TERATOLOGICAL EVALUATION OF ETHANOL, PENTOBARBITAL, AND COMBINATIONS OF THESE, IN THE RAT.

A Thesis

Presented to the Faculty of Graduate Studies,

University of Manitoba, in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

by

Lois Ann Kennedy

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To my parents,

and the many friends,

who have offered their encouragement.

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ABSTRACT

Acute alcohol intoxication has proven to be teratogenic in mice. Recent human and animal studies have revealed a pattern of fetal dysmorphogenesis which is directly attributable to chronic maternal alcohol ingestion during pregnancy. Because alcohol is frequently abused in conjunction with other drugs, in particular the barbiturates, experiments were designed to investigate the teratogenicity of short-term intoxication with combinations of ethanol and pentobarbital.

Pregnant rats were treated intraperitoneally with three doses of ethanol (0.56 to 1.4 g/kg), three doses of pentobarbital (5 to 25 mg/kg), or combinations of these, on days 9 through 12 of gestation. Maternal and fetal toxicity were evaluated.

There were microscopic changes in the placenta and maternal ovary which indicated that attention should be directed towards placental and ovarian dysfunction in considering the pathogenesis of the Fetal Alcohol Syndrome. Treatment with ethanol was also associated with a reduction in placental weight ($p < 0.05$).

Gestational intoxication with either ethanol or pentobarbital was associated with a temporary reduction in maternal weight gain ($p < 0.05$) but no significant difference in weight or length of the offspring at term. Other than minor ossification deficiencies, there were no external or visceral anomalies detected, however, and no treatment-related variation in fetal mortality.

Although pathological changes were observed in the liver and kidney

of ethanol treated mothers, such changes were not evident in the corresponding fetal tissues. Thus, at the doses used in this experiment, the fetus is spared, even in the presence of maternal toxicity.

With the exception of minor changes in the maternal kidney, and a non-specific stimulation of the reticulo-endothelial system, microscopic examination of the fetal and maternal tissues revealed no changes following short-term intoxication with pentobarbital.

In pregnant animals treated simultaneously with ethanol and pentobarbital, there was no consistent pattern of response in the parameters evaluated. It appears that, in the intact animal, the effects of the two drugs interacting are unpredictable. With respect to the offspring, there was no evidence that combinations of ethanol and pentobarbital, at the dose levels used here, were more teratogenic than either acting independently.

These studies suggested that placental and ovarian function should be carefully monitored in human pregnancies involving maternal alcohol abuse.

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"Foolish and drunken and hare brained women more often bring forth children like unto themselves, morose and languid".

Aristotle, cited in Burton (1621).

INTRODUCTION

1. INTRODUCTION

The use and abuse of drugs in modern society has reached epidemic proportions among individuals of child-bearing age. Hill (1973) found that in a sample of women from middle to high socioeconomic class, between three and 29 drugs, with a mean number of 10.3, were ingested during pregnancy. The National Institute of Drug Abuse (NIDA) conducted a study (D.A.W.N., 1976) which revealed that in the United States alcohol and diazepam are responsible for the greatest number of drug-related illnesses, and that barbiturates and morphine derivatives are implicated in 45% of deaths caused by drugs.

It has been known since 600 B.C. that parental abuse of alcohol can have a deleterious influence on the outcome of pregnancy (Haggard and Jelinek, 1942). In 1726, soon after the lifting of distillation prohibition, the English College of Physicians reported to Parliament that parental drinking is a cause of "weak, feeble and distempered children". In 1834 a similar report was presented to the British House of Commons (Rosett, 1976). The results of experimental studies using dogs, mice, guinea pigs, rabbits and rats, have supported these clinical observations (Stockard, 1912; Ellis and Pick, 1976; Chernoff, 1977).

Recently the "fetal alcohol syndrome" has been described (Ferrier et al., 1973; Barry and O'Nullain, 1975; Jones et al., 1976), and attributed to chronic maternal alcohol abuse during pregnancy. A prospective study (Ouellette et al., 1977) involving 633 women revealed that infants born to heavy drinkers had twice the risk of abnormality

(including microcephaly and multiple anomalies) as those born to moderate or abstinent drinkers. Thirty-two percent of such infants had congenital defects compared to 14% in moderate and 9% in abstinent drinkers. A mouse model (Chernoff, 1977) and a beagle model (Ellis and Pick, 1976) have been reported, and Kronick (1976) has shown that acute alcohol intoxication is embryolethal and teratogenic in mice. Also, Obe and Herha (1975) have suggested that alcohol is mutagenic in vivo. Although much is now known about the harmful effects of chronic alcohol abuse during pregnancy, little attention has been directed to the more common problem of short-term abuse or binge drinking, or to the possible adverse effects of low levels of alcohol when taken in combination with other commonly used drugs.

A survey of Metropolitan Toronto showed that hypnotic-sedative drugs, largely composed of barbiturates, still rank first among all prescribed mood-modifying drugs and their consumption is steadily rising. The short and intermediate acting barbiturates, for example, pentobarbital, are the most widely prescribed sedatives and hypnotic agents, and are the most frequently abused (Devenyi and Wilson, 1971). As with alcohol, although chronic abuse or addiction is frequent, a "binge" of one night or a few days is also common. In vivo studies have shown that a single dose of sodium pentobarbital is associated with a high incidence of mutations in rabbit blastocysts (Shaver, 1975) and must therefore be considered to be a potential teratogen. In humans, prenatal exposure to anti-convulsant drugs, such as phenobarbital, is associated with increased incidence of congenital defects which Fredrick (1973) attributes to the drugs rather than to the epilepsy. No such studies have yet been carried out for the more frequently abused intermediate-acting

barbiturates.

In Canada, tranquillizers and barbiturates in combination with alcohol are increasingly encountered in forensic practice. Blood levels of barbiturate as low as 0.5 mg/100 ml combined with 0.1% alcohol have proved fatal (Gupta and Kofoed, 1966).

Experiments were designed to investigate the prenatal toxicity of short-term or binge abuse of ethanol and pentobarbital, using doses which produced a realistic range of behavioral impairment. In addition, the effect of low doses of ethanol and pentobarbital when administered together during pregnancy was investigated.

REVIEW OF RELATED LITERATURE

2. REVIEW OF RELATED LITERATURE

2.1 ETHYL ALCOHOL

2.1.1 Generalia

Generically, the word alcohol refers to the hydroxy derivatives of aliphatic hydrocarbons and certain aromatic structures. Colloquially, alcohol refers to beverages whose primary ingredient is ethyl alcohol or ethanol. It is a colourless liquid with a specific gravity of 0.783, a molecular weight of 46.07, and a characteristic odour and burning taste. Ethanol (CH_3CH_2OH) is a product of the process of fermentation in which yeasts, using their particular enzymes, derive energy from vegetable sugars (Lieber, 1976).

2.1.2 Absorption, Metabolism and Excretion

Because of its low molecular weight, infinite water solubility and relative solubility in lipids, it passes easily through biological membranes. Thus, ethanol is readily absorbed through the peritoneum, gastrointestinal tract and lungs, and is rapidly distributed throughout all body tissues including the brain. Absorption in the gut is so rapid that little if any ethanol reaches the duodenum. The presence of food in the stomach is the major factor in variations in the rate of absorption from this site but dilution, habituation and stress may also be involved. As soon as it reaches the blood it begins to disappear. Its rate of disappearance is linear and is the result of unchanged excretion (up to 10% via the lungs and body surface) or by its reduction to carbon dioxide and water (90 to 98%). The relative importance of these two pathways varies with the amount of ethanol ingested (Forney and Hughes, 1968).

The rate of metabolism in the mammalian organism is high and therefore plays an important role in calorie economy. In fact, ethanol ingestion heavily influences the metabolic pathways of the body by depressing them, especially those of the liver. Theoretically, the average person can metabolize 7g/hr ethanol, of which 90% is oxidized in the liver to acetaldehyde This is then oxidized completely to carbon dioxide and water in extrahepatic tissues. Ethanol can produce 1200 cal/24 hr which represents 80% of the energy required for basal metabolism. The partial metabolism of ethanol provides 70 - 90% of the energy used by the liver (Forsander, 1970).

Many organs in various species have been studied to determine the exact location and amount of extrahepatic ethanol metabolism. Although the problem is not yet clarified, the kidneys are primarily involved.

The literature on the metabolism of ethanol is voluminous. A brief summary will be presented here.

The hepatic metabolism of ethanol in vivo is accomplished by the alcohol dehydrogenase system (ADH) and the microsomal ethanol oxidizing system (MEO). The ADH system is the primary pathway and is known to be under genetic control in mice (Sheppard et al., 1968). The first step is the oxidation of ethyl alcohol to acetaldehyde. The enzyme liver alcohol dehydrogenase and a hydrogen acceptor such as NAD are required for this reaction. Acetaldehyde is then converted mainly to acetyl coenzyme A which is in turn converted primarily to CO_2 and H_2O via the Krebs citric acid cycle. If pyruvic acid is used as the hydrogen acceptor, still using NAD as the coenzyme, then blood lactate increases following ingestion of alcohol. Alcohol can also be oxidized directly into acetaldehyde and H_2O in the presence peroxide but this pathway is

of lesser significance.

An accessory pathway which comes into operation after blood alcohol reaches a certain level is the MEO system. At this level of intoxication the ethanol enters into competition with other drugs such as tranquillizers whose metabolism shares some elements of this system. In such cases the metabolism of these drugs is delayed and their effects enhanced.

The MEO system adapts to heavy ethanol ingestion by increasing its functional potential. This is reflected in an increase in the smooth endoplasmic reticulum of hepatocytes, and contributes to alcoholic tolerance and to cross-tolerance to other drugs such as barbiturates. This adaptation can be offset by progressive liver damage after prolonged, heavy drinking. Most of the systemic complications of chronic, excessive alcohol consumption relate directly to the metabolic process, in particular, the excessive accumulation of hydrogen ions and acetaldehyde (Lieber, 1976).

2.1.3. Toxicity of Ethanol

There are many complicating factors in the assessment of the toxicity of ethyl alcohol. For example, although ethanol's toxicity in the liver is independent of poor diet, it enhances malnutrition by causing inflammation in the stomach, pancreas and intestine thereby impairing digestion and absorption of nutrients, by potentially reducing the appetite for food, by providing empty calories without nutritive proteins, minerals and vitamins, and by interference with vitamin activation in the liver (Lieber, 1976).

Considerable information is available regarding experimental studies with ethyl alcohol using small laboratory rodents. Latven and Molitor (1939) determined the LD_0, LD_{50} and LD_{100}, as well as the minimum symptomatic dose of ethanol administered in two concentrations to mice, and also identified an increasing gradient of toxicity when ethanol was administered perorally (p.o.), subcutaneously (s.c.) and intravenously (i.v.). The Charles River Breeding Laboratories (1976) published the rate of alcohol metabolism in the mouse, rat and rabbit the $LD_{50}$and LD (g/kg) of ethanol using p.o., s.c., i.v., and intraperitoneal (i.p.) routes of administration for mouse, rabbit, rat and guinea pig, as well as the lethal ethanol blood levels for mouse and rat. Heistand et al. (1952) found that the toxicity of a given volume of ethanol administered i.p. to mice varied according to the dilution of the solution and according to previous treatments with ethanol. Wiberg et al. (1970) found that old rats (10-12 months) were much more sensitive to ethanol poisoning than young animals. Ernst et al. (1976) attributed this age toxicity relationship to a greater volume body water which resulted in lower blood alcohol concentrations. They also found an age difference in recovery from alcohol intoxication which increased with increasing dose. Lagerspetz (1972) found a diurnal variation in the effects of alcohol in mice. Haus and Halberg (1959) also observed that the lethal effect of 8 mg/g ethanol in mice showed diurnal differences. Attempts have been made to correlate these variations to diurnal changes in blood glucose, 5-HT metabolism, body temperature, the rate of voluntary ethanol consumption, illumination and activty cycles. Maling (1970) observed differences in LD_{50} of

ethanol administered orally, subcutaneously, intraperitoneally, and intravenously, which varied among rodent species, as did the rate of metabolism among commonly used strains of animals. Chesler et al. (1942) found that fetal rats do not possess the greater resistance of the newborn, but rather succumb to approximately the same toxic doses of alcohol as adult rats. They suggested that age differences in susceptibility to anoxia may be the underlying factor in this variability.

2.1.4. Tissue Effects of Ethanol

The literature relating to the effects of ethanol on the central nervous system is complex and contradictory. Although evidence suggests that ethanol exerts its effects by influencing the actions of various neurotransmitters and by altering cell membrane properties, it is clear that the complexity and the inter-relatedness of the nervous system make it virtually impossible to identify one locus or one mechanism to account for the observed effects (Kalant, 1975). Whether or not the morphological changes in the central nervous system of the chronically alcoholic adult (Wernicke's encephalopathy) or in the offspring of alcoholic mothers (Fetal Alcohol Syndrome) is due to alcohol itself cannot be ascertained because of many complicating factors, such as hypoxia, liver disorders and nutritional deficiencies (Robbins, 1974).

The heavy ingestion of alcohol is associated particularly with disease of the liver which is independent of poor diet, although malnutrition can be a complicating factor (Lieber, 1976). Fatty liver is observed in humans after merely a few days of alcohol consumption. Drunkenness is not a prerequisite for liver damage either in humans or experimental animals. Alcoholic hepatitis follows, in which decreased

liver function leads to inflammation, cell death and a mortality rate of from 10 to 30%. The final stage is cirrhosis which occurs as fibrous tissue replaces functional hepatocytes and gives rise to potentially fatal complications such as hyperlipemia, ketoacidosis, low blood proteins, clotting defects, portal hypertension, low blood sugar and vitamin levels, as well as high ammonia, bilirubin, lactic acid, uric acid and acetaldehyde levels. Death may be the result of hepatic coma, ascites, ruptured varices, kidney malfunction, gout, malfunction of cardiac and other muscle, and brain damage (Lieber, 1976).

The kidney excretes unchanged ethanol when excessive amounts are consumed and they have also been implicated in the extrahepatic metabolism of ethanol (Forney and Hughes, 1968). Increased diuresis is also observed following ingestion of alcohol. Further, the metabolism of large amounts of alcohol results in an excess of hydrogen ions and an accumulation of lactate. Lactic acidosis interferes with the excretion of uric acid from the kidney and promotes hyperuricemia (Lieber, 1976). Renal malfunction is one of the complications of chronic alcoholism and may represent prolonged adaptive changes. However, even a single large dose of ethanol has been reported to have produced microscopic changes in the rat as early as 18 hours after administration (Maling et al., 1967). Tost et al. (1971) suggested that alcohol directly produces an adjusted and reduced glomerular-tubular equilibrium in the kidney. It appears therefore, that both acute and chronic alcohol consumption can produce functional and morphological changes in the kidney.

Cardiomyopathy is related to excessive consumption of ethanol. The release of myocardial enzymes in apparently healthy alcoholics

suggests a direct insult to myocardial fibres (Wendt et al., 1966). Gothert and Thielecke (1976) studied the sympathetic nerve function in rabbit myocardium and proposed that the cardiomyopathy may be secondary functional changes in adrenergic innervation. Ultra-structural studies have demonstrated marked cytopathology in the myocardium of chronic alcoholics (Sander, 1970). Myocardial defects have also been reported in from 20% to 70% of children diagnosed as "fetal alcohol syndrome" (Jones et al., 1973, 1974; Jones, 1975; Hanson et al., 1976).

The hematopoietic tissues are affected by ethanol. Vacuolization of proerythrocytes, erythrocytes, promyelocytes and myelocytes has been reported in adults following acute intoxication and in neonates whose mothers received ethanol to offset premature labour (Lopez and Montoya, 1971). There was no nutritional deficit in any of these cases.

Alcoholic thrombocytopenia has been demonstrated in 88% of 42 alcoholic subjects which was dose-related and independent of liver damage (Paintal et al., 1975). Papara-Nicholson and Telford (1957) found hemoglobin levels in the first generation offspring of alcoholic guinea pigs to be 70 to 80% of normal which falls to 40 to 50% in the second generation. Obe and Herha (1975) suggested a mutagenic activity of ethanol in vivo after they demonstrated a significantly higher frequency of exchange aberrations in the peripheral leukocytes of 22 alcoholics.

Chronic alcoholics appear to be immuno-deficient as evidenced by their enhanced susceptibility to bacterial infections (Robbins, 1974). Loose et al. (1975) showed that macrophagic activity was not impaired, but did not eliminate the possibility of alterations in the interactions of macrophages with other immunocompetent cells. Caiazza and Ovary (1976)

failed to produce any impairment of cell-mediated or humoral immunity in guinea pigs at doses insufficient to produce liver damage. In light of the high number of infant deaths and failure-to-thrive among the surviving offspring of alcoholic mothers (Stockard, 1912; Haggard and Jelinek, 1942; Green, 1974; Hanson et al., 1976), it is conceivable that chronic maternal alcohol consumption has deleterious effects on not only her own immunocompetence, but also on that of her offspring.

When alcohol is administered in amounts beyond the metabolic capacity of the liver, the lungs excrete the excess. In these cases surfactant activity is affected as reflected by an increase in surface tension and a decrease in phospholipid content (Krishnan and Ramakrishnan, 1973). This is of significance to the neonate, but even more so to the offspring of alcoholic mothers who tend to be premature and with a higher incidence of mortality.

Ethanol-related changes in the responsiveness of the endocrine system, such as altered plasma adrenocorticoid levels and ADH secretion, have been reported. Priem et al. (1976) showed that alcohol significantly attenuates the normal plasma growth hormone response to insulin-induced hypoglycemia. This may be of significance in the etiology of growth retardation infants exposed prenatally to alcohol-induced hypoglycemia. Root et al. (1975) found that the biochemical and endocrine functions of four siblings of an alcoholic mother, all four displaying characteristic dysmorphic features and three with learning disabilities, were within normal limits. The growth retardation cannot, therefore, be explained by altered endocrine function as observed later in childhood.

2.1.5 Ethanol and the Maternal-placental-fetal Unit

During recent years it has become evident that age is a major determinant of drug effect. Although receptor maturity and sensitivity may sometimes account for this, it is most likely the result of differences in pharmacokinetic processes (Yaffe, 1976). Finnegan (1976) discusses lipid solubility, molecular weight and degree of ionization of the drug, as well as placental flow, placental metabolism of drugs, protein binding of drugs and aging of the placenta as factors which affect the distribution of drugs during pregnancy. Distribution in the fetal circulation can be influenced by selective uptake of drugs by specific tissues due to non-specific lipid solubility, specific binding of cellular constituents, active secretion of drugs by the yolk sac, permeability of specialized membranes and the distribution of the fetal circulation.

2.1.5.1. Specific responses of the pregnant female to pharmacologic agents

Metabolic and physiological processes change considerably during pregnancy which influences the metabolism, disposition and effects of drugs. The absorption of certain nutrients appears to be enhanced, but not much is known about the absorption of drugs. Total serum protein concentrations, especially albumin, decrease during pregnancy as do certain specific binding capacities. Total water content and body volume increase and certainly would alter the distribution of a drug. The kidney undergoes hypertrophic changes which shift the normal clearance values downward thereby modifying the renal excretion of drugs. *In vitro* studies have suggested a decreased capacity for oxidative and reductive metabolic pathways, whereas sulfation is increased. The underlying

mechanism for these changes is not understood but may be related to hormonal changes (Finnegan, 1976).

2.1.5.2. Biotransformation and transfer of ethanol by the chorioallantoic placenta

The placenta is an extremely important organ for normal fetal development. It is capable of catalyzing a large variety of reactions involving endogenous substrates and drug biotransformations. There is evidence that the placenta possesses the capacity for oxidative, reductive, hydrolytic and conjugative metabolic processes, but to differing degrees. Of the dehydrogenases that catalyze xenobiotic biotransformation reactions, NAD-dependent alcohol dehydrogenase has been reported to be present in the placenta (Juchau, 1972).

Many factors, related both to the drug and to the placenta appear to influence the placental transfer of pharmacologically active molecules. For example, the lipid solubility, degree of ionization and the molecular weight of the substance itself. Other factors include the blood flow, metabolism and age of the placenta (Mirkin and Singh, 1976).

Ho et al. (1972), in a radioautographic study using monkeys and hamsters, investigated the placental transfer of ethanol-1-^{14}C. They found that in the hamster, the percentage of unchanged ethanol in the fetus was higher late in pregnancy than prior to placental development. Whereas the placenta may act as a barrier to the metabolites of ethanol, it is apparently not a barrier to unchanged ethanol. They also found a slight tendency of ethanol and its degradation products to remain longer in fetal tissues than in maternal (probably due to low fetal levels of alcohol dehydrogenase) indicating that the pharmacologic

effects of ethanol are of longer duration in the fetus than the mother. Also of interest was the tendency during late pregnancy for high concentrations of alcohol to accumulate in the placenta and amniotic fluid.

2.1.5.3 Interrelation of fetal circulation and the placental transfer of ethanol

The status of both the uterine and fetal sides of the placental circulation is important in determining the exchange of drugs across the placenta. The administration of a drug may affect the maternal circulation either by producing generalized changes such as hypotension due to generalized peripheral vasodilatation, or may produce direct, local vasoconstrictive effects on the uterine circulation. In both situations, interference with normal uterine perfusion occurs which may alter the exchange of drugs. Changes in the fetal circulation may also play an important role in the response of a fetus to a drug. Direct and indirect effects on fetal hemodynamics may modify reflex circulatory responses to stress. Variations in venous flow rates in the umbilical cord will influence umbilical drug concentrations and, prior to administration of an anesthetic agent can also complicate the interpretation of drug distribution studies in the maternal-placental-fetal unit (Heyman, 1972). Differences between fetal and adult circulatory systems should also be considered. For example, 60 to 85% of the blood in the umbilical vein enters the fetal liver via the portal vein. Thus liver tissue may achieve concentrations far above other fetal tissues. The remaining blood enters the venous circulatory system where drug concentrations will be progressively reduced until very low levels are achieved in the arterial blood being distributed to the body organs (Mirkin and Singh, 1976). It is clear that the concentrations of a drug reaching

adult and fetal organs via the arterial blood may differ.

2.1.5.4 Fetal localization of ethanol

Once a substance has crossed the placenta it may become localized in specific fetal tissues or body compartments, excreted into the amniotic fluid, metabolized to some degree, or it may be returned to the maternal circulation. Factors which may influence general drug distribution in the fetus are as follows:

a. The permeability of specialized membranes such as the blood-brain barrier, renal tubule and the endothelium of the yolk sac placenta

b. The selective uptake of drugs due to non-specific lipid solubility, specific binding to cellular or plasma protein components, enzyme substrate interactions, or to active secretion by the yolk sac in the case of rodents;

c. The distribution of fetal circulation (Mirkin and Singh, 1976).

Ho et al. (1972) studied the tissue distribution of ^{14}C-ethanol in the monkey and hamster fetus. Radioactivity in the monkey fetus was localized in the liver, pancreas, kidney, lung, thymus, myocardium and gut wall at 15 and 90 minutes after administration of the isotope. By 90 minutes, the unchanged ethanol represented only 21% of the total radioactivity. Distribution in the CNS was well-defined at 15 and 90 minutes with high concentrations in the cerebellum. At 12 hours radio-activity remained in the CNS, bone marrow and pancreas. At all times, a higher level of radioactivity was detected in the cortex than in the white matter. The visual cortex revealed the highest concentration of

cortical tissues. It is interesting that ^{14}C-ethanol concentrated in all tissues in the fetus which are known to respond pathologically to ethanol in the adult.

2.1.5.5. Ethanol and fetal metabolism

There is great variability in the capacity of fetuses from different species to metabolize drugs, and this varies in any given species or individual with gestational age. The human fetus is able to catalyze drug oxidations during the first half of pregnancy (Rane et al., 1973). Pelkonen et al. (1973) found that the *in vitro* capacity of fetal liver to metabolize four substrates was much lower (2.4% to 36.1%) than adult liver. Cytochrome P-450 content, NADPH cytochrome C reductase activity in the fetal hepatic microsomes, and microsomal protein content were lower in fetal preparations, reflecting the lower drug-metabolizing capacity. Ultrastructural studies of the human fetal liver have shown that the endoplasmic reticulum, which is the morphological correlate of oxidative and conjugative metabolism is present during the first half of gestation (Zamboni, 1965). In contrast, in the rat, the rapid differentiation of hepatic endoplasmic reticulum occurs around birth (Dallner et al., 1966).

