant animals were about 200 mg% in mice and 25 to 50 mg% in rats and rabbits. However, blood alcohol levels were never ascertained for pregnant animals of these species.

A dog model of the fetal alcohol syndrome was presented by Ellis and Pick (1976), based on their method of producing physical dependence on ethanol in beagles (Ellis and Pick, 1973). Three levels of ethanol dosage were given daily to different groups of pregnant beagles throughout gestation. The administration of 5.5-6.0 gm per kg in fractional morning and evening doses resulted in peak morning ethanol blood levels of about 350 mg% and did not prevent ovum implantation but completely suppressed in utero tissue differentiation and development. A second group of animals received 4-5 gm per kg in a similar schedule of administration. Peak morning ethanol blood levels averaged 250 mg% and this dosage regimen permitted more advanced intrauterine development, followed by spontaneous abortion at 6 to 7 weeks of dead immature fetuses, or by retained dead abnormal fetuses at term. A third group received 3 gm per kg similarly administered, resulting in peak morning ethanol blood levels of 150 mg%. A fourth group, which received isocaloric sucrose solution, served as control. Both these latter two groups delivered normal litters at
term. Anatomical defects in the pups of the group receiving 4 to 5 g per kg daily of alcohol included cleft palate, brachygnathism, renal agenesis and skeletal anomalies. In recent experiments, Ellis and Pick (1979) have found that in their model fetal exposure to ethanol throughout gestation induces a wider spectrum, and in certain respects, greater severity of offspring anomalies than following exposure during only short gestational segments.

It has been suggested that paternal (rather than maternal) alcoholism may adversely affect fetal morphogenesis, although the reports are inconclusive (for references, see Klassen and Persaud, 1976). The results show increases in fetal mortality and reduced litter size when male alcoholic rats were mated with normal females (Klassen and Persaud, 1976). Klassen and Persaud (1978) have also demonstrated adverse effects of alcohol on spermatogenesis and testicular function in male rats treated by an oral self administration technique. Teratogenesis due to paternal alcohol consumption has not been reported, however.

Possible mechanisms of ethanol teratogenicity

It is strongly suggested from clinical and experimental reports that ethanol is a teratogenic agent capable of producing a number of developmental anomalies. An important issue is to determine the etiology of the fetal
alcohol syndrome.

Alcohol is a small, readily diffusible uncharged molecule, equally distributed in the body water. It easily crosses the placenta and, unlike many other drugs, alcohol distribution is not influenced by degree of lipid solubility. Autoradiographic techniques have demonstrated a rapid equilibration between the mother and fetus in humans (Idanpaan-Heikkila et al., 1971), monkeys and hamsters (Ho et al., 1972) and mice (Åkesson, 1974).

The teratogenic effects of a drug, such as alcohol, need not be a result of direct embryotoxicity since indirect effects can be equally harmful. Alterations in the normal maternal-fetal equilibrium may be critical to normal development. Ethanol may produce protein, vitamin, or acid-base imbalances. Placenta morphology could be altered although this parameter has yet to be investigated. A few reports (Jones and Smith, 1975; Tenbrinck and Buchin, 1975) showed no evidence of chromosomal aberrations in infants with the fetal alcohol syndrome, although the possibility of ethanol-induced chromosomal damage needs to be more thoroughly investigated.

In a series of reports, Rawat (1975, 1976, 1977, 1978, 1979) attempted to analyze certain biochemical parameters in fetal and neonatal rats exposed to alcohol in utero to investigate the effects of alcohol on the fetus at
a molecular level. The experimental protocol for alcohol administration was similar for all of the studies. Pregnant rats were given liquid Metrecal diet containing 6% (w/v) ethanol or isocaloric sucrose throughout gestation and lactation. The rats were observed to feed well on the liquid diet and to gain weight.

Chronic ethanol-feeding to pregnant rats by this method resulted in about a 30% decrease in the rate of $^{14}$C-leucine incorporation by the fetal cerebral ribosomes and a 60% decrease in the rate of $^{14}$C-leucine incorporation by the cerebral ribosomes from neonatal rats suckling on ethanol-fed mothers (Rawat, 1975). In addition, the brains from both ethanol-exposed fetuses and neonates showed a decrease in the amount of t-RNA formed compared to the corresponding controls. The cerebral content of both total RNA and DNA was significantly lower in the brains of ethanol-exposed developing neonates.

Ethanol consumption by pregnant rats produced a significant decrease in the fetal brain contents of acetylcholine and an increase in γ-aminobutyric acid (GABA) and glutamate (Rawat, 1977). There were no significant changes in levels of 5-hydroxytryptamine, norepinephrine or choline in fetal brains due to ethanol treatment, however. The brains of neonates suckling on ethanol-fed mothers showed
a significant decrease in acetylcholine levels and an increase in GABA and glutamate levels. In brains from both ethanol-exposed fetuses and neonates the activities of L-glutamate decarboxylase and GABA-aminotransferase were decreased. Such brain changes, as observed in these studies may be responsible for the observed neurological abnormalities and mental deficiency in infants born to chronic alcoholic mothers.

Maternal ethanol treatment resulted in an inhibition of the rates of protein synthesis by both fetal and neonatal livers in vivo and in vitro (Rawat, 1976). Incubation of adult rat livers with ethanol produced an inhibition of $^{14}$C-leucine incorporation into hepatic proteins, but this effect was not observed in fetal liver slices. The addition of pyrazole, an inhibitor of alcohol dehydrogenase, to the adult liver slices partially prevented this effect of ethanol addition. There was no significant effect on the rates of protein synthesis after pyrazole addition to the fetal liver slices, where the activity of alcohol dehydrogenase is very low or absent (Sjöblom et al., 1978). These results suggest the necessity of alcohol oxidation to acetaldehyde in the genesis of the effects on hepatic protein synthesis. A decrease in hepatic total RNA content RNA/DNA ratio and ribosomal protein content was also observed in the livers of ethanol-exposed fetuses, while
fetal hepatic DNA content was not significantly affected. In addition, a significant decrease in proteolytic activity and the activity of tryptophan oxygenase was observed in the livers of fetuses, neonates and adults exposed to this alcohol regimen.

Finally, prolonged ethanol consumption produced a significant increase in the hepatic contents of total lipids, triacylglycerols and plasma unesterified fatty acids (Rawat, 1978) and a decrease in cardiac total proteins and RNA both in fetuses and neonates (Rawat, 1979). Thus some of the congenital heart defects in the fetal alcohol syndrome may be the result of an inhibition in cardiac protein synthesis.

These animal experiments and many of those studies described previously have been performed under controlled nutritional conditions. This demonstrates that alcohol is embryotoxic in the presence of an adequate diet and rules out nutritional factors as the primary teratogen.

The importance given acetaldehyde, ethanol's primary metabolite, has been minimal, even though acetaldehyde is toxic and may be responsible for some of the effects of ethanol (Rahwan, 1975). Experimental studies on the placental transfer of acetaldehyde are scarce. Kesäniemi and Sippel (1975) administered ethanol (2g per kg, i.p.) to pregnant rats. They reported that only 25% of the
acetaldehyde content present in the maternal aortic blood could be found in the intact placenta and no acetaldehyde was found in the intact fetal tissue. The authors proposed that the placenta metabolizes acetaldehyde before it reaches the fetus, and that fetal liver aldehyde dehydrogenase, although considerably lower in activity than adult levels would then metabolize the small amount of acetaldehyde not metabolized by the placenta (Kesäniemi and Sippel, 1975).

These experiments, however, were performed in the near-term fetus. The rodent placenta continually changes with increasing gestation. What may hold true in late gestation when the functioning placenta is the chorioallantoic placenta, may not be true for earlier stages of gestation where the primary placenta is the yolk sac. Acetaldehyde is a simple, very lipid soluble molecule that would be expected to easily cross the placenta.

Randall et al. (1977a) maintained pregnant mice on a liquid diet containing 25% of the total calories as ethanol from either gestation day 5 through 10 or gestation days 12 through 18 and sacrificed them on gestation day 11 or 19, respectively. Acetaldehyde was measurable in fetal tissue at both time periods studied. Gestation day 11 fetal tissue contained 18% of maternal blood acetaldehyde and gestation day 19 fetal tissue contained 40% of maternal levels. In addition these dosage regimens produced a
significant number of fetal anomalies in the offspring.

