STUDIES ON THE POSTNATAL DEVELOPMENT OF THE RAT LIVER PLASMA MEMBRANE FOLLOWING MATERNAL ETHANOL INGESTION

by

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ABSTRACT

Studies on the developing rat liver and on the structure and function of the postnatal rat liver plasma membrane were carried out following maternal consumption of alcohol during pregnancy and lactation.

A developmental study of alcohol dehydrogenase (ADH) indicated that both the activity and certain kinetic properties of the enzyme from the progeny of alcohol-fed and pair-fed mothers were similar. Fatty liver, however, developed in the alcoholic progeny only after ADH appeared on day 19 of gestation.

Further studies on structural and functional changes were then undertaken on the postnatal development of the rat liver plasma membrane. Initial characterization with electron micrographs and isopycnic centrifugation showed no difference between the membranes of pups from both maternal groups. Radioligand binding studies performed using the hepatic alpha$_1$-adrenergic receptor as a plasma membrane probe demonstrated a significant decrease in receptor density in the alcoholic progeny, but no changes in binding affinity. Functionally, the hormonal stimulation by epinephrine of glycogen phosphorylase a in liver slices from alcoholic pups was found to be significantly reduced when compared with the controls. Finally, the fatty acid composition of constituent phospholipids and the cholesterol content of rat liver plasma membranes were determined. Although the postnatal development of the membrane lipids was not affected, specific adaptive changes were induced in the membrane lipids of the alcoholic progeny.

All these observations suggest that membrane alterations in the newborn may be partially responsible for the deleterious action(s) of maternal alcoholism at the molecular level.

RÉSUMÉ

Des études ont été effectuées sur le développment du foie de rat et sur la structure et la fonction de la membrane cytoplasmique postnatale de foie de rat à la suite de l'ingestion maternelle d'alcool pendant les périodes de grossesse et de lactation.

Une étude sur le développement de l'alcool déshydrogénase (ADH) n'a révelé aucune différence dans l'activité et certaines propriétés cinétiques de l'enzyme chez la progéniture d'animaux témoins et alcoolisés. Néanmoins, une accumulation d'acides gras dans le foie des rats alcoolisés a coïncidé avec l'apparition de l'ADH au dix-neuvième jour de la gestation.

Par la suite, plusieurs études ont été effectuées sur les membranes cytoplasmiques de foie de rat chez les nouveau-nés. Aucune différence n'a été détectée entre les membranes hépatiques des deux groupes d'animaux par voie de microscopie électronique et de centrifugation isopycnique. Des études de liaison ont indiqué une diminution du nombre des récepteurs alpha$_1$-adrénergiques sans que leur constante d'affinité soit modifiée. Au point de vue fonctionnel, une diminution de la stimulation hormonale avec l'adrénaline de l'activité glycogène phosphorylase a dans des coupes de foie a été observée chez les nouveau-nés alcoolisés. Finalement, la composition des acides gras provenant des phospholipides membraneux ainsi que le contenu de cholestérol ont été déterminées dans les membranes cytoplasmiques. Aucune différence n'a été détectée entre les deux groupes d'animaux dans la composition des lipides membraneux pendant le développement postnatal. Néanmoins, des changements d'adaptation dans la composition lipidique ont été observés chez la progéniture des rats alcoolisés.

L'ensemble des résultats suggère un rôle important pour les altérations membraneuses chez le nouveau-né dans les mécanismes moléculaires étant à l'origine des effets néfastes induits par l'alcoolisme maternel.

To the memory of my grandmother Dina and to my parents, who through their constant and unconditional support, taught me that the integrity of a human being is reflected in his enduring fight for the search of truth and freedom.

Seule la vérité peut affronter l'injustice. La vérité, ou bien l'amour.

Albert Camus

PREFACE

The work presented in Chapter 3 and Chapters 5-7 of the thesis has been published or submitted for publication in the following journals:

Chapter 3. Rovinski, B., Lau-You-Hin, G., Lee, H. and Hosein, E.A. (1984) submitted to Biochem. J.

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In accordance with regulations described in the general information booklet 1980 of the Faculty of Graduate Studies and Research and as approved by the Department of Biochemistry, papers already published or submitted for publication have been incorporated into the thesis.

References have been included at the end of each chapter.

LIST OF PUBLICATIONS

ABSTRACTS:

1. B. Rovinski and E.A. Hosein (1981). Effect of maternal alcohol ingestion during pregnancy and lactation on the postnatal development of hepatic adrenergic receptors in the newborn rat. Can. Fed. Biol. Soc. Proc. v. 24, 305 (Montreal, Quebec).

2. B. Rovinski, H. Lee and E.A. Hosein (1982). Effect of chronic alcohol feeding on rat liver plasma membranes. Lipid compositional analysis. Can. Fed. Biol. Soc. Proc. v. 25, 74 (Edmonton, Alberta).

3. B. Rovinski and E.A. Hosein (1983). The influence of chronic alcohol feeding to pregnant rats on epinephrine-stimulated phosphorylase a activity in the liver of the progeny. Can. Fed. Biol. Soc. Proc. v. 26, 234 (Ottawa, Ontario).

4. B. Rovinski and E.A. Hosein (1983). Lipid composition of postnatal rat liver plasma membrane subsequent to chronic maternal ethanol ingestion. Fed. Proc. v. 42, 885 (Chicago, Illinois).

5. E.A. Hosein, K. Bozic, B. Rovinski, H. Lee and M. Thakur (1983). Development of fatty liver in the progeny of rats chronically fed alcohol during pregnancy and lactation. Fed. Proc. v. 42, 2003 (San Francisco, California).

PAPERS:

1. H. Lee, E.A. Hosein and B. Rovinski (1983). Effect of chronic alcohol feeding and withdrawal on rat liver structure and function: A study with the binding of [^{3}H] prazosin to the membrane-bound alpha$_1$-adrenergic receptor. Biochem. Pharmacol. 32, 1321-1323.

2. B. Rovinski and E.A. Hosein (1983). Adaptive changes in lipid composition of rat liver plasma membrane during postnatal development following maternal ethanol ingestion. Biochim. Biophys. Acta 735, 407-417.

3. B. Rovinski, E.A. Hosein and H. Lee (1983). Effect of maternal ethanol ingestion during pregnancy and lactation on the structure and function of the postnatal rat liver plasma membrane: Assessment with [^{3}H] prazosin binding to the hepatic α_1-adrenergic receptors. Biochem. Pharmacol. 33, 311-317.

4. B. Rovinski and E.A. Hosein (1983). Chronic maternal ethanol administration in the rat decreases the stimulation by (-) epinephrine of glycogen phosphorylase a in the livers of the progeny during development. Substance and Alcohol Actions/Misuse, in the press.

5. B. Rovinski, G. Lau-You-Hin, H. Lee and E.A. Hosein (1983). Ontogenetic development of liver alcohol dehydrogenase and emergence of fatty liver in rat offspring following chronic maternal ethanol ingestion. Biochem. J., submitted.

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CHAPTER 1

Introduction

1. INTRODUCTION

1.1 HISTORICAL SURVEY

1.1.1 Use of Alcohol

Drinking of alcoholic beverages can be traced back to prehistoric times. Such beverages were probably discovered by chance when fruits, honey, cereals, berries, cactuses and other plant materials stored in pottery jars or rock cavities were left unattended in the sun and underwent natural fermentation (1).

Archaeological records from murals, wall paintings, vessels, and hieroglyphic texts indicate that fermented beverages were widely used by the earliest civilizations (2). Beer and wine, for example, were known in ancient Egypt as far back as the fourth millennium B.C., and they were often used for medical purposes (3).

Written records also provide evidence of the ubiquity and importance of fermented beverages in the earliest civilizations. For example, the medicinal use of beer and wine was widespread as indicated by the clay tablet records of the Sumerian Civilization and the medical papyri of the Egyptians (3). On the other hand, regulations regarding the use of alcoholic beverages were already found in the oldest known code of laws, that of Hammurabi of Babylonia, which specified in detail restrictions on the sale of wine (4).

Ultimately, alchemists sought to capture the so-called "spirit" of wine, and in about A.D. 800, an Arabian known as Jabir ibn Hayyan succeeded by developing the technique and art of distillation. Because the "spirit" was extracted in an invisible state, it was named "alcohol" which is an Arabic word meaning "finely divided spirit" (5).

The abuse of alcoholic beverages did not spare the magnificent Greek and Roman Civilizations. In Greece the cult of Dionysus and in Rome that of Bacchus, the God of wine, was often associated with sex orgies (3). In the Western Civilization, which emerged as a mix of these two cultures, alcohol was also an important component of daily life, and the advent of commercial distillation intensified the problems associated with drinking alcohol by providing a more potent intoxicant and a more dangerous pathogen (2).

Finally, an intensification of the consumption of alcoholic beverages has been associated with industrialization; this was often overlooked or neglected, and it was only at the end of World War II when the World Health Organization was created, that people seriously considered the consumption of alcohol as prejudicial to the health of individuals and the community (6). Since then, several legal measures have been enacted in various countries to restrain people from drinking (7). However, the history of the use of alcohol indicates that the custom of drinking is persistent, diffused, and widely pervasive in all known cultural groups. Therefore, it is imperative that more attention be given to alcohol abuse and its associated behavioral and biochemical derangements, which may be involved in the pathogenesis of alcohol-related diseases.

1.1.2 Alcohol as a Teratogen

The belief that alcohol consumption during pregnancy can affect the developing fetus can be traced back to antiquity (8). For example, the Bible says: "Behold, you are barren and have no children; but you shall conceive and bear a son. Therefore, beware, and drink no wine or strong drink ..." (Judges, 13:3-4).

The potential teratogenic effects of maternal alcoholism were also recognized by the early Greek and Roman Civilizations. Carthage and Sparta, for instance, had laws prohibiting the use of alcohol on the wedding night, for fear of conceiving a defective child (9). Plato (8) likewise, cautioned that "it is not right that procreation should be the work of bodies dissolved by excess wine, but rather that the embryo should be compacted firmly, steadily, and quietly in the womb ..." (Laws, 6:775).

Observations made during England's "Gin Epidemic" of 1720-1750 also indicated that drinking during pregnancy could damage the fetus, and in 1834 a report to the British House of Commons indicated that infants born to alcoholic mothers often appeared "starved, shrivelled and imperfect" (8).

In 1968, Lemoine and colleagues (10) were the first ones to describe a distinct pattern of malformations in the offspring of female alcoholics. It was not, however, until 1973 when Jones and Smith (11) described a pattern of multiple congenital abnormalities associated with maternal alcoholism, collectively named the "Fetal Alcohol Syndrome" (FAS), that the teratogenicity of alcohol consumption during pregnancy started receiving wide attention.

The characteristic cluster of features of FAS include pre- and postnatal growth deficiency; facial dysmorphic features, characterized mainly by underdevelopment of the mid-face; microcephaly; central nervous system dysfunction manifested principally as mental deficiency of varying degrees of severity.

Although intensive investigation of the effects of <u>in utero</u> alcohol exposure has been undertaken in the past decade (12,13), there is little

direct information on the cellular and biochemical basis of the mechanism(s) of the deleterious action(s) of alcohol at the molecular level.

1.2 PHYSICOCHEMICAL PROPERTIES OF ALCOHOL

Ethyl alcohol or ethanol* is the intoxicating agent in fermented and distilled alcoholic beverages.

Pure ethanol is a clear, colorless flammable liquid with a characteristic but weak odor and a strong, burning taste. The molecular composition of ethanol consists of a hydroxyl group and an alkane chain of two carbons:

$$CH_3\text{-}CH_2\text{-}OH \qquad (M.W.\ 46.07)$$

Ethanol has a boiling point of 78.5°C, a melting point of -117.3°C, and a density of 0.7893 g/cc at 20°C compared with water at 4°C (14).

The molecular composition of ethanol makes it soluble in both polar and non-polar media. The presence of the very polar hydroxyl (-OH) group confers upon the molecules of ethanol the ability to form strong hydrogen bonds and the capacity to be miscible in all proportions with water. Therefore, like the molecules of water, those of ethanol are polar with a strong tendency to association and orientation at interphases such as those between cell membranes and surrounding fluids (15,16). On the other hand, the alkane (CH_3CH_2-) portion of the ethanol molecule makes it soluble in lipid-dissolving liquids such as ether, chloroform and methanol, but relatively poorly soluble in lipids. Its partition coefficient between lipid and aqueous phases is about 0.1; therefore, the concentration of ethanol in tissue lipids will always be about 10% of that in the body water (17).

*The terms alcohol and ethanol are interchangeably used in the thesis.

Ethyl alcohol is industrially produced either by fermentation of carbohydrates or by catalytic hydration of the ethylene obtained from the cracking of petroleum (15). Except for alcoholic beverages, nearly all the ethyl alcohol used is a mixture of 95% alcohol and 5% water, known simply as 95% alcohol. In addition to its use in the preparation of alcoholic beverages, pure ethyl alcohol (100%), also known as absolute alcohol, is widely used as a starting material for the production of a variety of chemicals such as acetic acid, lacquers, varnishes, dyes, artificial fibers and fabrics, etc. It is also used as a fuel, and as a solving and extracting agent in the pharmaceutical and cosmetic industries (16).

1.3 PHARMACOLOGY OF ALCOHOL

Ethanol is an aliphatic alcohol, which is classified as a reversible general CNS depressant (18). It is usually ingested in the form of beer, wine, and distilled spirits, with the ethanol concentration varying from approximately 4% by volume in beer, to 12% in wines, and 40% to 50% in distilled spirits. Alcoholic beverages also contain a variety of other constituents, collectively categorized as congeners, which, although generally present in low concentrations, can be toxic (18).

1.3.1 Absorption

When ethanol is ingested by animals and humans, it is rapidly absorbed from the alimentary tract into the circulation by passive diffusion across the gastric and intestinal mucosa (18). The rate of absorption of ethanol depends upon several factors. For example, gastric absorption is increased when ethanol is taken on an empty stomach, whereas the presence of food in the stomach slows absorption by diluting the ethanol in the gastrointestinal tract and delaying stomach emptying (17). These and other factors, such as the volume, type, and dilution of the alcoholic beverage, affecting the rate of absorption of ethanol, are important determinants of the levels of alcohol in the blood. Because ethanol passes through the liver, which normally is working to capacity to metabolize it before it reaches the peripheral blood, a faster rate of absorption will result in higher blood alcohol levels and will lead to a greater degree of intoxication.

1.3.2 Distribution

After being absorbed, ethanol is rapidly distributed throughout the aqueous compartments of the body. Because of its hydrophylic properties, ethanol does not accumulate in adipose tissue, but will distribute in all

body tissues in a concentration which is dependent upon their water content (17).

The rate of distribution of ethanol into body tissues is also proportional to their blood supply. For example, after being absorbed, ethanol will rapidly reach peak concentrations in vascular tissues such as brain, liver, kidneys, lungs and placental membranes (18,19).

Several studies on pregnant rats (20), mice (21), hamsters (22), monkeys (22) and humans (23,24), have shown that ethanol readily crosses the placenta and is distributed in fetal blood in concentrations comparable to those found in the maternal circulation. Hence, the distribution of ethanol to the fetus may be an important determinant of the deleterious actions of *in utero* alcohol exposure (25,26).

1.3.3 Elimination

The metabolism of alcohol occurs mainly in the liver which is responsible for the elimination of about 90% of an ingested dose (27). However, other tissues such as kidneys (28,29), lungs (30), heart (28), pancreas (31), bone marrow (32), intestines (33-35), and even brain (36), have also been shown to contribute to the overall metabolism of alcohol *in vivo*. Although most of the alcohol ingested is eliminated by metabolic removal, 5-10% is eliminated unchanged through the skin, the expired air and the urine (17).

The pathways of ethanol metabolism are very similar in humans and animals (37,38). The hepatic metabolism of ethanol can be catalyzed *in vitro* by various enzyme systems through three different pathways: 1) the alcohol dehydrogenase (ADH) pathway located in the cytosol of the hepatocyte (39,40); 2) the microsomal ethanol-oxidizing system (MEOS) found in

the endoplasmic reticulum (41-44); and 3) catalase, which is located in the peroxisomes (45).

1) Alcohol Dehydrogenase (ADH)

Liver alcohol dehydrogenase (ADH; EC 1.1.1.1) is the main enzyme responsible for the metabolism of ethanol *in vivo* (46). It is a soluble, zinc-containing enzyme found in the cytosol (47), and it uses nicotinamide adenine dinucleotide (NAD^+) as coenzyme to catalyze the conversion of ethanol to acetaldehyde as follows:

$$CH_3\text{-}CH_2\text{-}OH + NAD^+ \xrightarrow{ADH} CH_3\text{-}CHO + NADH + H^+ \qquad (I)$$

This reaction is the initial and rate-limiting step in the hepatic metabolism of alcohol (38,46,48). However, the rate-limiting factor is generally considered not to depend upon ADH activity itself, but rather upon the capacity of the liver to reoxidize the NADH produced from the reduction of NAD^+ after alcohol is metabolized (46).

ADH is found in micro-organisms, plants, animals and humans (49). It exists in several genetically determined variants (17,46), and kinetic studies have shown that it has a K_m for ethanol of 0.5 to 2.0 mM (47,50). ADH has also been shown to metabolize other substrates and be inhibited by pyrazole (17,50).

Although ethanol elimination *in vivo* via the ADH pathway proceeds at a constant rate (17), when blood ethanol concentrations exceed values between 20 and 25 mM, other pathways not blocked by pyrazole have also been shown to participate in the metabolism of ethanol (46,48,51).

2) Microsomal Ethanol-Oxidizing System (MEOS)

Microsomal fractions of liver can oxidize ethanol in the presence of NADPH and oxygen to produce acetaldehyde as follows:

$$CH_3\text{-}CH_2\text{-}OH + NADPH + O_2 + H^+ \xrightarrow{MEOS} CH_3\text{-}CHO + 2H_2O + NADP^+ \quad (II)$$

This enzyme system has been well characterized (41-43) and shown to be responsible for a substantial part (10-20%) of the metabolism of alcohol *in vivo* in the liver, especially at high blood alcohol levels (51) because its Km for ethanol (10 mM) is higher than that of alcohol dehydrogenase (0.5 - 2.0 mM).

3) Catalase

The enzyme catalase abounds in liver peroxisomes and has the ability to oxidize ethanol *in vitro* (45,52) in the presence of a hydrogen peroxide-generating system as follows:

$$CH_3\text{-}CH_2\text{-}OH + H_2O_2 \xrightarrow{catalase} CH_3\text{-}CHO + 2H_2O \quad (III)$$

Catalase, however, has not been considered to be important in the metabolism of ethanol *in vivo*, since it cannot affect the rate of alcohol elimination *in vivo* or by tissue slices *in vitro* (46,52). Also, its metabolic role is probably limited by the slow rate of hydrogen peroxide production in normal hepatocytes (53).

In general, when alcohol is ingested the ADH pathway (Equation I) is the main enzyme system responsible for its metabolism *in vivo* (46). Chronic alcohol consumption, however, has been observed to induce an

increase in the rate of ethanol elimination in man (46,54) and in laboratory animals (46), which has been shown to be independent of ADH and rather associated with an induction of the MEOS (Equation II) pathway (46,51). Other factors such as increased mitochondrial reoxidation of NADH (55) and ethanol peroxidation by catalase (53) may also be implicated in the adaptive increase of alcohol metabolism after chronic consumption. Alterations in the elimination of ethanol and the activity of alcohol-metabolizing enzymes have also been reported and shown to be associated with gender (56-58), age (56), genetic factors (59,60), nutritional status (61,62), liver disease (46,63), and abuse of other drugs (64).

1.4 EFFECTS OF CHRONIC MATERNAL ALCOHOL CONSUMPTION ON THE FETUS AND THE POSTNATAL DEVELOPMENT OF THE PROGENY

1.4.1 Development of the Fetus and Neonate

The development of the fetus is linked anatomically, genetically and metabolically with that of the placenta, which is the vascular organ that unites the developing embryo to the maternal circulation. Rather than serving as a passive filter, the placenta plays a dynamic modulating role between the maternal environment and the developing fetus by maintaining an adequate exchange of oxygen, nutrients and waste by-products (65). Placental transfer, therefore, plays a vital role in the development of the embryo and fetus, and any toxic agent capable of either diffusing across the placental membrane or perturbing placental function may affect fetal development.

At birth, the interruption of the continuous transplacental fuel supply to the fetus imposes a new metabolic environment upon the newborn. The transition from placental nutrition to the onset of lactation requires a few hours, and during this time the metabolic substrates needed by the organism must be supplied by its own energy reserves (65). During this transitional period the liver plays a determinant role in the regulation of important homeostatic functions, such as the production of glucose which is the main oxidative fuel for the newborn, and this is possible because the liver is formed at an early stage in embryonic life (66).

During lactation the newborn is fed through the maternal milk which provides most of its nutrients. Changes in either milk content or secretion may, therefore, affect postnatal development. Moreover, since enzymic differentiation occurs very rapidly during the early stages of

postnatal development (66), maternal perturbations occurring during lactation can also induce alterations in the metabolic performance of the newborn.

1.4.2 Animal Studies on the Effects of Maternal Alcoholism

Since 1973, when the Fetal Alcohol Syndrome (FAS) was described as a distinct outcome resulting from in utero exposure to maternal alcoholism (11), intensive investigation of the effects of alcohol exposure during pregnancy has been undertaken (12,13).