2.1.6 Fetal Alcohol Syndrome

For centuries, maternal alcoholism has been implicated in defective or abnormal development of the offspring. Recent reports have cited an increase in premature deliveries, miscarriages, stillbirths and infant deaths, and, among survivors an increase in the incidence of epilepsy, growth and mental retardation, psychosis, birth defects and

a general failure to thrive, as characterizing the Fetal Alcohol Syndrome (Jones et al., 1973, 1974; Green, 1974; Ouellette et al., 1977).

Maternal alcohol ingestion can influence fetal development by affecting gametogenesis, placental function and/or the prenatal environment. The latter can be altered by such factors as malnutrition, physiological states, etc., all of which are part of a complex socio-economic situation. Furthermore, consideration must be given to the differences in the adult and fetal organisms, and to differences in pregnant and non-pregnant physiological conditions. It is difficult therefore to isolate the actual causal mechanism(s) by which alcohol exerts its effects on developmental processes.

From a review of the literature, there emerges a pattern of fetal dysmorphogenesis which Ulleland described in 1972 and which Jones et al., in 1973, named the Fetal Alcohol Syndrome. Subsequent reports have substantiated and offered further details regarding the relationship between maternal alcoholism and fetal anomalies. Characteristic defects include prenatal and postnatal growth deficiency, psychomotor retardation. Micropthalmia, short palpebral fissures, ptosis, epicanthal folds, myopia and strabismus have been described in the eye. Skeletal anomalies include maxillary hypoplasia, cleft palate, varying degrees of joint limitations and micrognathia. Altered palmar crease patterns and capillary hemangiomas are common ectodermal defects while clitoromegaly and vulvar hypoplasia have been reported in the reproductive system. Systolic murmurs occur early in infancy but tend to disappear spontaneously. Dysmorphogenesis of the central nervous system such as microcephaly, serious disorientation of neuronal and glial elements, and incomplete

development of the brain, is considered to underlie or at least contribute to the impaired motor functions, and the learning and behavioral disturbances. Minor ear anomalies have also been reported (Stockard, 1912; Ferrier et al., 1972; Ulleland, 1972; Jones et al., 1973, 1974; Green, 1974; Palmer et al., 1974; Barry and O'Nullain, 1975; Tenbrink and Buchin, 1975; Hanson et al., 1976). Although it is difficult to separate environmental (both prenatal and postnatal) from biological factors in the etiology of this syndrome several investigations suggest that it is the actual intake of alcohol which initially produces the damaging and apparently irreversible deficits in these children (Goodwin et al., 1973; Jones and Smith, 1973; Palmer et al., 1974). Nutritional deficit or imbalance is frequently associated with undergrown offspring and chronic alcoholism. Naeye (1965) however reported that morphological changes in malnourished children (pre- and postnatal malnutrition) relate primarily to the reduced size of many organs due to a reduced cell number and cytoplasmic mass. No birth defects have been reported and catch-up growth occurred with appropriate therapeutic measures. Thus, maternal dietary insufficiency alone cannot account for the permanent morphological and developmental anomalies related to chronic maternal alcoholism. Furthermore, a recent prospective study of 633 women, in which drinking and nutritional patterns were observed throughout pregnancy revealed that infants born to heavy drinkers had twice the risk of abnormality of those born to abstinent or moderate drinkers. Thirty-two percent of infants born to heavy drinkers demonstrated congenital anomalies (such as microcephaly), as compared to 14% in the moderate group and 9%

in the abstinent group. There was no significant difference in nutritional status among the three groups of women (Ouellette et al., 1977).

Experimental investigations have yielded much information regarding the etiology of these clinical observations. For example, extremely low levels of alcohol and acetaldehyde dehydrogenase have been shown in both human and rat fetus (Pikkarainen and Räihä, 1967; Räihä et al., 1967; Pikkarainen, 1971). Idänpään-Heikkilä et al. (1972) demonstrated, clinically, a rapid equilibrium of ethanol between mother and fetus, and a slow rate of elimination in the neonate. Alcohol which was not metabolized was excreted by the lungs and kidney, which, in the fetus would be re-circulated in the amniotic fluid.

Papara-Nicholson and Telford (1957) reported that the offspring of alcoholic guinea pigs had low birth weights, poor locomotion and co-ordination, anemia, and sometimes were blind and spastic. Examination of their brains revealed flat gyri, shallow tissues, edema, vascular dilation, hemorrhages and delayed myelination in parts of the cerebral cortex and basal ganglia. By the fourth generation of alcoholism the remaining offspring were sterile. Chesler et al. (1942) found that the newborn rat was more resistant to the lethal effects of ethanol than was the adult and that the fetal rat was less resistant than the newborn and approximately of equal resistance as the adult. Kesäniemi (1974) found that elimination of ethanol in vivo was equal in pregnant and non-pregnant rats but that peripheral blood acetaldehyde concentrations were higher in pregnant animals. Detectable aldehyde-oxidizing capacity has been found in the kidney, adrenal gland and gastro-intestinal

tract of the human fetus although the liver accounts for about 90% of the total capacity (Pikkarainen, 1971).

The fetus is therefore exposed for longer periods of time both to unchanged alcohol crossing the placenta and re-circulating in the amniotic fluid, as well as to the acetaldehyde produced by fetal and maternal metabolism, than is the adult. This has been documented by Ho et al. (1972). Thus, the sympatho-mimetic effects of acetaldehyde and the enzyme inhibiting effects of ethanol, may contribute to the deleterious effect of chronic ethanol consumption on the growth and development of the offspring.

2.2 PENTOBARBITAL

2.2.1 Generalia

The barbiturates include a large number of drugs with a central structure of malonylurea (barbituric acid). Further substitutions to this molecule result in a class of drugs which are central nervous system depressants with effects ranging from mild sedation to deep anesthesia. Chemical alterations of the molecule also alter such pharmacological properties as distribution, metabolism, excretion, interaction with other drugs, onset and duration of action, etc. (Forney and Hughes, 1968).

Barbiturates are classified according to the onset and duration of their action which is a function of lipid solubility. Lipid solubility is altered by substituting various groups for the hydrogen atom on carbon 5 of the barbituric acid ring (Meyers et al., 1974).

2.2.2 The use and abuse of barbiturates

The ultra short-acting barbiturates such as hexobarbital and thiopental, are used as agents to induce rapid, superficial anesthesia for short surgical procedures. They play no part in drug abuse. The short-and intermediate-acting barbiturates such as pentobarbital, secobarbital and amobarbital, produce effects ranging from two to four hours and are the most widely prescribed sleeping pills and sedatives. These are the most frequently abused. The long-acting barbiturates (phenobarbital, barbital, diethylbarbital) act for periods greater than six hours, are generally used as sedatives, hypnotics or anti-convulsant and are frequently abused. In Metropolitan Toronto, hypnotic-sedative drugs, which are largely barbiturates are the most commonly prescribed mood-modifying drugs and their consumption is steadily rising. In addition to chronic abuse and addiction, these drugs are frequently abused sporadically,or for short periods of time, a pattern analogous to alcohol abuse (Devenyi and Wilson, 1971).

2.2.3 Absorption, Distribution, Metabolism and Excretion.

The barbiturates readily penetrate the extracellular and intracellular fluid compartments of the body. The route of administration, however,will influence the effect produced by a given dose by altering the initial distribution of the drug in the body. Three important factors affecting the distribution and fate of barbiturates are lipid solubility, protein binding and extent of ionization of the particular drug form (Harvey, 1975).

The more highly lipid-soluble compounds have a more rapid onset of action since they penetrate the blood-brain barrier more

rapidly. They tend to be more rapidly degraded metabolically and are almost completely reabsorbed by the renal tubule (Harvey, 1975).

A fraction of the barbiturates in the blood is bound to plasma protein, chiefly albumin. The intensity of CNS depression can be increased by the presence of acidic drugs such as aspirin or sulfon mides which displace barbiturates from the plasma proteins. Since the cerebral spinal fluid is virtually protein free, barbiturate concentrations in this compartment are generally only slightly less than in an ultra-filtrate of plasma. In body tissues, concentrations are generally as high or slightly higher than in plasma. With the exception of adipose tissue the capacity of tissues to concentrate barbiturates depends largely on protein binding. Somewhat higher concentrations are found in liver and kidney than in other tissues (Harvey, 1975).

Three processes are responsible for the termination of the central depressant effects of the barbiturates: physical redistribution, metabolic degradation and renal excretion. All reduce the plasma concentrations of active drug and result in its withdrawl from the site of action in the central nervous system (Harvey, 1975).

Most barbiturates are transformed primarily in the liver, into forms which are pharmacologically inactive and which can be excreted. There are four transformation routes: oxidation of radicals at C5 (the most important pathway), N-dealkylation, desulphuration of thiobarbiturates, and the destruction of the barbituric acid rings (Parke, 1971). Being a short-acting compound, pentobarbital is degraded entirely in the liver with only a negligible amount excreted unchanged in the urine (Meyers et al., 1974). At any given rate of blood flow,

the clearance of barbiturates is altered by changes in urine pH (Wilson et al., 1975). In rats, the metabolites of pentobarbital are also excreted into the bile in high concentration along with low concentrations of the unchanged drug. Thus a small fraction of pentobarbital may also appear in the feces (Harvey, 1975).

2.2.4 Mechanisms of Action

The barbiturates reversibly depress the activity of all excitable tissues albeit at different doses and concentrations. Thus, in sedative doses, there is very little effect on skeletal, cardiac or smooth muscle compared to the CNS effects. In prolonged anesthesia, however, serious deficits in cardiovascular and other peripheral functions can occur. Oxygen consumption in various tissues, mitochondrial respiration and the activities of several enzymes can be depressed by several concentrations of barbiturates. Although the actual mechanism is not known, it is probable that excitability in each tissue is depressed by an action on or in a membrane, and that the ultimate mechanisms are quite similar in the different tissues (Harvey, 1975).

2.2.5 Pharmacological Effects

2.2.5.1 Behavioral effects: Central nervous system

Whereas the action of a drug on the organic substrate of behavior is quite consistent, many of the effects described are subjective and vary according to the situation and from individual to individual. Barbiturates can produce the following range of effects: sedation, disinhibition, relief from anxiety, ataxia and nystagmus, sleep and finally anesthesia. Barbiturates are selective depressants of the

ascending reticular activating system and this explains the loss of consciousness induced. Other central neurons in the medulla and hypothalamus are also particularly sensitive (Meyers et al., 1974).

It is generally believed that the synapse is the site of action of hypnotic compounds since chemical transmission across neuronal and neuroeffector junctions is far more susceptible to barbiturate interference than is conduction along nerve or muscle fibres. Different central synapses appear to be affected in different ways. Barbiturates are also known to selectively depress sympathetic ganglia in concentrations which have no detectable effect on nerve conduction, neuroeffector junctions, or cardiovascular or smooth muscle (Exley, 1954).

2.2.5.2 Respiration

Barbiturates are respiratory depressants affecting both the drive to breathe and the mechanism responsible for the rhythmicity of respiratory movements. The three physiological influences maintaining respiration are differentially affected by barbiturates: The neurogenic drive, possibly arising in the reticular activating system is the most sensitive. Since the peripheral chemoreceptors are less sensitive to barbiturates than is the respiratory centre, with increasing doses respiration is maintained by the hypoxic drive and only later does medullary depression occur. In the deeply anesthetized subject, shock may occur as the result of vasomotor depression (Harvey, 1975).

2.2.5.3 Liver

In therapeutic doses, the barbiturates stimulate certain hepatic functions. However barbiturates combine with cytochrome P-450 and thus competively interfere with the biotransformations of a number of

substrates of this enzyme. These include endogenous substrates such as steroids or exogenous substances such as other drugs. Thus, endocrine imbalance or adverse drug interactions can occur. On the other hand barbiturates produce a non-specific increase in the activity of the hepatic microsomal enzyme system, which enhances the metabolism of many drugs and steroids. This inducing effect is accompanied by considerable hypertrophy of the endoplasmic reticulum (due to enzyme content), a moderate increase in liver weight which requires several days of treatment to become maximal, and accounts for part of the tolerance and cross-tolerance observed (Harvey, 1975).

2.2.5.4 Kidney

In anesthetic doses, pentobarbital exerts direct effects on the resorptive processes in the renal tubular epithelium such as the depression of sodium and glucose reabsorption (Blake, 1957). These direct effects may be partially overshadowed by changes in renal hemodynamics which are secondary to systemic hypotension, and to the stimulation of anti-diuretic hormone secretion. The net effect is a decrease in urine flow.

2.2.5.5. Tolerance and dependence

When activation of the drug-metabolizing enzyme systems in the liver occurs, a drug-disposition tolerance results which is manifested by the more rapid detoxification of barbiturates, a decrease in sleeping time, and an increase in the average dose required to maintain a given tissue concentration. This type of tolerance can be demonstrated in animals after one or two administrations and is most pronounced

for those agents with durations of action determined by the rate of metabolic degradation (Harvey, 1975).

Pharmacodynamic tolerance involves adaptation of nervous tissue to the presence of the drug. Tolerance to the hypnotic effects of barbiturates however does not significantly increase the lethal dose (Harvey, 1975).

Maynert and Klingman (1960) have reported that the plasma concentration at the time of awakening from barbiturate intoxication depends on the dose administered; the larger the dose the higher is the plasma concentration at the time of return of consciousness. This suggests that the central nervous system may become resistant to the effects of the drugs even during a single administration and the phenomenon has been called "acute tolerance". The mechanism of acute tolerance is unknown.

The development of addiction, or psychic and physical dependence, is largely defined by the presence of an abstinence syndrome upon withdrawal of the drug. The severity of the syndrome varies, depending on the depth, duration and continuity of intoxication prior to withdrawal, and on the rate at which the drug is removed from the tissues by metabolic degradation and excretion (Harvey, 1975).

2.2.6 Barbiturates and Pregnancy

2.2.6.1 Microsomal enzyme oxidizing systems

Difference in drug-metabolizing capacity has been reported between pregnant and non-pregnant rats (King, 1964), adult and fetal livers in humans (Pelkonen et al., 1973), and fetal and newborn rabbits (Fouts, 1973). Although exceptions can be cited, the liver is the site

where most xenobiotic drugs and chemicals are metabolized most extensively. Extrahepatic metabolism may be important however, in explaining tissue-specific, drug-induced pathology or toxicology.

Earlier work indicated that most hepatic drug-metabolizing enzymes were either absent or at barely detectable levels in the fetus and newborn of common laboratory animals. The rate of development of these drug metabolic pathways appeared to depend both on the animal species and on the particular drug substrate being used to assay these hepatic enzymes. In most cases adult levels of hepatic drug-metabolizing activity were reached only slowly, up to several weeks after birth (Fouts and Adamson, 1959; Jondorf et al., 1959). In contrast to laboratory rodents, drug metabolizing enzymes are present in the human fetus in the first half of pregnancy but generally at much lower levels than in the adult. Human fetal drug-metabolizing activity lies between five and 25% that of rat liver, and human adult capacity lies below that observed in adult rats (Pelkonen et al., 1973). Methodological problems and uneven functional distribution within the liver however, make such experiments very difficult to interpret.

Ioannides and Parke (1975) found that the barbiturates with low rates of metabolism and long half-lives,such as pentobarbitone, were the most potent inducers. Attempts to stimulate hepatic drug-metabolizing enzymes in fetal and newborn animals, however, has yielded variable results. Phenobarbital administered to pregnant rabbits failed to induce the fetal microsomal enzymes until just before birth. This response increased even more postnatally. The lack of response of fetal enzymes to inducers may be related to a deficiency in the

synthesizing systems, the lack of a proper stimulus, the inability of the fetal liver to bind the inducer, or to the presence of a repressor or inhibitor of fetal enzyme synthesis (Hart et al., 1962).

2.2.6.2 Placental transfer and fetal distribution

There have been many investigations regarding the placental transfer and accumulation of barbiturates in the fetal organism. Unfortunately the use of barbiturates with very different metabolic properties, in different mammalian species, widely varying doses and methods of analysis make it difficult to integrate the results. Fabre (1933) in dogs; Dille (1936) in rabbits, cats and guinea pigs; Windle and Becker (1941) in cats and guinea pigs; and Persaud (1964) in rabbits, have shown that there is a rapid transfer of barbituric acid derivatives through the placenta to the fetus. Ploman and Persson (1957) studied placental transfer of amylobarbitone in man following intramuscular injection into the mother and found that an approximate equilibrium between mother and fetus was reached during the first 30 minutes, and that the rate of drug elimination from the blood was about the same in mother and fetus. An accumulation of barbiturate was found in the placenta, and, more pronounced, in the liver and brain tissues. In the brain, a much higher concentration was found in the fetal 4th ventricle which is close to the respiratory centre, than in the cerebral cortex.

Of particular relevance is the study of King (1964) of the levels of pentobarbital (60 mg/kg administered subcutaneously) to non-pregnant, pregnant and fetal rats. The pregnant rat was observed to be less susceptible to the depressant effects of pentobarbital than

than the non-pregnant, but the duration of anesthesia was considerably lengthened during pregnancy. King made the following observations:

1. Pentobarbital disappeared from the blood of pregnant rats relatively more slowly than from the blood of non-pregnant females. From the eighth to the 12th hour after injection, the blood level remained significantly higher in the pregnant animal.

2. Brain levels remained significantly higher in the pregnant rat from the sixth hour. Two to four hours after injection fetal brain levels were 90% of maternal values, and up to 12 hours fetal levels remained approximately 75% of that of the mother.

3. Similarly the drug levels remained higher in the liver of the pregnant rat than in the non-pregnant rat. Fetal liver had a slightly higher affinity than did the fetal brain.

4. Pentobarbital was present in small amounts in amniotic fluid, but its disappearance from this site was slower than from fetal tissues.

Thus, when the non-pregnant female was given the drug, the brunt of detoxification fell upon the liver at once. The respiratory, circulatory, and temperature regulatory mechanisms were severely depressed until detoxification was achieved within a few hours and the animal awakened relatively early. In the pregnant rat, on the otherhand, the liver did not receive the full burden of the drug as rapidly. It crossed the placental barrier and pooled in fetal tissues and in amniotic fluid, only to be fed back into maternal tissues gradually. The fetal tissues played no role in detoxification, but merely served to decrease the load

thrown upon the mother at one time. Thus, maternal barbiturate levels remained high and sleep was prolonged.

In light of the higher barbiturate levels observed during pregnancy, the failure of the pregnant animal to become as severely depressed as the non-pregnant rat remains enigmatic. A possible cause could lie in alterations of protein-binding in pregnancy (King, 1964)

2.2.6.3 Effects on progeny

Kuenssberg and Knox (1973) reported a several-fold increase in congenital malformations following gestational exposure to anticonvulsant drugs. Fredrick (1973) reported that the offspring of epileptic women not taking anticonvulsants have the same incidence of malformations as the general populations. Loughnan et al. (1973) and Barr et al. (1974) described several children with hypoplasia and irregular ossification of the distal phalanges with nail dysplasia born to epileptic mothers using diphenylhydantoin and phenobarbital throughout pregnancy.

In vivo experiments using sodium pentobarbital have demonstrated a significantly higher incidence of cell divisions resulting in chromosomal aberrations (Setala et al., 1964). Shaver (1975) found that pentobarbital administered to female rabbits post coitum produced a slight delay in oocyte maturation and a greater frequency of chromosomally abnormal blastocysts. It is also possible that fetal hypoxia, resulting from altered placental hemodynamics, might be involved in the etiology of congenital anomalies associated with the use of barbiturates during pregnancy.

Persaud (1969) studied the effect of hexobarbital, sodium barbital and thiopental, administered intraperitoneally to rats on different gestational days and failed to elicit any teratogenic effect. Similar

results were observed in rabbits (Persaud, unpublished).

In mice, however, treatment with 65 to 330 mg/kg sodium barbital produced 30% fetal mortality, compared to 1.1% in controls. In addition, 59% of the experimental offspring had congenital malformations such as stunting, exencephaly, hydrocephalus, anopthalmia, cleft palate, spina bifida, umbilical hernia and limb defects (Persaud and Henderson, 1969). Walker and Patterson (1974) have demonstrated that barbiturates are significant cleft palate teratogens.

It appears that pentobarbital is potentially embryotoxic and teratogenic but that there are considerable species differences in susceptibility to this drug.

2.3 DRUG INTERACTIONS

2.3.1 General Principles

When two or more chemical agents are introduced into the circulation simultaneously several types of interactions may occur. If one compound offsets the action of the other, antagonism results. If these agents are pharmacologically equivalent it is theoretically possible to have complete negation of action. In practice, this is unlikely to happen. The effects of two agents may be additive, both qualitatively and quantitatively. Finally a synergistic or potentiation interaction may occur (Forney and Hughes, 1968).

Drug interactions are usually classified as either pharmacokinetic or pharmacodynamic in nature. Interactions in which one drug affects the absorption, distribution, metabolism or elimination of another drug and thereby affects the concentration at action sites are generally called pharmacokinetic interactions. Alternatively, interactions in which one drug displaces another at its action site or changes the responsiveness

of drug-action site complexes are referred to as pharmacodynamic interactions. It is obvious that pharmacokinetic interactions can have profound effects on the subsequent pharmacodynamic responses.

Some factors which can influence drug interactions are as follows:

a. Absorption from the gastrointestinal tract can be altered by changes in pH, gastric motility, (and thus the mixing, absorption and passage of its contents), and by the formation of ionized complexes that are more slowly absorbed.

b. Drug distribution can be altered by the displacement of one drug by another from plasma proteins and from tissue receptor sites.

c. Drug elimination by the kidney can be altered by changes in urine pH, the administration of diuretics or by chemicals which influence the urinary blood flow or the functioning of transport systems. The importance of any such action however, will vary according to the predominant mechanism by which a drug is normally eliminated.

d. Alterations in enzyme activities can significantly alter the effects of drugs but only to the extent that metabolism contributes to the total body clearance of a drug. Species, strain and individual differences exist which frequently result in differences in the biological half-life, and thus the pharmacological effect, of a drug. The availability of extrahepatic enzymes and the alteration of these by foreign compounds appears generally to contribute little to total body clearance.

e. The relationship between drug metabolism and urinary excretion can be affected by drug interactions since most drugs are eliminated not only by being excreted but also by being converted to metabolites which are more readily excreted.

f. The relationship between an enzyme that catalyzes the metabolism of a drug and the tissue clearance value of metabolism is complex. The complexity is increased when two or more drugs act simultaneously.

g. The binding of drugs to plasma protein will alter not only the excretion of drugs by the kidney but also their metabolism. Again the presence of two or more compounds would complicate this even more (Gillette, 1976).

The study of drug interactions during pregnancy is further complicated because the maternal-placental-fetal unit responds in a unique fashion (Finnegan, 1976).

2.3.2 Alcohol-Pentobarbital Interactions

The frequency with which alcohol and pentobarbital are used and abused in our society increases the probability that they will be used simultaneously. Alcoholics are particularly prone to intermittent or continuous abuse of barbiturates (Devenyi and Wilson, 1971). This raises the concern of possible altered actions and an increased toxicity.

In general, the acute simultaneous administration of various psychoactive drugs, including the so-called "drugs of abuse," all produce the combination of effects of the two agents, both qualitatively and quantitatively. However, the effects of drug interactions are not predictable. The combined short-term use of ethanol and pentobarbital may result in an additive interaction, an acute acquired, functional tolerance (Mellanby, 1919; Leblanc et al., 1975), or, an acute withdrawal syndrome. With more prolonged administration possibilities for functional and dispositional tolerance, cross-tolerance and sensitization arise (Patel and Lal, 1973).