It is unlikely that the higher fetal acetaldehyde levels observed on day 19 resulted from fetal production since fetal liver alcohol dehydrogenase activity at this time is reported to be only 4% of adult levels (Räihä et al., 1967). Randall et al. (1977a) suggest that hormonal differences, known to exist between early and late pregnancy, could alter ethanol metabolism and affect blood acetaldehyde levels. Differences in placental maturation or fetal liver aldehyde dehydrogenase activity during the progression of gestation are also possible explanations. Sjöblom et al. (1978) have reported fetal rat liver aldehyde dehydrogenase activity to be only 5% and 16% of adult rat liver activity at 15 and 20 days of gestation, respectively. Low aldehyde dehydrogenase activity may thus explain the accumulation of acetaldehyde in the fetus in gestation following maternal alcohol consumption.

Véghelyi et al. (1978) reported that in the human situation, acetaldehyde passes across the decidua but after chorioallantoic placenta formation it is metabolized by the placenta. Therefore, after the third month no acetaldehyde is found in the fetus. Since the first 3 months of pregnancy, the period of embryogenesis, may be the most sensitive period to teratogenesis, the passage of acetaldehyde to the fetus would be critical at this time.
Indeed, O'Shea and Kaufman (1979) demonstrated the teratogenicity of acetaldehyde in a mouse model. Pregnant female mice were injected intravenously with saline or 0.1 ml of saline containing 2% (v/v) or 1% (v/v) of acetaldehyde in the morning on days 7, 8 and 9 of gestation. Females were autopsied on either day 10 or day 19. The higher dose of acetaldehyde was calculated to produce levels of acetaldehyde slightly above those seen in human chronic alcoholics during intoxication. Acetaldehyde-treated females had more resorptions both at mid-gestation and at term, although no significant difference in maternal weight gain was observed. All the treated embryos examined on day 10 were smaller and had a higher incidence of developmental delay, as compared to controls. Acetaldehyde-exposed fetuses when examined on day 19 were still significantly smaller and weighed less than controls. The most commonly noted defects were anomalies of closure of the cranial and caudal region of the neural tube. These central nervous system abnormalities are interesting since microcephaly and mental deficiency have been seen in human infants suffering from the fetal alcohol syndrome (Jones and Smith, 1975).

Véghelyi et al. (1978) reported that alcohol itself is not cytotoxic, mutagenic, or teratogenic, but that acetaldehyde possesses all 3 properties. Alcohol in double the lethal human dose had no effect on sister chromatid
exchange frequency in Chinese hamster ovary cells (CHO cells). Acetaldehyde killed 100% of the CHO cells at an 880 \( \mu \text{M} \) concentration, and acetaldehyde concentration of 40 \( \mu \text{M} \) still affected cell progression with sister chromatid exchange being double the control value. In three alcohol addicts under chronic disulfiram treatment and after consuming 0.2 ml alcohol per kg, blood acetaldehyde levels rose to above 100 \( \mu \text{M} \). In the lymphocytes obtained during this episode, sister chromatid exchange increased over background in all 3. These changes were not observed in the same patients before they drank alcohol and in other disulfiram-treated addicts who had taken no alcohol. In lymphocytes from 7 subjects under the acute influence of alcohol with blood alcohol levels ranging from 100 mg% to 400 mg%, no sister chromatid exchange was observed.

Véghelyi et al. (1978) also found that the administration by gastric intubation of 10 ml per kg of 40% ethanol to pregnant rats daily from day 7 to day 16 of gestation had no or only a very slight effect on the rate of fetal resorption and on the weight development of living fetuses as compared to non-treated controls. Disulfiram (150 mg/kg) administered alone to another group of pregnant rats on the same days was also with little or no effect. However, the combined action of disulfiram and alcohol caused a very significant increase in fetal resorption and skeletal retardation, and a significant decrease in fetal weight.
This combined action was ascribed to high acetaldehyde levels.

Véghelyi et al. (1978) suggest that blood acetaldehyde levels of above 40 μM in pregnant women will produce a deleterious effect on the fetus. Normal healthy mothers would not be expected to exceed 30 μM acetaldehyde blood concentrations after acute alcohol consumption. However, if acetaldehyde oxidation is abnormal in the subject, or if disulfiram is taken concomitantly, then blood acetaldehyde levels may exceed these concentrations. In fact 1 mM blood acetaldehyde levels may occur in subjects under the effect of disulfiram while they are drinking. Véghelyi et al. (1978) believe acetaldehyde to be teratogenic by lesioning the fetus directly in the first two months of gestation and by affecting placental function during the further course of pregnancy. They strongly suggest that prospective mothers be screened for blood acetaldehyde levels after alcohol consumption, and if levels surpass 30 μM, the women should be advised against bearing a child, or if they are pregnant, to consider abortion.

Véghelyi and Osztovics (1978) sighted clinical cases in support of their hypothesis. They reported that a heavily drinking mother after having 6 miscarriages gave birth to a child afflicted with the fetal alcohol syndrome. During the early weeks of this pregnancy she was on disulfiram treatment but confessed to occasionally consuming a
little alcohol. Another female addict had a child with the fetal alcohol syndrome. On consuming 0.4 g of alcohol per kg, her blood acetaldehyde level rose to 140 μM.

Thus, as indicated by these reports, a role for acetaldehyde in ethanol teratogenicity is indeed implicated.

**Statement of the Problem**

A contemporary aspect of ethanol toxicity concerns its teratogenic action, which has been established in laboratory animals and in humans (for review see Clarrren and Smith, 1978; and see INTRODUCTION). Although no mechanism has been established to explain the teratogenicity of ethanol, evidence has been presented in one human study indicative of interference with cellular migration in the brain during embryogenesis (Jones and Smith, 1975). Recent evidence suggests that high maternal acetaldehyde blood levels may be a requirement for ethanol-induced teratogenicity (Véghelyi et al., 1978; Véghelyi and Osztovics, 1978). Increased acetaldehyde concentrations might be expected to result in tetrahydroisoquinoline (TIQ) or tetrahydro-β-carboline (TBC) biosynthesis.

It was speculated, therefore, that the teratogenic anomalies produced by ethanol could be, in part, the result of interference with embryonal catecholaminergic or serotonergic mechanisms as a consequence of aberrant biosynthesis
of TIQ or TBC alkaloids. Indeed, Azevedo and Osswald (1977) recently reported that chronic administration of 2 epinephrine TIQs resulted in selective degeneration of the adrenergic nerve terminals of adult rats and of the whole adrenergic neurone of newborn rats. In addition, teratogenicity of opiates and psychoactive drugs has already been reported in the literature (Schardein, 1976; Shepard, 1976).

The purpose of the following studies was, therefore, three-fold:

(1) Assays have been developed for the TIQs, salsolinol and tetrahydropapaveroline. A gas chromatographic/electron-capture (GC-EC) assay for salsolinol was developed by O'Neill and Rahwan (1977b) capable of detecting 8 ng of the alkaloid per g of mouse brain tissue. Riggin and Kissinger (1977) reported the development of a high performance liquid chromatography assay with a lower limit of sensitivity of 2 ng salsolinol or tetrahydropapaveroline per g of rat brain. With the evidence that the presence of picogram quantities of the TIQs introduced in the CNS may induce alcohol drinking in rats (Myers and Melchior, 1977a; and see INTRODUCTION) it became apparent that a more sensitive assay for the TIQs was needed. Therefore, to investigate the TIQ hypothesis of ethanol teratogenicity the development of a more sensitive analytical assay for the detection and quantitation of the TIQ, salsolinol, in neonatal rat tissue was undertaken.
(2) The alcohol molecule easily crosses the human and rodent placenta (see INTRODUCTION). The passage of acetaldehyde in early and late gestation has been demonstrated in a mouse model (Randall et al., 1977a), although Kesäniemi and Sippel (1975) did not detect placental transfer of acetaldehyde in very late gestation in the rat. If a TIQ, such as salsolinol, is to be considered the teratogenic metabolite of ethanol, it follows that the compound must be shown to cross the placenta or be formed in the fetus in order to exert a direct toxic effect on the fetus. Therefore, a study was undertaken to determine the placental transfer of salsolinol during embryogenesis or the "sensitive" period of development in the rat.