The adverse effects of maternal alcoholism have been demonstrated in humans through clinical (11-13,25,67,68) and epidemiological (69) studies, and in animals through controlled laboratory experiments (12,13,70,71). Animal studies have the advantage of enabling investigators to control for variables that are difficult to monitor in human studies, such as the assessment of the role of alcohol-related undernutrition by means of pair-feeding techniques, as well as the regulation of timing and dose of alcohol exposure.

1.4.2.1 Teratogenic and Developmental Effects

Numerous studies with several animal species have demonstrated that maternal alcohol consumption has deleterious effects on the fetus and the neonate. These include anatomical abnormalities (26,72-79), intrauterine growth retardation (26,75,77,80-84), and postnatal growth (76-81,83, 85-90) and behavioral (78,91-95) deficiencies.

Sandor and Elias (96) reported for the first time evidence of ethanol-induced teratogenicity in chick embryos. They injected a mixture of alcohol-distilled water into an air chamber at various incubation times and found an increased embryo mortality as well as loss of weight and central nervous system dysfunctions in the surviving embryos when

compared to the water-injected controls.

More appropriate studies were subsequently performed with animals having a uterine development. For example, Chernoff (26) administered Metrecal-based diets containing 15-35% ethanol-derived calories to CBA and C3H mice for at least 30 days before and throughout gestation. In this study females were killed on day 18 of gestation, and an examination of the alcoholic offspring revealed an increased incidence of prenatal death and a pattern of anatomical malformations similar to those recorded for FAS children, which was exacerbated in the CBA strain and exhibited a dose-related effect. Studies by Randall et al. (73,74) and those by Kronick (72) have also shown that chronic maternal alcoholism is embryolethal and teratogenic in mice.

The rat has also been widely used as an animal model of maternal alcoholism. Tze and Lee (85), for example, observed that fecundity, litter size, and litter weight were reduced in Sprague-Dawley rats given alcohol (30% w/v) in their drinking water prior to and during pregnancy when compared to their pair-fed controls. Studies by Abel (80) and Abel and Dintcheff (81) demonstrated that when pregnant rats are intubated with ethanol (30% w/v), the alcoholic progeny exhibits intrauterine and postnatal growth retardation with a degree of severity which depended on the dosage and the period of fetal exposure to the drug. Investigations by Henderson et al. (83), Lochry et al. (76) and Sigh and Snyder (90) also revealed an increased incidence of growth deficits and reduced survival rates in rats exposed to alcohol in utero.

All these studies demonstrated that prolonged in utero exposure to maternal alcoholism can produce developmental malformations in the progeny. The variability and severity of the adverse effects seemed to

depend upon the species, dose and time of exposure to alcohol.

Although the exact mechanism(s) responsible for the deleterious effects of *in utero* alcohol exposure are not known, several hypotheses, such as impairment of umbilical circulation (97,98), placental dysfunction (99-101), fetal hypoxia (71), and delayed cellular maturation (92,102,103), have been advanced. However, it is still not clear whether other factors, including alcohol-associated nutritional deficits, the toxicity of acetaldehyde, and genetic metabolic differences, may play a role in the deleterious actions of ethanol (104).

1.4.2.2 Biochemical and Physiological Changes

Several neurochemical (105-108), endocrinological (108-110) and neural (111,112) abnormalities have been reported in the offspring of animals exposed to alcohol *in utero*. For example, Rawat (105) reported that maternal ethanol consumption (30% of the total calories) during gestation and lactation decreased the levels of brain dopamine, acetylcholine and histamine in fetal and neonatal rats, while those of GABA and glutamate were increased. Druse and Hofteig (106) showed that *in utero* alcohol exposure (6% v/v) induced a delayed myelination in rat offspring during postnatal development, and Thadani (108) reported that maternal alcoholism affected the development of noradrenergic synaptic function in fetal and neonatal rats.

Other studies indicated that different biochemical and physiological anomalies, such as decreased protein synthesis (83,113), depressed immunological reactivity (114), and altered hepatic metabolic functions (115-117), can also be present in the offspring of animals exposed to ethanol *in utero*.

1.5 LIVER AS A DIRECT TARGET OF ALCOHOL TOXICITY

Prolonged alcohol ingestion is known to produce a variety of biochemical and pharmacological changes in the liver (118,119), as well as in other tissues (119). Some of the major disorders and abnormalities associated with excessive alcohol consumption include central nervous system impairments such as the Wernicke-Korsakoff syndrome (associated with thiamine deficiency) and degeneration of the brain cortex and cerebellum (120); liver disease (121); gastrointestinal lesions and disturbances (120); pancreatic damage (122); cardiomyopathy (123); increased incidence of infectious diseases such as pneumonia and tuberculosis (120); derangements of the endocrine system (124); hematologic disturbances (125); increased susceptibility to various types of carcinomas (126); alterations of drug metabolism (119,120); a decrease of the cellular elements produced by the bone marrow (127); nutritional deficiencies due to an inappropriate intake of vitamins and minerals as well as to alterations in the absorption, distribution, metabolism, storage, and excretion of many essential nutrients (128); fetal malformations and birth defects and anomalies (19,20,129).

Alcohol misuse, therefore, has a pervasive and potentially detrimental effect on the body. The deleterious effects of alcohol, however, relate not only to its own toxicity, but also to its metabolic course. The liver plays a determinant role in the metabolism of ethanol (21,118; Section 1.3), and this relative organ specificity of ethanol metabolism explains why the liver is most significantly damaged and physiologically deranged as a result of alcohol ingestion.

The hepatotoxicity of chronic ethanol consumption has been widely documented (44,118,119,130). A variety of hepatic lesions, ranging from

fat accumulation, necrosis and inflammation to fibrosis and cirrhosis, have been observed in humans (121,131) and laboratory animals (132,133) following prolonged alcohol exposure.

Prolonged ethanol ingestion has also been shown to have deleterious effects on hepatic protein, carbohydrate, and lipid metabolism (130,134). These hepatic metabolic alterations can in turn induce general metabolic derangements which include hypoglycemia (130); hyperuricemia (135); hyperlipemia and fatty liver (131-133); a hypermetabolic state associated with an increase in oxygen uptake (136,137).

Most of the metabolic effects of alcohol are related to its own metabolic course. The marked increase in the [NADH/NAD^+] ratio in hepatocytes actively oxidizing ethanol (40; Section 1.3), the toxicity of the metabolites of ethanol such as acetaldehyde (17), and the adaptive metabolic changes following chronic ethanol intake (40,138), have been implicated in the pathogenesis of alcohol-related diseases (119).

The liver, therefore, is one of the primary targets of the toxicity of ethanol, and characterization of the structural and functional changes underlying the hepatotoxicity of alcohol constitutes an important step in the elucidation of the mechanisms responsible for the deleterious effects of prolonged alcohol consumption.

1.6 STRUCTURE AND FUNCTION OF BIOLOGICAL MEMBRANES

1.6.1 Membrane Composition and Organization

1.6.1.1 Membrane Components

Biomembranes consist almost entirely of lipids and proteins (139,140), and their relative amounts vary considerably among the membranes of different organelles. For example, the myelin sheath contains about 75 per cent of the dry weight as lipid, while the mitochondrial inner membrane contains about 25 per cent lipid, and in each case the remainder consists mainly of protein (139).

In addition to lipids and proteins, biomembranes also contain carbohydrates, which are minor constituents and mainly associated with the plasma membrane rather than with internal membranes (139;140). Moreover, carbohydrates are always covalently attached to both lipid and protein molecules in the form of glycoproteins and glycolipids, respectively (139). A considerable amount of water (about 20 per cent of the total mass) and chelated ions have also been found to be tightly associated with biological membranes (140).

Most of the lipid molecules found in biological membranes are amphipatic, which means that they have a dual nature, one which is polar or hydrophilic and is stable in an aqueous solution, while the other is non-polar or hydrophobic and compatible with a non-aqueous environment (140-142).

The lipid composition of biomembranes is characterized by the presence of phospholipids, sphyngolipids and cholesterol. The most abundant lipids are the phospholipids such as phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI), with much smaller amounts of sphingolipids

(139,141,142). Cholesterol, on the other hand, is less abundant and found almost exclusively in the plasma membrane of mammalian cells (139). The relative amount of the various types of lipid components is characteristic of the kind of membrane system, the organ, and the species (141).

Biological membranes contain a wide variety of proteins which are generally classified according to the ease with which they are removed from the membranes: those loosely attached to the membrane surface and easily removed by mild extraction procedures are called peripheral proteins, while those tightly bound to the lipid components and requiring detergents or organic solvents for extraction are called integral proteins (141). Peripheral membrane proteins are hydrophilic and bind to polar surfaces of other membrane proteins and lipids, while the most abundant (about 60-70 per cent of the total membrane protein) integral membrane proteins have hydrophobic regions which allow them to intercalate in several ways with the lipid core of the membranes (139,141).

1.6.1.2 Current Models of Membrane Structure

It is generally recognized that lipids are responsible for the structural integrity of biological membranes. The dual nature of membrane lipids, with their hydrophilic heads and hydrophobic tails, is important to the organization of biomembranes (139,143). Since the polar heads of the lipids can interact with water, and their non-polar tails are mutually attracted by van der Waals forces, the lipid molecules are ideally suited to form an interface between a nonaqueous lipid environment within the membrane and the aqueous intra- and extracellular phases in contact with the two membrane surfaces. This notion originated around the turn of the century with the work of Overton (144), and subsequent

observations by Langmuir (145) and Gorter and Grendel (146) led to the concept that a lipid bilayer might be present in biological membranes. Conclusions drawn from those studies seemed to indicate that the lipid components of biomembranes are arranged in a bilayer composed of two monolayers apposed to each other, with the hydrophobic ends of the molecules facing inward, or toward each other, and the hydrophilic groups facing outward, or toward the aqueous phases adjacent to the surfaces of the double layer (143).

The suggested bilayer arrangement of lipids in the early models of biomembrane structure was further expanded by Danielli and Davson who proposed a general structural scheme for biological membranes characterized by a lipid bilayer coated at its aqueous interfaces with layers of protein (147). This scheme was subsequently modified and refined by Robertson who advanced the unit-membrane model (148). These models, however, led to the assumption that nonlipid constituents could not be present in the hydrophobic core of the bilayer.

More recently, the realisation that proteins can be associated with the hydrophobic membrane lipid phase, together with evidence of the fluid packing of the lipid hydrocarbon chains and of lateral diffusion of membrane components, led to the postulation of the fluid-mosaic model by Singer and Nicolson (149). This model states that the basic structure of biological membranes is a two-dimensional solution of globular proteins dispersed in a matrix of fluid lipid bilayer. Moreover, in this membrane model, lipids and proteins are free to diffuse laterally through the membrane bilayer. Although investigators have not yet agreed on a unified model of membrane structure, the fluid-mosaic model has now

generally been accepted as the most satisfactory postulation of the structural characteristics of biological membranes.

1.6.1.3 Asymmetry of Biological Membranes

The functional asymmetry of biological membranes has been shown to reflect an underlying structural asymmetry (150,151), which is maintained by a low frequency of transmembrane diffusion.

Early studies with red cell membranes (153,154) and several observations in other membrane systems (155,156) indicated an absolute asymmetry in the orientation of proteins in these membranes. Such an absolute protein asymmetry appears during biosynthesis and is responsible for the functional asymmetry of biological membranes (151).

Like the asymmetry of proteins, that of carbohydrates appears to be absolute. In plasma membranes, for example, many proteins and lipids are glycosylated and found exclusively at the extracellular surface of the membrane (139,151).

Membrane lipids are also distributed in an asymmetric way (151). However, unlike the protein and carbohydrate components of the membrane, the asymmetry is not absolute. Most of the knowledge of phospholipid asymmetry in biomembranes is based on studies of red cells (157,158), and of viral (159) and bacterial (160) membranes. In general, phosphatidylcholine (PC) and sphingomyelin predominate at the extracytoplasmic side of the bilayer, while phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE) are found at the cytoplasmic side (150,151).

The maintenance of lipid asymmetry is due to the fact that translocation of lipid molecules across biomembranes ("flip-flop") does not in general occur (151). Lipid asymmetry is an important structural feature

of biomembranes because it may play a functional role in regulating the properties of membrane proteins, in maintaining a balanced charge distribution, and in modulating the fluidity of both halves of the membrane bilayer (150,152,161).

1.6.2 Lipid and Protein Dynamics in Biological Membranes

The currently accepted model of membrane structure (149; Section 1.6.1) postulates a dynamic, asymmetric lipid matrix of phospholipids and cholesterol, with globular proteins embedded across the membrane to various degrees.

Although the functional properties of biological membranes are associated primarily with the dynamics of protein components (162), there is substantial evidence that these functions may be markedly influenced by lipid composition (163-167). In general, modulation of the functional integrity of biomembranes is manifested in terms of lipid fluidity, lipid-protein and protein-protein interactions (142).

1.6.2.1 Protein Dynamics

Integral membrane proteins have been shown to exhibit two distinct types of mobility within bilayer membranes. First, proteins can undergo a fast rotational diffusion about a vertical axis perpendicular to the membrane bilayer (168). This has been extensively studied on the visual pigment protein rhodopsin (168,169), the cytochrome c oxidase in mitochondrial membranes (168,170), and the cytochrome P450 in liver microsomes (168,171). A second mode of mobility of integral membrane proteins consists of a fast lateral diffusion within the plane of the bilayer membranes. This was first shown by Frye and Edidin (172), and subsequent experiments by others (173-175) conclusively demonstrated and quantitated the lateral diffusion of integral membrane proteins. In these studies,

the so-called fluorescence photobleaching and recovery method (FPR) has been applied to various kinds of biological membranes to obtain values for the lateral diffusion coefficient of integral proteins (139).

The rotational and lateral diffusion of integral membrane proteins are restricted by the physical state of the lipid bilayer, and lateral diffusion is also limited by the interaction of integral proteins with cytoskeletal components and peripheral proteins (139).

1.6.2.2 Lipid Dynamics

Artificial and biological bilayer membranes undergo reversible transitions from an ordered, quasi-crystalline to a disordered, fluid liquid-crystalline state at characteristic critical temperatures (139,142,168). These dynamic features of lipid bilayers have an influence on the synthesis, structure and function of biological membranes (139).

The motions of lipids in artificial and biological bilayer membranes have been investigated in detail using a variety of physical techniques such as electron spin resonance (ESR), nuclear magnetic resonance (NMR) and fluorescence polarization (139,168). Three kinds of motional modes have been described. One of the modes consists of intramolecular movements such as rotations of fatty acyl chains about single C-C bonds as well as rotational diffusion of individual fatty acyl chains and lipid molecules (139,168). A second dynamic feature of lipids in biomembranes is their fast lateral diffusion within the plane of the bilayer (176). Finally, a transmembrane movement of phospholipids generally called "flip-flop", although extremely slow in model membranes, red cell membranes, and viruses (177-180), appears to occur in bacterial membranes (181,182).

Lipid motions are important determinants of cellular function, and they can be manifested in biomembrane properties such as bilayer fluidity and viscosity, lipid phase transitions, and lipid-lipid as well as lipid-protein interactions. Moreover, all these dynamic features are influenced by the nature of the phospholipid headgroups, the type, length, and degree of unsaturation of the fatty acyl chains, and the amount of cholesterol present in the membranes (139,142,168).

1.6.3 Lipid-Protein Interactions

Phospholipid bilayers can exist in two different physical forms: a quasi-crystalline solid state and a liquid-crystalline fluid state (139; Section 1.6.2). Biological membranes, however, are in the fluid state under physiological conditions (139-142).

The "fluidity" of the lipid bilayer is usually defined in terms of the membrane microviscosity, which refers to the resistance to motion by the bilayer of intrinsic membrane components and extrinsic probe molecules (142,184). Membrane fluidity, therefore, refers to the degree of molecular motion throughout the bilayer, and it is influenced by the degree of lipid-protein interactions.

Integral membrane proteins display extensive hydrophobic and electrostatic interactions with the surrounding lipids (183,185). These interactions were investigated by using spin-label probes (185,186) which indicated the presence of an immobilized lipid layer surrounding intrinsic proteins. This shell of lipid surrounding the protein is usually referred to as "lipid annulus" or "boundary lipid". Most integral membrane proteins are solvated by the lipids of the bilayer, and the perturbation of lipid dynamics by proteins seems to affect mainly those lipids found within the annular domains (139,185). Overall, the

effect of protein on fluid biological membranes seems to be restricted to an increase in the viscosity of the bilayers (184,185).

On the other hand, the modulation of protein structure and function by lipid dynamics is more relevant because it has a greater impact on membrane function. In fact, biological functions of membranes, such as membrane-bound enzyme functions (188,189), receptor binding (190-192), transport (193), and membrane-associated enzyme activities (166,167), have been shown to be influenced by the membrane lipid composition.

The structure and function of biological membranes is therefore influenced by the dynamics of lipid-protein interactions, and this suggests that the perturbations of the lipid bilayer by agents that can affect the composition and physical state of the membrane lipids may lead to alterations in membrane function.

1.7 INTERACTIONS OF ALCOHOL WITH CELL MEMBRANES

Evidence for the notion that alcohol exerts its effects by acting on the lipids of cell membranes first appeared when Meyer and Overton (144,194) showed that simple chemical molecules such as ethanol could act as anesthetics, and that their narcotic effects reflected their lipid solubility. Further work by Seeman (195,196) indicated that many anesthetic drugs, including ethanol, could protect red blood cells against hemolysis in hypotonic solutions, and that their anesthetic potency fit the Meyer-Overton concept. All these observations established a definite correlation of the anesthetic potency of alcohol with its solubility in membranes.

Since prolonged alcohol ingestion produces a variety of biochemical and pharmacological changes in a wide variety of tissues (119,120), it is likely that the disorders and abnormalities induced by chronic alcohol administration may result from the interactions of alcohol with cell membranes.

It is now generally believed that some of the physiological, biochemical and behavioral effects of alcohol are exerted through nonspecific interactions with cell membranes which may in turn result in expansion, disordering, and disorganization of the membrane lipid bilayer (197-199).

1.7.1 Membrane Lipid Fluidization

Various physical techniques have been used in the past to show that ethanol, like many other nonspecific general anesthetic molecules, can disorder membrane lipids and induce an increase in the fluidity of biomembranes (200).

Nuclear magnetic resonance studies by Metcalfe et al. (201) showed that benzyl alcohol increased the fluidity of erythrocyte membranes; the disordering effect was also observed on the lipids extracted from the membranes.

Another technique that has been used to study the disordering effect of ethanol on bilayer membranes is fluorescence polarization. This technique consists of the incubation of membrane samples with a lipid-soluble fluorescence dye that, when irradiated with polarized light, emits light with an intensity which is a function of the fluidity of the membrane lipids (200). The use of fluorescent probes in various investigations indicated that ethanol induced a fluidization of mouse synaptic membrane lipid bilayers (202-204).

Fluidity of biomembranes can also be examined by electron paramagnetic resonance (EPR) techniques. Spin-label probes are incorporated into membranes, and an examination of the absorption of energy by the probe when placed in a magnetic field gives information about its mobility which is indicative of the degree of rigidity of the membranes (17). EPR experiments by Chin and Goldstein (205) demonstrated for the first time that intoxicating concentrations of ethanol added in vitro caused an increase in the fluidity of erythrocyte and synaptosomal membranes from mice. Subsequent studies demonstrated that ethanol can also fluidize rat liver mitochondrial (199,206) and microsomal (207) membranes.

Since the fluidity of cell membrane lipids is an important factor in membrane function (188-193), alcohol-induced changes in membrane lipid fluidity may affect cellular function.

1.7.2 Lipid Phase Transitions

As previously mentioned (Section 1.6.3), biomembranes can exist in two different physical forms: a quasi-crystalline solid state and a liquid-crystalline fluid state. Lipid bilayers can undergo transitions from one state to the other at specific critical temperatures which may be influenced by various environmental factors (139,168).

Lipid phase transitions determine the physical state of phospholipid bilayer membranes (139). Therefore, since the activity of membrane-bound proteins may be dependent on the fluidity of the membrane lipids (188-193), changes in the lipid transition temperature may also alter cellular function.

Studies using differential scanning calorimetry on rat liver mitochondrial lipids (208) and investigations of Arrhenius plots of the activity of rat liver mitochondrial membrane-bound enzymes (199,208,209), demonstrated that ethanol can induce changes in membrane lipid phase transitions. Thus, these results indicate that ethanol may also affect membrane-associated functions by altering lipid phase transitions.

1.7.3 Effects of Chronic Ethanol Administration on Membrane Structure and Function

Ethanol is known to act primarily within the lipid bilayer of biological membranes, inducing a general molecular disordering effect which may cause alterations in membrane-associated cellular functions (Section 1.7.3). Since ethanol seems to act by disordering biomembranes, it is possible that cellular adaptation to its chronic presence may be mediated by alterations in the physical properties of the lipid bilayer.

The observations by Sinensky (210) that bacteria can regulate the fluidity of their membranes in response to environmental changes led him

to postulate the homeoviscous adaptation concept, which states that cells can adjust their lipid composition as an adaptive response to variations in their environmental milieu. This concept was further expanded by Hill and Bangham (211) who proposed a general hypothesis concerning the changes that might lead to general-depressant drug dependency. They suggested that adaptations to general central nervous system depressants, such as alcohol, are manifested through changes in membrane lipid composition so as to maintain the normal degree of fluidity of the membrane.