Investigations into the possible sites or mechanisms of actions of various agents are of primary concern to the study of drug interactions. The fact that in many varied investigations, ethanol, anesthetics (including barbiturates) and even hypoxia are usually approximately additive in their effects on a given system leads to one or more of the following conclusions:

a. Each agent has a different site(s) of action, on the same or on a different cell.

b. Even in the case of a common receptor site, the concentration of these sites is probably far in excess of the relative concentrations of each agent such that there is not detectable competition for these sites.

c. The number of receptor sites that must be occupied for a given or a maximal drug action is less than the total number available (Smith, 1976).

2.3.2.1 Effects of ethanol on drug metabolism

Interactions of ethanol with other drugs vary from one individual to another with the amount of ethanol consumed and with the duration of use of large amounts of ethanol. Some of the observed effects may result from the alteration of the metabolism of endogenous or exogenous substances which share a common metabolic pathway with ethanol. For example, ethanol, *in vitro* is a competitive inhibitor of pentobarbital hydroxylase, and *in vivo*, ethanol administration decreases the urinary excretion of p-hydroxy phenobarbital and therefore increases the tissue concentrations of phenobarbital (Mezey, 1976).

In contrast, ethanol can also be an inducer, not only of its own metabolism, but also of the metabolism of other drugs. In animals its administration results in increases of smooth endoplasmic reticulum, cytochrome P-450, cytochrome c reductase, and of the activity of a number of microsomal enzymes. In humans ethanol has been shown to induce pentobarbital hydroxylase. Ethanol, however, is a weak inducer, producing only a one-fold increase in enzymatic activity and it fails to stimulate liver growth (Mezey, 1976). Increases in microsomal enzyme activity in the rat occur as early as 48 hours and are maximal after two to three weeks of ethanol administration. Mezey (1976) has reported however, that after six weeks increases in microsomal enzyme activity are no longer apparent, which coincides with increasing fatty infiltration and hepatocellular injury. Furthermore, rats which become tolerant to ethanol have cross-tolerance to other drugs such as pentobarbital. This may be due to an increased metabolic capacity or to a decreased CNS sensitivity (Hatfield et al., 1972).

2.3.2.2. Toxicity of ethanol-pentobarbital combinations

Several studies have emphasized that a considerable number of fatal barbiturate poisonings are accidental deaths where survival might have been possible if alcohol had not been present (Jetter and McLean, 1943). The effects of ethanol-barbiturate combinations have been variably described as additive (Gruber, 1955; Aston and Cullumbine, 1959; and Graham, 1960), potentiating (Dille and Ahlquist, 1937; Milner, 1968), non-synergistic (Haggard et al., 1940), and antagonistic (Allergi, cited in Coldwell, 1970). Obviously, these conclusions contribute little

towards an understanding of the underlying mechanisms, or the real clinical and teratological significance of the problem.

Coldwell et al. (1970) found that the combined administration (ip) of ethanol (3 g/kg, 15% w/v) and pentobarbital (30 mg/kg), shortened the induction time, lengthened the sleeping time, and raised the brain levels of acetaldehyde and acetone compared to animals treated only with pentobarbital. Rats given pentobarbital only had significantly higher brain and serum barbiturate levels at the time they regained the righting reflex than was present in the combined treatment, suggesting that CNS depression is dependent on factors other than brain barbiturate levels. The decay profiles of serum barbiturate concentrations were unaltered by ethanol, and brain and blood ethanol levels were unaffected by pentobarbital. It therefore appears that at least the immediate interactive effects are not due to the delayed metabolism or excretion of ethanol.

Thomas et al. (1972) studied the effect of ethanol on the distribution of ^{14}C-pentobarbital in the rat. At three hours after treatment, but not at one hour, the ethanol treated rats had higher levels of tissue radioactivity than controls (^{14}C-pentobarbital only). No particular redistribution between blood and tissues was found but it was noted that ethanol inhibited the excretion of radioactivity during the first six hours after treatment. The investigators postulated that the lower excretion rates and consequent higher tissue levels were due to inhibition of pentobarbital metabolism by ethanol.

Wiberg et al. (1970) after extensive investigations of ethanol-barbiturate interactions, concluded:

a. Ethanol (3g/kg, ip.) with barbiturates markedly reduce blood pressure with a concomittant reduction in urine formation and renal clearance of barbiturates.

b. Ethanol produces a dose-related decrease in body temperature (as much as two to three degrees), an effect which would likely decrease the hepatic metabolism of both barbiturates and ethanol;

c. Ethanol depresses the respiratory rate and lowers the blood pCO_2, which would likely further reduce ethanol and barbiturate metabolism.

d. Pentobarbital enhances the activity of purified rat liver alcohol dehydrogenase but retards the metabolism by liver slices.

e. Ethanol is more toxic in older rats (12 to 14 months) and there is an increased sensitivity to ethanol-barbiturate mixtures; and,

f. Ethanol alters the distribution of barbiturates in body compartments.

Some of the conclusions drawn by the above investigators are clearly contradictory. These discrepancies could be explained by either methodological problems or by differences in the doses used, but serve to emphasize the complexity of the problem of drug interactions and the difficulties in interpreting the results of such studies. There are no known investigations of the effects of combined doses of ethanol and barbiturates during pregnancy, or on the outcome of pregnancy.

PRELIMINARY INVESTIGATION

3. PRELIMINARY INVESTIGATION

3.1 INTRODUCTION

The administration of test-substances to experimental animals in toxicological experiments presents many methodological problems. Toxicity is known to vary according to any one or combination of the following: number of doses administered, dose dilution, solvent vehicle, route of administration, degree of tolerance or habituation, animal species, as well as the age, sex and health status of the individual animal and environmental factors (Wiberg et al., 1970).

A number of experimental studies involving ethyl alcohol in rodents have been undertaken. Latven and Molitor (1939) determined the LD_0, LD_{50}, LD_{100} of ethanol in mice, and the minimum symptomatic dose, when administered in two concentrations. An increasing gradient of toxicity was also identified when ethanol was administered perorally (p.o.) subcutaneously(s.c.) and intravenously (i.v.). Heistand et al. (1952) found that a given volume of ethanol administered intraperitoneally (i.p.) to mice varied according to the dilution of the solution and to previous treatments with ethanol. Wiberg et al., (1970) found that older rats (10-12 months) were much more sensitive to ethanol poisoning than younger animals. Lagerspetz (1972) found a diurnal variation in the effects of alcohol in mice as did Haus and Halberg (1959). Attempts have been made to correlate these variations to diurnal changes in blood glucose, 5-HT metabolism (Lagerspetz, 1972), body temperature (Haus and Halberg, 1959), rate of voluntary consumption, illumination (Eriksson, 1971), and activity cycles (Lagerspetz, 1972). Maling (1970) has observed differences in the LD_{50} of ethanol when administered orally, subutaneously, intraperitoneally and intravenously. Variations in LD_{50} and in the rate

of metabolism were also noted among commonly used strains of laboratory rodents.

Preliminary investigations were carried out to establish the following:

a) the relative toxicity of three different doses of ethanol administered intraperitoneally to rats in different dilutions.

b) A desirable range of ethanol intoxication for subsequent experiments.

3.2 MATERIALS AND METHODS

Virgin female Sprague-Dawley rats, weighing 250-300 grams, were used in this experiment. Ethanol was mixed in physiological saline and administered in three different concentrations - 30%, 20% and 10% ethanol v/v (Table 1). Four millilitres of each solution were administered daily on four consecutive days, in two intraperitoneal injections. A control group was treated with physiological saline. Each treatment group consisted of two animals. The animals were killed the day after the last treatment and examined for signs of peritonitis. Samples of liver and kidney were fixed in Bouin's solution and stained routinely with H&E for microscopic examination.

3.3 RESULTS

3.3.1. Behavioral Observations

It was always possible to distinguish between the animals of the four treatment groups on the basis of behavioral patterns. The group treated with 30% ethanol (E30) were comatose and were atonic for variable periods up to four hours. With subsequent treatments this group appeared progressively more irritable and wasted, sometimes showing abdominal spasms following the injection.

Table 1. Treatment schedule. Preliminary investigation.

Treatment group	Treatment	Mean daily dose
1. E30	4 cc. (i.p.) of 30% ethanol (v/v)	3.5 g/kg
2. E20	4 cc. (i.p.) of 20% ethanol (v/v)	2.1 g/kg
3. E10	4 cc (i.p.) of 10% ethanol (v/v)	1.1 g/kg
4. S.C. saline control	4 cc. (i.p.) of physiological saline	0.0 g/kg

The animals treated with 20% ethanol (E20) became hypotonic with very delayed righting reflexes. Locomotor ataxia was severe with only sliding and crawling being observed for as long as three hours.

The animals treated with 10% ethanol (E10) appeared slightly ataxic but were generally active.

There were no changes in motor co-ordination or irritability in the saline treated animals.

3.3.2. Gross External and Visceral Observations

The animals treated with the highest dose of ethanol (E30) displayed progressive physical wasting and a deterioration in the state of the coat which became dull and ruffled. In the intermediate dose group the changes were less dramatic. Neither wasting nor coat changes were observed following treatment with the lowest dose of ethanol (E10) or physiological saline (S.C.)

Weight change and evidence of peritonitis, as indicators of ethanol dilution toxicity, are summarized in Table 2. There was no apparent dose-related effects among the ethanol treated animals, although as a group they clearly lost more weight (mean loss = 21 grams) than did the corresponding controls (mean loss = 5 grams) over the treatment period.

Postmortem examination one day after the last treatment revealed no generalized hyperemia or ascites in any animal. Minimal fibrous adhesions were found in some cases in the E30, E20 and S.C. groups, but not in all animals.

Table 2. Weight changes and signs of peritonitis associated with different dilutions of ethanol administered intraperitoneally.

Treatment Group	Mean Weight Change	Signs of Peritonitis		
		Fibrous Adhesions	Ascites	Hyperemia
E30	-19.0 grams	minimal gastro-hepatic adhesions in one animal	none	at injection site in one animal
E20	-29.0 grams	gastrohepatic adhesions in one, minimal in other	none	none
E10	-15.5 grams	none	none	none
SC	- 5.0 grams	minimal in one animal*, none in other	none	none

* necrosis and hematoma in abdominal wall at injection site

3.3.3. Histological Observations

The kidneys and livers of ethanol treated and saline control animals were studied histologically. The most severe lesions were observed in rats exposed to the highest dose of alcohol, and both the extent and severity of the damage were less at the lower dose levels. However, no attempt was made to establish a quantitative dose-response relationship. None of the tissue changes described for the alcohol treated tissues were present in the saline controls.

At low magnification there was no apparent difference between the liver parenchyma of saline and ethanol treated animals. At higher magnifications, however, there were areas demonstrating subtle changes. Although there was no consistent or homogeneous increase in the fatty nature of experimental livers, there was evidence of early fatty change in some hepatocytes of the centrilobular area in those animals treated with the two highest doses of ethanol (Figure 1.).

Many cells with large, spherical and very prominent inclusions of varying density, which frequently obscured the nucleus, were also observed throughout the liver parenchyma of the 20% and 30% ethanol treated animals. These were in contrast to the smaller, more vesicular nuclei of the neighboring hepatocytes and were always found to be associated with large accumulations of pigment fragments in the adjacent parenchyma and blood channels, and with increased numbers of macrophages. Pigment-laden cells and pigment accumulation were not observed in livers of animals treated with 10% ethanol or saline, but an increased number of macrophages was noted in the former.

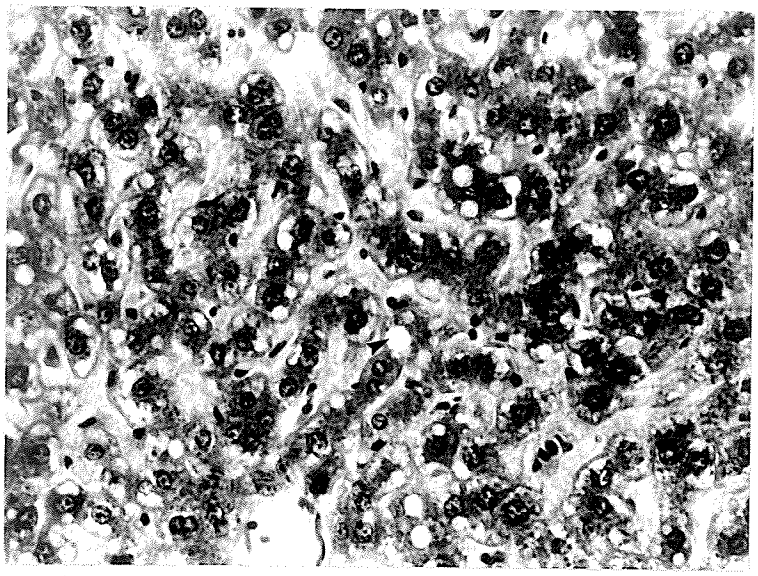
Other changes in the livers of alcohol treated rats were related to the contents of the blood channels. These vessels were frequently

Figure 1. Preliminary investigation. Early fatty change observed in the liver of rats following treatment with 30% ethanol on four consecutive days.
x 307

Figure 2. Preliminary investigation. Changes in the kidney following treatment with ethanol on four consecutive days.

a. Extensive cortical necrosis. Note that the glomeruli are intact even in regions of severe tubular degeneration. Dosage: 30% ethanol.
x 120

b. Epithelial damage observed in the straight tubules of the medulla. Dosage: 10% ethanol.
x 378



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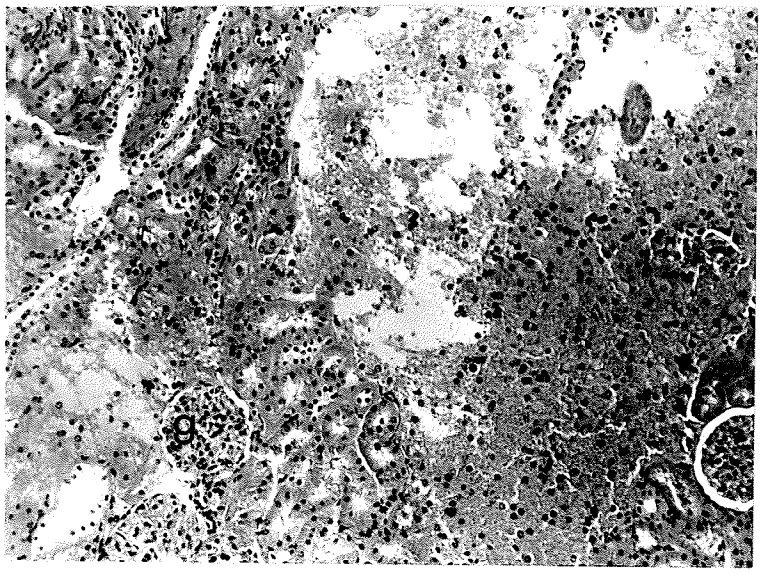

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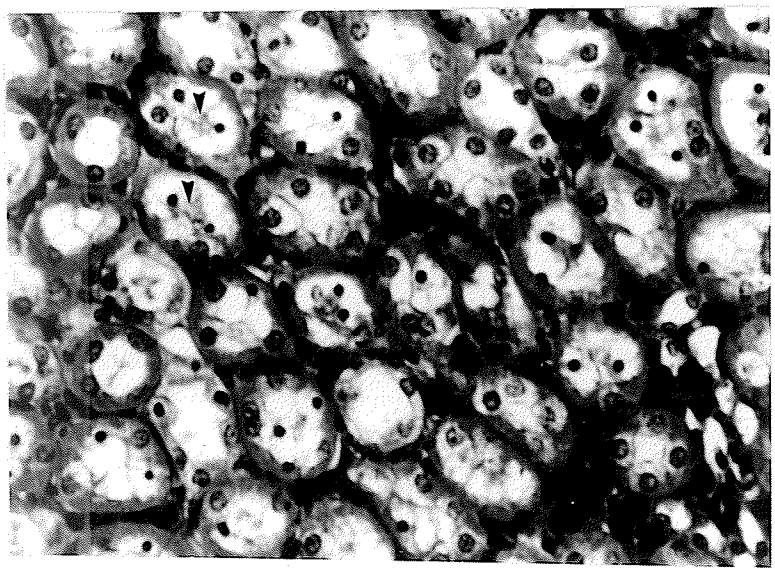


b

engorged with tightly packed chains of erythrocytes suggesting congestion and cell damage. Large numbers of pigment fragments or shells were noted in the sinusoids as well as the major vessels. An increased number of macrophages and monocytes were also present in these vessels, as well as in the perivascular connective tissue. Nucleated cells were observed adhering to the endothelium of some arterioles, which was suggestive of either stasis or an inflammatory reaction.

The most pronounced changes observed in the kidneys of ethanol treated animals were related to the vascular system. Compared to controls, the ethanol exposed kidneys appeared hyperemic. There were areas of vascular engorgement and disruption, of severe hemorrhage and necrosis, and other areas where blood components were scattered in the interstitium. Periarteriolar cuffing was also observed.

Changes were evident in the renal parenchyma. Whereas the glomeruli appeared to be unaffected even in large necrotic areas, the tubular epithelium was variably damaged (Figure 2). Some tubules were completely denuded of epithelium and others were lined with epithelium in varying stages of degeneration. Many lumina were filled with cellular debris. The straight tubular segments appeared to be the most severely affected. Evidence of regeneration was noted in the form of low, attenuated, but apparently healthy cells.

3.4 DISCUSSION

The purpose of these investigations was to study the teratogenicity, in the rat, of short-term or acute alcohol abuse, and of the interaction between low doses of alcohol and sodium pentobarbital, another

drug commonly used or abused by women of child-bearing age. Because of the different metabolic capacities between and within species, the varying effects of age, sex, concentration of the dose, route of administration and various environmental factors on the toxicity of ethanol, a preliminary investigation was designed to establish a desirable range of ethanol dose levels by assessing the toxicity of various doses, and, to assess the influence of the dilution of the dose and the route of administration on the toxicity of ethanol in the rat.

The dose range selected for the preliminary investigation (from 1.12 g/kg to 3.35 g/kg) was considerably below the LD50 of 5.0 g/kg for a single dose of ethanol when administered ip. to the rat (Maling, 1970). Even so, at the highest dose the experimental animals were comatose for up to four hours post-injection, stupor was produced by the intermediate dose, and severe locomotor ataxia was observed at the lowest dose. Weight loss over the treatment period was four times greater among the ethanol treated animals than among controls, suggesting that ethanol was largely but not solely responsible for the weight loss. The loss of weight among ethanol treated rats was not dose-related. However, physical wasting and locomotor impairment were clearly dose-related.

The post-mortem visceral examination revealed no evidence of a general peritonitis which could be attributed to the alcohol or its concentration. It is likely that the minimal fibrous adhesions and occasional instances of hyperemia and hematoma observed in some animals was a response to the repeated needle punctures rather than to the toxicity of ethanol itself.

The livers and kidneys of ethanol treated animals were studied

microscopically. There were signs of early fatty change in the liver parenchyma near the major arterioles and of a direct cytotoxic effect of ethanol on the hepatic cords adjacent to major bile channels. The arterioles appeared to be congested with swollen erythrocytes. The accumulation of pigment in the blood vessels and liver sinusoids was suggestive of a hemosiderosis which may be local, due to long-standing congestion of the blood in the liver, or systemic, due to abnormal breakdown of red cells, or both. Abnormal amounts of hemolysis could be attributed to central circulatory congestion and/or a direct cytotoxic effect of ethanol.

Systemic hemosiderosis is merely a morphological expression of iron overload; in this case, it was likely the result of excessive hemolysis Hemosiderosis produces little or no tissue damage or functional impairment (Robbins, 1974). Since the excretion of iron is strictly limited, excess iron binds with proteins with no heme complex and is stored as ferritin or hemosiderin. These forms localize initially in the cells of the reticuloendothelial system where erythrocytes are broken down, such as the Kupffer cells in the walls of liver sinusoids.

The vascular changes in the kidney might be attributed to a direct toxic effect of ethanol on the circulatory endothelium and/or musculature resulting in congestion and hemorrhage. Evidence supporting this would be the periarteriolar cuffing which was suggestive of an inflammatory process. On the other hand, severe alteration of central hemodynamics known to be associated with acute alcohol intoxication could have produced hypoxic damage to the endothelium and local hemorrhage.

The parenchymal changes indicated an acute tubular nephrosis (ATN) which may be of the nephrotoxic or ischemic type. Nephrotoxic ATN would

be the result of the direct cytotoxicity of ethanol on the tubular epithelium. In this case the proximal convoluted tubules (PCT) are most affected, the distal tubules are generally spared, and the tubular basement membrane remains intact. On the other hand ischemic ATN typically affects primarily the distal tubular segments and follows any shock or event which leads to peripheral circulatory insufficiency. In both cases the nephrosis is a reversible disorder compatible with complete recovery in the absence of any significant damage to the PCT and the presence of pronounced changes in the distal tubular segments. It seems likely that the observed ATN was secondary to long-standing renal vascular congestion, ie., was ischemic in etiology. This is consistent with the observations of Tost et al. (1971) that ethyl alcohol reduces renal blood pressure and that the hypotensive effect is more marked when the infusion is directly into the renal artery than intravenously. The deleterious effects of acute alcohol intoxication on the rat kidney may well have been secondary to severe circulatory shock.

Heistand et al. (1952) have shown that ethanol toxicity, as measured by LD_{50}, is inversely proportional to the dilution or concentration of the ethanol solution, up to 60%. Wiberg et al. (1970) suggested that this relationship is due to the cell damage produced by the high concentrations. Furthermore, they stated that even when ethanol is administered intraperitoneallyto mice in 20% concentrations, an irreversible, fatal chemical pertonitis results, the signs of which do not become evident before 12 hours. At 15% w/v concentrations this effect is not seen. For this investigation ethanol concentrations of 10%, 20% and 30% were administered intraperitoneally to rats to assess the peritoneal toxicity of each.

Lethal doses of ethanol also vary according to the route of administration. Latven and Molitor (1939) found that ethanol toxicity was greatest when administered i.v., with s.c. intermediate and p.o. the least toxic route. Maling (1970) found that LD_{50} decreased following ethanol administration p.o., ip, and i.v.. To reproduce in the rat, acute alcohol abuse as seen in man, would require voluntary ingestion. However the Metrecal method described by Klassen and Persaud (1976) and Chernoff(1977) for the study of chronic alcoholism, is not readily applicable to acute or short-term studies. Oral administration by intubation necessitates a general anesthesia which presents the problem of possible drug interaction. Furthermore, in p.o. administration, ethanol absorption is delayed and incomplete due to severe gastrointestinal damage, which inhibits nutrient absorption (Krawitt, 1974) and produces lower blood alcohol levels (Rasmussen, 1940). Direct administration into the bloodstream (i.v.) has the most toxic effect and ensures immediate, high blood alcohol levels. However, it too requires a general anesthesia and repeated daily injections could prove traumatic. Subcutaneous (s.c.) injection provides adequate but slow absorption requiring very small volumes and therefore high concentrations of ethanol. Since ethanol is a potent irritant it was anticipated that repeated injections could be unnecessarily traumatic for the animals. Thus, in order to avoid possible drug interactions and to ensure complete and rapid absorption with minimum trauma and irritation to the animal, the intraperitoneal route was selected The preliminary investigations revealed that after two daily i.p. injections of ethanol on 4 consecutive days, there was no evidence of severe peritonitis related to any of the treatments. Individual animals in both experimental and control groups showed minimal fibrous adhesions or focal

hyperemia which was attributed to repeated needle punctures.

CONCLUSIONS

1. The rapid onset of distinctly treatment-related levels of intoxication, as evidenced in locomotor impairment, indicated that the intraperitoneal route of administration allowed a rapid and efficient absorption of ethanol into the blood stream with minimum trauma to the animal.

2. Since there were no gross signs of general or severe peritonitis, it was concluded that the intraperitoneal administration of ethanol, in concentrations of 10%, 20% and 30% (v/v), at the dose levels described, would be a useful experimental model for subsequent investigations.

3. Acute ethanol intoxication induced in the rat by mean daily doses ranging from 1.12 to 3.35 g/kg was associated with liver and kidney damage. It is likely that these changes were due to a direct cytotoxic effect of ethanol and to an altered hemodynamic state associated with acute alcohol intoxication.