(3) As mentioned above, a direct teratogenic effect of salsolinol would necessitate placental transfer of the compound or formation of the agent in the fetus. A study was therefore undertaken to examine fetal formation of salsolinol following maternal administration of alcohol in rats during the embryogenesis period.

It was hoped that these proposed studies would shed some light on the possible etiological role of the TIQs in the yet unresolved problem of the fetal alcohol syndrome.
CHAPTER II

MATERIALS AND METHODS

Development of a Radioenzymatic/Thin Layer Chromatographic Assay for Salsolinol in Neonatal Rat Tissue (Nesterick and Rahwan, 1979)

Rationale

Since it has been reported (Collins et al., 1973) that salsolinol is a substrate for mammalian catechol-O-methyltransferase (COMT) as well as a competitive inhibitor of the O-methylation of catecholamines, the possibility of development of a sensitive thin-layer chromatographic/radioenzymatic (TLC/RE) assay for detection, separation and quantitation of trace amounts of salsolinol and catecholamines became apparent. This endeavor was greatly facilitated by the recent commercial availability of a radioenzymatic assay kit (CAT-A-KIT, Upjohn Diagnostics, Kalamazoo, Michigan) for catecholamines, which is based on the method of Passon and Peuler (1973). The method described below is a modification of the commercial kit, adapted for the incorporation of salsolinol into the assay. Salsolinol (SAL), dopamine (DA), epinephrine (EPI), and norepinephrine (NE) are simultaneously converted to their corresponding meta \(^{3}\text{H}\)-methoxy-derivatives (\(^{3}\text{H}\)-salsoline, \(^{3}\text{H}\)-3-methoxytyramine, \(^{3}\text{H}\)-metanephrine, and \(^{3}\text{H}\)-normetanephrine,
respectively) by the catalytic action of COMT in the presence of $[^3\text{H}]-S$-adenosylmethionine ($[^3\text{H}]-\text{SAM}$). The $[^3\text{H}]$-O-methylated derivatives are extracted and separated by TLC. $[^3\text{H}]-\text{normetanephrine}$ and $[^3\text{H}]-\text{metanephrine}$ (but not $[^3\text{H}]-\text{salsoline}$ or $[^3\text{H}]-3$-methoxytyramine) are susceptible to periodate oxidation which converts the former two derivatives into $[^3\text{H}]-\text{vanillin}$; this additional chemical characteristic serves to differentiate between the $[^3\text{H}]$-O-methylated derivatives of EPI and NE on the one hand and those of DA and SAL on the other.

**Chemicals and equipment**

All chemicals were of the highest obtainable commercial grade and were used without further purification. Demineralized, double-distilled water was used in reagent preparation. The following chemicals were used: CAT-A-KIT (Catecholamines Radioenzymatic Assay Kit $[^3\text{H}]$, Upjohn Diagnostics, Kalamazoo, Mich.), salsolinol HBr (Aldrich Chemical Co., Milwaukee, Wis.), salsoline HCl (ICN Pharmaceuticals, Inc., Plainview, N.Y.), reduced glutathione (Sigma Chemical Co., St. Louis, Mo.), Liquifluor (New England Nuclear Corp., Boston, Mass.), isoamyl and t-amyl alcohol (Baker Chemical Co., Phillipsburg, N.J.), methylamine 40% aqueous solution (Aldrich Chemical Co., Milwaukee, Wis.), and toluene (Ashland Chemical Co., Columbus, Oh.).
Vacutainer tubes, containing 100 μl of a solution (pH 6-7) composed of 90 mg EGTA/ml and 60 mg reduced glutathione/ml, were purchased from Upjohn Diagnostics (Kalamazoo, Mich.). Glassware was siliconized by immersion for 1 min in a 1% solution of Prosil (VWR Scientific Co., Columbus, Oh.), and then rinsed in distilled water and dried at 150°C.

Prescored silica gel GF TLC plates, 20 x 20 cm, 250 μM thick (Analabs, North Haven, Conn.) were used in conjunction with a 16 channel TLC Multispotter (Analytical Instrumentation Specialties, Libertyville, Ill.).

Other equipment included: a Polytron (Kinematia, Switzerland), glass disposable borosilicate tubes (16 x 125 mm) with screw caps (VWR Scientific Co.), 12 x 3 7/8 x 10 inch standard developing tanks (VWR), Whatman No. 1 filter paper (VWR), ultraviolet lamp equipped with short (254 nm) and long (366 nm) wavelengths (Ultra-Violet Products, San Gabriel, Calif.), Dubnoff metabolic shaking incubator (VWR), table-top centrifuge (Sorvall, Inc., Norwalk, Conn.), RC2-B refrigerated centrifuge (Sorvall, Inc.), LS-75 ultracentrifuge (Beckman Instruments, Palo Alto, Calif.), and an LS-35 liquid scintillation counter (Beckman Instruments).

The following reagents were included in the CAT-A-KIT:
Reagent 1 (Catecholamines Standard Solution): Each ml contains 100 μg each of ¹-NE, ¹-EPI, and DA in acid glutathione solution. Diluted 1:10,000 before use.

Reagent 2 (Stabilizing Solution): Acidic glutathione solution, diluted 1:10,000 with distilled water before use.

Reagent 3 (Buffer Solution): For buffering the enzyme reaction in the assay. Contains tromethamine, EGTA, and MgCl₂.

Reagent 4 (³H-SAM, Methyl Donor): S-Adenosyl-L-Methionine (³H-Methyl), 5 μCi ³H/10 μl, in dilute H₂SO₄/EtOH (acetaldehyde-free).

Reagent 5 (COMT Enzyme Preparation): Rat liver COMT (in excess of assay needs), tromethamine, glutathione, benzylhydroxyamine HCl, and dithiothreitol.

Reagent 6 (Stopping/Carrier Solution): Contains 4 mM each of normetanephrine, metanephrine, and methoxytyramine, in pH 11 borate buffer containing EDTA.

Reagent 7 (Oxidizing Reagent): Sodium metaperiodate 4% w/v solution.

Reagent 8: Glycerol 10% v/v solution.

Reagent 9 (Control Human Plasma): Contains assayed levels of catecholamines (DA, EPI, and NE), EGTA and glutathione.
Separation of O-methylated derivatives of salsolinol, dopamine, epinephrine and norepinephrine by TLC

In order to determine whether the O-methylated derivative of salsolinol (salsoline) was separable by TLC from the O-methylated derivatives of EPI, NE, and DA, 5 ml of Reagent 6 were spiked with 4.6 mg of salsoline HCl to provide a 4 mM concentration of salsoline. The resulting mixture of salsoline, methoxytyramine, metanephrine and normetanephrine was extracted (Peuler and Johnson, 1977) into 2 ml of toluene:isoamyl alcohol (3:2), and the aqueous and organic phases separated. The O-methylated compounds were extracted from the organic phase into 0.1 ml of 0.1 N acetic acid. The acid layer was separated and washed with 1 ml of toluene:isoamyl alcohol (3:2), and the organic wash discarded. Absolute ethanol (0.1-0.15 ml) was added to the acid extract to clear the solution. The ethanolic-acid extract was spotted on the silica gel TLC plates using the TLC Multi-spotter set at low speed and 60°C. The distance between the solvent front and points of application was set at 16 cm. After allowing to cool, the plates were developed in 44 ml of t-amyl alcohol:toluene:40% methylamine (6:2:3) for approximately 2.5 hrs in developing tanks lined with Whatman No. 1 filter paper and pre-equilibrated for 10 mins with the developing solvent. The plates were allowed to dry, and the four zones were visualized under
UV light (254 nm).

**Radioenzymatic reaction**

The following radioenzymatic assay is a modification of the method of Peuler and Johnson (1977). Fifty μl aliquots of each sample (e.g. plasma, tissue) to be analyzed for SAL, DA, EPI and NE, were mixed with 10 μl of diluted acidic glutathione stabilizing solution (Reagent 2) and 40 μl of a reagent mixture composed of equal parts (10 μl each) of distilled water, buffer solution (Reagent 3), $^3$H-SAM (Reagent 4), and COMT (Reagent 5). The mixtures were incubated at 37°C for 60 min in a metabolic shaker. To each mixture was then added 50 μl of the buffered carrier solution (Reagent 6) and 5 μl of a salsoline carrier solution (freshly prepared by dissolving 46 mg salsoline HCl in 5 ml of pH 11 borate buffer containing 27.8 mg EDTA/ml). The [H]-O-methylated derivatives and their non-radioactive carriers were then extracted and separated by TLC as described above. The radioactive zones on the TLC plates were scraped into individual scintillation vials.