There is now considerable evidence that chronic ethanol administration may lead to structural and functional alterations of membranes from different cellular organelles (199,206-209,212-216). These have been generally interpreted to reflect adaptive changes which may be partially responsible for the development of physical dependence on and tolerance to ethanol.

1.7.3.1 Membrane Lipid Composition

Several studies on cellular membranes from various microorganisms provided for the first time evidence of a possible relationship between alterations in membrane lipid composition and development of cellular tolerance to the chronic presence of ethanol.

Studies by Ingram et al. (217-219) demonstrated that Escherichia coli exhibits significant changes in fatty acid composition following growth in media containing 0.34 M ethanol. These changes occurred prior to the resumption of growth and consisted mainly of an increase in the proportion of unsaturated fatty acids. Similarly, Nandini-Kishore et al. (220) reported an increase in the level of unsaturation of Tetrahymena pyriformis during growth in the presence of 0.35 M ethanol. They also found that the lipid pattern reverted to normal when ethanol was removed

from the media. Other studies have also reported a general ethanol-induced increase in unsaturated fatty acids in membranes from different microorganisms (221,222). All these changes in fatty acid composition have been proposed as being adaptive because they facilitate growth and survival in the presence of ethanol (223). However, since the increase in the level of unsaturated fatty acids would actually tend to fluidize lipid bilayers (139,143,168), and since ethanol has a disordering effect on biomembranes (197,200-204), the observed pattern of increased fatty acid unsaturation in microorganisms grown in the presence of ethanol is still unclear.

Several studies have shown that the lipid composition from membranes from various mammalian cells is also altered in response to prolonged exposure to ethanol (224-238). For example, long-term exposure to ethanol may result in an increased cholesterol/phospholipid molar ratio in synaptic plasma membranes from mice (224,225,231) and brain microsomal membranes from rats (234) physically dependent on ethanol. Other important changes induced by chronic alcohol administration reveal a relative increase in the level of fatty acid saturation in synaptosomal membranes from mice (226,227) and liver mitochondrial membranes from rats (206). Finally, other studies (216,230,237) have reported a substantial decrease in the arachidonate/linoleate (20:4/18:2) ratio in rat liver mitochondrial membranes, which could result in a decreased level of polyunsaturated fatty acids. There is definite evidence that these changes in polyenoic fatty acid composition are due to ethanol-induced decreases in desaturase activities (239).

The reported increase in cholesterol/phospholipid molar ratio (224,225,231,234) and the decrease in polyunsaturated fatty acids

(216,226,227,230,237) may lead to alterations in the physical structure of the membranes which could become more resistant to the disordering effects of ethanol (240). Moreover, as suggested by Hill and Bangham (211), these changes in lipid composition may provide a molecular basis for the development of physical dependence on and tolerance to ethanol at the cellular level.

1.7.3.2 Membrane-Associated Biochemical Functions

There is now conclusive evidence that alcohol exerts its effects by acting on the lipids of cell membranes, and under chronic conditions, it has been proposed that the presence of ethanol may induce adaptive changes in the physical properties of biomembranes (Section 1.7.3.1), which may contribute to alterations in membrane function.

Several studies have shown that chronic alcohol administration can also induce alterations in membrane-associated biochemical functions, such as modulation of the activities of membrane-bound enzymes (241-248) and transport systems (247,249-252), and regulation of the binding properties of membrane-bound receptors (253-265).

These multiple effects of ethanol on the activity of membrane-associated enzymes, transport systems and receptors have been attributed to the disordering effects of ethanol on membrane lipid organization (205,211; Section 1.7.3.1). Moreover, ethanol has been shown to alter the activity of a variety of neurotransmitters in the central nervous system (266-268), and since most of these studies were done on neuronal membranes, it is possible that the depressant action of ethanol on the central nervous system may be partially modulated by changes in the physical structure of these membranes.

Although alcohol-induced changes in membrane lipid composition have generally been regarded as an expression of tolerance at the cellular level (Section 1.7.3.1), alterations in membrane-associated functions may also be implicated in the cellular mechanisms responsible for the development of physical dependence on and tolerance to ethanol.

1.8 THE RAT LIVER PLASMA MEMBRANE

The liver plasma membrane is the organelle located on the hepatocyte surface, delineating the boundaries of the cell by serving as the interface between the cell and its environment. The plasma membrane, therefore, forms a continuous barrier around the cell which controls the intracellular milieu, thus protecting the cell from fluctuating or adverse conditions in the environment (269).

In addition to its function as a barrier, the liver plasma membrane has been shown to participate in a wide variety of hepatic cellular functions such as transport, intercellular communication, cell adhesion, recognition of hormones, viruses, drugs and toxins (270). Another important property of the plasma membrane is its immunogenicity which expresses, through histocompatibility antigens, the genetic individuality of the cell (139).

The plasma membrane fraction of rat liver, originally prepared by Neville (271), has been extensibly characterized (272-278). It is a complex organelle differentiated into three major anatomical and functional regions: the vascular blood sinusoidal and the biliary bile canalicular regions, both separated by a third smoother and contiguous region of gap junctions attaching adjacent hepatocytes (279).

The blood sinusoidal region of the rat hepatic plasma membrane is involved in the recognition, uptake and degradation of metabolites; the binding of several hormones, cations and metabolites; the immunogenicity of the cell (270). The contiguous region also contains hormone receptors, and it plays an important role in intercellular communication, cell adhesion and tissue permeability (270). The bile canaliculi constitute the loci where various biliary constituents such as bilirubin

and bile acids are transported from the parenchymal cells into the bile (279).

The rat liver plasma membrane contains about 60% of its dry weight as protein, 30% as lipid, and 10% as carbohydrate, the latter found principally on the external surface covalently attached to both proteins and lipids (139). These membrane components have also been shown to be asymmetrically distributed between the two surfaces of the plasmalemma (280).

The chemical composition of rat liver plasma membrane components (270,272-278) is characterized by the presence of a complex pattern of polypeptides resolved electrophoretically; a high cholesterol/phospholipid ratio; a set of phospholipids asymmetrically distributed, with phosphatidylcholine (PC) and sphingomyelin being the major lipids on the external membrane surface, whereas phosphatidylethanolamine (PE) and phosphatidylserine (PS) are mainly located on the cytoplasmic side of the membrane.

The importance of the liver plasma membrane as the primary site of action of many drugs and toxins, coupled with its metabolic role in a wide variety of hepatic cellular functions (270,281), make the rat liver plasma membrane an excellent model for the study of the mechanisms of action of toxins and drugs of abuse.

1.9 SCOPE AND OBJECTIVES

During the past decade a substantial amount of evidence has accumulated demonstrating the adverse effects of chronic maternal alcohol consumption on the fetus and the postnatal development of the progeny. Animal models of maternal alcoholism have been developed by several investigators who have conclusively shown that *in utero* alcohol exposure can induce developmental malformations and biochemical anomalies in the fetus and the neonate. There is, however, little direct information on the cellular and biochemical basis of the mechanism(s) of the deleterious action(s) of alcohol at the molecular level.

It is now generally believed that alcohol exerts its adverse effects partly by acting on cell membranes, and there is considerable evidence that chronic ethanol administration may alter the structure and function of membranes from various cellular organelles.

In view of this evidence, the present study was undertaken in an attempt to investigate the effects of maternal ethanol administration on the postnatal development of a model membrane system. Since liver tissue is one of the primary targets of alcohol toxicity, studies on the developing rat liver and on the structure and function of the postnatal rat liver plasma membrane were carried out following chronic maternal ethanol consumption. The experiments are presented as papers prepared for publication, and the results obtained are discussed in detail.

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CHAPTER 2

Maternal Alcohol Administration in the Rat: Diet Composition and General Characteristics

2.1 MATERNAL ALCOHOL ADMINISTRATION

The liquid diet regimen used in feeding experiments is essentially a modification of the diet currently used in chronic studies in our laboratory (1), and that used by Rawat (2) in his studies with pregnant rats.

Virgin female Sprague-Dawley rats weighing 200-225 gm were purchased from Canadian Breeding Farms (St. Constance, Québec) and housed in polycarbonate shoe-box cages. They were maintained under constant temperature (22 ± 1°C) and humidity (31-40%) conditions with a 12 hr light cycle (7 a.m. to 7 p.m.), and were given water and Purina Rat Chow ad libitum. After an adjustment period of 1 week they were mated overnight with male Sprague-Dawley rats on a one to one ratio. The appearance of sperm in the vaginal washings the following morning established day 1 of pregnancy. The pregnant rats were randomly divided into two groups. Both had their diet changed to a totally liquid low-fat Metrecal-sucrose diet providing 1 kcal/ml, which was supplemented with 3 g/l Vitamin Diet Fortification Mixture. The designated experimental alcohol group was switched to a new diet containing ethanol. This was introduced gradually so that ethanol first provided 10% of the total calories in that diet. After every 2 days, calories derived from ethanol were increased to 20, then 30 and finally 37% of the diet (Table 1). By the end of the first week of pregnancy, animals in this group were consuming a Metrecal-ethanol liquid diet in which proteins contributed 16%, fat 5%, carbohydrate 42%, and ethanol 37% of the total calories (Table 2). The pair-fed control animals were maintained on the Metrecal-sucrose diet such that the total calories consumed equalled that of the alcoholic animals (≃ 85-95 kcal/day; 1 cal = 4.184 J), and this was achieved by matching

TABLE 1

Feeding Protocol

Days of Pregnancy	Experimental Alcoholic Group (≃ 90 kcal/day)	Pair-fed Sucrose Group (≃ 90 kcal/day)
1	10%	37%
2		
3	20%	
4		
5	30%	
6		
7 to 21	37%	

TABLE 2

Composition of the Diets

	Treatment	
	Control (kcal/100 ml)	Alcoholic (kcal/100 ml)
Protein	16	16
Fat	5	5
Carbohydrate	42	42
Ethanol	-	37
Sucrose	37	-
Vitamins and Minerals[1]		

[1]Provided in the diet (3 g/l) by Vitamin Diet Fortification Mixture (ICN Nutritional Biochemicals, Canada).

ethanol-derived calories with sucrose. Both groups of rats were maintained on their respective diets throughout pregnancy and lactation. All animals were weighed every 2-3 days throughout. The newborn pups were weaned at 21 days and maintained on the liquid diets fed to their respective mothers, until they were 30 days old.

2.2 GENERAL CHARACTERISTICS

The general characteristics of the animal model under investigation are described in detail in Chapter 5.

The average food intake for animals in the maternal alcoholic group was 247 ± 17 (S.E.M., n = 5) kcal/kg body weight/day, and the average ethanol intake ranged from 10 to 15.7 g/kg body weight/day (Chapter 5, Table 1).

Although no significant differences were found in maternal weight gain, litter size, and average litter weight at birth between animals in the alcoholic and pair-fed sucrose control groups, newborn pups of alcohol-fed mothers gained significantly less weight than pups from the maternal pair-fed sucrose group (Chapter 5, Table 2 and Figs. 1 and 2).

In pregnant and lactating mothers, blood ethanol concentrations (measured between 8 and 10 a.m.) varied between 35 and 40 mM. Blood alcohol levels in newborn pups of alcohol-fed mothers ranged between 4 mM at day 5 to 40 mM at day 25.

2.3 REFERENCES

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APPENDIX 1

Metrecal Liquid Supplies

		Per 100 ml
Protein	g	5.9
Fat	g	1.3
Carbohydrate	g	15
Linoleic acid	g	0.53
Ash	g	1.3
Calories	g	95
Vitamin A	I.U.	540
Vitamin D	I.U.	11
Vitamin E	I.U.	1.5
Vitamin C	mg	3.2
Vitamin B_1	mg	0.17
Vitamin B_2	mg	0.19
Niacin	mg	2.1
Vitamin B_6	mg	0.22
Folic acid	mg	0.022
d-Pantothenic acid	mg	0.58
Vitamin B_{12}	mg	0.001
Biotin	mg	0.005
Sodium	mg	105
Potassium	mg	244
Chloride	mg	200
Calcium	mg	204
Phosphorus	mg	160
Magnesium	mg	33
Iron	mg	1.5
Iodine	mg	0.015
Copper	mg	0.21
Zinc	mg	1.3
Manganese	mg	0.27

APPENDIX 2

Vitamin Diet Fortification Mixture

A mixture of the following vitamins in dextrose:

	Grams
Vitamin A concentrate (200,000 units per gram)	4.5
Vitamin D concentrate (400,000 units per gram)	0.25
Alpha tocopherol	5.0
Ascorbic acid	45.0
Inositol	5.0
Choline chloride	75.0
Menadione	2.25
p Amino benzoic acid	5.0
Niacin	4.5
Riboflavin	1.0
Pyridoxine hydrochloride	1.0
Thiamine hydrochloride	1.0
Calcium pantothenate	3.0
	mgms
Biotin	20.0
Folic acid	90.0
Vitamin B_{12}	1.35

CHAPTER 3

Ontogenetic Development of Liver Alcohol Dehydrogenase and Emergence of Fatty Liver in Rat Offspring Following Chronic Maternal Ethanol Ingestion

SYNOPSIS

A developmental study of alcohol dehydrogenase activity in rat liver was carried out. Comparisons made between this activity and certain kinetic properties of enzyme preparations from the progeny of alcohol-fed and sucrose pair-fed mothers indicated that chronic maternal ethanol consumption during pregnancy and lactation did not affect the ontogenetic development of the enzyme in the progeny. Fatty liver, however, developed in the alcoholic progeny, and a positive correlation was established between the appearance of alcohol dehydrogenase activity and the emergence of fatty liver in the offspring of alcoholic mothers.

INTRODUCTION

The liver plays a predominant role in the metabolism of ethanol. At low concentrations, 75-80% of the hepatic ethanol metabolism is carried out by alcohol dehydrogenase (ADH; EC 1.1.1.1) which is believed to be the rate limiting step (Hawkins & Kalant, 1972; Lieber, 1973; Thurman & McKenna, 1975). However, at high concentrations other enzymatic systems also contribute to the oxidation of ethanol. These include the microsomal ethanol oxidizing system (Lieber & DeCarli, 1972) and catalase (Keilen & Hartree, 1945). Limited oxidation of ethanol has also been demonstrated in a number of organs. However, such extra-hepatic oxidation never accounts for more than 10-15% of total ethanol metabolized (Bernstein, 1982). This relative organ specificity of ethanol metabolism explains why the liver is most significantly damaged and physiologically deranged as a result of ethanol ingestion.

The hepatotoxicity of chronic ethanol consumption has been widely documented (Lieber et al., 1975; Isselbacher, 1977; Nalpas & Berthelot, 1982). Prolonged ethanol ingestion has deleterious effects on hepatic protein, carbohydrate, and lipid metabolism (Ginsberg et al., 1974; Isselbacher, 1977), and some of these metabolic alterations may possibly result from products of ethanol metabolism or alterations in cell organelles (Cederbaum et al., 1974). In fact, previous studies from this (Hosein et al., 1980; Lee & Hosein, 1982) and other laboratories (Matsuda et al., 1979; Waring et al., 1981) have shown that, in the rat, chronic ethanol consumption may lead to alterations in the membrane structure and function of various liver cell organelles.

Recently, we have undertaken a study of the effects of chronic maternal ethanol consumption on the developing rat liver. Results showed

that growth and survival of progeny from the alcohol-treated rats were adversely affected (Rovinski et al., in the press). Since ethanol readily diffuses across the placenta, the fetus will be constantly exposed to any unmetabolized maternal ethanol present in the amniotic fluid. ADH, being the enzyme involved in ethanol metabolism, can be considered a possible defence mechanism in the fetal tissue against ethanol. It was therefore important to determine in our animal model the ontogenetic pattern of ADH activity in the fetal liver and the effects of chronic maternal ethanol ingestion on the in utero and postnatal development of its activity.

Although various studies of the development of ADH activity in the human (Pikkarainen & Räichä, 1967), guinea-pig (Räichä et al., 1967) and rat liver (Räichä et al., 1967; Rawat, 1976; Sjöblom et al., 1978, 1979; Horton & Mills, 1979) have been reported, only two have investigated the effects of maternal ethanol consumption. In the study by Rawat (1976), a liquid diet similar to that used in our regular feeding protocol was used. However, this author did not describe the analytical method utilized for the enzyme assay, and moreover since the enzyme activity was not measured in the control animals, the effect of maternal ethanol consumption on the development of ADH in the progeny cannot be determined from these studies. In the work of Sjöblom et al. (1979), ethanol was administered in a sucrose solution as the drinking fluid complementing a solid semi-synthetic diet for 4 weeks before the animals were mated; and up to 12 days after birth. The blood ethanol levels at the time of sacrifice were not measured, and ADH activity, measured in the total homogenate, was studied only up to 12 days after birth when, according to

the authors (Sjöblom et al., 1978), enzyme activity is only 40% of adult values.

In the present investigation, a complete developmental study of ADH activity has been carried out in fetal and postnatal rat liver cytosolic preparations, and comparisons were made between this activity and certain kinetic properties of enzyme preparations from the progeny of alcohol-fed and sucrose pair-fed mothers.

Finally, a variety of hepatic lesions, ranging from fat accumulation to fibrosis and cirrhosis, have been observed in humans (Lieber, 1983) and laboratory animals (Lieber & Rubin, 1969; Goheen et al., 1983) following prolonged alcohol exposure. Therefore, we also decided to investigate whether fatty infiltration develops in fetal and neonatal hepatic tissues following chronic maternal ethanol ingestion.

MATERIALS AND METHODS

Chemicals

The ethyl alcohol (100%) used in feeding experiments was purchased from Consolidated Alcohol Co. Ltd. (Toronto, Ont., Canada). Potassium chloride was purchased from J.T. Baker Chemical Co. (Phillipsburg, N.J., U.S.A.). Semicarbazide hydrochloride and sodium pyrophosphate were obtained from Fisher Scientific Inc. (Montreal, Que., Canada). Glycine (sodium salt) and NAD^+ were supplied by Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Vitamin Diet Fortification Mixture was obtained from ICN Nutritional Biochemicals (Montreal, Que., Canada), and Metrecal (vanilla flavor) was prepared by Mead Johnson (Belleville, Ont., Canada).

Chronic Maternal Ethanol Feeding

Virgin female Sprague-Dawley rats weighing 200-225 g were purchased from Canadian Breeding Farms (St. Constance, Quebec) and given water and Purina Rat Chow ad libitum for one week. Rats were then mated overnight and the appearance of sperm in the vaginal washings established day 1 of pregnancy. The pregnant rats were randomly divided into two groups and their diets changed to a nutritionally adequate low-fat Metrecal liquid diet providing 1 kcal/ml and supplemented with 3 g/l Vitamin Diet Fortification Mixture. The diet consumed by the designated alcoholic group contained ethanol which provided 37% of the total calories. The control group was pair-fed with the same Metrecal liquid diet, but with sucrose isocalorically substituted for ethanol. Both groups of rats were maintained on their respective diets throughout pregnancy and lactation. All pups were weaned at 21 days postnatally. Under these conditions, the intake of total nutrients and calories for both the maternal alcoholic and pair-fed control groups fulfill the nutritional requirements of

laboratory rats (NRC Subcommittee on Laboratory Animal Nutrition, 1978). The average ethanol intake ranged from 10 to 15.7 g/kg body weight/day, and blood ethanol concentrations (measured between 8 and 10 a.m.) in the progeny of ethanol-fed mothers ranged between 4 mM at the end of the gestational period to 40 mM at 25 days of age. The postnatal weight gain of the newborn pups of ethanol-fed mothers was reduced by 33% when compared to the corresponding pair-fed control pups. Litters were sacrificed between 8 and 10 a.m.

Tissue Preparations

The livers were dissected out and homogenized in ice cold 0.1 M KCl solution with a Potter-Elvehjem homogenizer to obtain 15% (w/v) homogenates. The homogenates were then fractionated by differential centrifugation as described by Messiha et al. (1979) to obtain the cytoplasmic fractions in which ADH activity was determined. Enzyme assays were performed on the same day the tissues were excised.

Blood Ethanol Measurements

Blood samples were collected from the neck after decapitation, and the ethanol concentration was measured enzymically (Bernt & Gutmann, 1974).

Alcohol Dehydrogenase Activity

ADH activity was measured as described by Rachamin et al. (1980), with minor modifications. The reaction mixture contained 2.1 ml of semicarbazide/glycine/pyrophosphate buffer, pH 8.7, 0.1 ml of 12.5 mM NAD^+ solution, 0.1 ml of 250 mM ethanol, and 0.2 ml of the cytoplasmic fraction. NADH formation at 22°C was followed for 5 min at 340 nm in a Beckman Model 25 spectrophotometer. Under these conditions enzyme activity was linear both with respect to time and the amount of cyto-

plasmic fraction used. ADH activity was expressed as nmoles of NADH formed/min/mg protein. Protein was determined by the method of Lowry et al. (1951) using recrystallized bovine serum albumin as standard.

Electron Microscopy

Small liver samples were fixed overnight in 2.5% glutaraldehyde in 0.1 M Na^+ cacodylate buffer, pH 7.3. The next day, the samples were washed three times with 0.1 M Na^+ cacodylate buffer, then post-fixed in a mixture containing 1.5% K^+ ferrocyanide and 1% O_sO_4 for 2 hr. The samples were dehydrated in turn with 70%, 90% and 100% ethanol, then with propylene oxide. They were finally embedded in Epon. Ultrathin sections were cut to approximately 700-800 Å with an LKB Ultratone III, and were stained successively with 2% aqueous uranyl acetate and with lead citrate (Reynolds, 1963). The electron micrographs were taken with a Philips 300 electron microscope.