4. Based on the observations of behavioral intoxication, physical wasting and the microscopic changes observed in the liver and kidney of ethanol treated rats, it was decided to reduce by one half, the amount of ethanol to be administered in subsequent experiments.

METHODS AND MATERIALS

4. MATERIALS AND METHODS

4.1 ANIMALS

Albino Sprague-Dawley rats of the Holtzman strain were housed in groups of three in wire-mesh cages under controlled environmental conditions (temperature, approximately 21^{o}C; relative humidity approximately 50%; and a 12 hour light-dark cycle). They were maintained on Teklab Mouse and Rat Diet and water, both available ad libitum.

All handling was done by one person. Experimental treatments and killing were performed during the first three hours of the light cycle.

Male rats were placed with virgin females (200-250 g) overnight and the following morning vaginal smears were taken. The first day of gestation was considered to be the day on which spermatozoa were found in the vaginal smear.

4.2 EXPERIMENTAL DESIGN

Each experimental treatment group consisted of six pregnant rats. For each experimental group a control group consisting of three animals, were treated concurrently with equal volumes of physiological saline.

The test-substances were administered on days nine through 12 of gestation. Each daily treatment consisted of two consecutive intraperitoneal (ip.) injections of varying combinations of the test substances (ethanol, pentobarbital, or, ethanol and pentobarbital). Following each treatment the animals were observed for behavioral changes.

On day 20 of gestation, the animals were killed by ether administration. At autopsy the viscera were examined for signs of peritonitis, and the uterine horns and contents were removed and opened for

inspection. The total number of implantation sites were recorded as resorptions, dead or live fetuses. Maternal liver, kidney and ovary, all placentas, and 75% of fetuses were placed immediately in Bouin's fixative. The remaining fetuses were eviscerated and placed in absolute alcohol for subsequent staining with Alizarin Red S. (Dawson, 1926).

Maternal weights were recorded on day one of gestation, on each of the four treatment days (days nine through 12), and on the day of sacrifice (day 20).

4.3 ASSESSMENT OF TOXICITY

4.3.1. Maternal Toxicity

A general assessment of the toxic effects of the various treatments in the pregnant animal was made by observing the degree and duration of locomotor impairment following the administration of the test-substances.

A one-way analysis of variance and multiple comparison analyses (Rollwagen, 1973) were used to study the effects of the various treatments on maternal weight gain. The difference in maternal weight from the first to the last day of treatment (Day 9-12) was used as a measure of the immediate effects of treatment. Maternal weight changes over the treatment period relative to the number of live fetuses recovered on day 20 (Day 9-12/number of live fetuses) was also considered. The long-term effect of treatment was assessed by observing the variations in maternal weight gain from the first day of treatment to the end of gestation (Day 9-20) and the same measure relative to the number of live fetuses recovered on day 20 (Day 9-20/number of live fetuses).

The livers, ovaries and kidneys of the pregnant rats were processed routinely stained with H & E, and examined for microscopic changes.

4.3.2. Fetal Toxicity

The following criteria were used to assess the effects of the test substances on the intrauterine growth and development of the offspring.

a. Fetal mortality. Resorption sites and dead fetuses were recorded and expressed as percentages of the total number of implantation sites as observed on day 20 of gestation.

b. Growth morphometry. Measures of fetal growth and development, including weight, crown-rump length and placental weight on day 20, were recorded and analyzed using a nested analysis of variance (Finn, 1972).

c. Gross morphology. Each fetus fixed in Bouin's solution was examined under a dissecting microscope for external abnormalities and then cut into serial sections as described by Wilson (1965) for visceral examination.

Fetuses fixed in absolute alcohol were stained with Alizarin Red S (Dawson, 1926). The skeletal systems were examined for congenital deformities and variation in the rate of ossification.

d. Histological studies. Placentas, livers, kidneys, and adrenal glands of those fetuses fixed in Bouin's solution were processed routinely, stained with H&E, and examined for microscopic changes. In the ethanol studies, fetal bone was also examined and additional placentas were stained with PAS for further examination of mucoprotein content.

4.4 TREATMENT SCHEDULES

4.4.1. Ethanol

The dose levels of ethanol used to investigate the prenatal toxicity of short-term intoxication are indicated in Table 3.

4.4.2. Pentobarbital

The dose levels of pentobarbital used to investigate the prenatal toxicity of pentobarbital are shown in Table 4.

4.4.3. Ethanol-pentobarbital Combinations

The dose levels for the combined treatment with ethanol and pentobarbital which were used to investigate the prenatal toxicity of their interaction at low doses are presented in Table 5.

Table 3. Treatment schedule. Prenatal toxicity of ethanol.

Treatment Group	Number of animals	Treatment
SC 30	3	2 cc. saline
30% E	6	2 cc. ethanol (30% v/v) mean daily dose = 1.4 g/kg
SC 20	3	2 cc. saline
20% E	6	2 cc ethanol (20% v/v) mean daily dose = 1.1 g/kg
SC 10	3	2 cc saline
10% E	6	2 cc ethanol (10% v/v) mean daily dose = 0.56 g/kg

Table 4. Treatment schedule. Prenatal toxicity of pentobarbital.

Treatment Group	Number of animals	Treatment
SC 25	3	saline
25 P	6	25 mg/kg pentobarbital
SC 15	3	saline
15 P	6	15 mg/kg pentobarbital
SC 5	3	saline
5 P	6	5 mg/kg pentobarbital

Table 5. Treatment schedule. Prenatal toxicity of ethanol-pentobarbital combinations.

Treatment group	Number of animals	Treatment
SC E10 P5	3	saline
E10 P5	6	2 cc 10% E + 5 mg/kg P
SC E10 P15	2	saline
E10 P15	5	2 cc 10% E + 15mg/kg P
SC E10 P25	3	saline
E10 P25	5	2 cc 10% E + 25 mg/kg P
SC E20 P5	3	saline
E10 P5	4	2 cc 20% E + 5 mg/kg P
Controls (pooled)	11	saline

RESULTS

5. RESULTS

5.1 ETHANOL

5.1.1 Maternal Toxicity

5.1.1.1. Behavioral effects

A dose-related effect was observed in the different treatment groups with respect to locomotor impairment. Those rats receiving 30% ethanol (mean daily dose = 1.4 g/kg) occasionally lost their righting reflex for up to 15 minutes but never lost their abdominal tonus. They displayed varying degrees of hypotonia and ataxia and generally appeared to be in a state of stupor for up to four hours after treatment. Those rats receiving 20% ethanol (mean daily dose = 1.1 g/kg) displayed locomotor ataxia and hypotonia for up to four hours after treatment. Although movements were slow and ataxic the animals were active in contrast to the 30% group. The 10% ethanol treated group (mean daily dose = 0.6 g/kg) displayed no locomotor ataxia or hypotonia. They were generally more active and exploratory than the controls. The saline treated animals showed no impairment in locomotion or change in activity levels.

5.1.1.2. Maternal weight

The analysis of variance and the multiple comparison tests revealed no significant difference in weight gain between the mothers of the three control groups (Table 6). This observation justified the pooling of the controls into one group (N=9).

a. A one-way analysis of variance was used to analyze the weight changes observed in the different treatment groups over the treatment period (Figure 3 and Table 7). Due to a significantly higher variance within the

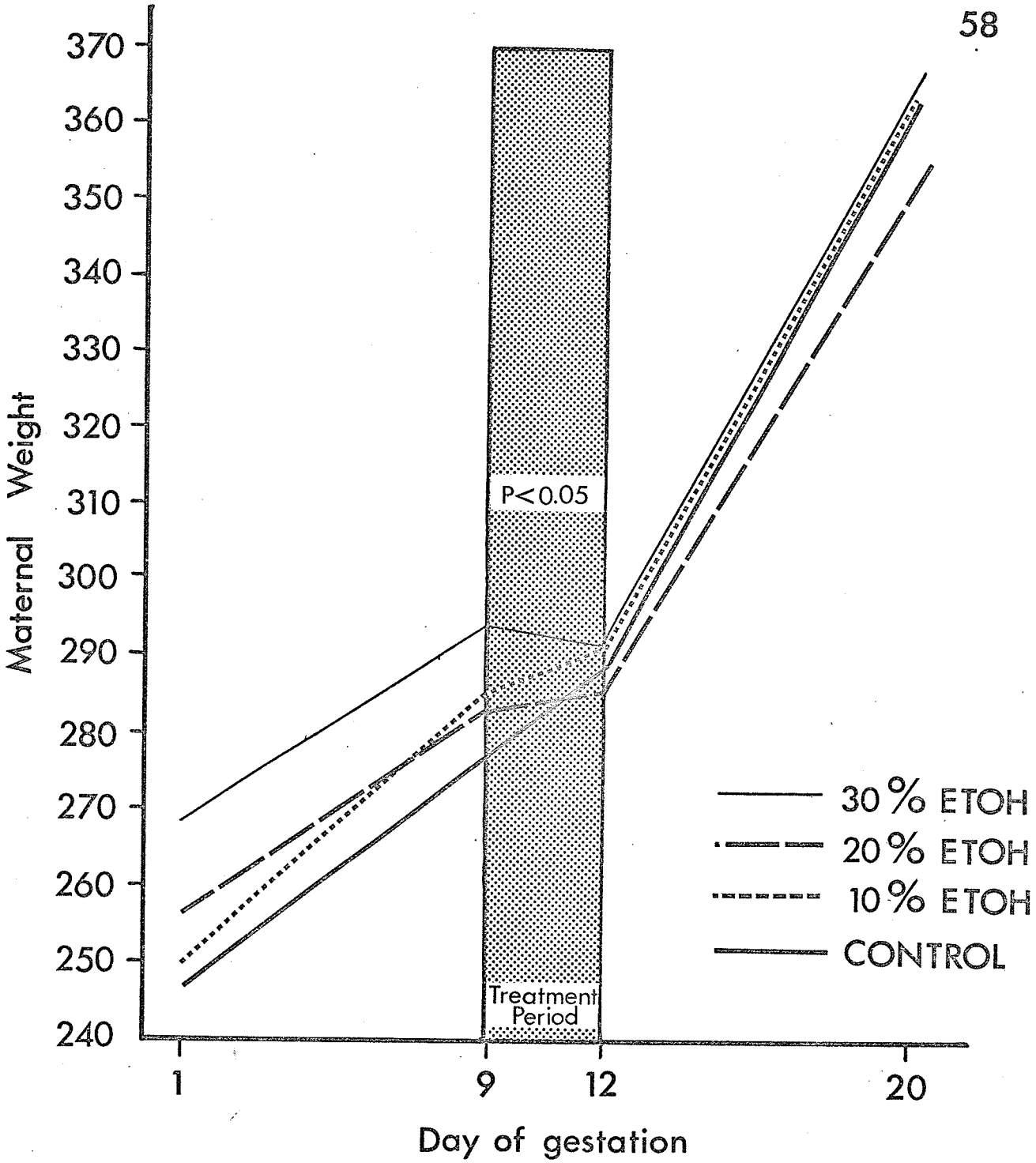


Fig. 3 PRENATAL TOXICITY OF ETHANOL: COMPARISON OF MATERNAL WEIGHT GAIN BETWEEN TREATMENT GROUPS.

Table 6. Prenatal toxicity of ethanol. A comparison of maternal weight gain among saline treated control animals.

Treatment Group	N	Mean maternal weight change (g)	
		D9-12	D9-20
SC 30	3	+9	+95
		S.E. = 3.3	S.E. = 7.4
SC 20	3	+10	+68
		S.E. = 2.3	S.E. = 6.5
SC 10	3	+14	+88
		S.E. = .9	S.E. = 8.2
"F" ratio observed		1.614	3.372
Significance/F.95 (2,6)=5.14		N.S.	N.S.

Table 7. Comparison of maternal weight gain among pooled control animals and ethanol treated animals

Treatment Group	Mean Maternal Weight (g) Change				
	N[1]	D9-12	D9-12/n[2]	D9-20	D9-20/n
Control (pooled)	9	+11.0	+1.0	+84.0	+6.0
30% Ethanol	6	-3.3	-0.3	+69.0	+5.8
20% Ethanol	6	+2.5	+0.2	+68.0	+6.2
10% Ethanol	6	+7.0	+0.5	+77.0	+6.4
"F" ratio observed		4.477	4.082	1.873	0.740
Significance $F_{.95}$ (3,23) = 3.03		p<0.05	p<0.05	N.S.	N.S.

1 N = number of rats in group

2 n = number of live fetuses

30% group, an analysis of variance was repeated for control, 20% and 10% groups only, to avoid distortion of the analysis (violation of equality of variance assumption). This revealed a significant difference in maternal weight gain during the treatment period between these three groups ($p < 0.05$). Multiple comparison tests (see Table 8) further revealed that there was significant variation between the control and 20% ethanol treated rats ($p < 0.05$). The mean of the 30% group was then tested against the mean of the control group using Welch's t-test for unequal variances and found to vary significantly ($p < 0.05$). A comparison of the means of 30% : 20%, 30% : 10%, and 20% : 10% was made using a multiple t-test with a pooled error variance. This was justified since they were not significantly different and no significant variation was found.

b. Maternal weight change over the treatment period relative to the number of live fetuses recovered on day 20 (day 9-12/n) was found to vary among treatment groups (Table 9). Dunn's multiple comparison test revealed a significant difference between the saline treated controls when compared with the 30% ethanol treated animals ($p < 0.05$).

c. Analysis of variance revealed no significant variation in weight gain among the treatment groups when the final weight (day 20) was considered in relation to day nine, the first day of treatment (Table 7). Thus, the weight loss observed during the treatment period was recovered by the end of gestation.

d. Analysis of variance revealed no significant difference among the different treatment groups when weight changes from day nine to 20 were related to the number of live fetuses (Table 7).

Table 8. Comparison of maternal weight gain among control and ethanol treated animals over the treatment period (days 9 to 12 of gestation).

Multiple Group Comparison	Required value(p=0.05)	Observed Value	Significance
Control vs. 30% E	2.447	2.677	5% Welch's t(6)
Control vs. 20% E	2.61	2.840	5% Dunn (3,20)
Control vs. 10% E	2.61	1.373	N.S. Dunn (3,20)
30% vs. 20% E	1.812	-1.134	N.S. t(10)
30% vs. 10% E	1.812	-1.977	N.S. t(10)
20% vs. 10% E	2.61	1.339	N.S. Dunn (3,20)

Table 9. Comparison of maternal weight gain, relative to the number of live fetuses recovered on day 20, among control and ethanol treated animals over the treatment period (day 9-12/n)

Multiple Group Comparison	Observed Value	Significance Dunn .95 (6,24) = 2.88
Control vs. 30% E	3.412	$p < 0.05$
Control vs. 20% E	1.969	N.S.
Control vs. 10% E	1.176	N.S.
30% vs. 20% E	1.318	N.S.
30% vs. 10% E	2.041	N.S.
20% vs. 10% E	0.124	N.S.

5.1.1.3 Gross visceral observations

Evidence of peritonitis observed at autopsy eight days after the last treatment is summarized in Table 10.

Table 10. Signs of peritonitis observed in animals eight days following intraperitoneal injections of saline or ethanol.

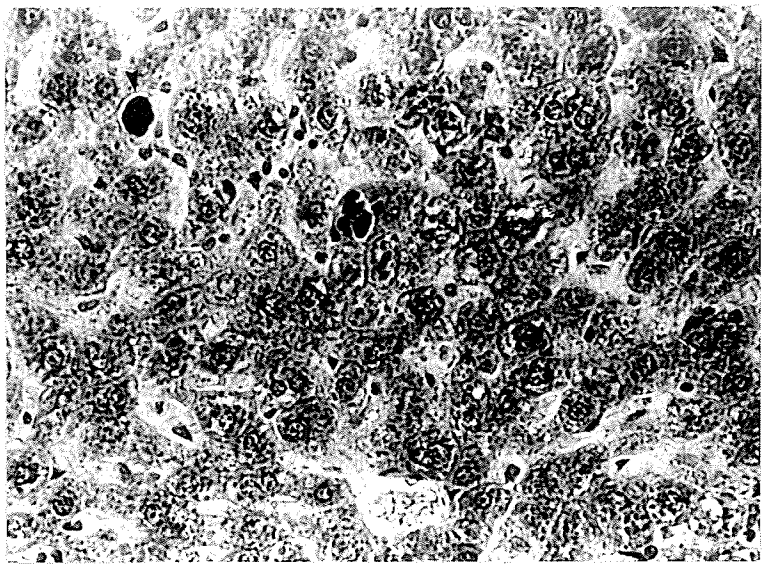
Observation	Saline	30%	20%	10%
Hyperemia	-	-	-	-
Ascites	-	-	-	-
Adhesions	-	4/6	1/6	-
Balloon liver	-	4/6	-	-

5.1.1.4 Microscopic observations

There was no evidence of fatty change in the liver of any of the experimental animals nor was there evidence of the massive accumulation of pigment in the parenchyma or in the vascular channels. This is in contrast to the observations noted in the Preliminary Investigation. In general, the most noticeable changes were observed in the livers of animals treated with the highest dose of ethanol (1.4 g/kg 30%). These changes were less severe, less frequent and even absent in the 20% group (1.1 g/kg), and virtually absent in the livers of animals receiving 0.56 g/kg ethanol (10%). The changes observed in the livers of animals treated with 30% ethanol are as follows:

Figure 4. Aggregates of inclusion-laden macrophages in maternal liver following acute intoxication with ethanol (30%) during pregnancy.
x 307.

Figure 5. Periarteriolar cuffing and hemorrhage, parenchymal macrophages and tubular epithelial damage observed in the maternal renal cortex following acute intoxication with ethanol (10%).
x 120.



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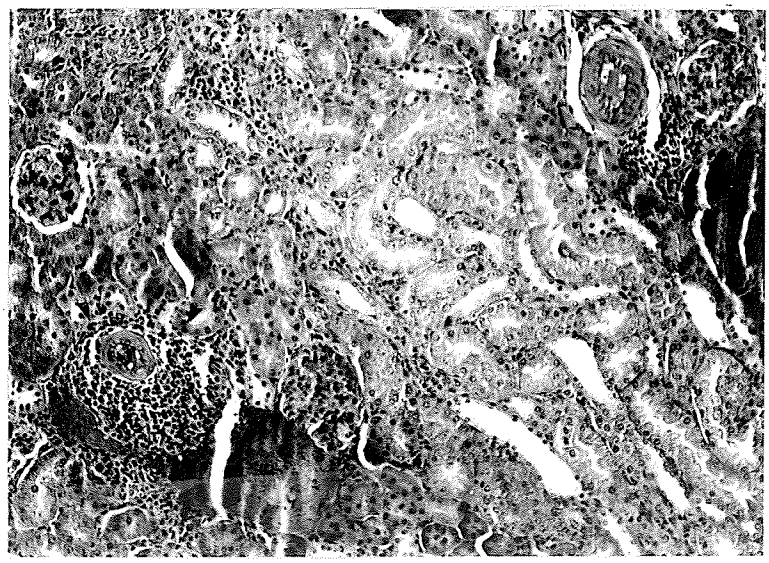

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a) An increase in nucleated cells in the blood channels. These were observed "pavementing" the endothelium but were more typically free and evenly distributed among the erythrocytes.

b) The presence of distinct clumps of macrophages filled with inclusions within the parenchyma (Figure 4). These cells were usually tightly packed together with the nucleus obscured. In this characteristic arrangement they appeared more like inclusion bodies than cells. However there were also more diffuse clumps of cells which were not as engorged, with inclusions which more clearly revealed their macrophagic nature.

c) The sinusoids and blood vessels of control and experimental livers frequently contained debris such as washed out or degenerating erythrocytes, and specks of unstained particles (which might be dust or pigment). Since there was no observable lesions associated with these changes in control tissues, they were interpreted as reflecting a normal process of degeneration and recycling of erythrocytes. In the experimental tissues however there were additional changes. The concentration and size of the pigment fragments in the blood were much greater, and there was as well a general distribution of pigment throughout the parenchyma. Furthermore, the presence of cellular debris and ghost cells among the degenerating erythrocytes suggested that there was also a treatment-related toxic effect in the experimental tissues.

d) Although there were numerous mitotic figures in the control livers their number was markedly increased in the cords of ethanol (30%) treated rats, especially near the central veins.

In the liver of animals treated with 20% ethanol there were macrophage clumps but these were fewer, more diffusely arranged and not as engorged as in the 30% group. There also appeared to be more mitotic figures in these livers than in the controls.

In the livers of the 10% ethanol treated animals the only noticeable difference from the controls was the presence of some diffuse macrophage clumps.

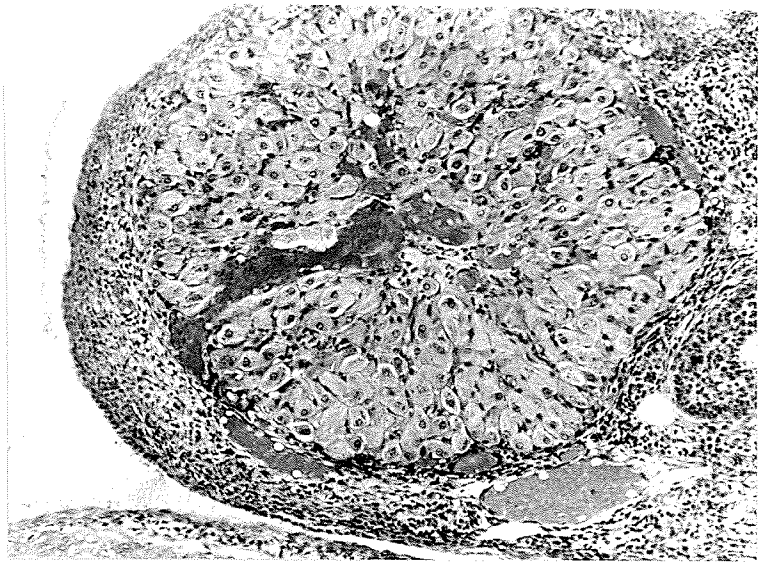
Compared to the controls, the kidneys of ethanol treated rats displayed changes which were again related to the vascular system and to the parenchyma (Figure 5). Parenchymal changes were observed in both the cortex and the medulla but involved only the tubular portion of the nephron. Generally, there was a dose-related effect, with the highest dose of ethanol being associated with the most extensive and severe lesions.

Changes observed in the vascular system included marked peri-arteriolar cuffing, venous disruption and hemorrhage, and the presence of pigment fragments in the larger vessels of some tissues.

Morphological changes in the parenchyma involved only the tubular portion of the nephron. In the cortex, degeneration of the convoluted tubules was observed but associated glomeruli were never affected. Closer examination revealed that it was primarily the proximal convuluted tubule which was affected. In the highest dose group, ghost cells and debris virtually filled the lumina of these tubules. The most pronounced

Figure 6. Congestion in the sinuses of the corpus luteum and minimal luteolysis around a central sinusoid following treatment with 10% ethanol.
x 120

Figure 7. Delay or deficiency of ossification in the supraocciput of fetuses exposed prenatally to ethanol. Control fetus on the left.



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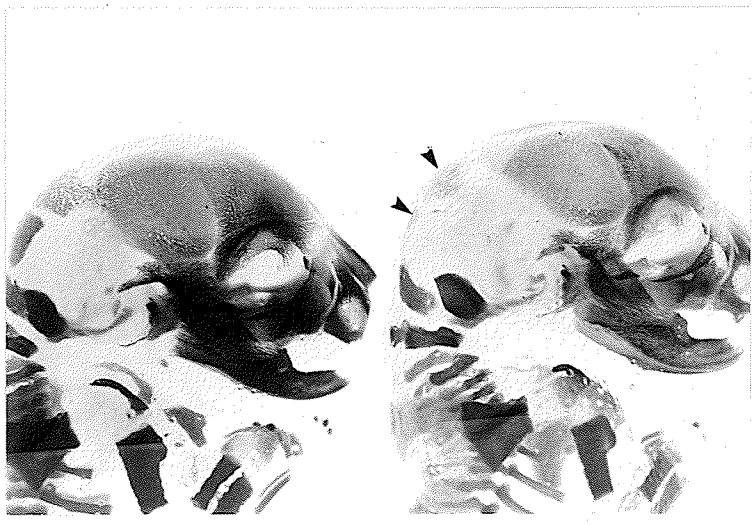

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changes however were observed in the medullary tubules. Marked degeneration and sloughing off of the tubular epithelium were consistently observed in the kidneys of all ethanol treated animals, especially at the base of renal papilla. The tubules of the papilla however never displayed any sign of damage although debris was sometimes present in their lumina. Severe distension of isolated tubules was observed in this area and the most extensive hemorrhages also occurred here.