The $^3$H-metanephrine and $^3$H-normetanephrine were eluted from the silica in their respective scintillation vials by vigorous mixing with 1 ml of 0.05 M NH$_4$OH. The $^3$H-metanephrine and $^3$H-normetanephrine were then each converted to $^3$H-vanillin by periodate oxidation as
described by Peuler and Johnson (1977) and in the CAT-A-KIT Procedures Manual. Fifty µl of sodium metaperiodate solution (Reagent 7) was added to each vial. The vials were shaken periodically. Five minutes after the addition of periodate, 50 µl of glycerol solution (Reagent 8) was added. Following the oxidation, 1 ml of 0.1 M acetic acid was added to each vial with vigorous mixing. To each vial was then added 10 ml of toluene/Liquifluor (1000:50 v/v) scintillation cocktail with vigorous mixing, and the radioactivity counted in a Beckman LS-35 liquid scintillation counter with an efficiency for $^3$H of approximately 58%.

The $[^3]$H-salsoline and $[^3]$H-methoxytyramine were eluted from the silica in their respective scintillation vials by vigorous mixing with 1 ml of 0.05 M NH$_4$OH. Ten ml of toluene:isoamyl alcohol:Liquifluor (700:300:50 v/v/v) scintillation cocktail were added to each vial with vigorous shaking, and the radioactivity counted in a Beckman LS-35 liquid scintillation counter with an efficiency for $^3$H of approximately 39% (38.7-40%).

Aqueous standard curve

A salsolinol HBr stock solution was prepared (10 µg SAL/10 ml distilled water, with 60 mg reduced glutathione/ml), and various dilutions of this stock solution were made from which 50 µl aliquots were assayed
by the radioenzymatic procedure described above. The concentrations analyzed were 0, 50, 500, 1000 and 1500 pg salsolinol/50 μl sample. Each sample was analyzed in triplicate.

**Plasma standard curve**

A salsolinol HBr stock solution was prepared (2.5 mg SAL/10 ml distilled water, with 60 mg reduced glutathione/ml). Various dilutions of this stock solution were made from which 2 μl aliquots were used to spike 50 μl samples of human plasma (Reagent 9) which were subsequently assayed by the radioenzymatic procedure described above. The amounts of salsolinol added to the 50 μl plasma samples were 0, 5, 30, 50, 230, and 500 pg. Each sample was analyzed in duplicate to quadruplicate. An aliquot of an appropriate blank, which consisted of the contents of the vacutainer tubes (see Chemicals and equipment) used for plasma collection diluted with distilled water, was also assayed without salsolinol spiking. In all plasma samples, the [3H]-O-methylated derivatives of endogenous DA, EPI and NE were also assayed and compared to the standard values provided with the CAT-A-KIT. Adult rat plasma was analyzed similarly.

**Tissue standard curve**

Sprague-Dawley neonatal rats were used to prepare the tissue standard curve. The pups were immersed in
liquid nitrogen, and stored at -20°C until used. At the time of assay, each frozen pup was weighed, minced, and homogenized in 8 ml of 0.1 N HClO₄ (containing 5 mM glutathione) by use of a Polytron tissue shearer. The tissue homogenates were spiked with various concentrations of salsolinol (with a volume not exceeding 150 μl). The concentrations of salsolinol ranged from 100 pg to 48 ng/g tissue. The polytron was rinsed with an additional 3 ml of 0.1 N HClO₄ which was then added to the spiked homogenate. Each homogenate was centrifuged at 100,000 xg at 4°C for 1 hr. The supernatant was decanted and retained. The pellet was rehomogenized in 6 ml of 0.1 N HClO₄, recentrifuged at 100,000 xg at 4°C for 1 hr, and the supernatant decanted. The two supernatants were combined and stored at -20°C until assayed. Upon thawing, the supernatant was subjected to a final centrifugation at 39,000 xg for 1 hr at 4°C, and any residue discarded. Duplicate aliquots of the supernatants were assayed according to the radioenzymatic procedure described above. Perchloric acid blanks (containing 5 mM reduced glutathione) without salsolinol spiking nor tissue were also assayed.

Verification of the identity of trace amounts of tissue salsolinol

Two sets of experiments were designed to verify the identity of [³H]-salsoline, particularly at the lower limits of sensitivity of the radioenzymatic assay for
(a) The first set of experiments was designed to ascertain that the radioactivity detected on the TLC plate zone corresponding to the \( R_f \) of salsoline, at the lower limits of sensitivity of the tissue standard curve, was contributed exclusively by a COMT-dependent end-product (presumably \([^{3}H]\)-salsoline). Neonatal rat homogenates were prepared as described above and spiked with salsolinol to give a concentration range of 100 to 2000 pg salsolinol/g tissue. The samples were subjected to the radioenzymatic assay described above in presence of \(^3\text{H}-\text{SAM}\) but with the omission of COMT. After extraction and development on TLC plates as described above, the zones on the TLC plates corresponding to the \( R_f \) of salsoline were scraped, eluted, and counted, in order to determine if a significant amount of radioactivity (above background) could be detected as compared to similar samples which had been O-methylated in presence of COMT.

(b) The second set of experiments was designed to further insure that the tissue-extracted radioactivity on the TLC plate at the \( R_f \) corresponding to salsoline was contributed by a single product (\([^{3}H]\)-salsoline). Bidirectional TLC in two additional solvent systems was performed on two salsolinol-spiked tissue samples (1000 pg salsolinol/g tissue) which were taken through the entire
radioenzymatic assay procedure (in presence of $^{3}$H-SAM and COMT) as described above. The TLC plates were then developed first in α-amy1 alcohol:toluene:40% methylamine solution (6:2:3) as previously described, and the zones corresponding to the tritiated O-methylated derivatives of SAL, DA, EPI and NE visualized under UV light. The plates were allowed to dry and then turned 90° and re-developed in one of the following solvent systems: isopropanol:n-butanol:water:formic acid (60:20:19:1) or 1-butanol:methanol:1 N formic acid (60:20:20), and the solvent front allowed to travel a distance of 16 cm. The plates were allowed to dry and again visualized under UV light. The fluorescent spot in the salsoline lane was scraped from each plate, as were additional 1 cm increments of silica below and above the fluorescent spot, from the origin to the solvent front. Elution and counting of radioactivity were performed as described above.

**Determination of Endogenous Salsolinol in Neonatal Rat Tissue (Nesterick and Rahwan, 1979)**

A set of experiments was designed to determine if any endogenous salsolinol was present in the neonatal rat, which may contribute to the [3H]-salsoline counts resulting from exogenously added salsolinol in the assay. Nine tissue homogenates, with no exogenously added salsolinol, were taken through the entire radioenzymatic procedure
(with $^{3}$H-SAM and COMT). Duplicate 50 μl aliquots of each tissue homogenate were subjected to the methylation, extraction, and TLC separation procedures as described above.

Assessment of the Placental Transfer of Salsolinol (Nesterick and Rahwan, Submitted)

Animal experimentation

Sprague-Dawley rats, purchased from Lab Supply, Indianapolis, Indiana, were used in the studies. Animals were housed in stainless steel cages with Purina Lab Chow and tap water available ad libitum. The animals were maintained on a 12 hr light-dark cycle at a temperature of approximately 23°C and humidity of approximately 50%. One male was placed with 3 or 4 females per cage and female rats were checked each morning for the presence of sperm in the vaginal smear. The day of sperm identification was referred to as day 1 of pregnancy. Those females identified as sperm-positive were then removed and housed separately under the conditions described above.

On the 15th day of pregnancy, animals were injected (i.p.) with a dose of 2mg/kg of an aqueous solution of salsolinol hydrobromide in a volume not exceeding 0.4 ml. The rats were divided into 3 groups and were sacrificed by decapitation at 30, 45 or 60 min post injection. A group of control animals was injected (i.p.) with
demineralized double-distilled water and sacrificed 45 min after injection.