Statistical Analysis

All results are expressed as means ± S.E.M., and the level of significance of the difference between treatment groups was assessed by two-tailed Student's t-test.

RESULTS AND DISCUSSION

The ontogenetic development of liver ADH activity in the progeny of ethanol and pair-fed control rats is shown in Fig. 1. ADH activity, although very low, was first detectable at day 19 of gestation in the fetal livers of rats from both maternal groups. At 21 days of gestation the specific activity of ADH increased to 12% of the adult values and was identical in both groups of animals. After birth, the enzyme activity increased almost linearly in both groups of animals from 3 days of age when the activity was about 25% of the adult values up to 14 days of age. At this age, ADH activity reached a plateau and adult values were consistently obtained between 14 and 18 days postnatally. Although we used a different feeding protocol and a modified analytical method for the enzyme assay, our observations on the developmental pattern of ADH activity in the rat are similar to those reported in previous studies (Räihä et al., 1967; Rawat, 1976; Horton & Mills, 1979). However, in another investigation (Sjöblom et al., 1978) the ADH activity was found to reach adult values at 47 days of age. This discrepancy may be attributed to the different ADH assay used or, alternatively, to the particular strain of rat employed.

The results shown in Fig. 1 also indicate that no significant differences in specific ADH activity could be demonstrated between the pups of mothers fed the different diets. Thus it appears that in our animal model, chronic maternal ethanol ingestion during pregnancy and lactation does not lead to alterations in the ontogenetic development of ADH activity despite the fact that the blood alcohol values varied between 4 and 10 mM during the last 3 days of gestation, increased to 20 mM at the end of the first postnatal week and reached values between

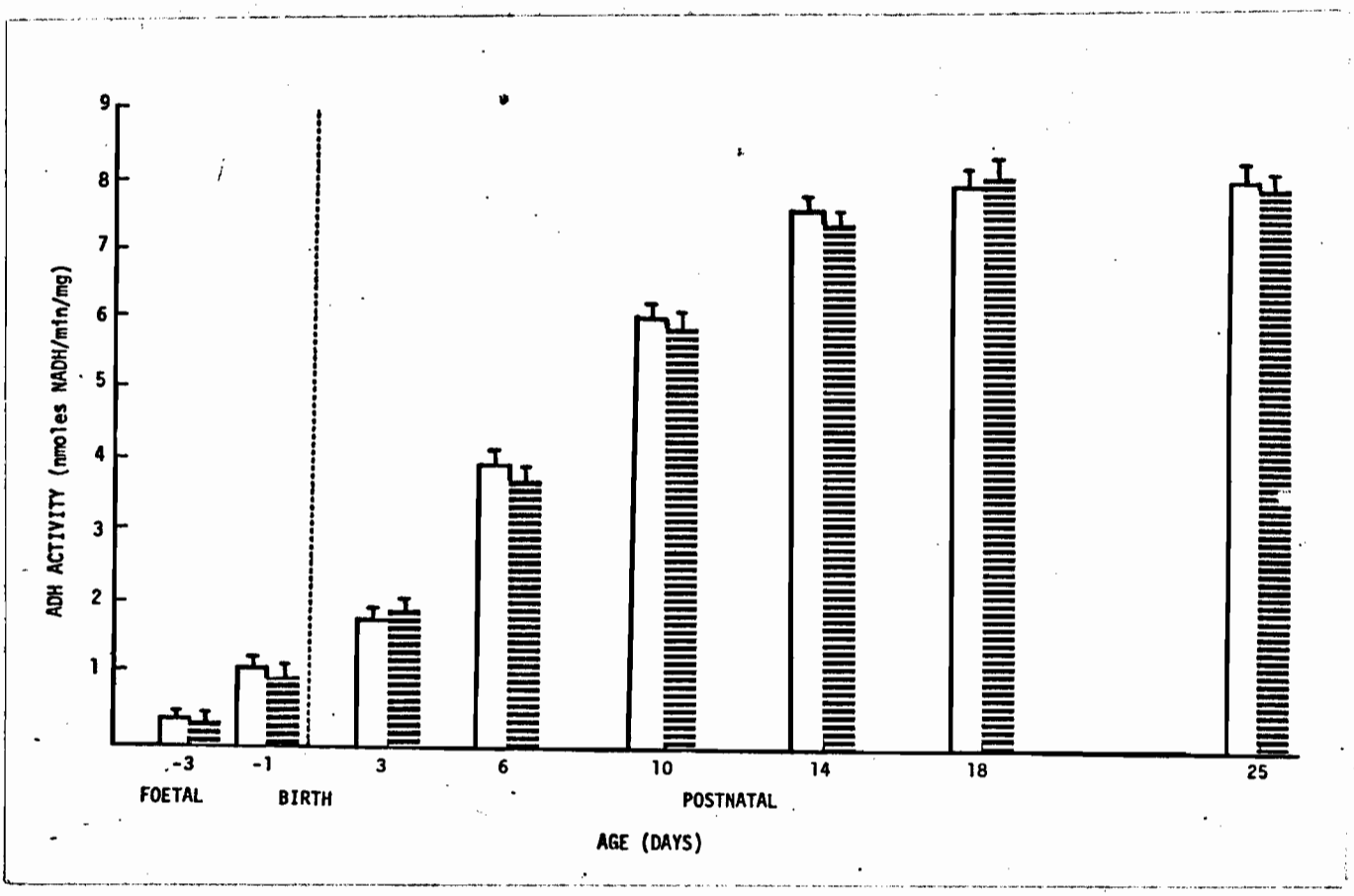

ADH ACTIVITY (nmoles NADH/min/mg)
9
8
7
6
5
4
3
2
1
-3
-1
3
6
10
14
18
25
FOETAL
BIRTH
POSTNATAL
AGE (DAYS)

FIGURE 1

Ontogenetic development of liver alcohol dehydrogenase in the rat. ADH activity was determined in control (open bars) and alcoholic (striped bars) pups as described in the text. Values are means ± S.E.M. from three to six experiments.

35 and 40 mM at 18-25 days of age. Whether this is the case for the rate of ethanol metabolism *in vivo* is uncertain, although in various studies (Räihä *et al.*, 1967; Rawat, 1976) the developmental changes in ADH activity were found to correlate closely with the ethanol-oxidizing capacity of liver slices. Since we employed a pair-feeding regimen, and since adult values of the specific activity of ADH in the progeny of both maternal groups are similar to those found in adult rats of the same strain fed a standard laboratory diet (Messiha & Hughes, 1979), it is highly unlikely that nutritional imbalances secondary to ethanol treatment could have masked any changes induced by ethanol treatment on the specific activity of ADH.

The results shown in Table 1 indicate that no significant differences were found in the apparent K_m values for NAD^+ and ethanol, as determined by the method of Lineweaver & Burk (1934), between liver preparations from pups of both maternal groups at all ages studied. These results seem to indicate that during the period of development studied, the enzyme preparations were composed of a similar isoenzyme pattern which did not seem to be altered by chronic maternal ethanol ingestion.

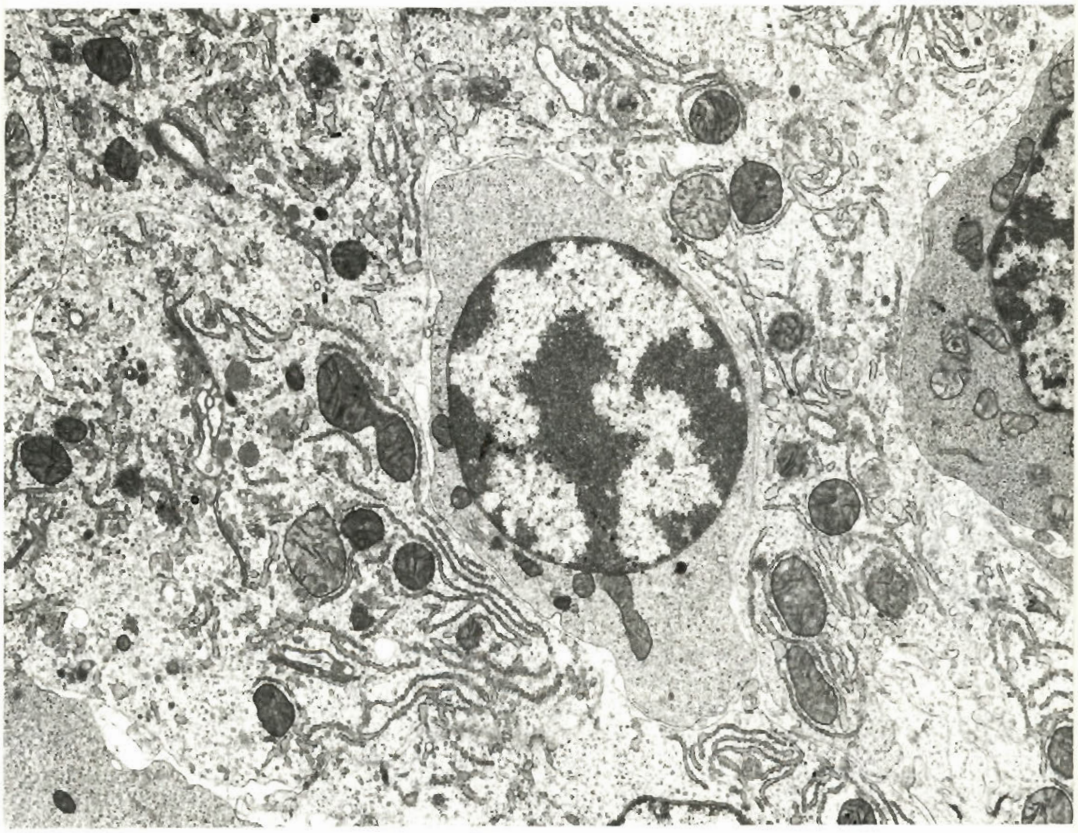
As shown in Figures 2 and 3, although offspring from pair-fed control mothers exhibited normal liver morphology, a marked accumulation of fat was first detected in the livers of alcoholic offspring at day 19 of gestation and persisted after birth. It is interesting to notice that a positive correlation can be established between the appearance of ADH activity (Fig. 1) and the emergence of fatty liver (Fig. 3) in alcoholic offspring. Because metabolic derangements in the liver during ethanol administration are believed to be caused primarily by changes in the

Table 1. Effect of maternal ethanol administration on the apparent K_m values for ethanol and NAD^+ in the progeny during development.

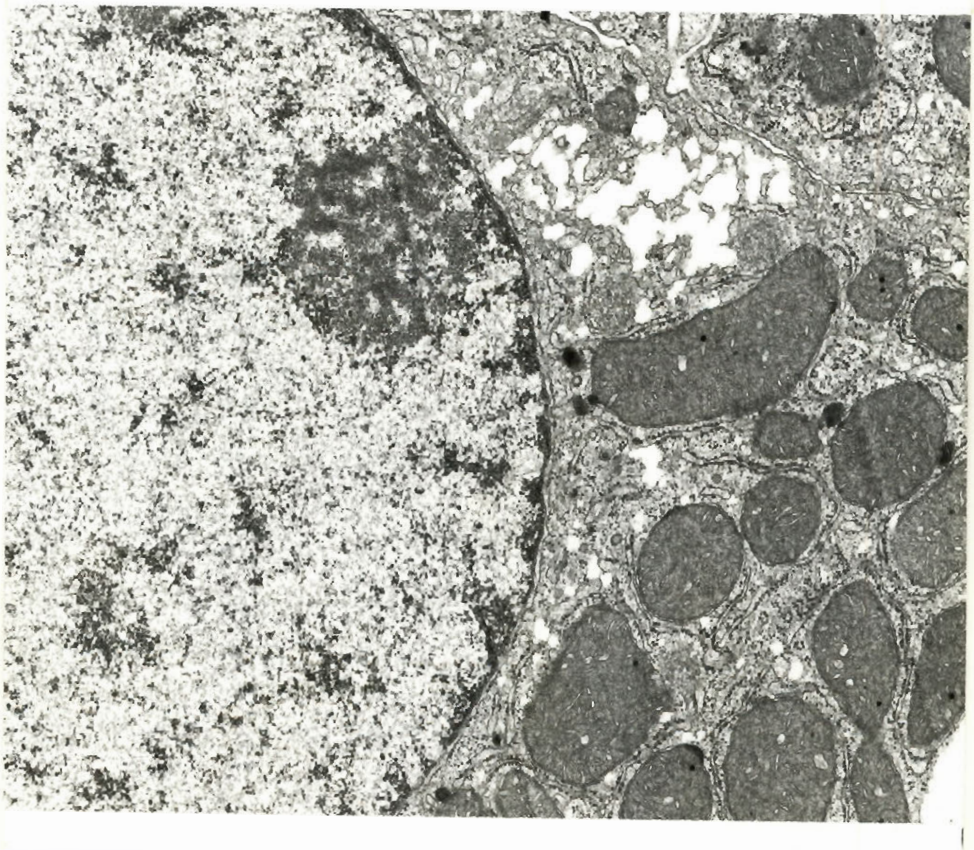
Livers from pups of various postnatal ages were dissected out and homogenized in ice cold 0.1 M KCl to obtain 15% (w/v) homogenates which were fractionated by differential centrifugation as described by Messiha et al. (1979) to obtain the cytoplasmic fractions. ADH activity was determined by the method of Rachamin et al. (1980) as described in the text, and the apparent K_m values for ethanol and NAD^+ were determined by the method of Lineweaver and Burk (1934). Results are means ± S.E.M. from four to six experiments.

AGE (days)		K_m for NAD^+ (mM)	K_m for ETHANOL (mM)
3	Control	0.06 ± 0.01	0.20 ± 0.03
	Alcoholic	0.04 ± 0.02	0.21 ± 0.05
10	Control	0.04 ± 0.02	0.20 ± 0.03
	Alcoholic	0.04 ± 0.01	0.24 ± 0.02
18	Control	0.05 ± 0.01	0.24 ± 0.04
	Alcoholic	0.04 ± 0.01	0.22 ± 0.03

A



B



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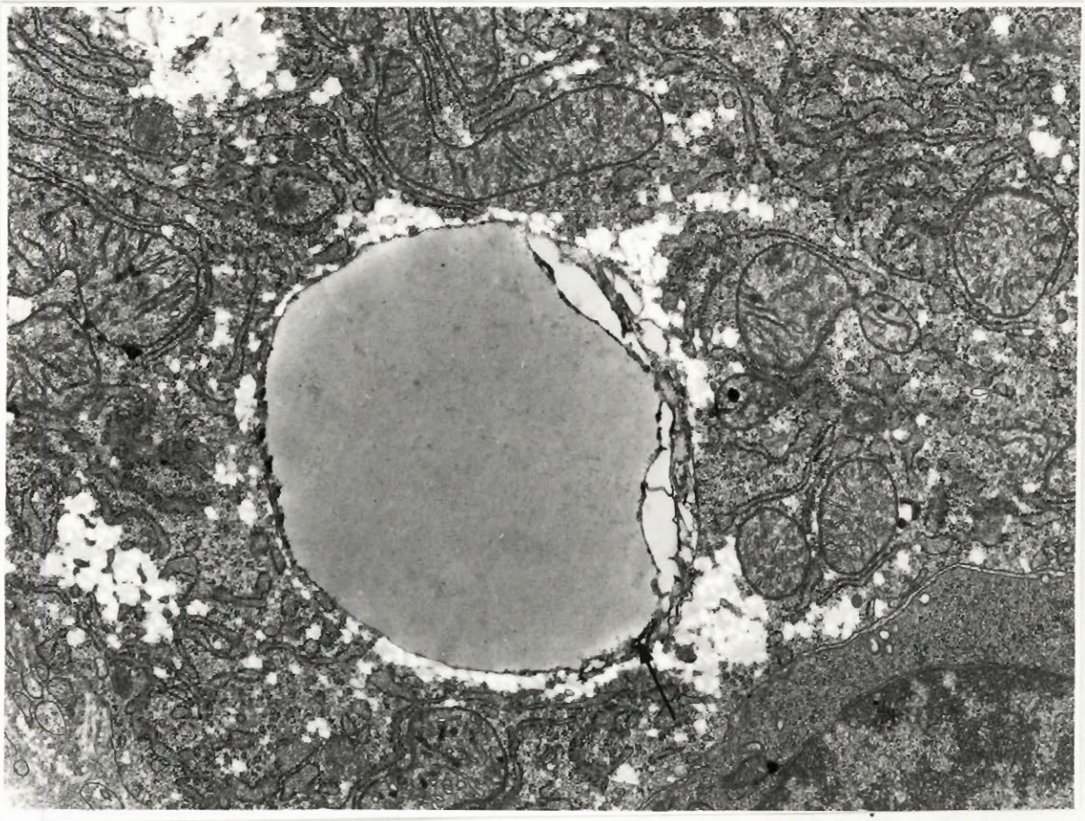

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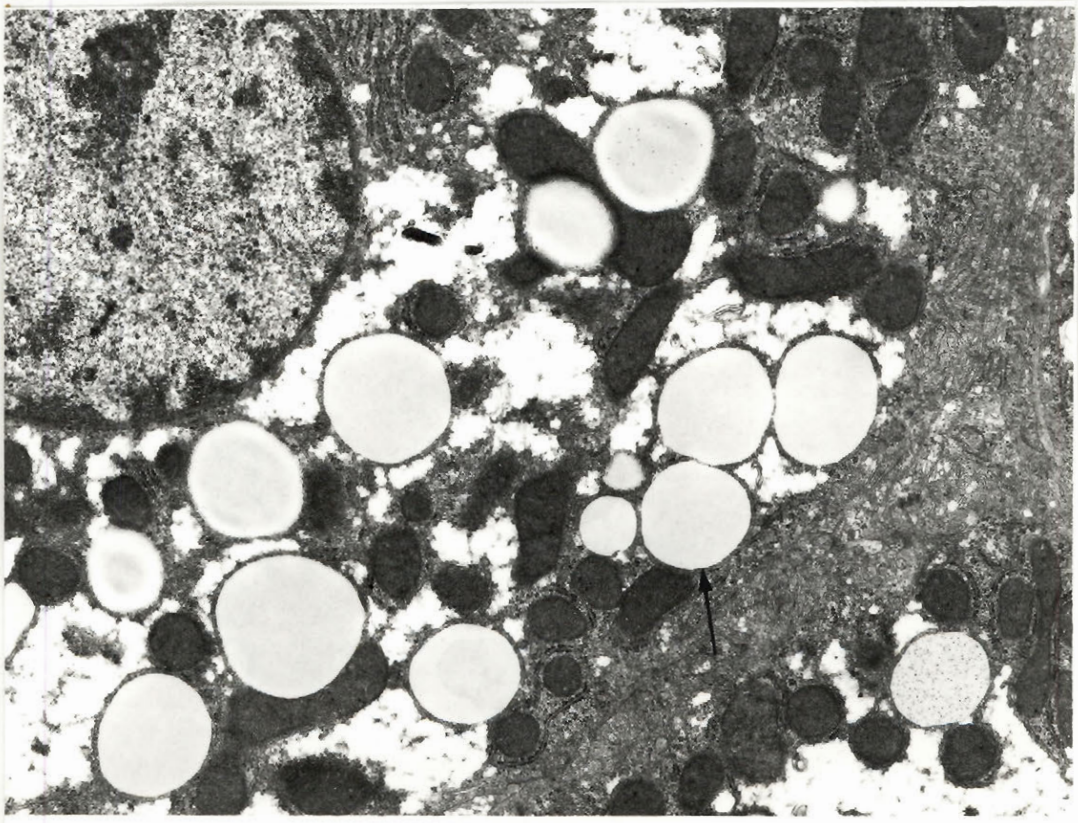

FIGURE 3

Electron micrographs of livers from the progeny of alcoholic mothers. Liver tissue from (A) 19-day-old alcoholic fetal rats (x39,280) and from (B) 25-day-old alcoholic pups (x32,400) was isolated and processed for electron microscopy examination as described in Materials and Methods. Distinct fatty droplets can be observed in both samples.

[NADH/NAD^+] ratio (Lieber & DeCarli, 1977), and since maternal ethanol ingestion in the rat was shown to produce an increased [NADH/NAD^+] ratio in the hepatic cytoplasmic and mitochondrial fractions of the progeny (Rawat, 1976), one might expect that changes in the hepatic redox state of the progeny may lead to hepatic lesions, such as fatty liver, and to alterations in the metabolic capacity of the liver during development.

In summary, our results indicate that chronic maternal ethanol administration during pregnancy and lactation did not affect the ontogenetic development of ADH activity in the progeny. Fatty liver, however, developed in the alcoholic progeny, and it is suggested that changes induced by ethanol metabolism in the hepatic redox state of alcoholic offspring (Rawat, 1976) may be important in contributing to the adverse effects of chronic maternal ethanol ingestion on the development of the progeny.