There was no morphological difference observed in the follicular development or the supporting stroma of the ovaries of ethanol or saline treated rats. However the corpora lutea were distinctly congested in the peripheral venous channels, the luteal sinusoids and the large central sinusoid. In the tissue immediately adjacent to the congested central sinusoids, there was evidence of damage to the luteal cells themselves. The presence of necrosis and the increased numbers of macrophages were indicative of cell death (Figure 6). No changes were observed in luteal cells which were more distant from the central sinusoids even where congestion was observed.

5.1.2. Fetal Toxicity

5.1.2.1 Resorption rates

Resorption rates expressed as a percentage of the total implantation sites observed on day 20 are presented in Table 11.

5.1.2.2 Gross abnormalities

Whole fetuses from all treatment groups were examined for external malformations and then sectioned serially using the freehand razor blade technique described by Wilson (1965) and examined for

Table 11. Resorption rates following maternal treatment with ethanol or saline on days nine through 12 of gestation

Treatment Group	Implantation sites	Number of resorptions	Percent
Saline controls	116	4	3.45%
30% ETOH	76	3	3.95%
20% ETOH	69	2	2.9%
10% ETOH	74	5	6.76%

visceral anomalies. There were no external or visceral abnormalities observed in any of the experimental fetuses. One case of unilateral anophthalmia was noted in a control fetus.

Approximately 25% of the fetuses from each rat were cleared and stained with Alizarin Red S (Dawson, 1926) in order to study skeletal development. Compared to controls, those fetuses whose mothers were treated with 30% ETOH showed the most severe and frequent ossification delays and irregularities. Ossification delay was most common in the supraoccipital bone, sternebrae and metacarpal/metatarsal bones of fetuses exposed prenatally to ethanol (Figure 7). There was no actual skeletal deformity observed. Irregular ossification patterns such as unilateral delay in supraoccipital and interparietal ossification were frequently observed. Other less frequent observations included delays in the ribs and in the longbones of the hindlimb.

The offspring of mothers treated with 20% and 10% ETOH showed very few and much less severe developmental delays related to the same bones as the 30% ethanol treated group.

5.1.2.3 Growth morphometry

A nested analysis of variance was used to detect any differences among the weights, lengths and placental weights of the offspring of the treatment groups. Whereas there was no significant treatment-related difference in the crown-rump lengths, there was a significant variation ($p < 0.001$) in the lengths of fetuses between the rats within the treatment groups (see Table 12). The same pattern of variation was observed with respect to fetal weights (Table 13).

Placental weights varied significantly ($p < 0.05$) between treatment groups (Table 14). Further analysis revealed that the source of the variation was the difference between the 30% ethanol treated rats and their saline controls (Table 15), the mean placental weight of the experimental group being less than the controls ($p < 0.05$).

5.1.2.4. Microscopic observations

The liver of rat fetuses at day 20 of gestation is a highly vascular tissue which appears to be primarily a hematopoietic organ. Cords of hepatocytes are indistinct and the liver sinusoids are filled with immature blood cells. Megakaryocytes are abundant around blood vessels. The livers of fetuses of ethanol treated mothers were indistinguishable from those of saline treated controls.

At day 20 of gestation the fetal kidney is differentiating rapidly as evidenced by the numerous mitotic figures but is still an immature metanephric structure.

Table 12. Variation in fetal length related to prenatal exposure to ethanol.

	df.	MS	F value	Significance
Between treatments	5	0.0751	0.0917 $\left(\frac{0.0751}{0.0819}\right)$	N.S. $F_{.05}(5,21) = 2.68$
Between rats	21	0.0819	5.648 $\left(\frac{0.0819}{0.0145}\right)$	$p < 0.001$ $F_{.001}(21,207) = 3.26$
Within litters	207	0.0145		

Table 13. Variation in fetal weight related to prenatal exposure to ethanol.

	df.	MS	F value	Significance
Between treatments	5	0.3046	0.658 $\left(\frac{0.3046}{0.5463}\right)$	N.S. $F_{.05}(5,21) = 2.68$
Between rats	21	0.5463	5.8478 $\left(\frac{0.5463}{0.0934}\right)$	$p < 0.001$ $F_{.001}(21,206) = 3.26$
Within litters	206	0.0934		

Table 14. Variation in placental weight related to prenatal exposure to ethanol.

	df.	MS	F value	Significance
Between treatments	5	0.141	3.2 (0.141/0.044)	$p < 0.05$ $F_{.05}(5,21) = 2.68$
Between rats	21	0.044	0.88 (0.044/0.05)	NS $F_{.05}(21,207) = 1.57$
Within litters	207			

Table 15. Variation in placental weight between ethanol treated animals and their respective controls. Multiple comparison analysis.

Treatment group comparison	df	Hypothesis MS	Observed F	Significance*
SC30 : 30% E	1	0.2195	4.39	$p < 0.05$
SC30 : SC20	1	0.0088	<1	N.S.
SC30 : 20% E	1	0.1528	3.056	N.S.
SC30 : SC10	1	0.1716	3.432	N.S.
SC30 : 10% E	1	0.1524	3.056	N.S.

* Critical rate $F_{.95}(1,21) = 4.32$

The kidney at this time is composed of a few immature metanephric units forming at the corticomedullary junction. Both the tubular and glomerular components of the nephrons are few and surrounded by loose undifferentiated mesenchyme. The vessels of the blood circulatory system are poorly developed. There was no morphological difference between the kidneys of fetuses of saline or ethanol treated mothers.

Fetal bone was examined in an attempt to study hematopoiesis. At day 20 of gestation in the rat, the bone marrow cavity is undergoing embryonic bone formation leading to spicule formation. Osteoclasts are abundant suggesting further erosion and sculpturing. The marrow cavity was a very vascular tissue containing mainly mature, non-nucleated erythrocytes and no megakaryocytes. Hematopoiesis obviously occurs mainly in the liver at this point in gestation. There was no morphological difference in the bone marrow cavities between the offspring of saline and ethanol treated mothers.

The fetal adrenal gland at term is predominantly cortex with a distinct zona glomerulosa and well-defined cords of zone fasciculata. Zonation between the zona reticularis and the medulla however is indistinct and this region is collectively referred to as the "zona reticulo-medullaris" (Thliveris and Connell, 1973). The medulla consists of a few scattered medullary cells, sympathetic ganglia and anastomosing blood sinusoids.

There were no marked morphological differences in the adrenal glands of fetuses exposed prenatally to ethanol when compared to the controls.

The mature rat placenta, after about day 17, is composed of a trophoblastic labyrinth with fetal and maternal circulation, and a basal zone which contains only maternal blood. The basal zone has three cellular elements, namely, the chorionic giant cells, small basophils, and nests of glycogen cells. Overlying the basal zone is a layer of maternal decidua basalis. The fetal blood circulates in the trophoblastic septa of the labyrinth and is separated from the maternal sinusoids by cytotrophoblastic epithelium (Davies and Glasser, 1968).

Towards the end of gestation normal degenerative changes were observed in control tissues such as cytolysis, liquefaction and hyalinization of the giant cells, necrosis of the glycogen nest cells, capsularization of the small basophils with an acidophilic, PAS-positive material and fibrosis in the decidua basalis. These reflect a normal regression of placental activity.

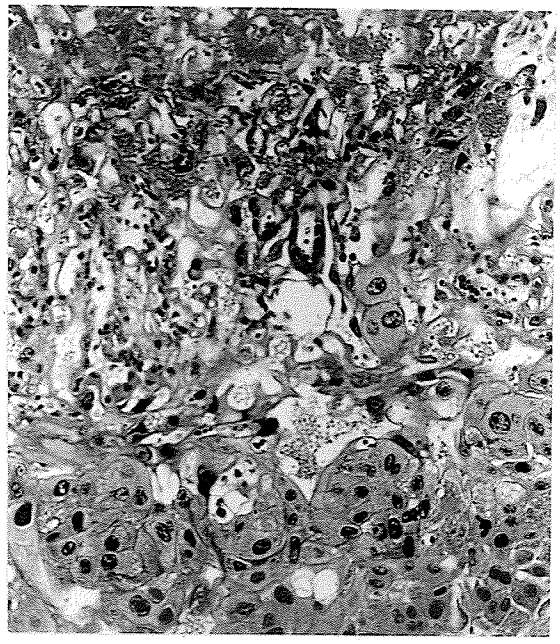
In the placentas of ethanol treated animals, these degenerative changes were much more extensive and pronounced than in control placentas (Figure 8). This was evident in tissues stained both with H&E and PAS. Severe vascular congestion was observed in the maternal labyrinthine sinusoids at the interface with the basal zone, and of the maternal sinusoids in the basal zone. A large number of nucleated blood elements (macrophages, immature monocytes, and polymorphs) were present in the basal sinusoids. Polymorphs were observed laminating the edges of the sinusoids and emmigrating in large numbers into the surrounding tissues. Cytological changes in the basal zone frequently accompanied these hematological changes. There was a distinct increase in the frequency and severity of giant cell liquefaction/hyalinization, as well as

Figure 8. Placental changes observed following acute maternal intoxication with ethanol.

a. Normal maternal and fetal circulation at the junction between the labyrinth and the basal zone.
x 120

b. Severe vascular congestion in the maternal sinusoids of the basal zone and at the basal-labyrinthine junction following treatment with 10% ethanol.
x 120

c. Degeneration of blood cells in a fetal blood vessel.
Dosage: 30% ethanol.
x 768



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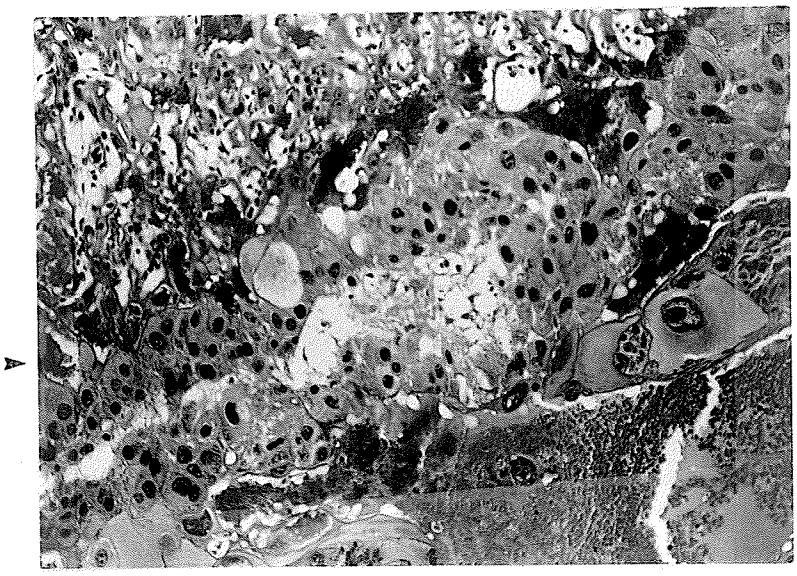

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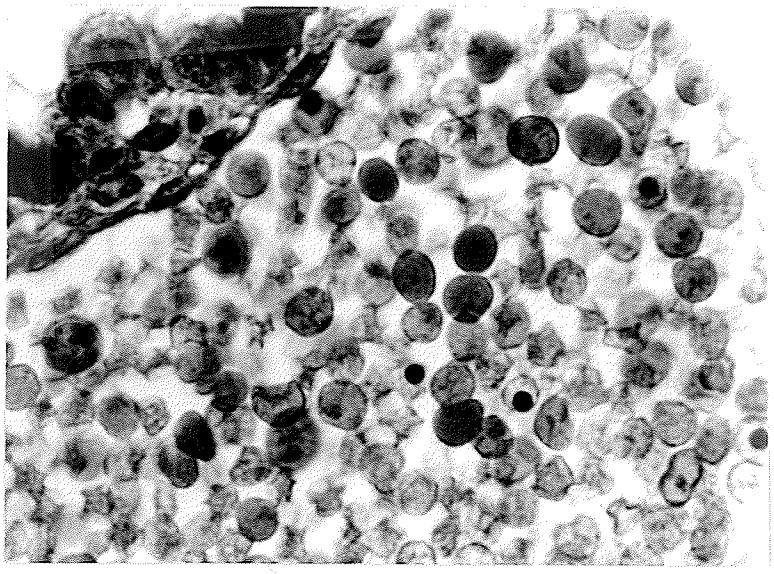

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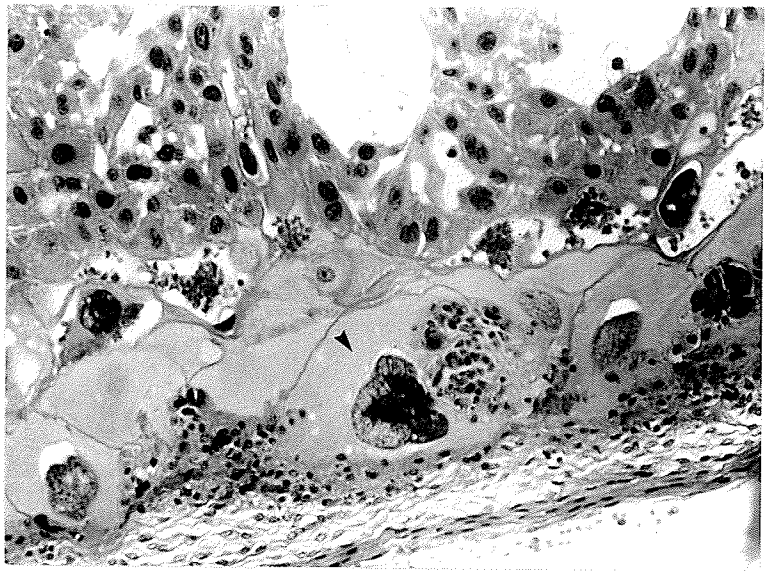
Figure 8 continued

d. Degenerative changes observed following treatment with 30% ethanol. Giant cells contain cellular debris. Macrophages and leukocytes are abundant immediately below the maternal decidua.
x 192

e. A giant cell with cytoplasm displaying extensive vacuolization and containing many blood cells. (Dosage: 30% ethanol).
x 480



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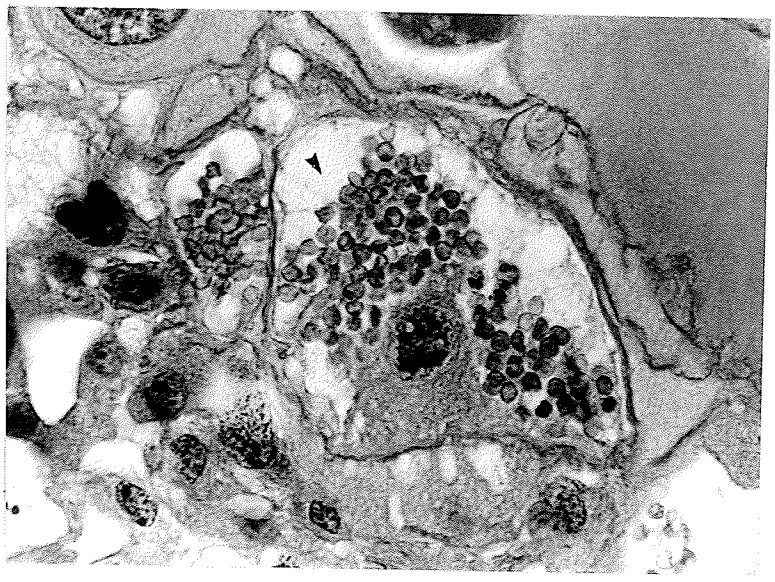


e

the debris contained within these cells. Large areas of amorphous eosinophilic/PAS-positive material were closely related to the cytological and hematological changes.

In the large fetal blood vessels near the fetal surface of the placenta, large numbers of blood cells appeared to be degenerating.

5.2 PENTOBARBITAL

5.2.1 Maternal Toxicity

5.2.1.1 Behavioral effects

Although there was considerable individual variation, a dose-related effect was observed in the different treatment groups with respect to locomotor impairment. There were no changes in locomotor patterns in the saline treated controls. A summary of behavioral changes related to treatment with pentobarbital follows:

25P. Intoxication ranged from deep sedation to coma for up to two hours. At four hours the animals were awake but sedated. Tremors were observed which may have been due to hypothermia.

15P. There was a fast loss of righting reflex and locomotor coordination but no loss of tonus or consciousness. By 1½ hours the animals were active but ataxic and had slow righting reflexes. They were ostensibly normal by three hours after treatment.

5P. These animals displayed locomotor ataxia (crawling) and delayed to absent righting reflex for up to 1½ hours. There was sedation but no hypnosis or atonia for up to three hours.

5.2.1.2. Maternal weight

Maternal weight gain (Figures 9 to 11) was evaluated using an analysis of variance. Since there was a significant difference ($p < 0.05$) between the control groups for both days 9-12 and days 9-20 (Table 16) they were treated as separate treatment groups in subsequent analyses. Tukey's multiple comparison identified SC15 as the source of the variation among the control groups (Table 17). Analysis of variance of maternal weight gain in the six treatment groups (Table 18) revealed a significant variation ($p < 0.01$) following the treatment period (days 9-12). A multiple comparison analysis (Table 19) revealed a significant difference between SC25 and 25P ($p < 0.05$) but not between 15P or 5P and their respective controls (Table 19, part a.). Since SC15 was previously identified as being significantly different from the other controls, 15P was compared to these and found to differ significantly from SC25 ($p < 0.05$) but not from SC5 (Table 19, part b.). There was no significant variation in weight gain among the experimental groups (Table 19, part c.) although the variation between 25P and 15P was much less than between 25P and 5P, suggesting a dose-related, albeit statistically insignificant, effect of the treatments.

Although there was a significant variation ($p < 0.05$) in maternal weight gain from the first day of treatment to the end of gestation (Table 18), the multiple comparison analysis revealed that this difference existed only between 15P and SC5 (Table 20) which is not an appropriate comparison. Therefore, it appears that the treatment effects on maternal weight which were observed at day 12 were eliminated by day 20.

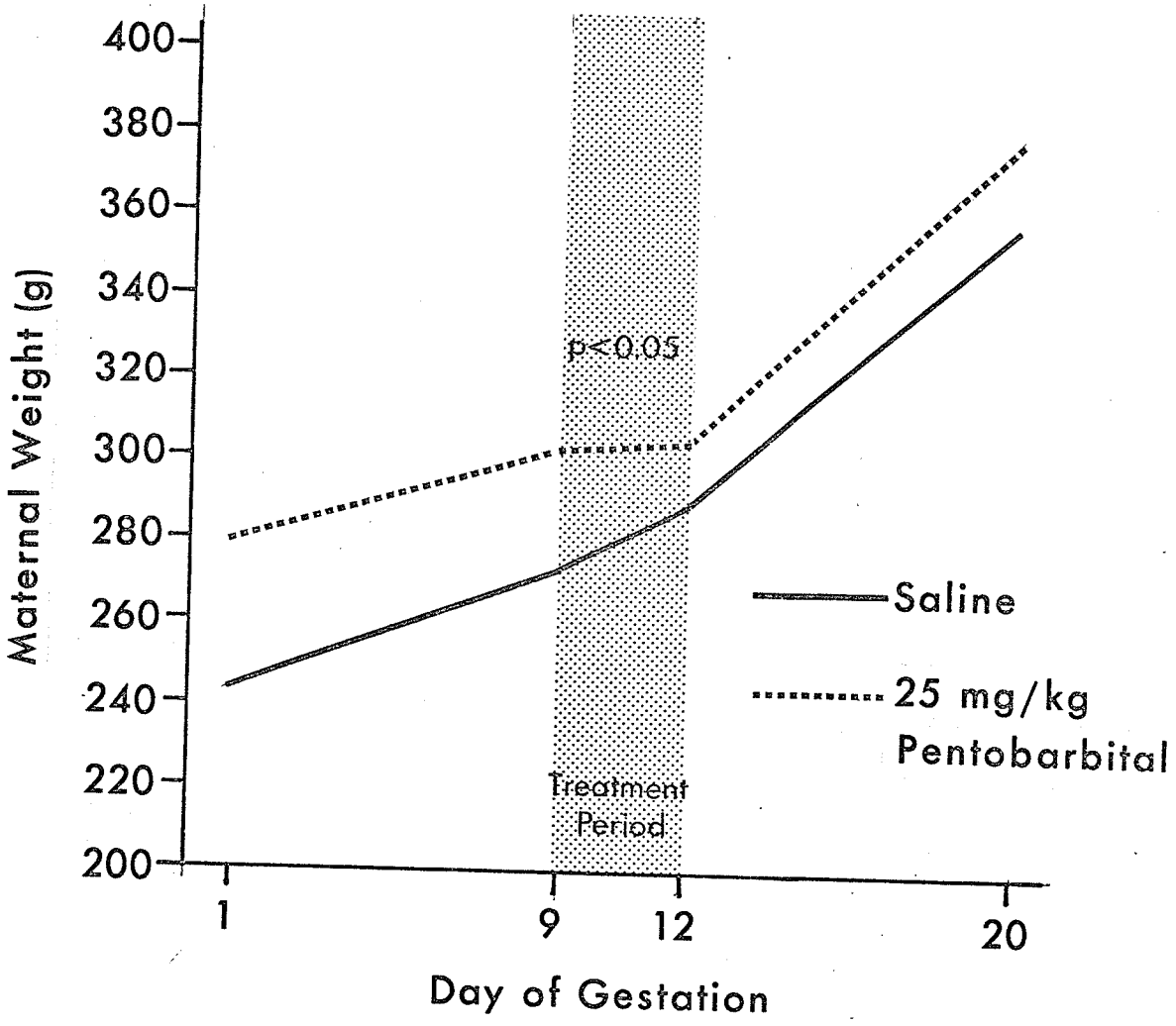


Fig.9 Maternal weight gain related to treatment with 25 mg/kg pentobarbital on days 9 through 12 of gestation

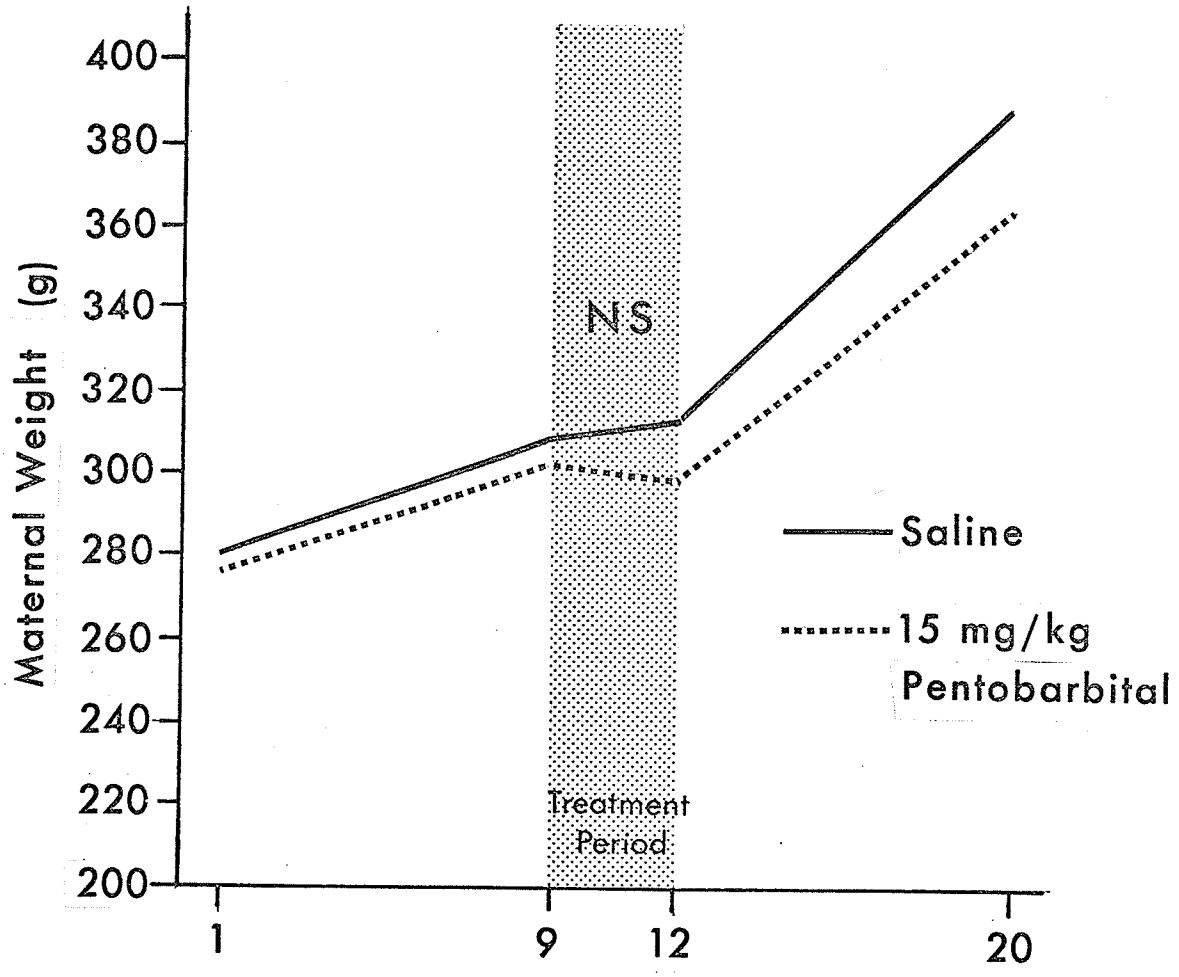


Fig.10 Maternal weight gain related to treatment with 15 mg/kg pentobarbital on days 9 through 12 of gestation.