Animals were immediately opened and the number of feti noted. Three feti per animal, randomly chosen, were excised and separated from the uterus, placenta and amniotic sac. The feti were rinsed quickly in demineralized double-distilled water, gently blotted on filter paper, and frozen in aluminum foil packets in liquid nitrogen. The process of removal and freezing 3 feti took approximately 1 min and the frozen tissue was stored at -20°C.

The 3 frozen feti, that had been pooled from each animal, were weighed, minced and homogenized in 4 ml of 0.1 N HClO₄ (containing 5 mM reduced glutathione) by use of a Polytron tissue shearer. The homogenates were centrifuged at 100,000 x g at 4°C for 1 hr. The supernatant was decanted and retained. The pellet was rehomogenized in 3 ml of 0.1 N HClO₄, recentrifuged at 100,000 x g at 4°C for 1 hr, and the supernatant decanted. The 2 supernatants were combined and stored at -20°C until assayed.

Salsolinol assay

Upon thawing, the supernatants were adjusted to a pH 3-9 by dropwise addition of 2 N NaOH to insure adequate buffering of the subsequent enzyme incubation mixture.
To determine salsolinol content, quadruplicate aliquots of the supernatants were assayed according to the radioenzymatic/thin-layer chromatographic procedure (Nesterick and Rahwan, 1979) described above, and employing an authentic salsolinol internal standard.

Two 50 μl aliquots of each supernatant were mixed with 10 μl of diluted acidic glutathione stabilizing solution. Ten μl of both a catecholamines standard solution (Reagent 1) and a salsolinol solution (100 μg/ml) were diluted 1:10,000 before use. Ten μl of this diluted mixture (containing 100 pg each of SAL, DA, NE and EPI) was added to another two 50 μl aliquots of each supernatant. All four 50 μl aliquots for each supernatant were then subjected to the radioenzymatic assay as previously described. Perchloric acid blanks (containing 5 mM reduced glutathione) were also assayed. The salsolinol content of each supernatant was then assessed by the following formula:

\[
\text{Salsolinol Concentration (pg/ml)} = \frac{\text{CPM (sample) - CPM (blank)}}{\text{CPM (sample + std) - CPM (sample) \times sample volume (ml)}} \times \text{salsolinol std (pg)}
\]
Assessment of Salsolinol Formation after Acute Alcohol Administration (Nesterick and Rahwan, Submitted)

Preliminary studies of alcohol disappearance*

Ethanol (30% w/v) was administered to Sprague-Dawley female rats in a dose of 3g/kg by oral intubation. Eight groups of animals were included in the study and were sacrificed at 15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, or 6 hr after injection. Blood samples for each animal were collected at the time of sacrifice and were assayed for ethanol content by the method of Lundquist (1959) which is described below.

Animal experimentation

Sprague-Dawley rats, purchased from Lab Supply, Indianapolis, Indiana, were used in these studies. Animals were housed in stainless steel cages with Purina Lab Chow and tap water available ad libitum. The animals were maintained on a 12 hr light-dark cycle at a temperature of approximately 23°C and humidity of approximately 50%. Male and female rats were mated as described above.

Ethanol was administered on either the 14th, 15th, 16th or 17th day of pregnancy. The protocol was as follows. On the day of treatment, rats were given either 3 g/kg of ethanol (30% w/v) or demineralized double-

* The participation of Yiu-Kai Fung is gratefully acknowledged.
distilled water by oral intubation 3 times daily at 7:00 am, 10:30 am and 2:00 pm. One hour after the final intubation the animals were sacrificed, and blood samples were collected in vacutainer tubes and kept on ice until assayed. Animals were immediately opened and the number of feti noted. Two (16th and 17th days of gestation) to three (14th and 15th days of gestation) randomly chosen feti per animal were excised and separated from the uterus, placenta and amniotic sac. The feti were rinsed quickly in demineralized double-distilled water, gently blotted on filter paper, frozen in aluminum foil packets in liquid nitrogen, and stored at -20°C.

**Salsolinol assay**

The feti that had been pooled from each animal were homogenized, centrifuged, and assayed, as described in the studies on the placental transfer of salsolinol.

**Ethanol assay**

Maternal blood ethanol levels were assayed according to the method of Lundquist (1959). The assay is based on the following reaction:

\[
\text{alcohol} + \text{NAD} \xrightarrow{\text{alcohol dehydrogenase}} \text{NADH} + \text{acetaldehyde}
\]

NADH is produced stoichiometrically and its absorption at 340 nm is monitored as a measurement of
alcohol in the sample.

Reagents for the assay included:

Buffer solution (pH 8.6)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na pyrophosphate</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Semicarbazide HCl</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.5 g</td>
</tr>
<tr>
<td>NaOH (2 N)</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Water</td>
<td>300.0 ml</td>
</tr>
</tbody>
</table>

Yeast Alcohol Dehydrogenase 10.0 mg/ml  
(Sigma Chemical Co., St. Louis, Mo.)

β- NAD 10.0 mg/ml  
(Sigma Chemical Co., St. Louis, Mo.)

Blood samples were prepared by deproteinization in 9 volumes of 3.4% HClO₄. A 50 μl aliquot of the deproteinized filtrate was added to a 3 ml aliquot of a freshly prepared mixture of buffer, NAD, and the enzyme (300:10:3). The optical density at 340 nm was read in a spectrophotometer (Gilford 250) after 60 min. Analysis and quantitation was performed by comparing the optical density obtained with the blood sample to that of known amounts of alcohol.

Statistical analysis

Values for the salsolinol content of fetuses from control and salsolinol- or ethanol-treated mothers were compared by means of the Student t-test (Sokal and Rohlf, 1969) and the Wilcoxon rank sum test (Wilcoxon and Wilcox, 1964).
CHAPTER III

RESULTS

Development of a Radioenzymatic/Thin-Layer Chromatographic Assay for Salsolinol in Neonatal Rat Tissue. (Nesterick and Rahwan, 1979)

Separation of O-methylated derivatives of salsolinol, dopamine, epinephrine and norepinephrine by TLC

Plate I illustrates the separation of the O-methyl derivatives of NE, EPI, DA and SAL in four samples, by the TLC method described under MATERIALS AND METHODS. The four distinct spots observed under UV light represent normetanephrine, metanephrine, 3-methoxytyramine, and 7-O-methylsalsolinol (salsoline) with average \( R_f \) values of 0.22, 0.35, 0.49, and 0.54, respectively.

Aqueous standard curve

Figure 4 represents the standard curve for varying concentrations of aqueous salsolinol solutions expressed in terms of DPM and CPM of \(^{3}\text{H}\)-salsoline formed. The data demonstrates linearity of the assay from the highest tested concentration of 1500 pg salsolinol/50 \( \mu l \) sample (30 ng/ml) to the lower limit of sensitivity of less than 50 pg salsolinol/50 \( \mu l \) (<1 ng/ml). The aqueous blank with no salsolinol averaged 198 ± 4 CPM/50 \( \mu l \) (508 ± 9 DPM/50 \( \mu l \)) for
Plate 1. Thin-layer chromatographic separation of normetanephrine ($R_f = 0.22$), metanephrine ($R_f = 0.35$), 3-methoxytyramine ($R_f = 0.49$), and salsoline ($R_f = 0.54$). A total of 20 experiments were performed, including the four shown in the figure. (Reverse negative taken under UV light; the points of application are at the bottom of the photograph).
Figure 4. Aqueous standard curve for salsolinol, expressed in terms of DPM or CPM of $[^3\text{H}]$-salsoline formed. Each point represents the mean ± S.E.M. of three observations.
GROSS DPM (x10^3)

SALSOLINOL (pg/50μl sample)

SALSOLINOL (ng/ml sample)

Figure 4
the area on the TLC plate corresponding to the $R_f$ of salsoline. As reported by Upjohn (CAT-A-KIT Procedure Manual) and as observed with our use of the CAT-A-KIT, it is common to obtain a blank value of as much as 185 CPM/50 μl sample for the area on the TLC plate corresponding to the $R_f$ of the O-methylated derivative of DA, as compared to a blank of 30-40 CPM/50 μl sample for the areas on the TLC plates corresponding to the O-methylated derivatives of EPI and NE. This is likely due to the fact that $[^3H]$-methoxytyramine is extracted from a basic solution whereas $[^3H]$-metanephrine and $[^3H]$-normetanephrine are extracted from acidic solution. Since salsoline is a dopamine derivative, and since $[^3H]$-salsoline and $[^3H]$-methoxytyramine are extracted by the same procedure and counted in the same cocktail, it was not surprising that the blank for $[^3H]$-salsoline resembled that of $[^3H]$-methoxytyramine.