ACKNOWLEDGEMENTS

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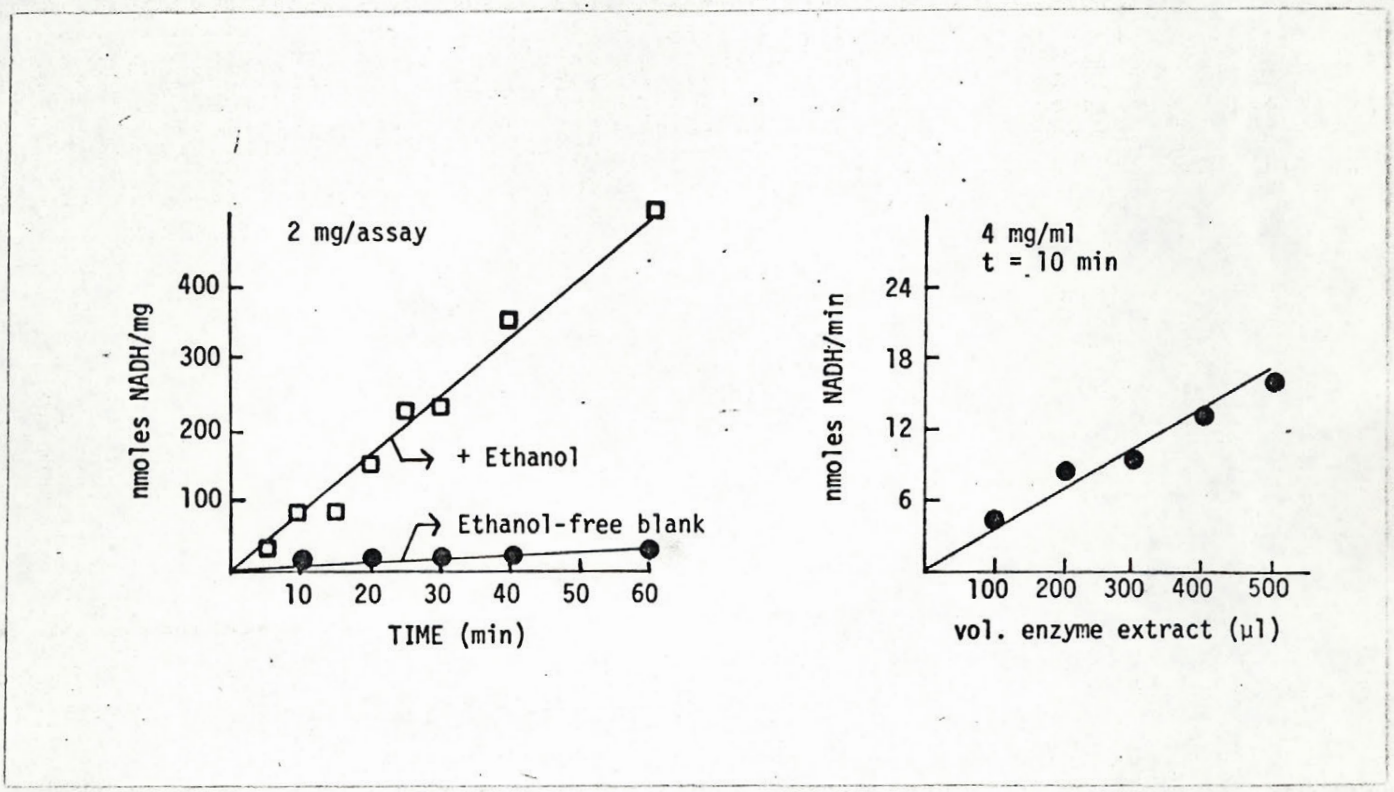
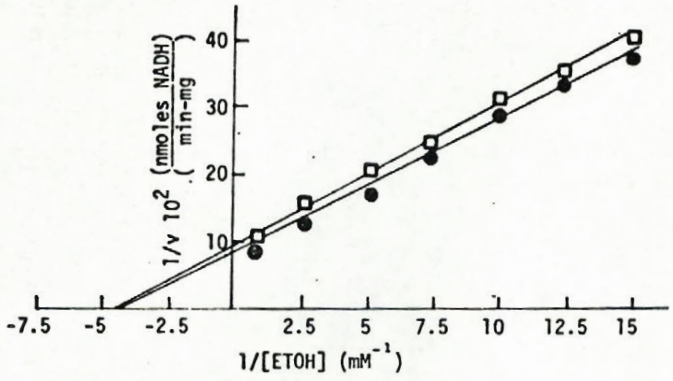

2 mg/assay
nmoles NADH/mg
400
300
200
100
+ Ethanol
Ethanol-free blank
10
20
30
40
50
60
TIME (min)
4 mg/ml
t = 10 min
nmoles NADH/min
24
18
12
6
100
200
300
400
500
vol. enzyme extract (µl)

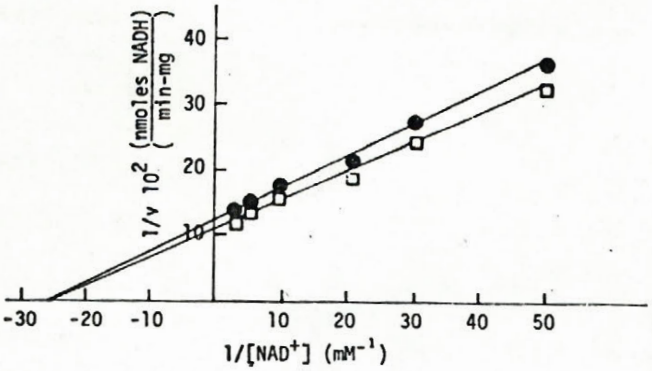
APPENDIX 1

Time course and protein dependence of liver ADH activity from the 18-day-old progeny of control rats. Values represent the means of three experiments.

A


1/v 10² (nmoles NADH / min-mg)
40
30
20
10
-7.5
-5
-2.5
2.5
5
7.5
10
12.5
15
1/[ETOH] (mM⁻¹)

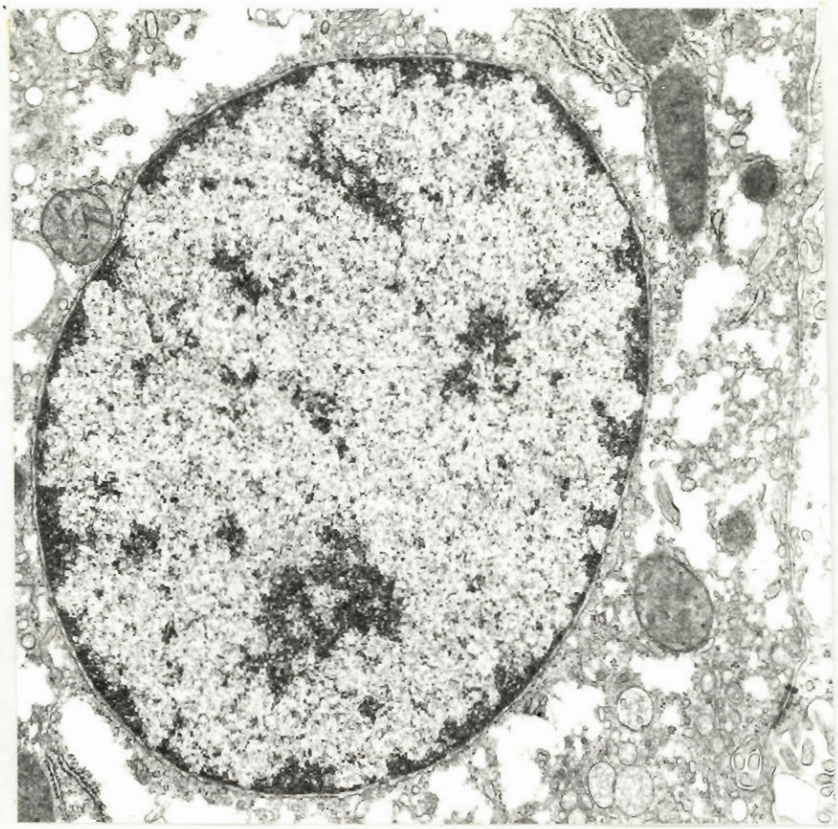
B


1/v 10² (nmoles NADH / min-mg)
40
30
20
10
-30
-20
-10
10
20
30
40
50
1/[NAD⁺] (mM⁻¹)

APPENDIX 2

Lineweaver-Burk plots of (A) the effect of ethanol concentration and (B) the effect of NAD^+ concentration in 18-day-old pups from alcoholic (●) and pair-fed control (□) mothers on liver ADH activity. All values represent the means of four to six experiments.

A



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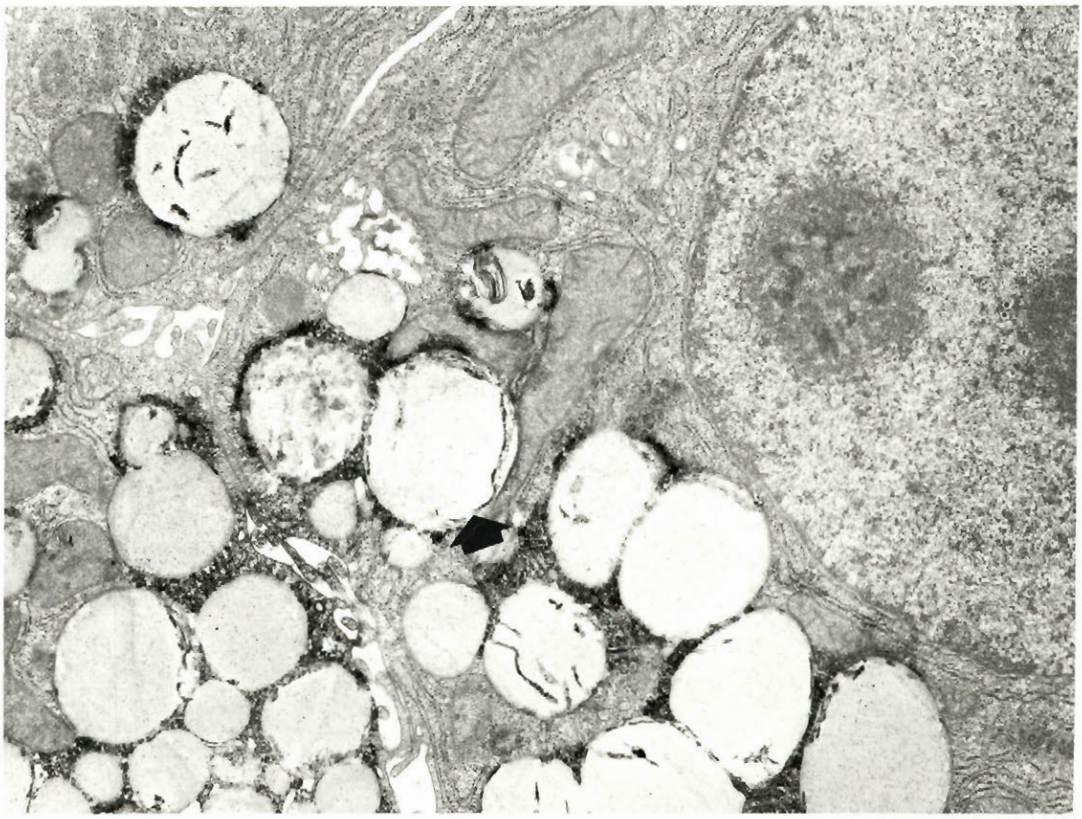


APPENDIX 3

Electron micrographs of livers from newborn rats. Liver tissue from 1-day-old control (A) and alcoholic (B) pups was processed for electron microscopy examination as described in Materials and Methods. Distinct fatty droplets can be observed in the newborn alcoholic pups. (Magnification, x39,280).

CHAPTER 4

Isolation of Liver Plasma Membranes

4.1 ISOLATION PROCEDURE

Livers of newborn pups from the alcoholic and pair-fed control groups were rapidly excised and hepatic plasma membranes isolated essentially by the method of Neville (1) as described by Wolfe et al. (2). Briefly, the livers were minced in 20 vol. of ice-cold 1.0 mM $NaHCO_3$ and homogenized with six up-and-down strokes in a Potter-Elvehjem homogenizer. Homogenates were filtered through four layers of cheese-cloth and centrifuged for 10 min at 4000 g. The pellet was resuspended in 1.0 mM $NaHCO_3$ and 69% (w/w) sucrose was added to produce a final concentration of 47.5% (w/w). Sucrose, 42.3% (w/w), was then layered over the resuspended pellet, and the samples were centrifuged at 100,000 g for 2 hr. The partially purified membranes which floated on top of the 42.3% sucrose were removed, washed, and resuspended twice with 50 mM Tris buffer, pH 7.5.

4.2 ELECTRON MICROSCOPY

Aliquots of plasma membranes were pelleted in bottleneck Beem capsules and fixed overnight in 2.5% glutaraldehyde in 0.1 M Na^+ cacodylate buffer, pH 7.3. The next day, the pellets were washed three times with 0.1 M Na^+ cacodylate buffer, then post-fixed in 1% O_sO_4 in Palade buffer for 1 hr. The samples were dehydrated in turn with 70%, 90% and 100% ethanol, then with propylene oxide. They were finally embedded in Epon. Ultrathin sections were cut to approximately 700-800 Å with an LKB Ultratome III, and were stained successively with 2% aqueous uranyl acetate and with lead citrate (3). The electron micrographs were taken with a Phillips 300 electron microscope.

As shown in Figures 1 and 2, hepatic plasma membranes from 25-day-old control and alcoholic pups are similar, and appear mostly vesiculated and minimally contaminated with other cell organelles.

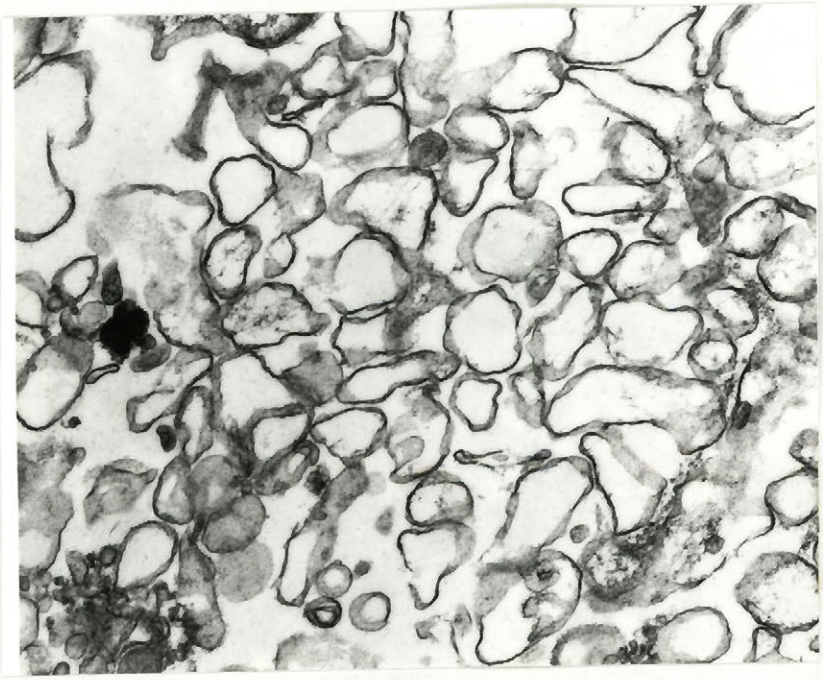


FIGURE 1

Electron micrograph of rat liver plasma membrane from the control progeny. Aliquots of liver plasma membranes from 25-day-old control pups were processed for electron microscopy examination as described in the Text. (Magnification, x78,000).

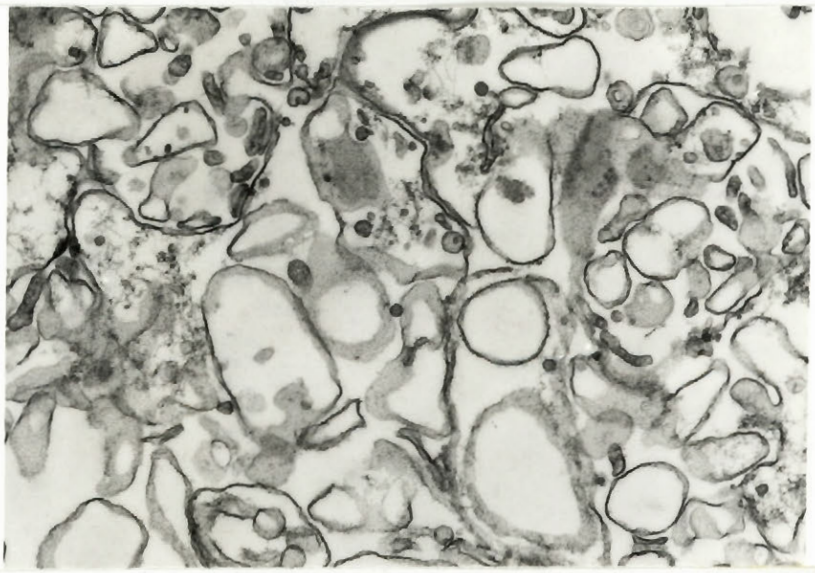


FIGURE 2

Electron micrograph of rat liver plasma membrane from the alcoholic progeny. Aliquots of liver plasma membranes from 25-day-old alcoholic pups were processed for electron microscopy examination as described in the Text. (Magnification, x78,000).

4.3 MARKER ENZYMES

Marker enzymes for various cellular organelles were assayed in the isolated hepatic plasma membrane fractions (4,5). As shown in detail in Chapter 7 (Table I), hepatic plasma membrane fractions from both alcoholic and pair-fed control pups are highly enriched in 5'-nucleotidase, a specific plasma membrane marker. The extent of microsomal and mitochondrial contamination, assessed with the measurement of the relative specific activities of glucose-6-phosphatase and monoamine oxidase, respectively, is minimal (Chapter 7, Table I). Moreover, the relative specific activities of the marker enzymes are independent of either age or diet treatment.

4.4 EQUILIBRIUM DENSITY MEASUREMENTS

The buoyant density of liver plasma membranes from alcoholic and control pups was also characterized in order to investigate whether alcohol feeding led to alterations in the physical properties of the membranes.

Membrane aliquots (0.25-0.50 mg protein) were layered on top of a preformed 30-50% (w/w) sucrose gradient and spun for 24 hours at 100,000 g using a SW 27.1 rotor. After centrifugation 0.3 ml aliquots were collected, and the sucrose density in each aliquot was determined at room temperature with a Bausch & Lomb refractometer. The activity of 5'-nucleotidase, a specific plasma membrane marker, was also measured in each aliquot, and a density profile was obtained by plotting the sucrose density against the enzyme activity.

A typical density profile of hepatic plasma membranes from 15-day-old alcoholic and control pups is shown in Figure 3. The equilibrium density (the peak values obtained for the activity of 5'-nucleotidase) was similar in both membrane samples, and as shown in Table 1, maternal ethanol administration did not induce any alterations in the buoyant density of postnatal rat liver plasma membranes.

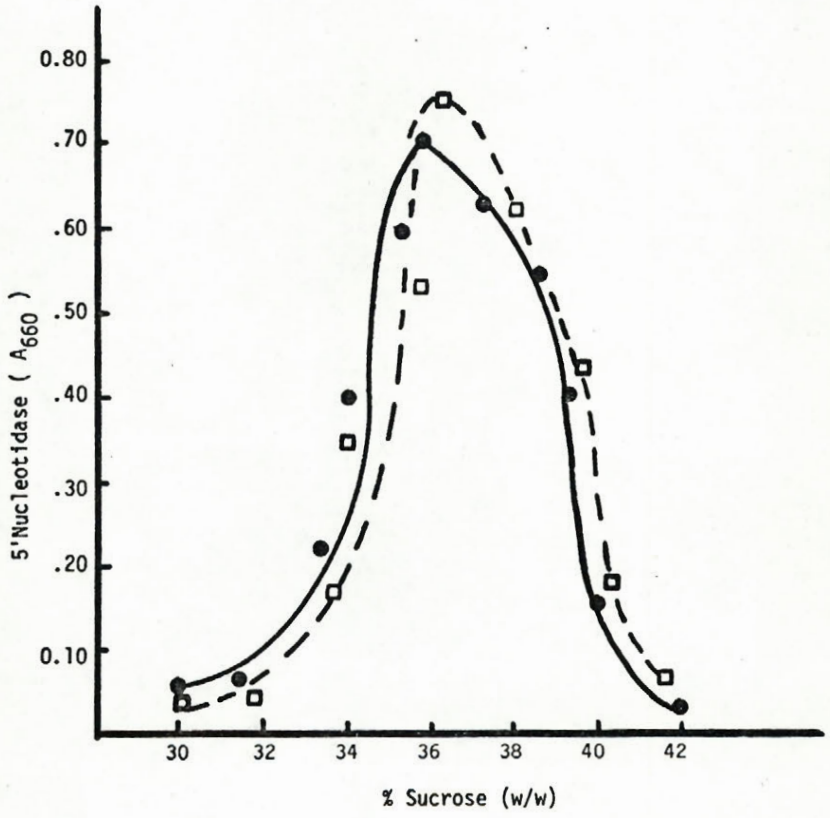

0.80
.70
.60
.50
.40
.30
.20
0.10
5'Nucleotidase (A_{660})
30
32
34
36
38
40
42
% Sucrose (w/w)

FIGURE 3

Typical density profile of liver plasma membrane from newborn rats.

Hepatic plasma membranes from 15-day-old control (□- - -□) and alcoholic (●——●) pups were subject to isopycnic centrifugation. A density profile was obtained by plotting the sucrose density of collected aliquot fractions against the activity of a plasma membrane marker enzyme as described in the Text.