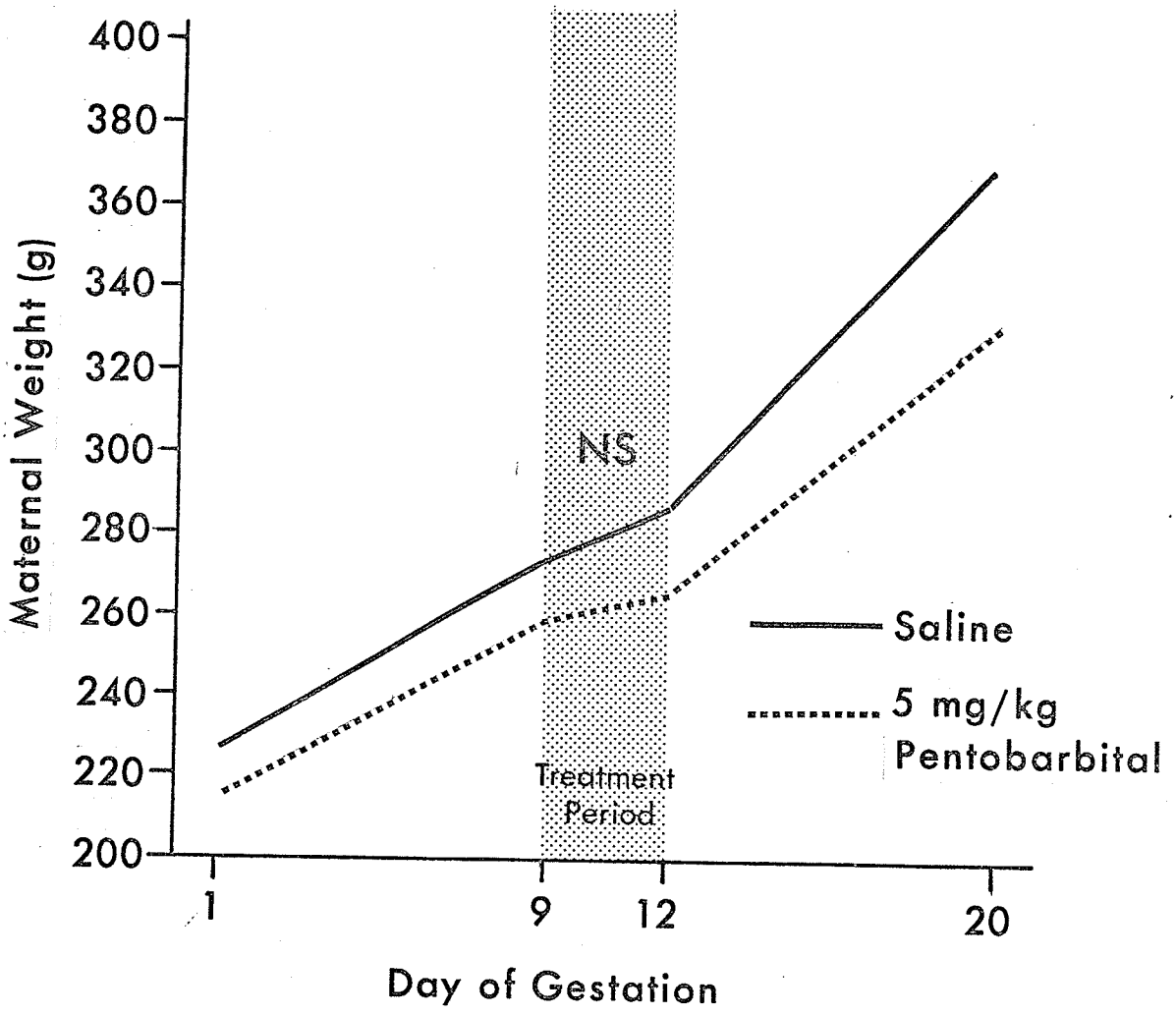


Fig.11 Maternal weight gain related to treatment with 5 mg/kg pentobarbital on days 9 through 12 of gestation

Table 16. Pentobarbital investigation. Variation in maternal weight gain among saline treated control animals.

Treatment group	Number of animals	Mean maternal weight gain (g)	
		Day 9-12	Day 9-20
SC25	3	+15.3	+91.0
SC15	3	+1.7	+80.3
SC5	3	+11.7	+96.0
Observed "F" ratio		6.26	9.55
Significance	$F_{.05}(2,6) = 5.14$ $F_{.01}(2,6) = 10.9$	$p<0.05$	$p<<0.05$

Table 17. Pentobarbital investigation. Multiple comparison of maternal weight gain among saline treated controls.

Group Comparison	Observed Value	Significance*
Day 9-12		
SC25 : SC15	4.12	NS
SC25 : SC5	1.93	NS
SC15 : SC5	6.05	$p<0.05$
Day 9-20		
SC25 : SC15	4.83	$p<0.05$
SC25 : SC5	1.3	NS
SC15 : SC5	3.54	NS

* Tukey's $_{.05}(3,6) = 4.34$

Table 18. Pentobarbital investigation. Analysis of variance in maternal weight gain among treatment groups.

Treatment group	Number of animals	Mean maternal weight gain (g)	
		Day 9-12	Day 9-20
SC25	3	15.33	88.3
P25	6	0.16	74.5
SC15	3	1.67	80.3
P15	6	0.33	62.0
SC5	3	11.67	96.0
P5	6	6.0	72.0
Observed "F" value		4.71	2.72
Significance		$p<0.01$	$p=0.05$
		$F_{.01}(5,21)=4.04$	$F_{.05}(5,21)=2.719$

Table 19. Comparison of maternal weight gain among saline and pentobarbital treated animals over the treatment period (days nine to 12 of gestation).

Comparison	Observed value(6,21)	Significance*
a. Control vs. experimental		
SC25 - 25P	5.338	$p<0.05$
SC15 - 15P	0.469	N.S.
SC5 - 5P	1.994	N.S.
b. 15P vs. other controls		
SC25 - 15P	5.279	$p<0.05$
SC5 - 15P	3.989	N.S.
c. Between experimentals		
25P - 15P	0.072	N.S.
25P - 5P	2.514	N.S.
15P - 5P	2.443	N.S.

* Tukey's critical value $_{.05}(6,20) = 4.45$

Table 20. Pentobarbital investigation. Multiple comparison of maternal weight gain among treatment groups from the first day of treatment to the end of gestation (day 9-20).

Comparison group	Observed value	Significance*
25P : SC25	1.86	N.S.
15P : SC15	2.47	N.S.
5P : SC5	3.23	N.S.
15P : SC5	4.58	$p<0.05$

*Tukey's $_{.05}(6,20) = 4.45$

5.2.1.3. Microscopic changes

In the animals treated with pentobarbital the liver occasionally appeared swollen and bulbous, and had peritoneal fibrous adhesions. Microscopic examination of these "balloon" livers revealed that the bulbous, swollen character and the adhesions were associated with a noticeable shortening and/or thickening of the liver capsule (Figure 12) as well as hyperemia and cellular changes suggestive of inflammation in both the adjacent capsule and the adjacent liver parenchyma. These changes were likely caused by a peritoneal inflammatory response to the pentobarbital rather than direct damage to the liver parenchyma.

In the experimental livers examined there was an increase in the number of macrophages in both the hepatic sinusoids and blood vessels. Otherwise there was no noticeable difference in the maternal liver parenchyma itself at day 20, which could be related to the treatment.

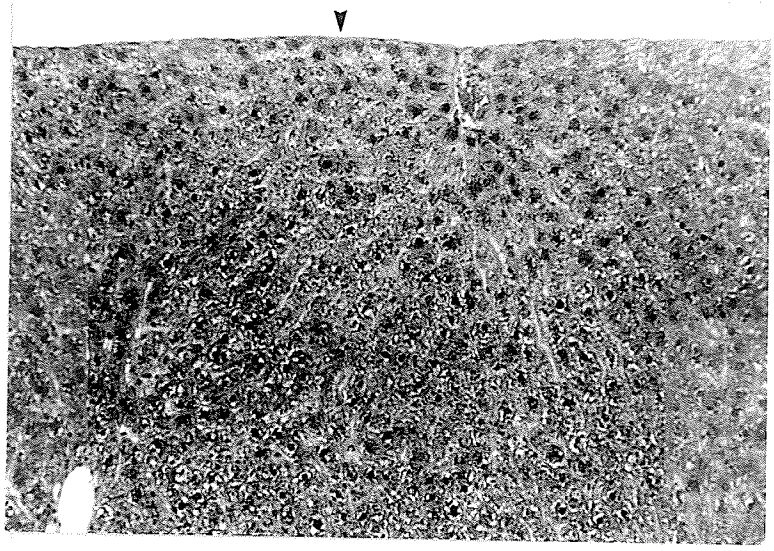
In the kidneys of those animals treated with pentobarbital there was no apparent tubular damage. The glomeruli of animals treated at the highest dose level, however, frequently appeared to be engorged with blood, displayed fibrotic changes, and contained degenerating podocytes (Figure 13). Macrophagic infiltration of the adjacent areas was frequently associated with these glomerular changes. Occasionally complete nephrons were observed to be degenerating. Similar changes were observed in the glomeruli of the animals treated with the two lower doses of pentobarbital. Whereas erythrocytes are normally seen in some control glomeruli both the frequency and severity of the vascular engorgement, as well as the macrophagic infiltration, increased with

Figure 12. Changes in the fibrous capsule of the liver associated with intraperitoneal administration of pentobarbital.

a. Control capsule.
x 120

b. Focal shortening and thickening of the capsule.
x 120

c. Capsular thickening, fibrous adhesion and inflammatory cells within the capsule and in the subcapsular area.
x 120



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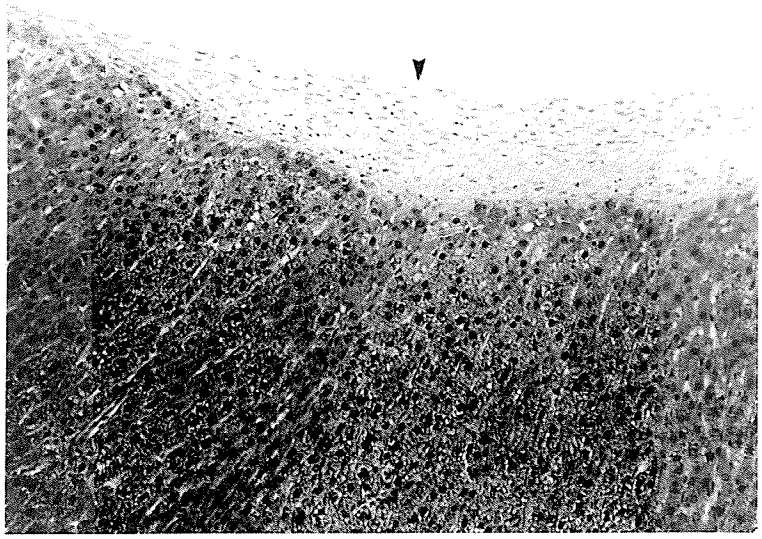

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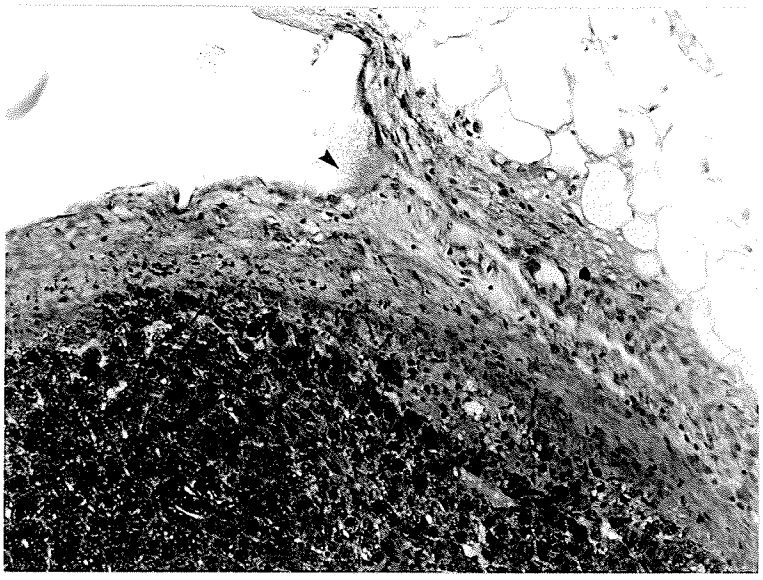

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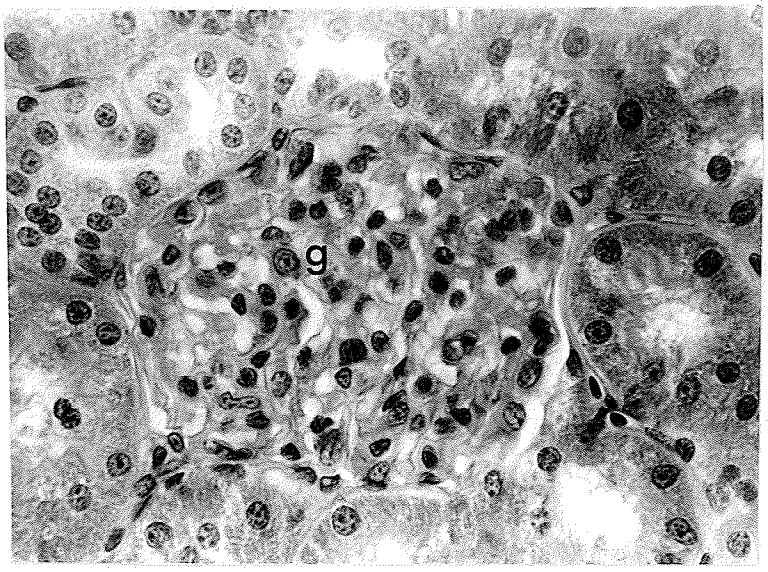
Figure 13. Changes in the maternal kidney following treatment with pentobarbital.

a. Glomerulus in a control kidney.
x 480

b. Glomerulus from an experimental kidney displaying hyperemia and podocyte degeneration.
Dosage: 15 mg/kg pentobarbital.
x 480



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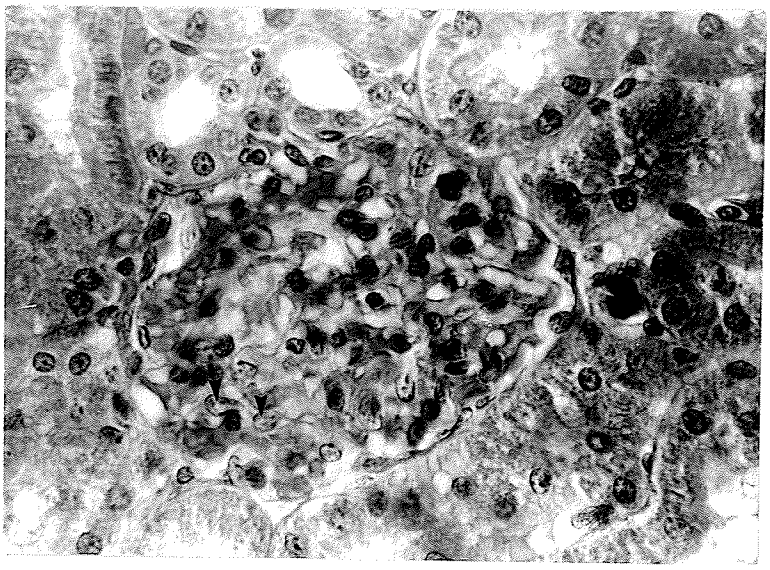

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the increasing doses of pentobarbital.

In the ovaries of experimental animals treated with the highest dose, there was frequently a massive vascular engorgement in the peripheral sinusoids of the corpora lutea, and sometimes also in the central sinusoids and in the vessels of the normal ovarian stroma. Otherwise there appeared to be normal follicular development and the cells of the corpora lutea appeared unaffected. There were no observable differences between the ovaries of control animals and those animals treated with the two lower doses of pentobarbital.

5.2.2. Fetal Toxicity

5.2.2.1 Resorption rates

The number of resorptions, expressed as a percentage of the total implantation sites observed on day 20 are presented in Table 21. No treatment-related effects were present and all were within the normal range.

5.2.2.2 Gross abnormalities

There were no gross external or visceral anomalies observed among the fetuses exposed prenatally to pentobarbital. However there were two control fetuses with multiple anomalies which were considered to have occurred spontaneously.

Approximately 25% of the fetuses of each litter were cleared and stained with Alizarin Red S for skeletal assessment. There were no malformations observed in any of the fetuses. The fetuses exposed prenatally to the highest dose of pentobarbital (25P) demonstrated a slight delay in ossification in the sternebrae and in the preskeleton of the

Table 21. Resorption rates observed following exposure to saline or pentobarbital on days nine through 12 of gestation.

Treatment group	Implantation sites	Number of resorptions	Percent
saline	123	6	4.88%
25 mg/kg pentobarbital	83	5	6.02%
15 mg/kg pentobarbital	81	2	2.5%
5 mg/kg pentobarbital	59	3	5.1%

Table 22. Variation in fetal length related to prenatal exposure to pentobarbital.

	df	MS	F value	Significance
Between treatments	5	0.0819	0.55	NS
Between rats	21	0.1478	5.68	$p < 0.001$
Within litters	204	0.026		

Table 23. Variation in fetal weight related to prenatal exposure to pentobarbital.

	df	MS	F value	Significance
Between treatments	5	0.935	1.375	NS
Between rats	21	0.680	8.19	$p < 0.001$
Within litters	204	0.083		

Table 24. Variation in placental weight related to prenatal exposure to pentobarbital.

	df	MS	F value	Significance
Between treatments	5	101.19	2.17	NS
Between rats	21	46.63	0.057	NS
Within litters	293	813.09		

distal limb segments. There was no difference between the middle dose group (15P) or the lowest dose group (5P) and the corresponding controls in this parameter of growth and development.

5.2.2.3 Growth morphometry

A nested analysis of variance revealed no significant difference in weight, crown-rump length or placental weight between the offspring of the six treatment groups (Tables 22 to 24). There was a significant variation ($p < 0.001$) in length and weight among offspring within treatment groups which is not attributable to the treatment which was administered.

5.2.2.4 Microscopic changes

There were no detectable morphological or developmental differences between the livers, kidneys or adrenal glands of the fetuses exposed prenatally to saline and pentobarbital. Nor was there any difference observed between the placentas of the four treatment groups.

5.3 ETHANOL-PENTOBARBITAL COMBINATIONS

5.3.1 Maternal Toxicity

5.3.1.1 Behavioral effects

In previous experiments pregnant rats were treated with low doses of ethanol or pentobarbital. In the present investigation combinations of these doses were administered. The behavioral effects of these treatments are summarized in Table 25. Since no quantitative assessment was made, it was not possible to determine whether the combinations of ethanol and pentobarbital were additive or synergistic with regard to locomotor impairment.

Table 25. A summary of behavioral effects of low doses of ethanol and pentobarbital administered singly and in combinations.

Pentobarbital (mg/kg)	Ethanol (percent concentration) 0	10	20	30
0	Controls	No locomotor ataxia or hypotonia. Generally more active than controls.	Locomotor ataxia and hypotonia lasting up to 4h. after treatment	Stupor, ataxia, hypotonia for up to 4h. Righting reflex lost occasionally for short period.
5	Ataxic, delayed righting reflex lasting up to 1½h. Sedation but no hypnosis up to 3h.	Ataxia, sedated to stupor but not comatose, up to 2h. Sleeping on side or back, no huddling behavior.	Delayed righting reflex, ataxic, or sleeping lightly up to 2h.	
15	Ataxia, loss of righting reflex. No hypotonia or coma. Active but ataxic at 3h.	Light coma but could be awakened. Awake, active, but ataxic at 3 to 4h.		
25	Deep sedation to coma lasting up to 2h. Tremors (hypothermia?). Sedated but awake at 4h.	Comatose and ataxic at 2 to 3h; some could be awakened but no righting reflex. Attempted activity at 5 to 6h was ataxic and agitated. Hyper-reflexia.		

5.3.1.2 Maternal weight

Changes in maternal weight observed in the different treatment groups were evaluated using an analysis of variance. Because there was no significant difference in weight gain among any of the control groups (Table 26) the controls were pooled for subsequent analyses.

Analysis of variance revealed no significant difference in maternal weight gain between any of the treatment groups (Table 27) throughout gestation.

5.3.1.3 Gross observations

Abdominal lesions in the epigastric region were observed in the drug-treated animals. These appeared to be self-inflicted. In each of treatment groups E10 P5 and E10 P15, there was one such animal. In E20 P5, all but one animal had abdominal lesions - one of these was found dead on day 17, having chewed into the abdominal cavity, and another had chewed as far as the muscle wall. One trial rat was then treated at dose levels E20 P15 and E20 P25. Each developed severe abdominal lesions. Visceral examination at autopsy revealed that balloon liver and peritoneal hepatic adhesions were frequently associated with these abdominal lesions. In the lowest dose levels, however, the visceral changes were observed in the absence of the external lesions.

5.3.1.4. Microscopic observations

Most of the changes detected in the livers of experimental animals were related to the blood vessels (Figure 14). Large accumulations of extravascular fluid were observed adjacent to large vessels and surrounding portal triads. Discontinuities of the venous endothelium with fluid leaking into the adjacent parenchyma were observed. There was

Table 26. Ethanol-pentobarbital investigation. Analysis of variation in maternal weight gain among saline treated control animals.

Treatment group	Number of animals	Mean maternal weight gain(g) Day 9-12	Day 9-20
SC E10 P5	3	14.3	83.7
SC E10 P15	2	10.5	81.5
SC E10 P25	3	10.7	77.0
SC20 P5	3	8.7	92.0
"F" ratio observed		0.389	0.929
Significance/$F_{.05}(3,7) = 4.35$		N.S.	N.S.