Plasma standard curve

Figure 5 demonstrates the linearity of the human plasma standard curve for salsolinol (measured in terms of $[^3H]$-salsoline formed) over the tested range of 5 pg/50 μl (100 pg/ml) to 500 pg/50 μl (10 ng/ml). Similar results were obtained with adult rat plasma. The plasma blank (with no salsolinol) averaged 219 ± 13 CPM/50 μl sample (561 ± 32 DPM/50 μl) ($n = 4$) for the area on the TLC plate corresponding to the $R_f$ of salsoline—a value
Figure 5. Plasma standard curve for salsolinol, expressed in terms of DPM or CPM of $[^3\text{H}]$-salsoline formed. Each point represents the mean ± S.E.M. of two to four observations.
which is not significantly greater than that observed for the aqueous blank (198 ± 4 CPM/50 μl).

For technical control purposes, the NE, EPI and DA contents of the plasma samples were also determined following the conversion of these catecholamines to their $^3$H-O-methylated derivatives, and the values obtained were well within the ranges reported by Upjohn (CAT-A-KIT Procedures Manual).

Tissue standard curve

Figure 6 demonstrates the linearity of the tissue standard curve for salsolinol (measured in terms of $[^3$H]-salsolinol formed) over a range of 500 pg salsolinol/g tissue to 48 ng salsolinol/g tissue. At the lower portion of the curve (< 500 pg salsolinol/g tissue) salsolinol can still be detected but its accurate quantification is no longer evident. The perchloric acid blank (with neither tissue nor salsolinol) averaged 140 ± 7 CPM/50 μl sample (358 ± 18 DPM/50 μl) ($n = 5$) for the area on the TLC plate corresponding to the $R_f$ of salsoline.

Verification of the identity of trace amounts of tissue salsolinol

In the MATERIALS AND METHODS section, 2 sets of experiments were described aimed at verifying the identity of $[^3$H]-salsoline, particularly at the lower limits of sensitivity.
Figure 6. Tissue standard curve for salsolinol, expressed in terms of DPM or CPM of $[^3H]$-salsoline formed. Each point represents the mean ± S.E.M. of six observations.
Figure 6

GROSS DPM (x 10^3)

GROSS CFM (x 10^3)

SALSOLINOL (ng/g tissue)

100

75

50

25

100

200

300

400

2  10  20  30  40

Figure 6
(a) Table 1 shows the results of the first set of experiments, designed to verify the COMT-dependency of the radioactivity detected on the TLC plate zone corresponding to the $R_f$ of salsoline at the lower level of sensitivity of the tissue standard curve (Figure 6). The results shown in Table I demonstrate that, as compared to tissue samples processed in the same manner in the presence of COMT (Figure 6), the exclusion of COMT resulted in insignificant radioactivity in the TLC zone corresponding to the $R_f$ for salsoline. It is thus evident that the radioactivity detected at the lower limits of sensitivity in the tissue standard curve (Figure 6) is only contributed by a tritiated O-methylated product (presumably $[^3H]$-salsoline) and is not due to the formation of any COMT-independent reaction product which may have utilized $[^3H]$-SAM as a $[^3H]$-methyl donor. It should also be mentioned that the TLC zones corresponding to the $R_f$ values of 3-methoxytyramine, metanephrine, and normetanephrine were likewise lacking in any significant radioactivity above background values.

(b) The second set of experiments, using bidirectional TLC in two additional solvent systems, was designed to further insure that the tissue-extracted radioactivity on the TLC plate at the $R_f$ corresponding to salsoline was contributed by a single product ($[^3H]$-salsoline). The results of these bidirectional
### TABLE 1

*Radioenzymatic Assay in the Absence of COMT*

<table>
<thead>
<tr>
<th>Concentration of salsolinol added to neonatal tissue (pg/g tissue)</th>
<th>Gross CPM/g tissue(^a)</th>
<th>Gross DPM/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>5916 ± 233</td>
<td>15169 ± 597</td>
</tr>
<tr>
<td>500</td>
<td>6069 ± 35</td>
<td>15561 ± 89</td>
</tr>
<tr>
<td>1000</td>
<td>4383 ± 175</td>
<td>11238 ± 448</td>
</tr>
<tr>
<td>2000</td>
<td>5992 ± 264</td>
<td>15364 ± 677</td>
</tr>
</tbody>
</table>

\(^a\)Mean value (± S.E.M.) of two observations. These mean values represent 84-120 CPM/50 μl sample, and correspond to the value of the perchloric acid blank (with neither tissue nor salsolinol).
chromatographic studies demonstrated that radioactivity on the TLC plate in the zone corresponding to the Rf of salsoline was contributed by a single product ([3H]-salsoline), since the bidirectional development in the two additional solvent systems did not resolve the [3H]-salsoline spot into any additional components. It should be pointed out that if any [3H]-isosalsoline (6-O-methyl-salsolinol) was formed along with the [3H]-salsoline (7-O-methylsalsolinol), then the former must have an identical mobility as the latter in all three solvent systems used in these experiments. It has been reported previously (Hamilton et al., 1978; Collins et al., 1973) that isosalsoline and salsoline exhibit identical retention times in gas chromatographic procedures.

**Determination of Endogenous Salsolinol in Neonatal Rat Tissue (Nesterick and Rahwan, 1979)**

In the set of experiments which was designed to determine if any endogenous salsolinol was present in the neonatal rat, an average of 19,944 ± 677 CPM/g tissue (51,138 ± 1735 DPM) (n = 9) was found in the zones on the TLC plates corresponding to the Rf of salsoline. Since this value falls on the non-linear lower portion of the tissue standard curve (Figure 6), the amount of endogenous salsolinol present in neonatal rats cannot be accurately quantitated, but it is significantly less than 500 pg/g
tissue ($P < 0.05$ by Student $t$-test) and significantly
greater than the values reported in Table I ($P < 0.001$ by
Student $t$-test). These findings are indicative of the
presence of traces of endogenous salsolinol in neonatal
rats who themselves and their parents had never been
exposed to exogenous ethanol.

**Assessment of the Placental Transfer of Salsolinol**
(Nesterick and Rahwan, Submitted)

The results of the placental transfer studies are
shown in Table 2. The average litter size for treated
and control animals was 12. Intraperitoneal administra-
tion of an acute dose of salsolinol (2 mg/kg) to pregnant
rats on day 15 of gestation did not significantly increase
the salsolinol content of the fetuses as compared to the
fetuses of control animals. The mean value for fetal
salsolinol content ($7.56 \pm 1.5$ ng/g) at 60 min post
injection, was not statistically different ($p > 0.1$) from
previous time periods or from the control value (Student
$t$-test and Wilcoxon rank sum test). At the most only
0.2% of the 2 mg/kg dose of salsolinol administered to the
dam was detectable in the fetus 1 hr after injection.
Endogenous salsolinol ($3.6 \pm 1.5$ ng/g) was detected in
the control fetuses of 15-day gestation age. The presence
of endogenous salsolinol ($< 0.5$ ng/g) in neonatal rats
has been described in the previous section.
**TABLE 2**

**Placental Transfer of Salsolinol in Rats**

Pregnant rats were injected (i.p.) with 2 mg/kg of salsolinol on the 15th day of pregnancy, sacrificed at 30, 45 or 60 min post injection, and the fetuses assayed for salsolinol.

<table>
<thead>
<tr>
<th>Treatment Group (n)</th>
<th>Fetal Salsolinol Content (ng/g) (Mean ± S.E.M.)</th>
<th>Percent of Maternal Dose of Salsolinol Transferred to Fetus</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (3)</td>
<td>3.60 ± 1.5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>30 min (1)</td>
<td>3.32</td>
<td>0.0</td>
<td>N.S.</td>
</tr>
<tr>
<td>45 min (3)</td>
<td>3.33 ± 1.9</td>
<td>0.0</td>
<td>N.S.</td>
</tr>
<tr>
<td>60 min (2)</td>
<td>7.56 ± 1.5</td>
<td>0.2</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

*The value "n" refers to the number of pregnant animals for each treatment group.