TABLE 1

Effect of Maternal Ethanol Administration on the Equilibrium Density of Postnatal Rat Liver Plasma Membranes

Age		Equilibrium Density: % Sucrose (w/w)
5	control	37.1 ± 0.4
	alcoholic	36.3 ± 0.5
15	control	36.4 ± 0.8
	alcoholic	36.0 ± 0.6
25	control	37.8 ± 0.5
	alcoholic	36.7 ± 0.8

Plasma membranes were isolated as described in the Text. Equilibrium densities after isopycnic centrifugation for 25 hr in a 30-50% (w/w) sucrose gradient were measured with a Bausch & Lomb refractometer at room temperature. Values represent means ± S.E.M. of four to six experiments.

4.5 REFERENCES

1. Neville, D.M. (1968) Biochim. Biophys. Acta 154, 540-552.

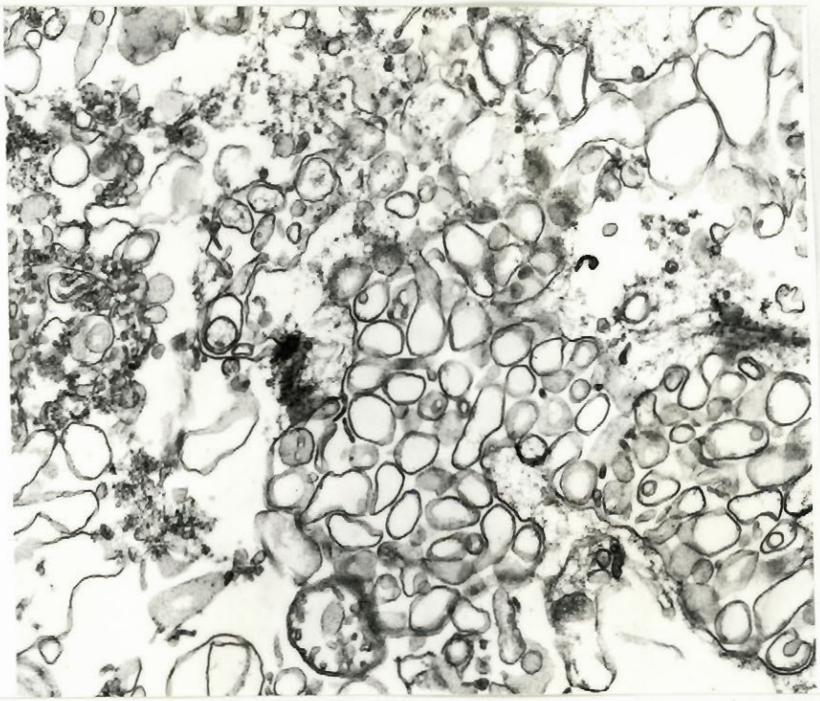
2. Wolfe, B.B., Kendall-Harden, T. and Molinoff, P.B. (1976) Proc. Natl. Acad. Sci. USA 73, 1343-1347.

3. Reynolds, E.S. (1963) J. Cell Biol. 17, 208-212.

4. Aaronson, N.N. and Touster, O. (1974) in Methods in Enzymology (Fleisher, S. and Packer, L., eds.), Vol. 31, pp. 90-102, Academic Press Inc., New York.

5. Sourkes, T.L., Missala, E.K., Bastomsky, C.H. and Fang, T.Y. (1977) Can. J. Biochem. 55, 780-795.

A



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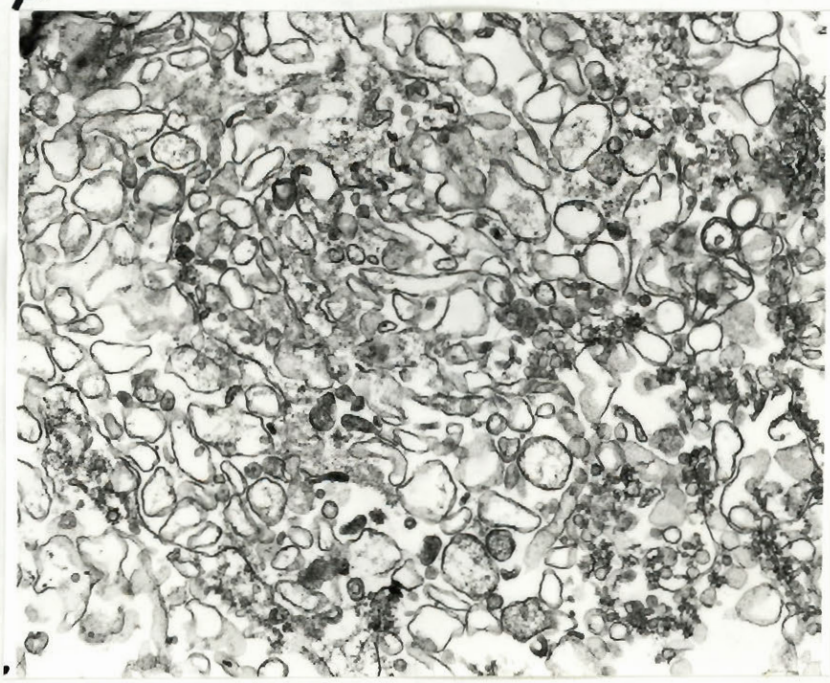

APPENDIX 1

Electron micrographs of liver plasma membranes from newborn rats.

Aliquots of liver plasma membranes from 1-day-old control (A) and alcoholic (B) pups were processed for electron microscopy examination as described in the Text. Both membranes appear mostly vesiculated. (Magnification, x50,000).

CHAPTER 5

Effect of Maternal Ethanol Ingestion During Pregnancy and Lactation on the Structure and Function of the Postnatal Rat Liver Plasma Membrane: Assessment with [^{3}H] Prazosin Binding to the Hepatic α_1-Adrenergic Receptors

ABSTRACT

A liquid low fat nutritionally adequate Metrecal diet in which alcohol contributed 37% of the total calories was given to pregnant rats and maintained during lactation. Control rats were pair-fed with an isocaloric sucrose-Metrecal diet. After birth, litters were killed at different ages (days 1-30) and the results showed that growth and survival of progeny from the alcohol-treated rats were adversely affected. Likewise, the wet weights of livers from such pups were consistently less than from the pair-fed controls. The yield of hepatic plasma membrane protein per wet liver weight was constant and independent of either age or diet. Using [^{3}H] prazosin as radioligand, equilibrium binding studies were carried out to monitor changes in the structure and function of the plasma membrane in the new born pups concomitant with the development of α_1-adrenergic receptors. Results obtained with the alcohol-fed pups showed that the binding affinity (K_D) was not altered throughout. However, the receptor density (Bmax) was decreased significantly. This decrease ranged from 60 to 70% in pups 6 to 15 days old; 45% at 20 days; and 30% in pups at 25 and 30 days of age. These observations suggest that maternal ethanol ingestion affected the postnatal development of rat liver plasma membranes. Furthermore, by using the hepatic α_1-adrenergic receptor as a metabolic probe, we deduce that a possible impairment exists in the capacity of the alcoholic progeny to respond to the hormonal action of epinephrine. Such a defect may contribute to impaired growth and metabolism in these young animals.

INTRODUCTION

A pattern of multiple congenital abnormalities associated with chronic maternal alcohol ingestion during pregnancy in humans was described by Jones and Smith [1] who collectively named them the "Fetal Alcohol Syndrome" (FAS). Animal models of FAS have since been developed in various laboratories [2-6], and most of these studies have implicated alcohol as the teratogen. However, there is little direct information on the mechanism(s) of the deleterious action(s) of alcohol at the molecular level.

Since alcohol readily diffuses across the placenta, the fetus will be exposed constantly to any unmetabolized maternal alcohol present in the amniotic fluid. The fact that liver alcohol dehydrogenase activity in the young rat only appears a few days prior to birth [7,8] suggests that fetal development in a dilute solution of alcohol is possible and dependent on the amount and duration of alcohol consumed by the mother.

When micro-organisms or mammalian cells are grown in the presence of alcohol, persistent changes in their plasma membrane lipid composition take place [9,10]. With the pregnant rat fed alcohol chronically, the fetus, if continuously bathed in a dilute solution of alcohol, might conceivably develop changes in membrane lipid composition analogous to those found in the microorganisms. Since the plasma membrane performs a wide range of important physiological functions, any induced alteration in its structure as a result of chronic alcohol consumption may, in turn, cause serious changes in those functions.

Many hormones regulate cellular metabolism by acting through plasma membrane-bound receptors. In the rat liver, epinephrine plays an important role in the regulation of carbohydrate metabolism, and its

actions are believed to be mediated through α_1-adrenergic receptors [11]. Hepatic membrane hormone receptors are intrinsic membrane-bound proteins. Previous work in this and other laboratories [12-14] has shown that membrane-bound proteins are highly sensitive to fluidity changes in their lipid microenvironment. The hepatic α_1- adrenergic receptors presumably are regulated in the same manner. Chronic perturbation of the plasma membrane through prolonged alcohol exposure may, therefore, be envisioned to affect the function of these receptor proteins.

Recent work in our laboratory with adult rats indicated that chronic alcohol feeding resulted in alteration of the liver plasma membrane as reflected by a significant diminution of α_1-adrenergic receptor density, without affecting its binding affinity [15]. In the present work, a similar series of experiments was performed to look for possible adaptive modifications in the liver plasma membrane of pups born of alcoholic mothers.

MATERIALS AND METHODS

Chemicals

Phentolamine HCl was a gift from Dr. G. Kunos (Department of Pharmacology and Experimental Therapeutics, McGill University). [^{3}H] prazosin (17.1 Ci/mmol) and liquifluor were obtained from New England Nuclear, Canada. Recrystallized bovine serum albumin was purchased from the Sigma Chemical Co. Sucrose, glacial acetic acid, toluene, and phenol reagent were obtained from Fisher Scientific Inc. NCS was purchased from the Amersham/Searle Corp. Vitamin Diet Fortification Mixture was obtained from ICN Nutritional Biochemicals, Canada. Metrecal was prepared by Mead Johnson, Canada. The ethyl alcohol (100%) used in feeding experiments was purchased from Consolidated Alcohol Co. Ltd., Toronto, Ontario.

Animal Treatment

Virgin female Sprague-Dawley rats weighing 200-225 gm were purchased from Canadian Breeding Farms (St. Constance, Quebec) and housed in polycarbonate shoe-box cages. They were maintained under constant temperature (22 ± 1°C) and humidity (31-40%) conditions with a 12 hr light cycle (7 a.m. to 7 p.m.), and were given water and Purina Rat Chow ad libitum. After an adjustment period of 1 week they were mated overnight with male Sprague-Dawley rats on a one to one ratio. The appearance of sperm in the vaginal washings the following morning established day 1 of pregnancy. The pregnant rats were randomly divided into three groups. Two groups had their diet changed to a totally liquid low-fat Metrecal-sucrose diet providing 1 kcal/ml, which was supplemented with 3 g/l Vitamin Diet Fortification Mixture. The designated experimental alcohol group was switched to a new diet containing ethanol. This was introduced gradually so that ethanol first provided 10% of the total

calories in that diet. After every 2 days, calories derived from ethanol were increased to 20, then 30 and finally 37% of the diet. By the end of the first week of pregnancy, animals in this group were consuming a Metrecal-ethanol liquid diet in which proteins contributed 16%, fat 5%, carbohydrate 42%, and ethanol 37% of the total calories. The pair-fed control animals were maintained on the Metrecal-sucrose diet such that the total calories consumed equalled that of the alcoholic animals ($\simeq$ 85-95 kcal/day; 1 cal = 4.184 J), and this was achieved by matching ethanol-derived calories with sucrose. Both groups of rats were maintained on their respective diets throughout pregnancy and lactation. The third group of pregnant females was kept as an additional set of controls on Purina Rat Chow and water ad libitum throughout pregnancy and lactation. All animals were weighed every 2-3 days throughout. The newborn pups were weaned at 21 days and maintained on the liquid diets fed to their respective mothers, until they were 30 days old.

Tissue Preparation

Newborn pups from the alcoholic and pair-fed control groups were killed at specific ages up to 30 days. The livers were rapidly excised and hepatic plasma membranes isolated essentially by the method of Neville [16] as described by Wolfe et al. [17]. Briefly, the livers were minced in 20 vol. of ice-cold 1.0 mM $NaHCO_3$ and homogenized with six up-and-down strokes in a Potter-Elvehjem homogenizer. Homogenates were filtered through four layers of cheesecloth and centrifuged for 10 min at 4000 g. The pellet was resuspended in 1.0 mM $NaHCO_3$ and 69% (w/w) sucrose was added to produce a final concentration of 47.5% (w/w). Sucrose, 42.3% (w/w), was then layered over the resuspended pellet, and the samples were centrifuged at 100,000 g for 2 hr. The partially puri-

fied membranes which floated on top of the 42.3% sucrose were removed, washed, and resuspended twice with 50 mM Tris buffer, pH 7.5. The membranes in the final resuspension were used for binding assay.

Equilibrium Binding Studies

α_1-adrenergic receptor binding was determined using [^{3}H] prazosin as the radioligand. Unless otherwise specified, all assays were conducted in triplicate. A typical binding assay contained 125 µl of membrane suspension (in 50 mM Tris buffer, pH 7.5), assay buffer (4 mM $MgSO_4$, 0.8 mM ascorbate, and 50 mM Tris, pH 7.5), and radiolabelled ligand in a final volume of 250 µl. Specific binding was defined as the difference between binding of the radioligand in the absence and in the presence of 10 µM phentolamine. Membrane (0.075 to 0.125 mg protein) suspensions were incubated for 15 min at 31°C, and the reaction was terminated by addition of 3 ml of ice-cold assay buffer. The incubation mixtures were rapidly filtered under vacuum through Whatman GF/C glass fiber filters. The filters were washed with two 6-ml rinses of ice-cold buffer, transferred to plastic vials, and dried overnight. Radioactive ligand was extracted from the filters by digestion with 300 µl NCS for 2-4 hr at room temperature (22 ± 1°C). Liquifluor (10 ml) and glacial acetic acid (15 µl) were added to the vials which were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer with an efficiency of 35%. Non-specific binding was usually 10-15% of the total radioactivity bound.

Protein Determination

Protein was determined by the method of Lowry et al. [18] using recrystallized bovine serum albumin as standard.

Statistical Analysis

Statistical significance between mean values was examined using the two-tailed Student's t-test. Least squares linear regression analysis was used to derive the Scatchard plots [19].

RESULTS

Nutritional and Reproductive Characteristics

The amount of food and ethanol consumed by animals in the maternal alcoholic group is shown in Table 1. The average food intake for animals in this group ranged from 180 to 300 kcal/kg body weight/day, and the average ethanol intake ranged from 10 to 15.7 g/kg body weight/day. The intake of total nutrients and calories for both the control and alcoholic maternal groups fulfilled the nutritional requirements of laboratory rats [20]. At the time of sacrifice, blood samples from suckling pups were collected from the neck and ethanol concentration was measured enzymically [21]. Blood ethanol levels varied between 4 and 10 mM during the last 3 days of gestation, increased to 20 mM at the end of the first postnatal week, and reached values between 35 and 40 mM at 18-30 days of age.

As indicated in Table 2, no significant differences were found in maternal weight gain, litter size, and average litter weight at birth between animals in the alcoholic and pair-fed sucrose control groups. However, as shown in Fig. 1 and Table 2, animals fed water and Purina Rat Chow ad libitum during pregnancy gained more weight than those in the pair-fed groups.

Postnatal Physical Performance

As shown in Table 2 and Fig. 2, newborn pups of alcohol-fed mothers gained significantly less weight than pups from either pair-fed sucrose or Rat Chow-fed control mothers. Pups belonging to the pair-fed sucrose group did not gain as much weight as those from the Rat Chow-fed control group, but this may be a consequence of the restriction in daily caloric intake due to pair-feeding.

TABLE 1

TOTAL FOOD AND ETHANOL INTAKE DURING GESTATION

Gestation Day	Energy intake (kcal/kg/day)	ETOH intake (g/kg/day)	Protein intake (g/kg/day)
1 - 6	180 ± 10[a]	10 ± 1[a]	6.4 ± 0.3[a]
7 - 14	300 ± 20	15.7 ± 0.9	11 ± 1
15 - 22	260 ± 20	13.8 ± 0.7	9 ± 1

[a] Values represent the means ± S.E.M. of five animals and they are expressed as daily intake per kg body weight.

TABLE 2

REPRODUCTIVE AND POSTNATAL PHYSICAL CHARACTERISTICS

Physical Parameters	Treatment Conditions: Ethanol	Treatment Conditions: Pair-fed	Water and Rat Chow ad libitum
N[a]	11	10	5
Viable Offspring	140	121	62
Number of Stillbirth	6	2	0
Average Offspring per litter (±S.E.M.)	12.7 ± 0.4	12.1 ± 0.8	12.4 ± 0.8
Average Weight (g) per pup at birth (±S.E.M.)	5.7 ± 0.1	5.8 ± 0.3	6.5 ± 0.1
Average Maternal Weight Gain (g) during pregnancy (±S.E.M.)	123 ± 5[b]	122 ± 5[b]	150 ± 10[b]
Average Body Weight Gain (g) per pup up to 30 days after birth (±S.E.M.)	38 ± 1[c]	60 ± 1	71 ± 4
% Survival	70	94	95

[a] Number of litters.

[b] N = 5 for all treatment conditions.

[c] Significantly different ($P < 0.05$) from control values as determined by the two-tailed Student's t-test.

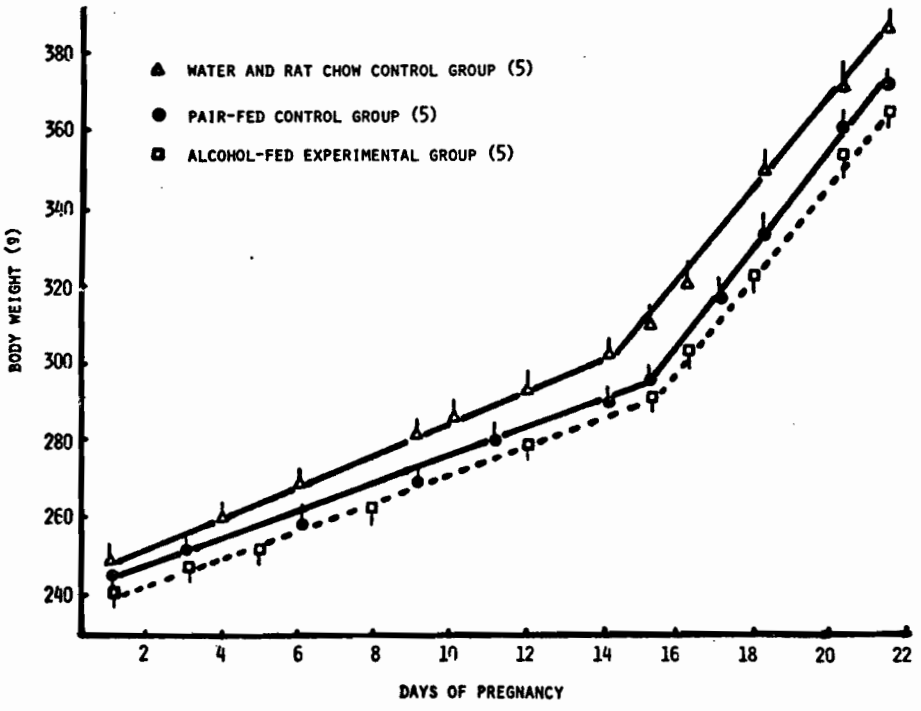
WATER AND RAT CHOW CONTROL GROUP (5)
PAIR-FED CONTROL GROUP (5)
ALCOHOL-FED EXPERIMENTAL GROUP (5)
BODY WEIGHT (g)
380
360
340
320
300
280
260
240
2
4
6
8
10
12
14
16
18
20
22
DAYS OF PREGNANCY

FIGURE 1

Body weight gain profile of pregnant rats. Pregnant rats were given either alcohol (□), sucrose (●) or water and Purina Rat Chow ad libitum (△) throughout pregnancy and lactation as described under Materials and Methods. The body weight gain was followed until time of delivery. Each value is the mean ± S.E.M. from five different animals kept under the corresponding diet.

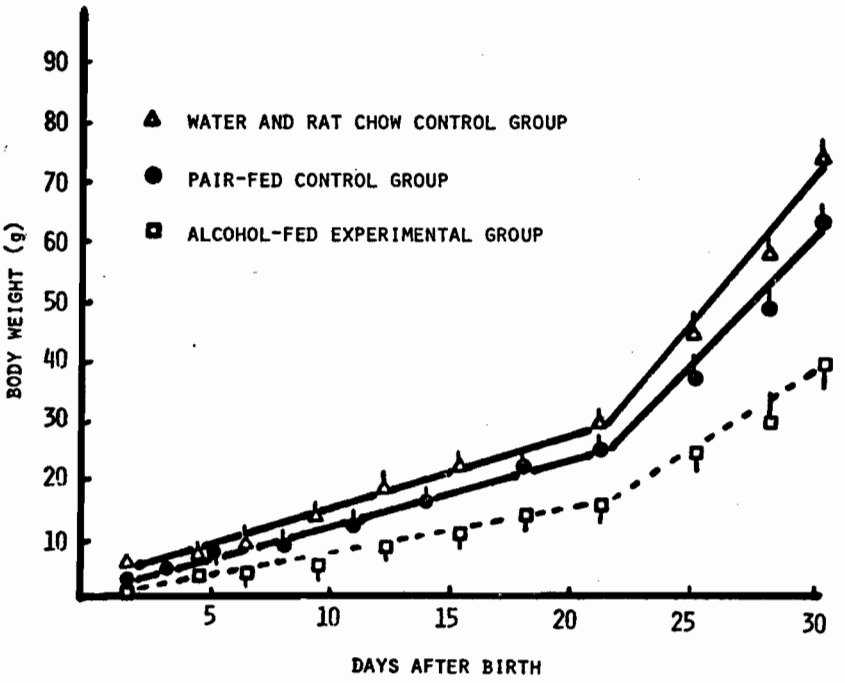
WATER AND RAT CHOW CONTROL GROUP
PAIR-FED CONTROL GROUP
ALCOHOL-FED EXPERIMENTAL GROUP
BODY WEIGHT (g)
90
80
70
60
50
40
30
20
10
5
10
15
20
25
30
DAYS AFTER BIRTH

FIGURE 2

Body weight gain profile of newborn rats. Maternal feeding of their respective diets continued throughout lactation and beginning of weaning period up to day 30. All pups were weighed up to 30 days postnatally. Each value represents the mean ± S.E.M from five different litters for each group.