Table 27. Ethanol-pentobarbital investigation. Analysis of variation in maternal weight gain among the treatment groups.

Treatment group	Number of animals	Mean maternal weight gain (g)	
		Day 9-12	Day 9-20
Pooled controls	11	11.1	83.7
E10 P5	6	11.0	81.5
E10 P15	5	5.6	62.8
E10 P25	5	3.4	79.6
E20 P5	4	5.8	84.8
Observed "F" value (df 4,26)		2.466	1.699
Significance/$F_{.05}(4,26) = 2.74$		N.S.	N.S.

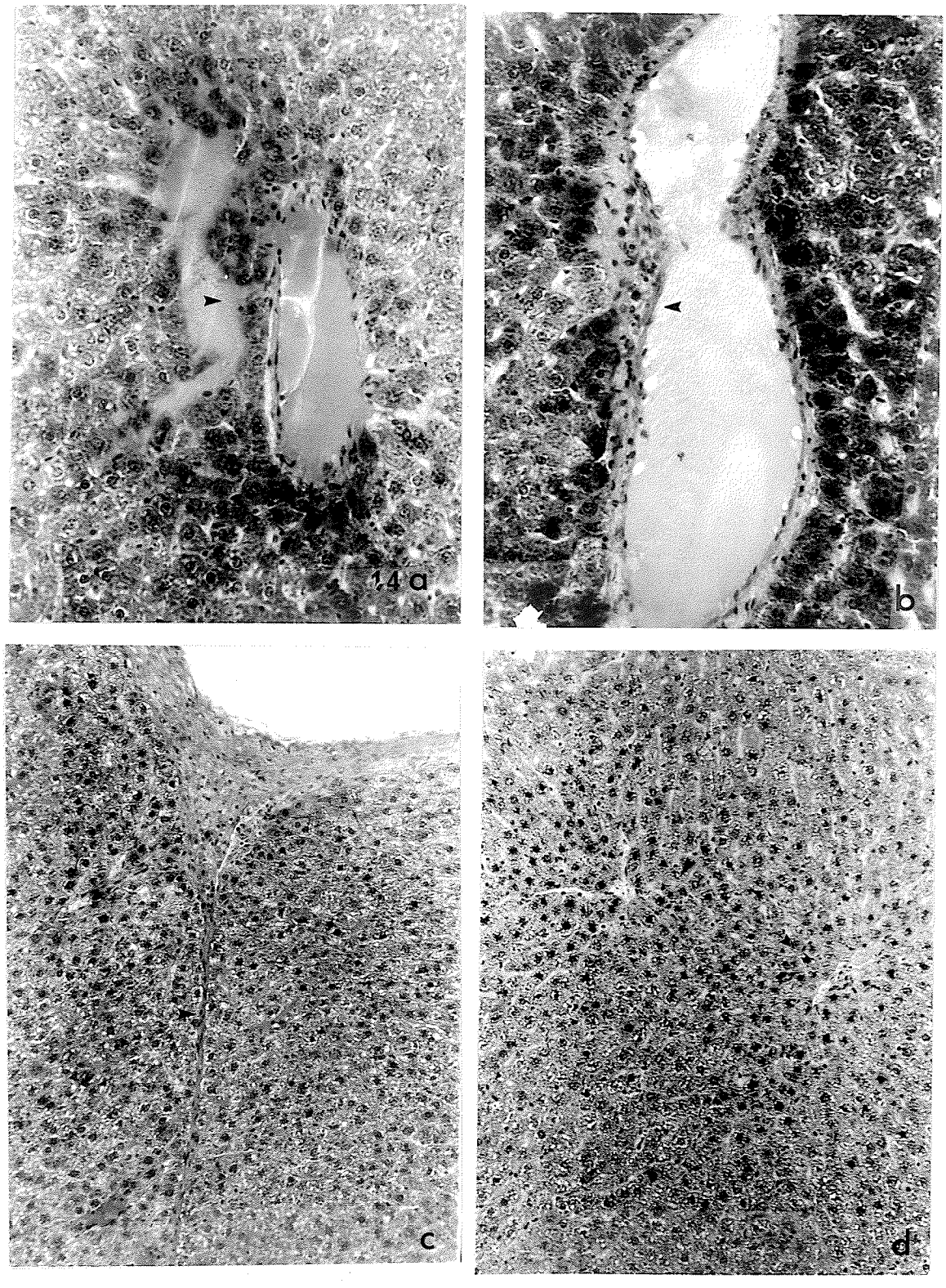
Figure 14. Changes observed in the maternal liver following treatment with ethanol and pentobarbital.

a. Extravasation through a break in a venous wall.
Dosage: E10P5.
x 192

b. Adventitial thickening in a vein.
Dosage: E10P5.
x 192

c. Fibrous adhesions between two liver lobes and capsular thickening.
Dosage: E10P15.
x 154

d. Increase of nuclear pyknosis around central veins.
Dosage: E20P5.
x 120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a distinct thickening of the adventitia of many large veins in such areas. Foci of degenerating hepatocytes could be found close to the vascular lesions but no parenchymal damage was observed elsewhere in the liver. There appeared to be no increase in the extent or severity of tissue damage related to the increasing doses of pentobarbital. All of these changes were observed in various combinations in the livers of rats treated with 10% ETOH and 5 mg/kg (E10 P5), 15 mg/kg (E10 P15), or 25 mg/kg (E10 P25) of pentobarbital.

In the livers of rats treated with 20% ETOH and 5 mg/kg pentobarbital E20 P5) there was a distinct increase in nuclear pyknosis in the hepatocytes surrounding the central veins.

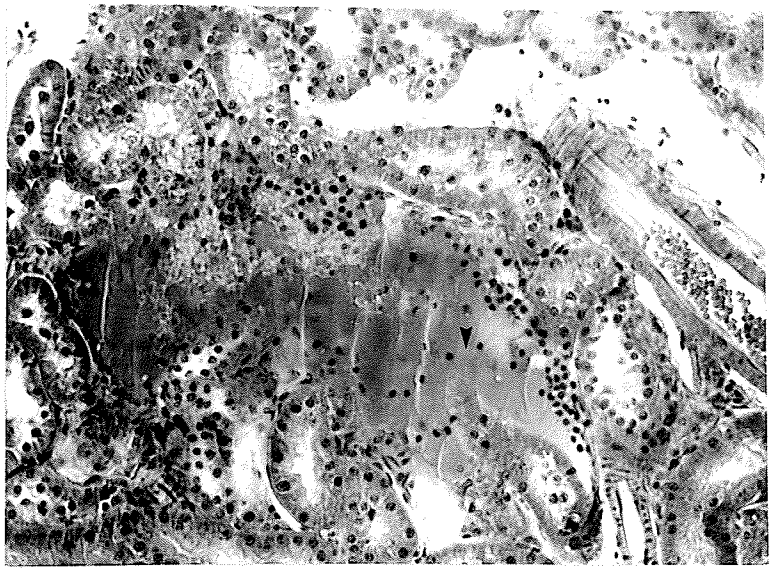
Microscopic examination of "balloon" livers revealed a significant increase in the thickness of the liver capsule and fibrous adhesions joining adjacent liver lobes.

There were noticeable changes in the kidneys of animals treated with ethanol and pentobarbital (Figure 15). In the cortex, glomeruli were frequently congested with blood and contained eosinophilic material. The intralobular vessels also appeared to be congested and more prominent than in control kidneys. In both control and experimental animals the renal tubules contained cellular debris. In the animals treated with the ethanol and pentobarbital, however, the epithelium of the convoluted tubules was degenerating, the lumina were filled with eosinophilic materials, and some nephrons were completely devoid of epithelium. Tubular epithelial cells were observed which were swollen and strongly eosinophilic. There were frequently interstitial areas between tubules which were filled with fluid and macrophages.

Figure 15. Changes in maternal kidney following treatment with ethanol and pentobarbital.

a. Extravasation with inflammatory cells, tissue necrosis and degeneration of tubular epithelium. Dosage: E10P25, x 192

b. Renal tubular degeneration. Pyknotic and extruded nuclei, swollen and leached cytosol, with many lumina filled with a homogeneous, eosinophilic material. Dosage: E10P15 x 192



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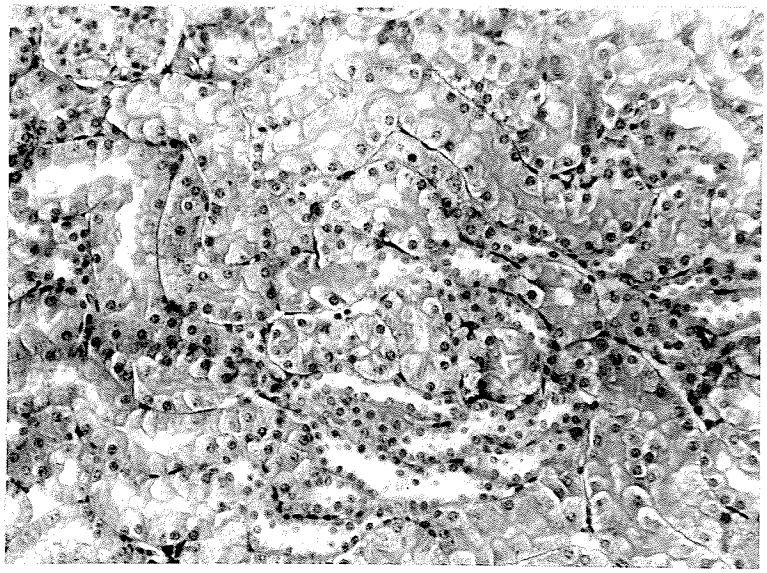

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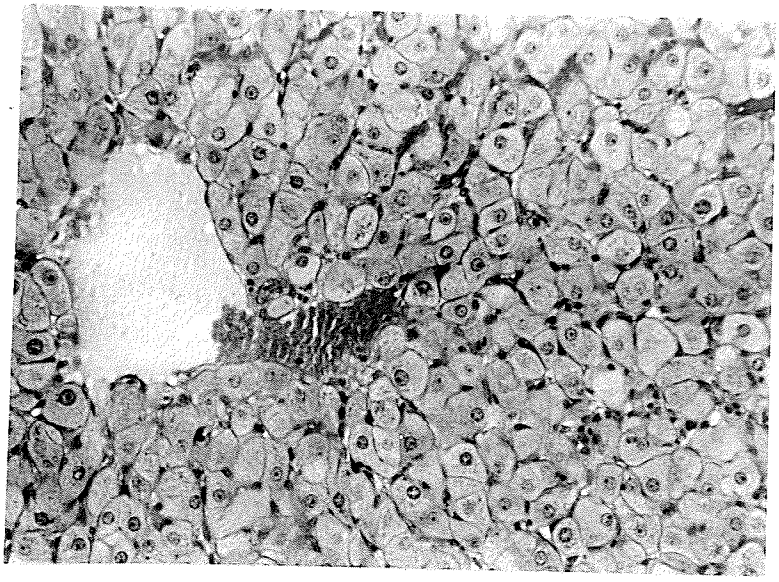
Figure 16. Changes in the corpora lutea associated with the administration of ethanol and pentobarbital.

a. Central sinusoid surrounded by luteal cells in a control animal.
x 192

b. Morphological luteolysis and fibrosis near a central sinusoid and patches of eosinophilia among luteal cells.
Dosage: E1OP5
x 192



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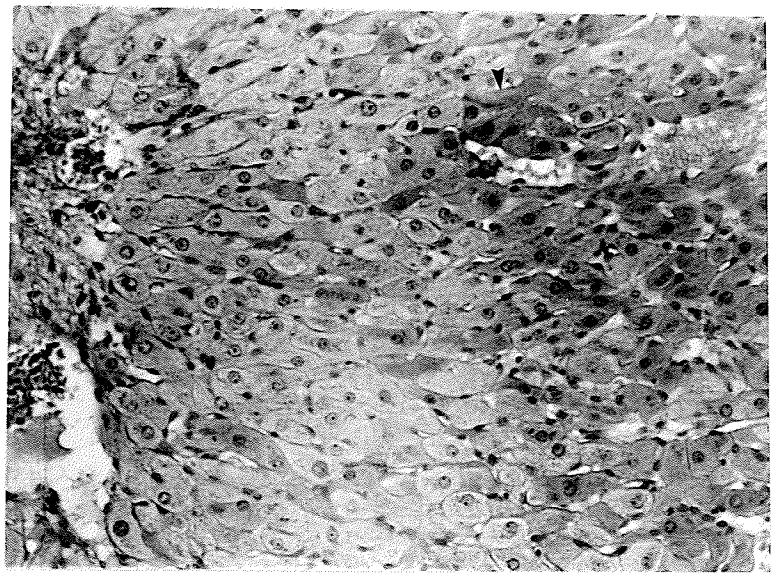

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In the ovaries of saline-treated controls there was evidence of normal term degeneration in the corpora lutea, for example necrotic changes in the central luteal cells and vascular congestion. Throughout the corpora lutea, the luteal cells were consistently similar, macrophages were evenly distributed throughout the parenchyma and the ovarian stroma was normal. In the ovaries of animals treated with ethanol and pentobarbital, however, there was an abnormal number of macrophages in some blood vessels. In some areas of the parenchyma, there were extensive areas of luteal cells with more homogenous and eosinophilic cytoplasm and nuclear changes (Figure 16), and evidence of fibrotic invasion of the central areas of the corpora lutea. These changes were observed only in experimental tissues.

5.3.2 Fetal Toxicity

5.3.2.1 Resorption rates

Numbers of resorptions expressed as a percentage of the total implantation sites observed on day 20, are presented in Table 28. The three high rates observed in control and experimental groups were due exclusively to a very high resorption rate in one rat within each group. Otherwise all the resorption rates fall within the resorption rates which occur spontaneously.

5.3.2.2 Gross abnormalities

Examination of fetuses under a dissecting microscope revealed no gross external or visceral anomalies in any of the control or experimental fetuses. Nor were there any skeletal defects or delays in ossification observed in experimental fetuses compared to their respective controls.

Table 28. Resorption rates observed in animals treated with saline or ethanol-pentobarbital combinations.

Treatment group	Number of animals	Number of implantation sites	Number of resorptions	Resorption rate
pooled controls	11	141	7	4.96%
E10 P5	6	80	8	10%*
E10 P15	5	57	9	15.79%*
E10 P25	5	60	2	3.3%
E20 P5	4	52	2	3.8%

* high % resorption due entirely to a high rate in one litter only.

5.3.2.3 Growth morphometry

A nested analysis of variance revealed no significant difference in weight, crown-rump length or placental weight between the offspring of the treatment groups (Tables 29 to 31). There was a significant variation ($p < 0.001$) in length and weight among the offspring within treatment groups which was not attributable to the treatment.

5.3.2.4 Microscopic changes

There were no detectable morphological or developmental differences in the livers, kidneys or adrenal glands of fetuses exposed prenatally to saline or combination of ethanol and pentobarbital.

The placentas of animals treated with the drug combinations, however, showed marked changes (Figure 17). The basal zone degeneration, which is characteristic of the normal term placenta, was much more extensive and severe. The degenerative changes were frequently localized in a definite, narrow and almost continuous layer of giant cells immediately below the maternal decidua in contrast to the more random, discontinuous patches of giant cell degeneration observed in controls. The maternal blood vessels of the basal zone were markedly more distended and congested, as were those projecting into the labyrinthine region. Congestion of maternal labyrinthine vessels at the interface with the basal zone was also observed. Large fluid-filled spaces were noted occupying the entire basal zone.

Consistent and clearly-defined changes were detected in the maternal vessels just under the fetal surface of the placenta. These were congested and distended, contained many nucleated cells and were enveloped with a thick irregular fibrous material in contrast to the

Table 29. Variation in fetal length related to prenatal exposure to ethanol-pentobarbital combinations.

	df	MS	F value	Significance
Between treatments	7	0.1129	1.73	NS
Between rats	23	0.065	3.65	$p < 0.001$
Within litters	221	0.0179		

Table 30. Variation in fetal weight related to prenatal exposure to ethanol-pentobarbital combinations.

	df	MS	F value	Significance
Between treatments	7	9.663	0.738	NS
Between rats	23	13.093	2.75	$p < 0.001$
Within litters	220	4.758		

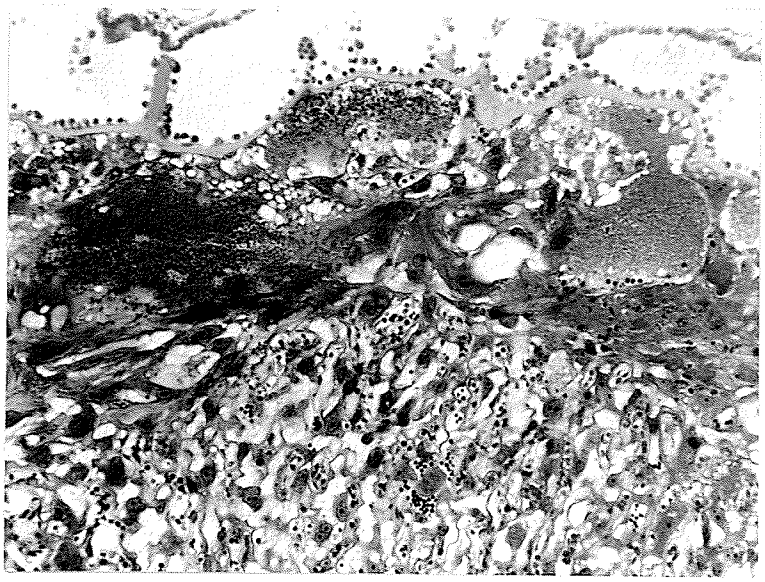
Table 31. Variation in placental weight related to prenatal exposure to ethanol-pentobarbital concentrations.

	df	MS	F value	Significance
Between treatments	1	26.39	0.37	NS
Between rats	23	71.37	0.08	NS
Within litters	327	885.2		

Figure 17. Changes in the placenta associated with the administration of ethanol and pentobarbital.

a. Severe congestion in maternal sinusoid at fetal surface of the placenta. There is a thick, eosinophilis fibrous material around the vessel and large accumulation of macrophages in the adjacent labrynth.
Dosage: E10P15
x 120

b. Thick fibrotic material surrounding a maternal sinusoid which is normally lined by small basophils.
Dosage: E20P5
x 192



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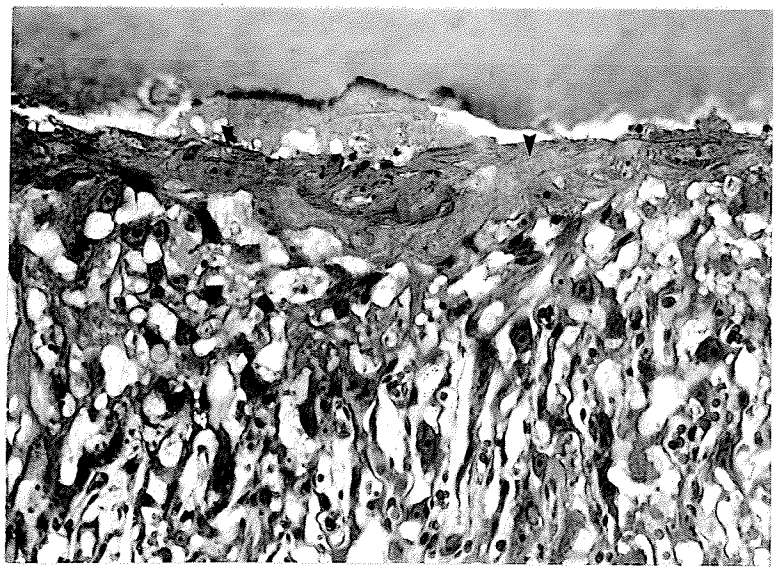

b

thin syncytium of small basophils which normally lines these channels. Increased numbers of macrophages were frequently seen in the parenchyma adjacent to these vascular changes.

DISCUSSION

6. DISCUSSION

6.1 ETHANOL

Intraperitoneal injection of ethanol produced changes in maternal behavior which were rapid in onset and which varied in intensity and duration with the dose of ethanol administered. Using locomotor impairment as an index of intoxication, it was deemed that the three doses of ethanol produced states of intoxication which satisfactorily represented three different degrees of short-term alcohol abuse or binge drinking. Because the ethanol was administered at the beginning of the light schedule and the daily period of severe intoxication never exceeded four hours in duration, the peak activity and feeding periods of the animals were not interrupted.

Patterns of maternal weight gain were studied as an indirect indication of food intake and general well-being of the animals. Multiple comparison analyses revealed a significant reduction in weight gain over the treatment period in animals treated with 30% and 20% ethanol, compared to control animals. This difference in weight gain was temporary, however, since at the end of gestation there was no significant variation in maternal weight relative to treatment. The number of live fetuses in each litter did not affect these observations. Changes in maternal weight gain, therefore, paralleled the dose-related effect of ethanol on behavioral impairment. In contrast, there was no treatment-related variation in fetal weight or crown-rump length in this investigation.

The nutritional status of the mother can influence the growth and development of her offspring (Naeye, 1965). Many studies in humans

(Ouellette et al., 1977) and in laboratory animals (Tze and Lee, 1975) have shown, however, that the effects of chronic maternal alcohol consumption on progeny occur independently of diet and are the direct result of the ingestion of alcohol. In these and other investigations (Jones et al., 1976; Chernoff, 1977) low fetal birthweights have been reported without any mention of changes in maternal weight gain.

Kronick (1976) studied the effects of single doses of ethanol during pregnancy in mice and found that maternal weight loss following treatment was temporary and that there was no significant reduction in the fetal weights at term associated with prenatal exposure to acute ethanol intoxication. This is consistent with the observations of the present investigation in the rat. Neither investigation determined, however, if there was an immediate effect of acute exposure to ethanol on fetal weight gain _in utero_. If, in fact, the normal term weights of these offspring do reflect catch-up growth, this would be inconsistent with the irreversible pre- and postnatal growth retardation observed in infants following chronic gestational exposure to ethanol (Jones et al., 1976).

Increased incidence of prenatal mortality and maldevelopment have been reported in mice following chronic (Chernoff, 1977) and acute (Kronick, 1976) exposure to ethanol during gestation. Chernoff (1977) reported a dose-response effect and strain differences in susceptibility to chronic alcohol intake. Anomalies at the lower dose levels included deficient occipital ossification and neural anomalies, and at the higher levels cardiac and eyelid dysmorphology were observed. Following acute prenatal exposure to ethanol, Kronick (1976) found that coloboma of the

iris and forepaw ectrodactyly were the most frequent defects. In the present experiment both external and visceral examination failed to reveal any congenital defect related to ethanol exposure. This could be explained by species variation in susceptibility, route of administration, differences in the duration of exposure and/or the level of alcohol intoxication. For example, Maling (1970) reported that the LD_{50} for ethanol in the mouse is 9.5 g/kg (p.o.) or 2.0 g/kg (i.v.), whereas in the rat it is 13.7 g/kg (p.o.), 5.0 g/kg (i.p.) or 4.2 g/kg (i.v.).

Further difficulty is encountered in interpreting these studies since different dose units and different criteria for defining intoxication were used to present the results. Chernoff (1977) administered ethanol in Metrecal liquid diet before and during gestation and reported non-pregnant blood alcohol levels of 73 to 398 mg/100 ml as the definition of the level of intoxication. Blood alcohol levels as low as 690 mg/100 ml are lethal in mice, whereas in rats 890 mg/100 ml, and in man 260 mg/100 ml represent the lower lethal limits (Maling, 1970). It would appear that the increased mortality and morbidity in this study were associated with chronic exposure to relatively low doses of ethanol. On the other hand, Kronick (1976) treated mice on one or two days with 0.030 ml ethanol per gram of body weight, intraperitoneally in a 25% v/v saline solution. This is roughly equivalent to 7.0 ml/kg of 95% alcohol, or, 6.0 g/kg, which was a massive dose, approximating the LD_{50} in mice, and could certainly explain the observed fetal mortality and morbidity. No criteria of intoxication were given except that none of the animals died as a result of the treatment.