*Values for fetal salsolinol content of each treatment group were compared separately to the control value, and to each other (p > 0.1).*
Assessment of Salsolinol Formation after Acute Alcohol Administration (Nesterick and Rahwan, Submitted)

Preliminary studies of alcohol disappearance

The results of the alcohol disappearance study are shown in Table 3. Blood alcohol levels increased to a peak value of 157.5 ± 10 mg% at 1 hr after oral administration of 3 g/kg of ethanol. At 3 1/2 hr, approximately one-half the maximum levels of ethanol were still present in the blood.

Determination of salsolinol formation

In preliminary studies described above, a 3g/kg oral dose of ethanol produced a maximum blood ethanol level of 157.5 ± 10 mg% in female rats after 60 min. To prevent accumulation of toxic levels of ethanol but to insure the continual presence of intoxicating ethanol levels in the blood, pregnant animals were intubated 3 times at 3 1/2 hr intervals with 3 g/kg of ethanol. Animals were sacrificed 1 hr after the last alcohol administration, since peak blood ethanol levels (and potentially maximal maternal formation of salsolinol and its placental transfer) would be expected at this time. The average litter size for treated and control animals was 10.

The results of the study are shown in Table 4. Animals were treated on day 14, 15, 16 or 17 of pregnancy.
### TABLE 3

**Rate of Ethanol Disappearance in Sprague-Dawley Female Rats**

<table>
<thead>
<tr>
<th>Time of Sacrifice(^a) (min)</th>
<th>n(^b)</th>
<th>Blood Ethanol Concentration (mg% ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>6</td>
<td>92.1 ± 6.7</td>
</tr>
<tr>
<td>30</td>
<td>6</td>
<td>111.9 ± 7.0</td>
</tr>
<tr>
<td>60</td>
<td>4</td>
<td>157.5 ± 10.0</td>
</tr>
<tr>
<td>120</td>
<td>4</td>
<td>131.4 ± 7.0</td>
</tr>
<tr>
<td>180</td>
<td>4</td>
<td>85.1 ± 7.5</td>
</tr>
<tr>
<td>240</td>
<td>5</td>
<td>69.3 ± 6.0</td>
</tr>
<tr>
<td>300</td>
<td>4</td>
<td>18.0 ± 1.5</td>
</tr>
<tr>
<td>360</td>
<td>4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^a\)The time of sacrifice of Sprague-Dawley female rats after oral intubation of 3g/kg ethanol (30% w/v).

\(^b\)The value "n" refers to the number of ethanol-treated rats sacrificed for each time period post injection.
TABLE 4

**Rat Fetal Salsolinol Content after Maternal Alcohol Treatment**

Ethanol was administered by oral intubation in 3 doses of 3g/kg each at 3 \(1/2\) hr intervals to pregnant rats on day 14, 15, 16 or 17 of gestation. Control animals were treated with distilled water using a similar dosage regimen on the 15th day of pregnancy. Animals were sacrificed 1 hr after the last injection.

<table>
<thead>
<tr>
<th></th>
<th>Total Salsolinol Content (ng/g) (Mean ± S.E.M.)</th>
<th>Maternal Blood Ethanol (mg%) (Mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>2.2 ± 0.73(^b)</td>
<td>124 ± 22(^c)</td>
</tr>
</tbody>
</table>

\(^a\)The value "n" refers to the number of pregnant animals in each group. The treated "n" represents a pooled group of animals treated on day 14, 15, 16 or 17.

\(^b\)Not significantly different from control (p > 0.1).

\(^c\)Maternal blood ethanol level at time of sacrifice.
However, the fetal salsolinol content did not differ significantly with maternal treatment during any of these 4 days of gestation, and the results were therefore pooled. At the time of sacrifice, maternal blood alcohol levels averaged $124 \pm 22$ mg%, yet alcohol administration failed to produce a significant increase in salsolinol content of the fetuses of treated dams as compared to untreated controls ($p > 0.1$ by the Student $t$-test and Wilcoxon rank sum test).
CHAPTER IV

DISCUSSION

Development of a Radioenzymatic/Thin-Layer Chromatographic Assay (Nesterick and Rahwan, 1979)

The development of an assay for the tetrahydroisoquinoline (TIQ) salsolinol, in whole neonatal rat tissue, was deemed necessary to study the role of salsolinol in alcohol teratogenicity with a more sensitive assay technique than those previously described (O'Neill and Rahwan, 1977b; Riggin and Kissinger, 1977). This need for increased sensitivity became evident with the demonstration that intraventricular infusion of minute quantities (as low as 400 pg) of TIQs, including salsolinol, mimics ethanol dependence and increases free-choice ethanol consumption over water in rats, suggesting a role for trace amounts of TIQs in ethanol addiction (Myers and Melchior, 1977a, 1977b; Myers and Oblinger, 1977; Melchior and Myers, 1977a, 1977b).

The radioenzymatic assay for salsolinol described above enables detection of trace amounts of salsolinol in tissue (0.1 ng/g) in the presence of catecholamines, although accurate quantitation of this alkaloid can only
be achieved at concentrations of 0.5 ng/g tissue or above (Fig. 6). Accurate quantitation of plasma salsolinol can be achieved at concentrations of the alkaloid as low as 0.1 ng/ml (Fig. 5).

This radioenzymatic method is, therefore, more sensitive than the previously developed gas chromatographic/electron-capture assay for salsolinol described by O'Neill and Rahwan (1977b) which has a lower sensitivity limit of 8 ng/g tissue and is also more sensitive than the gas chromatographic/electron-capture assay described by Riggin and Kissinger (1977) which has a lower limit of sensitivity of 2 ng salsolinol/g tissue.

Therefore, the assay for salsolinol described above is approximately 20 times more sensitive than other previously described assays for salsolinol. The identity of these trace amounts of salsolinol detected by the radioenzymatic procedure was verified by 2 sets of experiments, including an enzyme-dependent study (Table 1) and bidirectional chromatography, indicating that the radioactivity in the TLC zone corresponding to the $R_f$ value for salsoline was only contributed by a single tritiated O-methylated product and was not a result of any methodological artifacts or contamination.

With the use of the radioenzymatic procedure, the presence of endogenous salsolinol was demonstrated in concentrations of >0.1 to <0.5 ng salsolinol/g tissue
in neonatal rat tissue in the absence of prior exposure to ethanol. Accurate quantitation of the endogenous neonatal salsolinol was not possible, since the amount of radioactivity corresponding to the \( R_f \) value for salsoline was significantly higher than the perchloric acid blank value, but lay on the non-linear lower portion of the salsolinol tissue standard curve (Fig. 6). An explanation for this finding of endogenous salsolinol may reside in the observation that ethanol and acetaldehyde are formed endogenously in the rat gut (McManus et al., 1960), and it is possible that following absorption, the endogenous acetaldehyde may condense with dopamine to form traces of salsolinol. The fact that endogenous levels of TIQs have also been detected in the adrenal glands of untreated rats (personal communication, M. Collins) further substantiates these findings.

Assessment of the Placental Transfer of Salsolinol (Nesterick and Rahwan, Submitted)

If salsolinol is to be implicated in ethanol's teratogenicity, then it would be expected that this TIQ should be able to cross the placental barrier or be formed in fetal tissue following maternal alcohol administration. The results from the studies on the placental transfer of salsolinol indicate a negligible passage of this TIQ across the rat placenta during mid-embryogenesis. At the most only 0.2% (Table 2) of the 2 mg/kg dose of
salsolinol, which has previously been shown to be pharmacologically active (O'Neill and Rahwan, 1977a), was detected in the fetus 1 hr after injection.

Salsolinol is a molecule of relatively low molecular weight, and therefore might be expected to cross the placenta on the basis of its small size (Mirkin and Singh, 1976). Experiments by Brezenoff and Cohen (1973) suggest that peripherally administered TIQs do not cross the blood-brain barrier; however no experiments have been performed investigating the placental transfer of these compounds until this time. Catecholamines have been shown to accumulate in the rat placenta with apparent impaired transfer to the fetus (Mirkin and Singh, 1976).

Schardein (1976) states that the placental transfer of chemicals usually occurs about 45 min after maternal administration. Since our data indicate that there is no significant recovery of maternally administered salsolinol in the fetuses as late as 1 hr after administration (Table 2), it is doubtful that placental transfer of salsolinol would occur at a later time.