General Characteristics of Livers from New-Born Rats

The wet weight of livers from pups born of mothers pair-fed sucrose was consistently greater than those fed alcohol (Fig. 3). However, regardless of diet treatment and age, the liver wet weight to body weight ratio of pups from both pair-fed groups varied between 30 and 40 mg/g during the first month after birth. The yield of hepatic plasma membrane protein per wet liver weight was constant, independent of either age or diet, and close to 2.5 mg/g (Fig. 3).

[^{3}H] Prazosin Binding to Liver Plasma Membranes

The experimental conditions described for equilibrium binding studies were based on previous work done in our laboratory [15] where it was demonstrated that [^{3}H] prazosin binds with high affinity, in a saturable manner and stereospecifically to the α_1-receptors in the rat liver plasma membrane. The same conditions were found to characterize α_1-adrenergic binding in the hepatic plasma membrane of the newborn rat. Figure 4 shows a typical saturation curve and a Scatchard analysis of [^{3}H] prazosin binding to liver plasma membrane from 30-day-old control and alcoholic pups. Figure 5 represents the first characterization of the postnatal development of the physiologically relevant α_1-adrenergic receptors in rat liver using [^{3}H] prazosin as the radioligand. No detectable binding was observed at day 1 in both animal groups. Specific binding was detected at day 6, and this increased gradually in a sigmoidal fashion. This increase was relatively slow up to day 20 at which time the receptor density was approximately 20% of adult values for both the control and alcoholic progenies. After day 20, receptor number increased dramatically and at day 30 adult values [15] were obtained for both groups.

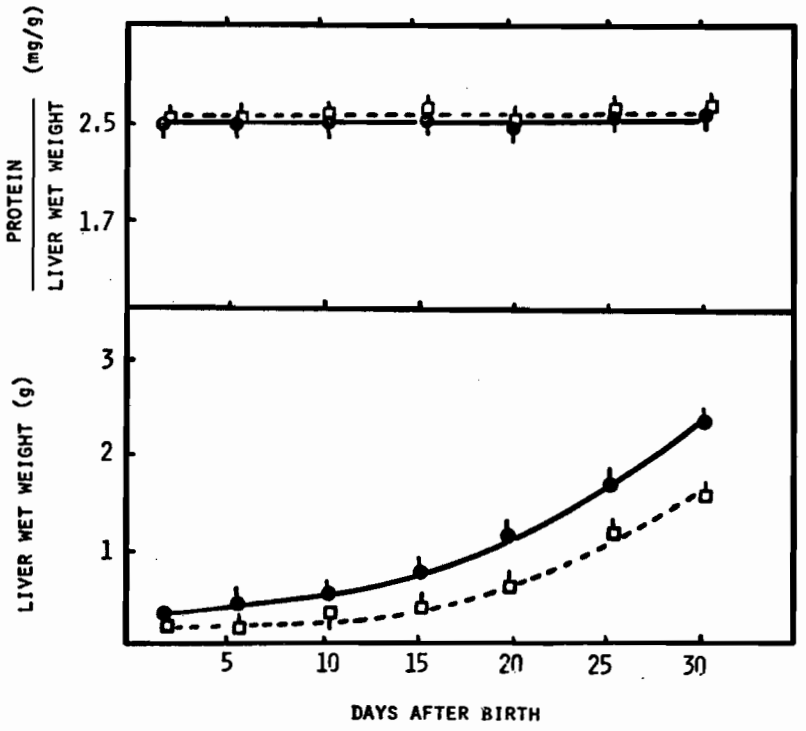
PROTEIN / LIVER WET WEIGHT (mg/g)
2.5
1.7
LIVER WET WEIGHT (g)
3
2
1
5
10
15
20
25
30
DAYS AFTER BIRTH

FIGURE 3

General developmental characteristics of livers from newborn rats belonging to maternal alcoholic and pair-fed sucrose groups. Newborn rats were weighed and decapitated at specific postnatal ages. The livers were rapidly excised and placed in 20 vol. of ice-cold 1.0 mM $NaHCO_3$. The wet weights were recorded before proceeding with the purification of hepatic plasma membranes. Total protein in the plasma membrane fractions was determined as described in the text. Each value is the mean of three to six experiments; □– – –□, alcoholic; ●——●, pair-fed.

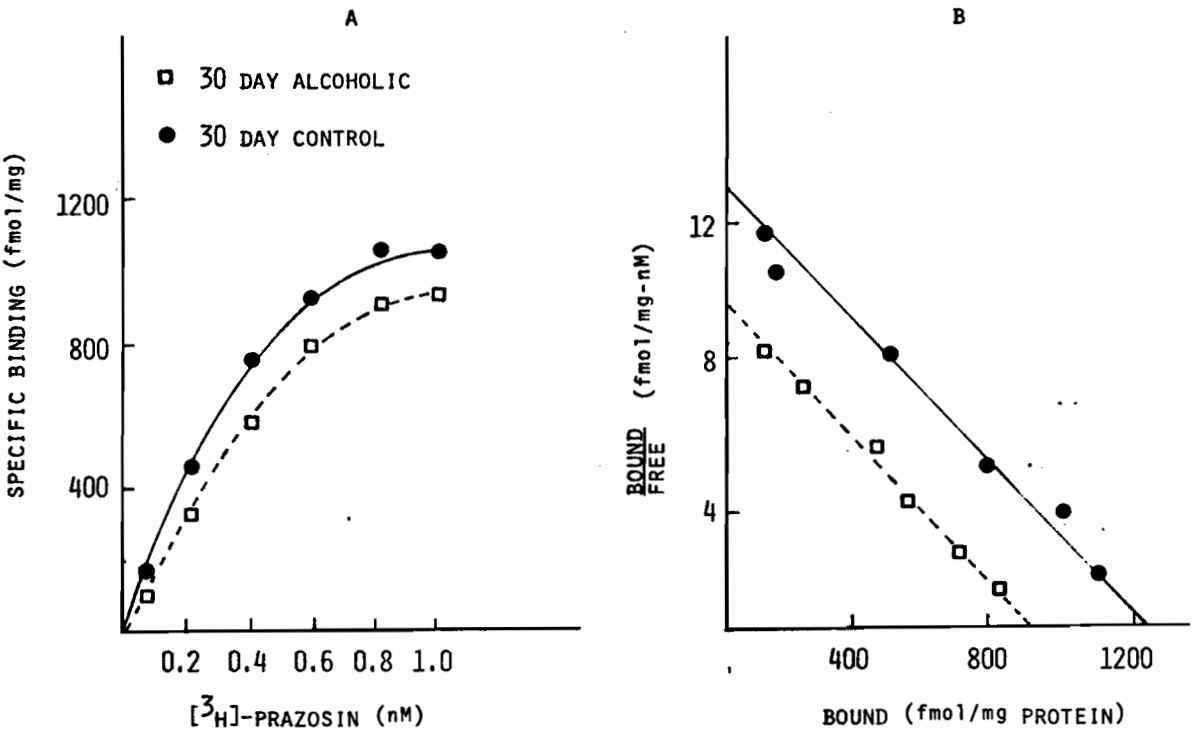
A
30 DAY ALCOHOLIC
30 DAY CONTROL
SPECIFIC BINDING (fmol/mg)
1200
800
400
0.2 0.4 0.6 0.8 1.0
[3H]-PRAZOSIN (nM)
B
BOUND/FREE (fmol/mg-nM)
12
8
4
400 800 1200
BOUND (fmol/mg PROTEIN)

FIGURE 4

Typical saturation curves of specific [^{3}H] prazosin binding to hepatic plasma membranes. The left panel (A) illustrates the dose-dependent binding of [^{3}H] prazosin to liver plasma membranes from 30-day-old rats born of alcohol (□---□) and pair-fed sucrose control (●——●) mothers. The right panel (B) shows the corresponding Scatchard plots for the saturation curves. B/F ratios for the [^{3}H] prazosin bound by membrane protein to free labelled ligand were plotted as a function of bound [^{3}H] prazosin (B). The slopes of the plots ($-1/K_D$) were determined by linear regression analysis and the number of binding sites (Bmax) was computed from the X intercepts of the plots. Values are the means of duplicate determinations from four experiments.

Although the postnatal developmental pattern of hepatic α_1-adrenergic receptors in newborn rats from the alcoholic group did not appear to differ from the pair-fed sucrose controls, there was consistently a significant decrease in the total number of receptors (Bmax) in the plasma membrane of pups in this group (Table 3 and Fig. 5). This decrease in receptor density was 60-70% in pups 6-15 days of age; 45% at 20 days; and approximately 30% in pups at 25 and 30 days. The equilibrium dissociation constant (K_D) did not change with postnatal age and was similar for both groups studied (Table 3 and Fig. 4). Chronic maternal ethanol administration during pregnancy and lactation has, therefore, a marked detrimental effect on the postnatal hepatic plasma membranes as reflected by a dysfunction in the development of the α_1-adrenergic receptors.

TABLE 3

COMPARISON OF THE EFFECTS OF CHRONIC ALCOHOL INGESTION DURING PREGNANCY AND LACTATION ON DIFFERENT CHARACTERISTICS OF SPECIFIC [^{3}H] PRAZOSIN BINDING TO RAT HEPATIC PLASMA MEMBRANES DURING POSTNATAL DEVELOPMENT

Days after birth	TREATMENT CONDITION: Control N[a]	Control K_D (nM)	Control Bmax (fmol/mg)	Alcoholic N[a]	Alcoholic K_D (nM)	Alcoholic Bmax (fmol/mg)
1	4	-	-	4	-	-
6	3	0.21 ± 0.01	100 ± 20	3	0.23 ± 0.04	40 ± 10[b]
9	3	0.25 ± 0.05	140 ± 20	3	0.08 ± 0.01	50 ± 10[b]
13	4	0.15 ± 0.02	190 ± 20	5	0.11 ± 0.05	60 ± 20[b]
15	2	0.30 ± 0.05	210 ± 20	4	0.33 ± 0.02	90 ± 10[b]
20	2	0.17 ± 0.02	270 ± 20	2	0.12 ± 0.02	150 ± 20[b]
25	3	0.15 ± 0.01	480 ± 50	3	0.10 ± 0.03	320 ± 20[b]
30	3	0.19 ± 0.01	1200 ± 100	3	0.16 ± 0.05	880 ± 10[b]

Equilibrium binding experiments were done as described under Materials and Methods. Scatchard analysis [19] was performed to determine the various binding characteristics. Values are given as the means ±S.E.M. for the number of experiments indicated.

[a] Number of experiments.

[b] Significantly different from pair-fed controls at $P < 0.05$ by the two-tailed Student's t-test.

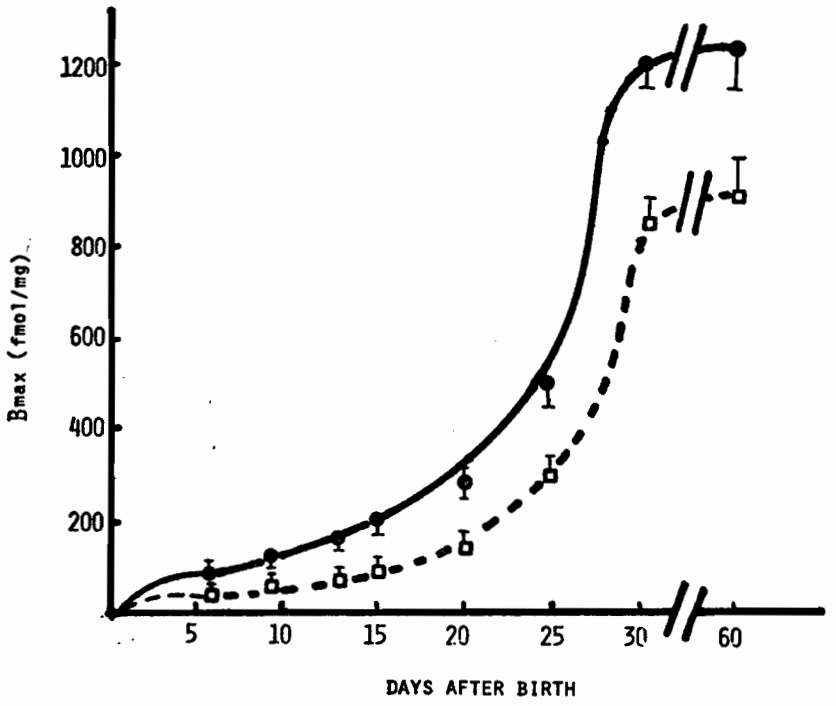
1200
1000
800
600
400
200
Bmax (fmol/mg)
5
10
15
20
25
30
60
DAYS AFTER BIRTH

FIGURE 5

Postnatal development of α_1-adrenergic receptors characterized by [^{3}H] prazosin. Hepatic plasma membranes from pups born of either alcoholic (□—□) or pair-fed sucrose control (●——●) mothers were isolated at the ages indicated and incubated with [^{3}H] prazosin for 15 min at 31°C, as described in the text. Scatchard plots were derived from specific equilibrium binding data to determine the total amount of binding sites (Bmax) and the dissociation constants (K_D). Values are the means of triplicate determinations from the number of experiments indicated in Table 3.

DISCUSSION

In the first part of this study, the effect of chronic ethanol feeding during pregnancy and lactation on maternal reproductive performance, as well as litter growth, was investigated. Maternal weight gain during pregnancy was greatest in the Rat Chow control group compared with the alcoholic and pair-fed sucrose control groups. Similar observations have been reported previously by other investigators [22,23]. Since in those as well as our present study the pair-fed sucrose controls had the same caloric intake as the alcoholic group, the lower maternal weight gain during pregnancy may be attributed to a restrictive effect of alcohol administration on daily caloric intake. Litter size was the same in all groups studied, in agreement with other authors [24-26]. No difference was found in the body weight of pups born of mothers kept on the different diets. This is in agreement with the recent work of Borges and Lewis [27] but contrasts with others [28,29] who reported decreased body weight of alcoholic pups at birth. Such discrepancies may be due to different methods of ethanol feeding, dosage, and duration of ethanol consumption.

Earlier studies with the rat demonstrated that offspring development and survival were impaired subsequent to maternal ethanol ingestion [22]. Our results showed that the number of stillbirths was considerably higher in the ethanol-fed compared to the pair-fed group. Furthermore, pups from alcoholic mothers exhibited a lower percentage survival and gained weight at a significantly lower rate than pups from pair-fed sucrose control and Rat Chow control mothers. A recent report [30] suggests that prenatal alcohol exposure can interfere with the development of normal suckling behavior. In addition, ethanol-consuming lactating mothers may

display significant deficits in maternal behavior towards alcoholic pups [31]. Both of these factors can contribute to a lowered survival rate, food intake, and thus decreased weight gain in our experimental pups.

In the second part of this work, the effect of chronic maternal ethanol feeding on the postnatal hepatic plasma membrane, as assessed with the development of α_1-adrenergic receptors, was investigated. Alpha-adrenergic receptors play an important role in the regulation of hepatic carbohydrate metabolism [32]. In adult rats, α_1-receptors comprise approximately 80% of the total rat hepatic α-adrenergic receptors, and they have been identified as the physiologically-relevant receptors which mediate the activation of glycogen phosphorylase [11]. [^{3}H] Prazosin, an α_1-adrenergic antagonist, has been shown recently in this and other laboratories to be a highly suitable and selective radioligand for identifying these receptors [11,15].

The results shown in Fig. 5 indicate that, in general, the postnatal development of the hepatic α_1-adrenergic receptors was quite similar for the pair-fed sucrose control and alcoholic pups, both following a sigmoidal pattern. Adult values for the total number of receptors (Bmax) were found in the 30-day-old pups in the pair-fed sucrose control and alcohol-fed groups (Bmax $\simeq$ 1200 $\pm$ 100 and 860 $\pm$ 20 fmol/mg protein, respectively) [15]. Butlen et al. [33] have described a biphasic pattern for the development of hepatic adrenergic receptors in rat pups fed water and Rat Chow ad libitum. In that study, the maximal binding capacity (Bmax) was observed in the 19-day-old fetal liver. A progressive decrease in Bmax ensued thereafter until the second week after birth. Bmax then increased again between 18 and 30 days after birth until the adult value was reached. In this study we were unable to detect any

significant binding with [^{3}H] prazosin 1 day after birth. This difference may be due to the fact that [^{3}H] prazosin binds specifically to α_1-receptors while [^{3}H] dihydroergocryptine used by Butlen et al. [33] binds to both α_1 and α_2 receptors [11]. Therefore, during the perinatal period most of the rat hepatic α-adrenergic receptors present may not be of the α_1 subtype.

Data presented in Table 3 and Fig. 5 showed that, between 6 and 30 days, receptor density (Bmax) for pups in the alcoholic group was decreased significantly compared with the pair-fed sucrose controls. No difference was observed in the binding affinity (K_D) during the entire postnatal period for both groups. This suggests that the fundamental functional characteristics of these receptors were not altered.

These results indicate that chronic maternal ethanol ingestion during pregnancy and lactation has, in some way, a detrimental effect on the postnatal rat liver plasma membrane, as reflected by a diminished density of α_1-adrenergic receptors. In the rat liver, α_1-receptors mediate the regulatory actions of epinephrine on glucose homeostasis [11]. In both humans and rats, glucose is the main oxidative fuel for the fetus and the newborn [34,35]. Most of the key enzymes regulating hepatic carbohydrate metabolism are known to be activated postnatally, and selective developmental changes in the control of carbohydrate metabolism have been shown to occur after birth [36-39]. Accordingly, a persistent reduction in the postnatal development of hepatic α_1-adrenergic receptors subsequent to maternal ethanol feeding may seriously affect the capacity of the liver to respond to the regulatory actions of epinephrine in those pups.

The precise mechanism(s) responsible for the diminished hepatic α_1-adrenergic receptor density from the experimental pups is not known. One likely explanation is that this anomaly represents the manifestation of some fundamental intrinsic difference between the control and alcoholic plasma membranes as was observed earlier in similar studies with adult male rats [15]. There is some experimental evidence to support this view. Cellular membranes are known to adapt to prolonged alcohol exposure by altering their lipid composition [9,10,40]. Lipid compositional changes could conceivably modify membrane lipid fluidity as well as the dynamics of lipid-protein interaction [14]. These may, in turn, alter the accessibility of the receptor to the ligand [41], i.e. some functional receptors may be masked. Thus, the decreased receptor binding to the experimental plasma membrane could represent another dimension of membrane adaptation to ethanol.

Alternatively, the decreased receptor density may represent insufficient assembly or insertion of these receptors into the outer surface of the experimental plasma membrane. Such a flaw could be the result of improper membrane biogenesis during fetal or postnatal development. Defective maturation of structural membrane components in the central nervous system has been demonstrated recently in neonatal rats suffering from hypothyroidism and undernutrition [42]. Similar defects might have occurred in the postnatal rat liver plasma membrane following prolonged alcohol exposure.

Regardless of the mechanisms involved, the enduring anomaly in the experimental plasma membranes must be presumed to have originated from chronic in utero ethanol exposure. Long-lasting membrane defects of this

nature would ultimately contribute to some of the abnormalities associated with chronic maternal alcohol ingestion.

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CHAPTER 6

Chronic Maternal Ethanol Administration in the Rat Decreases the Stimulation by (-) Epinephrine of Glycogen Phosphorylase *a* in the Livers of the Progeny During Development

SUMMARY

Previous results from this laboratory have shown that the progeny of alcoholic rats have diminished α_1-adrenergic receptors in the hepatic plasma membranes. Since these receptors mediate epinephrine action on glycogen metabolism, it was decided to determine whether this change might affect the activation of glycogen phosphorylase a in the livers of the alcoholic progeny. Pregnant female rats were divided into two groups of which one received a Metrecal-ethanol liquid diet throughout pregnancy and lactation. The pair-fed control group received a liquid sucrose-Metrecal diet over the same period. Phosphorylase a activity was determined in liver slices from the progeny during postnatal development. The basal hepatic phosphorylase a activity was identical between the control and experimental groups at 5, 15 and 25 days of age. Both epinephrine and phenylephrine were superior enzyme activators than was isoproterenol. Stimulation with epinephrine (10 μM) demonstrated a significantly diminished capacity of the enzyme in the alcoholic liver to be activated by the hormone. In every instance, the livers from 5, 15 and 25-day-old pups from alcoholic mothers displayed diminished epinephrine-stimulated phosphorylase a activity of about 30%, compared with the controls.