In the present study low doses (0.56 to 1.1 g/kg) in different dilutions were administered intraperitoneally on four consecutive days to pregnant rats and behavioral impairment was used to define the level of

intoxication. The absence of any significant deleterious effects on fetal growth and development or of any increased prenatal mortality could be explained by a very low level and short duration of exposure to ethanol relative to the two other studies, as well as a lower species susceptibility to ethanol as reflected in the much higher LD_{50} in the rat compared to the mouse. It is to be noted however that delayed or deficient occipital ossification was reported in Chernoff's study, thereby providing some measure of relatedness or overlap with the present investigation.

The effects of short-term alcohol intoxication during pregnancy were further studied microscopically. The tissue changes observed in the present study were subtle compared to those observed in the Preliminary Investigation. This difference can be accounted for by the lower doses of ethanol administered in this experiment, the larger fluid compartment available for drug distribution in the pregnant animal compared to the non-pregnant animal, and the fact that tissues for examination were recovered eight days rather than one day after the last day of treatment, thereby allowing time for recovery.

There was no evidence of fatty change in the maternal liver. An increase of nucleated cells in the liver sinusoids and the pavementing of the hepatic endothelium with leukocytes suggested the persistance of an inflammatory process. The increased frequency of mitotic figures in the liver parenchyma suggested that repair processes were underway and provided indirect evidence of a prior ethanol-related damage to the liver. Numerous hepatocytes displaying nuclear pyknosis were observed in the vicinity of the central veins which gave additional support to this hypothesis.

Extensive intravascular hemolysis was not observed in this study, however, there was evidence of an earlier state of systemic hemosiderosis, in the form of aggregates of inclusion-laden cells (Robbins, 1974). These cells were often so engorged that cytological characteristics were obliterated. They were most frequently observed in the livers exposed to the highest dose of ethanol and were absent in all control tissues. These cells were likely free or fixed reticulo-endothelial cells which became engorged with hemosiderin pigment and cellular debris during the period of treatment with ethanol. Engorged Kupfer cells are known to detach and become free in experimental or toxic situations (Stohr et al., 1969). Alcohol not only augments iron absorption, but also carries the threat of liver injury which eventually appears to enhance iron absorption (Robbins, 1974). Extensive hemolysis, with the release of iron from the heme pigment, would further contribute to the systemic hemosiderosis. In the case of acute ethanol intoxication the hemolysis may result as a direct cytoxic effect of ethanol on erythrocytes, or indirectly, as a result of severe central venous stasis and congestion. It is unlikely however, that aside from a possible hemolytic anemia, the observed changes in the maternal liver would have resulted in any long-term functional impairment. This assumption was supported by the absence of any effect on maternal or fetal growth parameters at the end of gestation.

In contrast to maternal liver, there was no morphological evidence of a toxic effect on the fetal liver. The fetal liver was either capable of a much faster recovery, or was, unlike the maternal liver, not susceptible to the toxic effects of ethanol. The latter is more likely the case since the fetal rat liver at day 20 of gestation is essentially a

hematopoietic organ, whereas the maternal liver is the major drug-metabolizing organ for the adult. Since it has been well documented that the exposure of the fetal liver to ethanol at least equals the exposure of the maternal liver (Ho et al., 1972), it is likely that the metabolic immaturity may have protected the fetal liver from the damage observed in the maternal liver in these experiments.

Similar observations were made in the kidney. Although the pattern of damage was the same as in the Preliminary Investigation, there was a dramatic reduction in the severity and extent of changes in the maternal kidney observed in the present experiments. Vascular changes were suggestive of an inflammatory process and severe venous congestion. Morphological changes in the parenchyma involved only the tubular portion of the nephron which would have been the most exposed to the unchanged ethanol that was being excreted. Since the tubules are generally the most active and thus the most sensitive portion of the nephron, and can take part in extrahepatic ethanol metabolism (Lieber, 1976), the observed changes may have been the result of a direct cytotoxic effect of ethanol on the renal tubular epithelium. However, it is also possible that the altered renal hemodynamics associated with ethanol infusion (Tost et al., 1971) results in an hypoxic state in the kidney and thus indirectly damages the tubular epithelium. As in the liver, the damage in the maternal kidney was neither extensive nor severe, repair processes were underway, and there was no evidence of scarring or fibrosis which would produce long-term functional impairment. The fetal kidney displayed no changes which parallelled those observed in the maternal kidney. Again, the functional and morphological immaturity of the fetal rat kidney, especially

of the tubular component, may have been the factor which protected the fetal kidney from the deleterious effects of exposure to ethanol.

Since the ovaries are involved in the maintenance of pregnancy, and since there is a documented increased incidence of premature deliveries among alcoholic women (Green, 1974), it was decided to examine the ovaries for morphological evidence of ethanol toxicity. Short-term ethanol intoxication had no detectable effects on the ovarian follicles or stroma of the rat. Minimal changes were noted in the corpora lutea of ethanol treated animals which, however, were not present in controls. The marginal blood vessels of the corpora lutea were frequently congested and necrotic changes were observed in the luteal cells surrounding the central sinus of many corpora lutea studied. The extent of the tissue changes observed would suggest little, if any, physiological luteolysis or endocrine impairment following short-term alcohol intoxication. It would be interesting to know, however, if this minimal luteolysis would be more extensive after chronic alcohol abuse, if it would also be indicative of a physiological luteolysis which would result in premature delivery, and if these regressive changes represent a direct effect of ethanol on the luteal cells or a withdrawal of endocrine support secondary to a more central effect of ethanol.

The changes observed in the placenta were difficult to interpret. Throughout gestation the placenta is maturing, then aging and undergoing structural and functional changes which presumably reflect the changing requirements of the developing organism. At term, morphological and metabolic properties suggest a reduction in placental function (Thliveris, 1976). In this experiment ethanol was administered at the time that the

primitive labyrinth was forming (Davies and Glasser, 1968). From this, it would have been expected that the labyrinth would be the most affected placental component. On the contrary, on day 20, the placentas which had been exposed to ethanol during their development, displayed advanced degenerative changes in the basal zone but no effect in the labyrinth. Although the chorionic giant cells of the basal zone do evolve from the basophils of the labyrinth (Davies and Glasser, 1968) there was no detectable reduction in cell number or basal zone thickness which would have been expected if the formative giant cells had been damaged as a result of the treatment. The observations made in day 20 tissues, however, were not the initial response to the treatment but rather the result of a complex and on-going process. Two factors should be considered when reviewing these placental changes, namely, the proximity of the chorionic giant cells to the maternal blood vessels, and the function of these cells.

Since ethanol readily crosses the placenta, the fact there were morphological changes in the basal zone and not in the labyrinth cannot be explained by differential ethanol concentrations in the maternal and fetal circulations. It is conceivable, however, that placental hemodynamics were affected by ethanol intoxication resulting in severe vascular congestion and stasis in the maternal sinusoids of the placenta as in other maternal organs (Tost et al. 1971; Lieber, 1976). Nothing is known about the fetal vascular response to ethanol. Congestion of the maternal vessels in the placenta, however, would have resulted in a prolonged exposure of the formative giant cells to blood ethanol.

Unfortunately, little has been reported about the normal function of these chorionic giant cells. Davies and Glasser (1968) have postulated an endocrine function based on ultrastructural characteristics. Being fetal cells in close proximity to the maternal decidua and blood vessels, it would be logical to attribute some protective function to these cells as well, since they likely form the placental barrier. Evidence in support of this protective function was seen in these experiments, in the form of giant cells filled with erythrocytes and other cellular debris, and giant cells actually in the process of phagocytosis. It appears therefore that the advanced degenerative changes in the chorionic giant cells and the basal zone which were observed in the ethanol-exposed placentas were related to their close proximity to the maternal sinuses. Rather than reflecting a direct cytotoxic effect of ethanol, these changes reflect an on-going, post-treatment clean-up of the cellular debris resulting from extensive intravascular hemolysis in the maternal vessels following ethanol intoxication.

Whereas the proximity of the giant cells to the maternal blood vessels was likely a key factor in the placental changes, another factor, metabolic capacity, may also have been involved.

The placenta has been shown to be capable of many drug biotransformations and to contain alcohol dehydrogenase (Juchau, 1972). There is also much controversy about whether unchanged ethanol or acetaldehyde is the compound exerting the toxic effects observed in alcohol abuse. Since in these studies, only those organs with proven ethanol metabolizing capacity, ie. the adult liver, the adult kidney and the placenta, displayed detectable tissue damage, it is conceivable that this capacity renders a tissue more susceptible to the deleterious effects of alcohol intoxication.

In the fetus, the metabolic, functional and anatomical immaturity of the liver and kidney may well have protected these tissues from parallelling the damage observed in the maternal rat. This would also suggest that it is the acetaldehyde, not the unchanged ethanol which is toxic to these particular tissues. However, it is reasonable to assume that all the factors discussed, namely, changes in the maternal hemodynamics producing severe stasis, a direct cytotoxic effect of ethanol or acetaldehyde, metabolic capacity and functional maturity,and, proximity to maternal blood may all play a role in producing the deleterious effects observed to be associated with acute ethanol intoxication,but to varying degrees in different organ systems.

In summary, it is evident from these experiments, that acute maternal ethanol intoxication during the period of organogenesis, at levels producing realistic behavioral impairment, has no long-term deleterious effect on the morphological parameters of fetal growth and development in utero. However, this study has not eliminated the possibility of post-natal functional and/or growth impairment subsequent to binge alcohol abuse. Furthermore, the results of these experiments have suggested new directions for the investigation of the pathogenesis of the fetal alcohol syndrome known to be associated with chronic maternal alcohol abuse, namely, placental and ovarian dysfunction throughout gestation.

6.2 PENTOBARBITAL

Maternal behavioral impairment was used to assess the degree of pentobarbital intoxication. There was a distinct dose-reponse effect observed ranging from deep coma in the animals receiving 25 mg/kg pentobarbital (25P) and to ataxia in the animals receiving 5 mg/kg (5P). Maternal weight gain over the treatment period was significantly reduced in the 25P group compared to their corresponding controls (SC25). There would also have been a significant weight reduction in the 15P group had their controls (SC15) not varied so greatly from the other control groups. Thus, the dose-response effect of pentobarbital on maternal weight gain paralleled that observed in behavioral impairment. The differences in maternal weight gain, however, were temporary, being completely eliminated by the end of pregnancy. Similarly, fetal weights on day 20 of gestation showed no variation which could be attributed to prenatal exposure to pentobarbital. Whether or not there was a temporary delay in fetal weight gain during the treatment period is not known. Other parameters of fetal development studied, such as mortality, crown-rump length, and placental weight showed no significant differences between the experimental and control groups. Furthermore, there were no external or visceral malformations observed in any of the litters. Although there were no skeletal anomalies observed, those fetuses exposed to the highest level of pentobarbital frequently displayed minor delays in ossification in the sternebrae and in the preskeleton of the distal limb segments.

Thus, with the exception of a temporary reduction in maternal

weight gain and minor ossification delays, short-term intoxication with doses of pentobarbital ranging from five to 25 mg/kg had no significant deleterious effects on the outcome of pregnancy. This is consistent with the results of other investigators using several animal species and much higher doses. For example, barbiturates elicited no significant malformations following either a single dose or daily doses ranging from 100 to 400 mg/kg during days three to 12 of gestation in rats (Persaud, 1965). Single doses of 200 mg/kg and consecutive daily doses from days one to 10 of gestation produced litters in the rabbit which were not significantly different from controls in number of implantations, incidence of resorptions, average size, and the incidence of malformations (Persaud, unpublished). Doses of sodium barbital ranging from 65 to 330 mg/kg on days one to six of gestation, however, produced a high incidence of fetal death and wide spectrum of congenital malformations (no skeletal defects) in mice (Persaud and Henderson, 1969). These observations were considered to be compatible with both the anti-mitotic activity and the inhibition of oxygen consumption attributed to the barbiturates. A single dose of pentobarbital (90 mg/kg administered during the period of palate closure proved to be a significant cleft palate teratogen in mice (Walker and Patterson, 1974). All of these doses far exceeded the range used in the present experiment.

With the exception of ultrastructural studies on the development (Zamboni, 1965; Dallner et al., 1966) and the induction (Fouts, 1973) of the smooth endoplasmic reticulum in the hepatocytes as an indicator of metabolic capacity, there have been no microscopic studies of pentobarbital toxicity reported.

Pentobarbital is metabolized exclusively in the microsomal enzyme oxidizing system of the liver although both the unchanged drug and its metabolites are excreted by the kidney (Harvey, 1975). In the maternal liver there was no evidence of a direct cytotoxic effect. Since induction of the microsomal system, as evidenced by increases in smooth endoplasmic reticulum, occurs rapidly following exposure to barbiturates (Fouts, 1973), there may have been changes resulting from the treatment which were not evident at the light microscopic level. Nevertheless, such changes if they did occur, caused no deleterious effects on pregnancy. Although there was a distinct increase of intravascular and parenchymal macrophages in both the livers and kidneys of animals treated with pentobarbital, there were no lesions associated with this increase, which probably reflected a non-specific stimulation of the reticulo-endothdial system in response to the administration of the test substance. In such cases, fixed macrophages become detached and free to move throughout the body.

In the renal glomeruli, there was evidence of fibrosis and vascular damage associated with pentobarbital intoxication. This observation is in agreement with the fact that not only inactive metabolites but also the unchanged pharmacologically active form of the drug are excreted by the kidneys. The renal tubules showed no changes. It is conceivable that the hyperemia and fibrosis within the glomeruli of the maternal kidney were the result of both a vascular and direct cytotoxic effect of gestational exposure to pentobarbital. No parallel changes were detected in the fetal liver or kidney,which was probably due to the functional and metabolic immaturity of the fetal tissues. All the changes found in

the mother were minor and without significance for the offspring.

The dramatic thickening in the liver capsule of animals treated with pentobarbital was most likely the result of peritoneal inflammation in response either to repeated peritoneal injections, or to peritoneal irritation by the pentobarbital itself. The absence of such changes in the control animals, and the even more pronounced effect in subsequent experiments using combinationss of ethanol and pentobarbital suggest that it was the test-substance which was the irritant, not the injection itself.

6.3 ETHANOL-PENTOBARBITAL COMBINATIONS

The treatment of pregnant rats with combinations of ethanol and pentobarbital at different dose levels results in no significant changes in maternal weight gain either immediately after the treatment period or at the end of gestation. This is in contrast to previous experiments in which animals treated with 30% and 20% ethanol and 25 mg/kg pentobarbital revealed a significant reduction in weight gain. However, the animals treated with 10% ethanol, and 5 mg/kg or 15 mg/kg pentobarbital showed no such reduction. The absence of a similar effect when combinations of the two drugs were administered suggested an inhibitory effect of one drug on the other with regard to this parameter, or, at least, no additive or synergistic effect of the combined treatment. This finding is difficult to interpret because the evidence of peritonitis and the behavioral impairment were distinctly greater when combinations of the two drugs were administered. In the previous experiments, maternal behavioral impairment displayed a clear dose-response effect for both

ethanol and pentobarbital.

In the present investigation, peritoneal irritation became increasingly severe with increasing doses of each drug. In the combinations using 10% ethanol, increasing levels of pentobarbital increased the severity of the irritation, and in the group treated with 20% ethanol., the addition of even 5 mg/kg pentobarbital produced severe irritation as evidenced by the external abdominal lesions. It appears, therefore that the addition of increasing doses of pentobarbital to a non-irritating concentration of ethanol (10%) resulted in a treatment which was much more irritating than either acting alone.

Despite the peritoneal irritation caused by ethanol-pentobarbital combinations, there was no deleterious influence on maternal weight gain, nor was there any treatment-related variation in fetal mortality, fetal length or weight, or placental weight. External and visceral examination revealed no gross abnormalities, and there were no serious skeletal abnormalities.

Miscroscopic examination of the maternal liver revealed damage that was primarily related to the walls of the blood vessels (endothelium and adventitia), with secondary damage to the parenchyma. The endothelial damage in the animals exposed to ethanol only was much less severe, less frequent, and no thickening of the adventitia was detected. Pentobarbital alone produced no such effects on the maternal liver. Acting in combination with ethanol, however, pentobarbital increased the damage done by the ethanol acting alone. Further evidence that it was the ethanol which was cytotoxic was seen in the increased nuclear pyknosis in the hepatocytes surrounding the central vein in the liver of rats

treated with the 20% ethanol concentration and the lowest dose of pentobarbital (20E 5P).

In the kidney, pentobarbital intoxication resulted in changes in the glomerulus, whereas in ethanol intoxication, the tubular epithelium was predominantly affected. From the microscopic examination of maternal kidney in the present study it appears that ethanol and pentobarbital acting in combination produce the same changes that each caused when administered independently. There was no detectable dose-response effect and the changes were not extensive.

In the normal term ovary and placenta regressive changes are seen which presumably reflect a declining function. In the animals treated with ethanol there was a slight increase in the degenerative changes observed in the corpora lutea and in the placenta compared to controls. No such changes were detected following pentobarbital intoxication. After treatment with combinations of the two drugs, these degenerative changes were much more pronounced in both the ovary and the placenta. This suggested that pentobarbital exacerbates the toxicity of ethanol although it has no detectable toxic effect itself.

These observations raise the question of the mechanism(s) by which these two drugs exert their effects when administered simultaneously. The literature pertaining to ethanol-barbiturate interactions is extensive and contradictory. Most studies have used single tissue, _in vitro_, and highly specific bioassays, which are fraught with methodological problems and contribute little to the general understanding of the problem. The present investigation attempted to compare the _in vivo_ effects of ethanol, pentobarbital and combinations of the two drugs, in the intact, pregnant animal. Morpholo-

gical and quantitative techniques were used to examine a broad spectrum of maternal and fetal parameters. From the results of these investigations, it appears that each parameter, and each organ system may respond uniquely to the simultaneous presence of the two drugs. For example, maternal weight gain appeared to be less affected by the combination of the two drugs than by each drug acting independently. Peritoneal irritation, microscopic changes in the liver, and the degenerative changes observed in the ovary and placenta which were minimal in ethanol intoxication,were markedly exacerbated by the presence of pentobarbital. In the kidney, exposure to the combination treatments resulted in the co-existence of changes associated with independent ethanol and pentobarbital intoxication. Treatment with three doses of ethanol, three doses of pentobarbital and four combination doses failed to elicit any serious deleterious effects on the fetus.

Since Coldwell et al. (1970) have shown that the decay profiles of serum barbiturate concentrations are not altered by ethanol, that barbiturates have no effect on blood ethanol levels and that pentobarbital has no effect on blood acetaldehyde in the intact rat, it is unlikely that the differences observed in this experiment were related to metabolic inhibition or stimulation. It is conceivable that the differential responses of individual organs or systems to ethanol, pentobarbital and combinations of these were related to differential effects on the hemodynamics of the maternal-placental-fetal unit to these three treatments. This could have resulted in a prolonged exposure of metabolically competent organs (maternal liver and kidney, or placenta, for example), or

highly vascular or lipid soluble tissues, to either the unchanged compounds or their metabolites. If fetal hemodynamics were not similarly affected, if fetal circulation or metabolic capacity was poorly developed, these could have been the factors protecting the corresponding fetal tissues from the toxic effects seen in the mother.

6.4 SOCIOMEDICAL CONSIDERATIONS

There are several differences between the state of acute alcohol intoxication induced in this investigation and that which would generally occur in a pregnant woman "on a binge". For example, the adult rat has several times the capacity of the adult human to metabolize ethanol, and is therefore capable of clearing ethanol from its tissues much more rapidly. Furthermore, the human fetus has some drug metabolizing capacity in the first half of gestation whereas this is not acquired in the rodent until the end of gestation.

Secondly, there are both structural and functional differences in the ovary and placenta between the two species. Despite these differences, alcohol is known to cross the placenta readily in many species and chronic maternal alcohol ingestion during pregnancy has produced comparable Fetal Alcohol Syndromes in dogs, mice, guinea pigs and humans, leaving little doubt as to the teratogenicity of ethanol in all of these species.

Thirdly, humans on a binge tend to take alcohol orally and over a longer period of time. In this particular experimental situation, a single dose was administered intraperitoneally, which would have resulted in a rapid absorption and a sudden high level of blood alcohol, but a more rapid elimination of the drug. The offspring of these animals would therefore have been exposed to higher levels of alcohol

for shorter periods of time, in contrast to a lower, more continuous exposure of the human fetus.

Despite the fact that maternal behavior was severely impaired at the highest dose level and there was demonstrable tissue damage in the mother, the fetus was largely spared from the characteristic morphological anomalies. Evidence of skeletal ossification deficiencies, however, suggests that the same mechanism which induces microcephaly and growth retardation in chronic prenatal exposure to ethanol is operating even during short periods of intoxication. The microscopic changes in the placenta and maternal ovary implicate placental insufficiency and/or a reduced endocrine support of pregnancy in the pathogenesis of growth retardation and the increased incidence of prematurity in pregnancies complicated by alcohol abuse. It is also important to note that the present study has not ruled out the possibility of subtle, postnatal functional and developmental deficits as consequences of acute intoxication with alcohol.

Most studies of this subject to date have been descriptive, and although maternal ingestion of ethanol has been implicated as the primary etiological factor, the pathogenesis of the Fetal Alcohol Syndrome is not understood. Further insight was gained, however, by comparing the effects of ethanol acting alone to the effects of ethanol-pentobarbital interactions, on a wide spectrum of parameters in the pregnant animal. The great variability in the response of the individual parameters to the presence of the two drugs suggests that it is not a simple question of whether the primary teratogen is the unchanged alcohol or one of its metabolites. Rather, each adult and fetal tissue seems

capable of its own unique response. Such factors as metabolic competence and vascularity, the hemodynamic changes in pregnancy as well as in the pharmacological response to ethanol have all been discussed with regard to their possible roles in the teratogenicity of ethanol. Whereas metabolic and vascular immaturity may protect the fetal rat from the damaging effects of ethanol during the period of organogenesis, the mid-trimester human fetus would not have the benefit of these protective mechanisms. For these reasons, the minimal teratogenic effects of acute intoxication with ethanol observed in these experiments may well underestimate the effects on the human fetus similarly exposed.

CONCLUSIONS

CONCLUSIONS

ETHANOL

1. Short-term intoxication with ethanol during pregnancy was associated with a temporary reduction in maternal weight gain which had been eliminated by the end of gestation. There was no deleterious effect of prenatal exposure to ethanol on fetal mortality rates or on the length and weight of the offspring observed at term.

2. Microscopic changes were observed in the corpora lutea and in the basal zone of the placenta which suggested that more attention should be directed towards ovarian and placental dysfunction in considering the pathogenesis of the fetal alcohol syndrome.

3. With the exception of minor delays in skeletal ossification, there was no detectable gross external or visceral congenital defect at the treatment levels used in this investigation.

4. Microscopic changes in the maternal liver and kidney were indicative of a long-standing venous congestion and inflammatory process. There were no microscopic changes in the corresponding fetal tissues, thereby demonstrating that maternal toxicity is not necessarily accompanied by fetal damage

PENTOBARBITAL

5. Short-term intoxication with pentobarbital, when administered in doses which induced moderate levels of intoxication in the pregnant rat, was associated with a temporary reduction in maternal weight gain

which was eliminated by the end of gestation. There were no deleterious effects of prenatal exposure to pentobarbital on fetal mortality rates, weights or lengths, or on placental weights. There were no gross external or visceral abnormalities, and no skeletal defects observed in the offspring.

6. With the exception of minor changes in the maternal kidney and a rather general, non-pathological stimulation of the reticulo-endothelial system, there were no microscopic changes in any of the maternal or fetal tissues examined.

ETHANOL-PENTOBARBITAL COMBINATIONS

7. There was no consistent pattern of response observed in the various maternal parameters used to assess the toxicity of ethanol-pentobarbital interactions. The results suggested that several mechanisms may be involved in the reaction of the intact organism to the presence of these two drugs, that each tissue is capable of a unique response, and that, for these reasons the effects of drug interactions are unpredictable.

8. From the results of the present investigation there is no evidence that at these dose levels, combinations of ethanol and pentobarbital are more teratogenic than either drug acting independently.

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