Assessment of Salsolinol Formation after Acute Alcohol Administration (Nesterick and Rahwan, Submitted)

Because of the inability of salsolinol to cross the placental barrier, the possibility that salsolinol may form in the fetus from placentally-transferred alcohol or acetaldehyde was investigated. Alcohol readily crosses
the placenta (Mirkin and Singh, 1976). However, fetal liver alcohol dehydrogenase activity is not present until day 18 or 19 of gestation in the rat, and is reported to be only 4% of adult levels at this time (Raihá et al., 1967), indicating that during early gestation the source of acetaldehyde in the fetus is most probably maternal.

Randall et al. (1977a) found increasing placental transfer of acetaldehyde during the course of gestation in rodents, whereas Kesäniemi and Sippel (1975) demonstrated no such transfer. This latter study, however, only investigated the placental transfer of acetaldehyde in the near-term fetus. Nevertheless, acetaldehyde teratogenicity has been conclusively demonstrated in mice (O'Shea and Kaufman, 1979) with the dysmorphogenesis bearing several resemblances to the human fetal alcohol syndrome—a finding which argues against a nonspecific toxic effect of acetaldehyde on the placenta.

In any case, the results of the present investigation (Table 4) demonstrate that acute maternal administration of ethanol on day 14, 15, 16, or 17 of gestation in a dosage regimen designed to maintain high blood concentrations of alcohol over a period of 8 hr (during which time the rats were ataxic but not anesthetized) did not result in an increase in fetal salsolinol content above endogenous levels. This finding provides evidence that salsolinol is not produced by the fetus from
placentally-transferred alcohol or acetaldehyde, and confirms that maternal salsolinol (if formed during alcohol ingestion) did not reach the fetus.

It may be argued that salsolinol could have escaped detection due to metabolic transformation and/or excretion prior to maternal sacrifice and analysis of the fetal tissue. This is unlikely, however, since dams were sacrificed 60 min after the last dose of alcohol when blood levels of ethanol were at a peak, and therefore, salsolinol formation should presumably have been maximal even in the presence of concomitant metabolic degradation of the TIQ by catechol-O-methyltransferase. Furthermore, since the fetuses were frozen in liquid nitrogen within one minute of their removal from the sacrificed dams, and were homogenized in perchloric acid immediately upon subsequent thawing, enzymatic degradation of salsolinol would be highly unlikely under these conditions.

Our present results also indicate that control fetal tissue from untreated dams contains endogenous salsolinol (Tables 2 and 4). The tissue salsolinol content appears to be slightly higher in the fetal tissue, as compared to the neonatal rat tissue. However, statistical analysis of the fetal and neonatal data cannot be performed in view of the qualitative nature of the data obtained from neonatal rats. Nevertheless, the presence of detectable amounts of salsolinol in fetal and neonatal tissue
warrants investigation into a possible physiological role for this TIQ during embryogenesis. Speculations for this role may include function as a neurofactor necessary for proper neuronal migration and CNS development, or possibly a role analogous to the role attributed to α-fetoprotein in sexual differentiation of the central nervous system (Bardin, 1979).

Various investigators have studied the role of TIQs in alcohol dependence (for review, see Rahwan, 1974, 1975). In in vivo studies involving acute or chronic administration of ethanol to laboratory animals, formation of TIQs could only be demonstrated under pharmacological conditions which enhanced TIQ biosynthesis or inhibited their metabolic degradation. On the other hand, when the same problem was approached without intervening pharmacological manipulations, no salsolinol formation could be demonstrated in the brains of mice rendered physically dependent on alcohol (O'Neil and Rahwan, 1977a)—a finding which was confirmed by others (Riggin and Kissinger, 1977; Hamilton et al., 1978) and was suggested to be a strong argument against a role for salsolinol in alcohol addiction.

The present investigation was undertaken to determine the involvement of salsolinol in the teratogenicity of alcohol. The results argue against a role for salsolinol in the mechanism of alcohol teratogenicity, but
indicate an endogenous presence of this TIQ in fetal and neonatal rat tissue. This latter finding may warrant further investigation into a possible physiological function for salsolinol, and further study into the possible presence and location of this and other TIQs in various species.
APPENDIX

Throughout the course of the work described in this dissertation, problems were encountered and experiments were performed which, due to complicating factors, were not followed to completion. These are reported here briefly, in that they may provide some additional helpful information.

With the development of the radioenzymatic/thin-layer chromatographic assay for salsolinol, an attempt was made to also incorporate the tetrahydroisoquinoline, tetrahydropapaveroline (THP, see Fig. 3) into the assay. Because of the very rapid oxidation of THP and its methylated derivatives during the spotting and developing of the TLC plate, it was impossible to determine which of the many spots on the TLC plate were actually the methylated derivatives of THP or products of oxidation. Attempts were made to prevent this oxidation by spotting the TLC plate, previously dried under vacuum for 24 hr, in an argon atmosphere and flushing the developing solvent and tank with argon gas. This treatment minimized the oxidation but did not prevent it entirely.
In addition to this concern, the actual identification of the methylated derivatives of THP was not known until very recently (Meyerson et al., 1979), even though it had been reported that THP was a substrate for COMT-catalyzed O-methylation as early as 1973 (Collins et al., 1973). Therefore, the non-radioactive methylated derivatives of THP could not be obtained for use as carriers in the radioenzymatic reaction, and it became apparent that the incorporation of THP into the assay was not feasible under the circumstances.

Early in the development of the radioenzymatic assay for salsolinol the spotting of the TLC plate was done by hand. However, it was extremely difficult to deliver the 250 μl sample volume containing the methylated products in a small, concise spot in a time interval short enough to prevent degradation of the products before TLC separation.

Therefore, in an attempt to reduce the sample to a more convenient volume for spotting, the samples were evaporated to dryness at 32°C under a nitrogen stream and then reconstituted in 15 μl of a mixture of absolute ethanol: 0.1 M acetic acid (1:1). These 15 μl samples were then spotted by hand on the TLC plate and subjected to the TLC separation procedure, as described in the MATERIALS AND METHODS. Assay of the control plasma sample (Reagent 9) provided by Upjohn, by this method of
reducing sample volume, still resulted in estimations of the plasma catecholamine values which were well within the ranges reported by the company when using the TLC Multispotter (Analytical Instrumentation Specialties, Libertyville, Ill.).

Fortunately, favorable economic factors enabled the purchase of the 16 channel TLC Multispotter, and the work reported in this dissertation on the development and use of the assay was done using this apparatus. This instrument is capable of spotting as many as sixteen 250 µl samples in 30 min or less. However, if purchase of the TLC Multispotter is not possible, it appears that the radioenzymatic assay can be performed adequately using the evaporation technique described above.

Other technical problems encountered in the development and use of this assay also merit mentioning. After TLC separation, it was observed that under 366 nm ultraviolet light a fifth spot (in addition to those seen under 254 nm ultraviolet light in Plate 1) was seen at an R_f value between that for normetanephrine and metanephrine. It was at first feared that this spot may have been a decomposition product. However, a series of experiments and NMR analysis revealed that this unknown entity was not a degradation product of the methylated derivatives of salsolinol, norepinephrine, epinephrine or dopamine, but a non-organic constituent of the non-radioactive
carrier solution of salsoline hydrochloride which would not interfere in the qualitative or quantitative nature of the assay.

Care in the analysis of the perchloric acid tissue blanks (see MATERIALS AND METHODS) must also be emphasized. From time to time these blanks when subjected to the radioenzymatic assay procedure may yield higher than normal radioactivity (e.g., >250 CPM/50 µl sample, for salsolinol). These abnormally high perchloric acid blank values may be the result of a contamination in the system or an unexplained problem with the COMT enzyme mixture as reported by the Upjohn Company (personal communication). The use of such high blank values in the calculation of salsolinol content of the tissue sample may lead to erroneous results. Therefore, reassay of these samples and blanks is recommended if this occurs.

Finally, because of the occasional occurrence of this problem the salsolinol content of a group of neonatal rats was reassessed during the conduction of the fetal tissue assays to determine if the assay sensitivity had undergone any changes. It was found that the endogenous neonatal salsolinol values were comparable to those obtained previously in the development of the assay.