INTRODUCTION

Ethanol is a general anesthetic which is known to affect the physical and chemical properties of biological membranes (8). Since ethanol is very lipophilic, some of its physiological and behavioral effects are believed to be exerted through nonspecific interactions with cell membranes which may in turn result in expansion, disordering, and disorganization of the membrane lipid bilayer (13).

Previous studies from this (11,17,18) and other (23,25,27) laboratories have shown that chronic administration of ethanol to various animal species elicited structural and functional changes in cell membranes of different tissues. These observations suggest that some of the membrane alterations represent adaptive changes which may be partially responsible for the development of physical dependence on and tolerance to ethanol.

More recently, we have undertaken a study of the structure and function of the liver plasma membranes from the progeny of alcoholic rats during postnatal development. Using the hepatic α_1-adrenergic receptor as a plasma membrane probe we found its density to be decreased in the progeny of rats consuming ethanol throughout pregnancy and lactation (26). These results indicate that chronic maternal ethanol ingestion may have serious consequences on the integrity and functional state of the hepatic plasma membranes in the alcoholic progeny.

In an effort to correlate the decreased α_1-adrenergic receptor density in the hepatic plasma membranes of the alcoholic progeny to a physiological event, we have examined the stimulation by (-) epinephrine of glycogen phosphorylase a in liver tissue slices. In normal rat liver this enzyme is known to be activated by catecholamines through adre-

nergic receptors of the α_1-subtype (2). Moreover, because glycogen phosphorylase a is believed to be the primary regulator of glycogen metabolism in the liver (15) and since glucose is the main oxidative fuel in the newborn (28,29), our study may also be informative with respect to the influence of maternal ethanol consumption on hepatic glycogen metabolism and glucose homeostasis in the newborn.

The results obtained indicate that the stimulation by (-) epinephrine (10 μM) of glycogen phosphorylase a is decreased in the livers of the alcoholic progeny. These observations are discussed in terms of the possible involvement of membranes in the development of some of the physical and metabolic anomalies in the alcoholic progeny.

MATERIALS AND METHODS

Chemicals

The ethyl alcohol (100%) used in feeding experiments was purchased from Consolidated Alcohol Co. Ltd. (Toronto, Ont., Canada). Vitamin Diet Fortification Mixture and glycylglycine were obtained from ICN Nutritional Biochemicals (Montreal, Que., Canada). Alpha-D-[U-^{14}C]-glucose-1-phosphate (0.39 Ci/mmol) and Liquifluor were supplied by New England Nuclear, Canada. (-) Epinephrine, (-) phenylephrine, (-) isoproterenol, EDTA, and alpha-D-glucose-1-phosphate were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Sodium fluoride was purchased from Merck and Co. (Montreal, Que., Canada). Caffeine was supplied by Eastman Organic Chemicals (Rochester, N.Y., U.S.A.). Metrecal was prepared by Mead Johnson (Belleville, Ont., Canada). All other chemicals were purchased from Fisher Scientific Inc. (Montreal, Que., Canada).

Chronic Maternal Ethanol Feeding

Virgin female Sprague-Dawley rats weighing 200-225 g were purchased from Canadian Breeding Farms (St. Constance, Que., Canada) and given water and Purina Rat Chow ad libitum for one week. Rats were then mated overnight and the appearance of sperm in the vaginal washings established day 1 of pregnancy. The pregnant rats were randomly divided into two groups and their diets changed to a nutritionally adequate low-fat Metrecal liquid diet providing 1 kcal/ml and supplemented with 3 g/l Vitamin Diet Fortification Mixture. The diet consumed by the designated alcoholic group contained ethanol which provided 37% of the total calories. The control group was pair-fed with the same Metrecal liquid diet, but with sucrose isocalorically substituted for ethanol.

Both groups of rats were maintained on their respective diets throughout pregnancy and lactation. All pups were weaned at 21 days postnatally. Under these conditions, the intake of total nutrients and calories for both the maternal alcoholic and pair-fed control groups fulfill the nutritional requirements of laboratory rats (NRC Subcommittee on Laboratory Animal Nutrition, 1978). The amount of food and ethanol consumed by animals in the maternal alcoholic group was previously described (26). Briefly, the average food intake for animals in this group ranged from 180 to 300 kcal/kg body weight/day during the first gestational week and the last two weeks of pregnancy, respectively. The average ethanol intake ranged from 10 to 15.7 g/kg body weight/day during the gestational period, and this resulted in intoxicating blood ethanol concentrations (measured between 8 and 10 a.m.) which varied from 35 to 40 mM. Upon withdrawal from alcohol all animals displayed tremor and hyperexcitability. Blood ethanol concentrations in the progeny of ethanol-fed mothers ranged between 4 mM at the end of the gestation to 40 mM at 25 days of age. Although the average maternal weight gain was similar in both groups throughout the study, the postnatal weight gain of the newborn pups of ethanol-fed mothers was reduced by 33% compared with pair-fed control pups (26).

Preparation and Incubation of Liver Slices

Rats from the progeny of alcoholic and pair-fed control mothers were sacrificed 5, 15 and 25 days after birth. The livers were rapidly removed and suspended in Krebs-Henseleit Buffer (KHB), pH 7.4. Liver slices, 0.5 mm in thickness, were prepared with a McIlwain mechanical tissue chopper and introduced into vessels with 2 ml of KHB. Vessels were shaken at 90 cycles/min and 37°C under an atmosphere of 95% O_2 - 5%

CO_2. After a 45 min recovery, the kinetics of activation of glycogen phosphorylase a was studied. Either (-) epinephrine (10 μM) or acid saline were added to the vessels and the reaction stopped in a mixture of ice and water at different times. The buffer was poured off and the slices homogenized with 2 ml of assay buffer (150 mM NaF, 0.5 mM caffeine, 100 mM glycylglycine, 2.5 mM EDTA, pH 6.1) as described by Hue et al. (12). This was followed by centrifugation at 8000xg for 10 min at 4°C to release the phosphorylase which was assayed by the method of Stalmans et al. (30) with slight modifications. Maximal stimulation (110% over basal levels) was achieved after 3 min incubation. Various concentrations of (-) epinephrine, (-) phenylephrine, and (-) isoproterenol were then used to study the effect of agonists on glycogen phosphorylase a activity.

Assay of Phosphorylase a

Phosphorylase a was assayed by a procedure which measured the incorporation of [U-^{14}C]-glucose-1-phosphate into glycogen (30). The incubation was started by the addition of the 8000xg supernatant extract (50 μl) to an equal volume of a solution containing 100 mM [U-^{14}C]-glucose-1-phosphate (390 mCi/mmole) and 2% (w/v) glycogen dissolved in assay buffer. After a 20 min incubation at 30°C, the reaction was stopped by spotting 50 μl aliquots of incubation medium onto Whatman No. 41 filter papers which were dropped into individual liquid scintillation counting vials containing 10 ml of 66% (v/v) ethanol to precipitate glycogen (3). The filter papers were kept in the ethanol solution at room temperature for 15 min. The ethanol was then decanted, and the procedure was repeated twice more by adding fresh ethanol. The samples were dried overnight and counted for radioactivity at an efficiency of 85% in the

same vials now containing 10 ml of Liquifluor. Under these experimental conditions, the rate of the enzyme reaction was linear both with respect to incubation time up to 60 min and protein concentration. In a typical experiment, the protein concentration as measured by the method of Lowry et al. (21), was 5-7 mg/ml. Enzyme activity was expressed as nmoles of ^{14}C-Glu incorporated into glycogen/min/mg protein.

Statistical Analysis

Results on the stimulation of phosphorylase a by (-) epinephrine (10 μM) are expressed as means ± S.E.M.; the level of significance of the difference between mean values was assessed by the two-tailed Student's t-test.

RESULTS AND DISCUSSION

We have previously demonstrated that in the perfused alcoholic rat liver the stimulation by glucagon and epinephrine of lactate gluconeogenesis is dramatically diminished (16). In this communication we have examined the influence of chronic ethanol feeding to pregnant rats on the stimulation by (-) epinephrine of glycogen phosphorylase _a_ in the liver of the progeny.

Results shown in Figure 1 represent initial studies aimed at characterizing the kinetics of activation of glycogen phosphorylase _a_ in liver tissue slices. The 15 day old progeny of alcoholic and pair-fed control rats were arbitrarily chosen for the kinetic studies. The data show that the basal activity of glycogen phosphorylase _a_ is constant with respect to incubation time and independent of diet treatment. This is similar to our previous findings with the perfused rat adult liver where it was demonstrated that the basal rates of the hormonal stimulation of lactate gluconeogenesis were the same in the control and alcoholic animals (16). Also, as shown in Figure 1, the kinetics of activation by (-) epinephrine (10 μM) of liver glycogen phosphorylase _a_ in the alcoholic progeny differs from that in the control group. Maximal rates of hormonal stimulation were rapidly achieved after a 3 min incubation period, but they were not similar in both groups of animals (110% over basal levels in the controls vs. 80% over basal levels in the alcoholics). This activation was transient, and the enzyme activity returned to basal levels 5-8 min after addition of (-) epinephrine. Similar results were obtained by Aggerbeck _et al_. (3) who studied the stimulation by (-) norepinephrine of phosphorylase _a_ in liver hepatocytes from rats fed a standard laboratory diet.

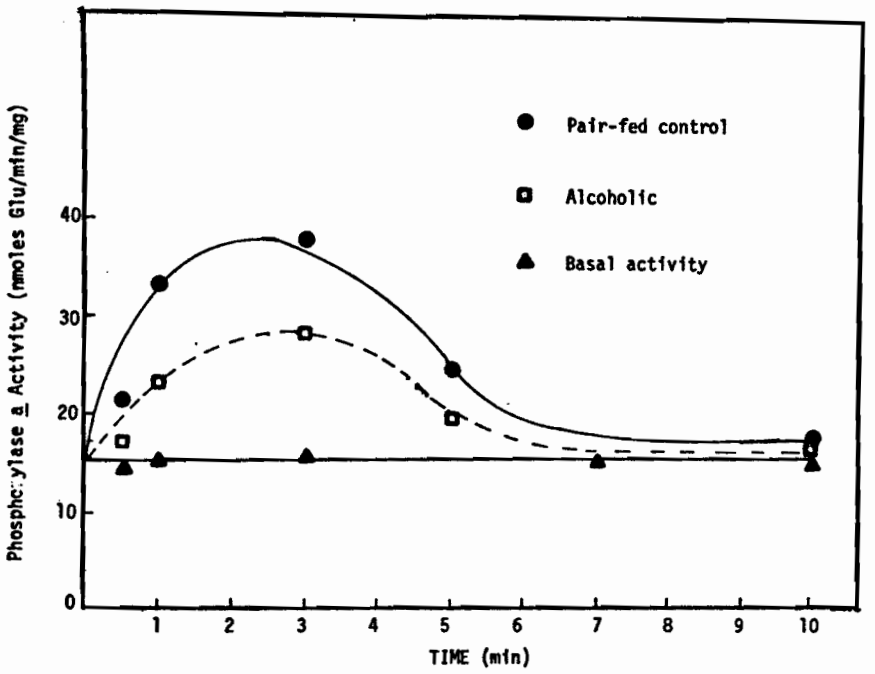
Pair-fed control
Alcoholic
Basal activity
Phosphorylase a Activity (nmoles Glu/min/mg)
0
10
20
30
40
1
2
3
4
5
6
7
8
9
10
TIME (min)

FIGURE 1

Kinetics of activation of glycogen phosphorylase a in liver slices from the 15-day-old progeny of alcoholic and pair-fed control rats. Liver slices (0.5 mm thickness) were preincubated in 2 ml of Krebs-Henseleit Buffer for 30 min at 37°C. Either (-) epinephrine (10 μM) or acid saline were added and the reaction stopped in a mixture of ice and water at different times. Phosphorylase a was assayed as described under Materials and Methods. Values represent the means of a quadruplicate determination. Basal values are the same for both groups of animals.

Data presented in Figure 2 demonstrate that in the 25-day-old progeny of control rats (this age group was also arbitrarily chosen for the preliminary characterization of the specificity of enzyme activation by various agonists), liver glycogen phosphorylase *a* is maximally stimulated at concentrations above 1 μM mainly by the α-agonists (-) epinephrine and (-) phenylephrine, and to a minor extent by the β-agonist (-) isoproterenol. These results indicate that in rat liver tissue slices glycogen phosphorylase *a* is stimulated by (-) epinephrine mainly through α-adrenergic receptors. Moreover, since Aggerbeck *et al.* (2) have shown that in rat liver hepatocytes (-) epinephrine activates glycogen phosphorylase *a* through adrenergic receptors of the α_1-subtype, it can be assumed that the stimulation by (-) epinephrine (10 μM) of phosphorylase *a* reported in this communication takes place almost exclusively through hepatic α-adrenergic receptors of the α_1-subtype.

Figure 3 represents a dose response curve of (-) epinephrine stimulation of glycogen phosphorylase *a* activity in liver tissue slices from the 15-day-old progeny of alcoholic and pair-fed control rats. Results from these experiments with a single age group show that above 1 μM (-) epinephrine, maximal enzyme activation is substantially diminished in the livers of the alcoholic progeny. It was therefore decided to study the activation by (-) epinephrine (at a concentration of 10 μM) of glycogen phosphorylase *a* in the livers of the progeny at various postnatal ages to investigate whether this change was induced by chronic maternal ethanol ingestion. The results are summarized in Table I. These data show that neither the basal nor the stimulated enzyme activity varied with age during development. These results are in agreement with those of Novak *et al.* (24) who found only minor changes in the activity of liver

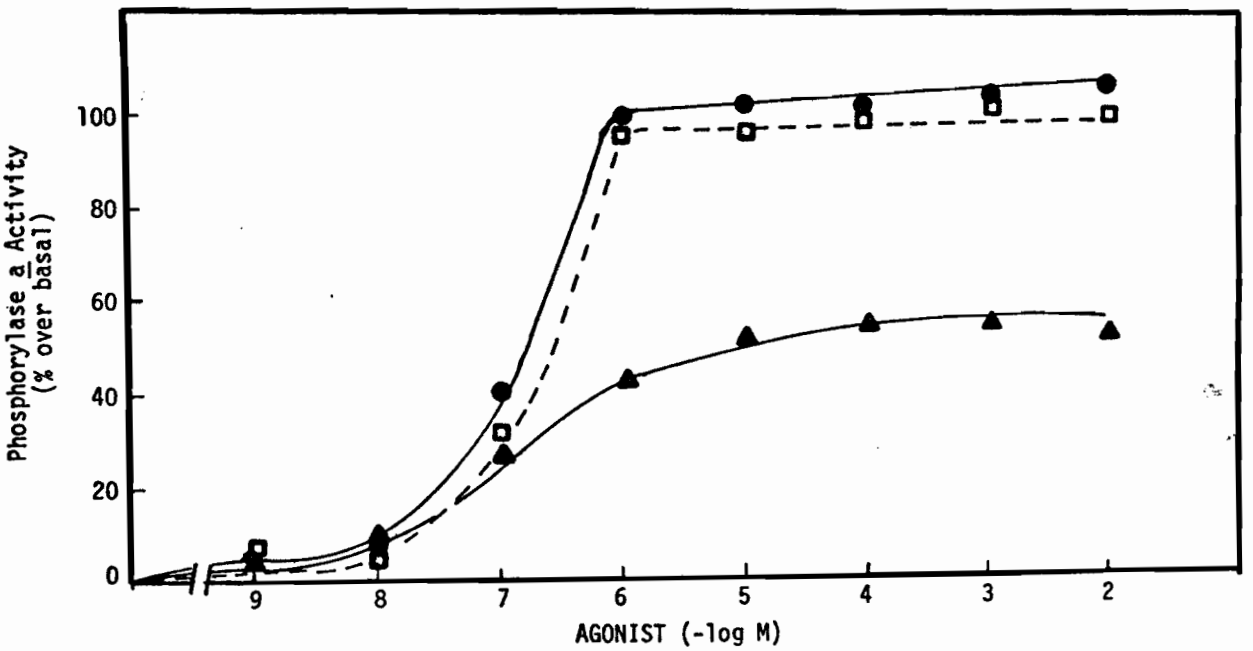
Phosphorylase a Activity
(% over basal)
100
80
60
40
20
0
9
8
7
6
5
4
3
2
AGONIST (-log M)

FIGURE 2

Effect of various agonists on liver glycogen phosphorylase a activity. Liver slices (0.5 mm thickness) from the 25-day-old progeny of control rats were pre-incubated in 2 ml of Krebs-Henseleit Buffer for 30 min at 37°C, and then incubated with (-) epinephrine (□), (-) phenylephrine (●), and (-) isoproterenol (▲) in various concentrations. Phosphorylase a activity was determined 3 min after addition of the agonists as described under Materials and Methods. Values represent the means of a triplicate determination from a representative experiment which was further replicated twice.

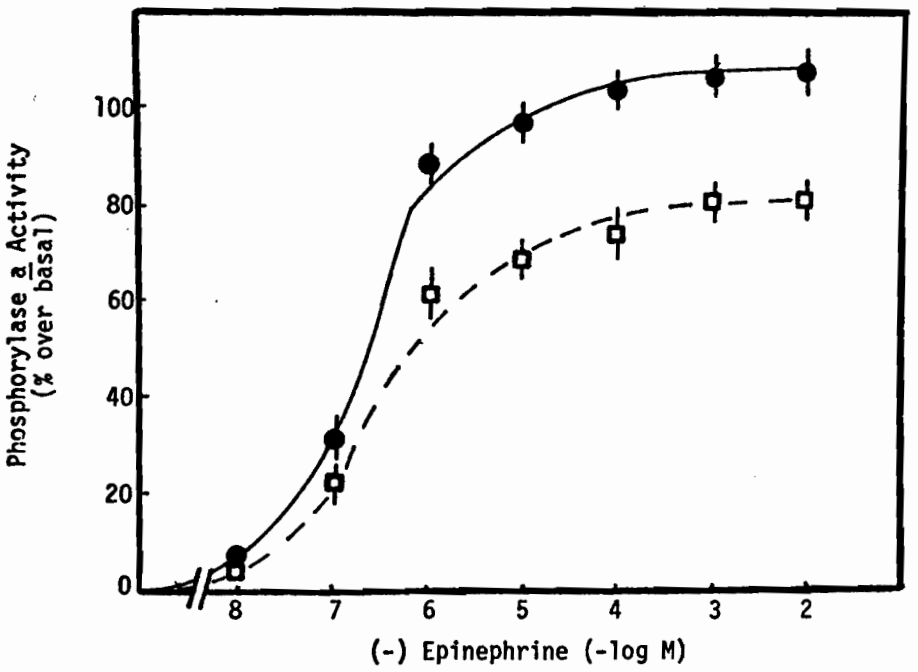
Phosphorylase a Activity
(% over basal)
100
80
60
40
20
0
8
7
6
5
4
3
2
(-) Epinephrine (-log M)

FIGURE 3

Dose response curve of (-) epinephrine stimulation of glycogen phosphorylase a activity in liver slices from the 15-day-old progeny of alcoholic (□) and pair-fed control (●) rats. Slices were incubated in Krebs-Henseleit Buffer, and phosphorylase a activity was determined 3 min after addition of the hormone. Each value is the mean ± S.E.M. from three different experiments. Each individual determination was done in triplicate.

glycogen phosphorylase a in the rat during postnatal development. The fact that basal adult levels of the enzyme activity are present in the 5-day-old rats from both groups (Table I) is not totally surprising. In fact, at birth there is a rapid mobilization of liver glycogen mediated through phosphorylase a and presumed to prevent hypoglycaemia until suckling and the onset of gluconeogenesis in the neonatal liver (7). In addition, since results in Table I also show a significant ($p < 0.01$ at 5 and 15 days of age; $p < 0.001$ at 25 days of age) decrease of about 30% in the stimulation by (-) epinephrine of liver glycogen phosphorylase a in the alcoholic progeny, it is possible that chronic maternal ethanol consumption may have seriously impaired hepatic glycogen metabolism in the newborn. Interestingly enough, similar conclusions regarding glycogen metabolism were formulated by Winston et al. (33) who also showed that subsequent to chronic ethanol ingestion, liver glycogen phosphorylase a is decreased in adult male and female rats. In those studies, however, the enzyme was not stimulated by any hormone because the authors were mainly interested in the interactions of ethanol ingestion and glucose homeostasis. Since results in Table I indicate that the basal activity of phosphorylase a is not affected by ethanol ingestion, the discrepancy between our results on the basal activity of the enzyme and those of Winston et al. (33) are probably due to the opposite direction in which the enzyme was assayed as well as the tissue preparation used. However, in previous studies we have shown (16) that the perfused alcoholic liver from adult male rats exhibited subnormal percentage stimulation by glucagon and epinephrine in the rate of gluconeogenesis when compared to the controls. These results indicated that chronic ethanol feeding affects the hormonal sensitivity of the liver.

TABLE I

Stimulation by (-) Epinephrine (10 μM) of Glycogen Phosphorylase a in Liver Tissue Slices From the Progeny of Alcoholic and Pair-Fed Control Rats

	5 days (6)		15 days (5)		25 days (6)	
	Control	Alcoholic	Control	Alcoholic	Control	Alcoholic
+ 10 μM (-) epinephrine*	33.8 ± 1.6	27.8 ± 1.8	30 ± 3	24.4 ± 0.5	30 ± 1.5	21.8 ± 1.6
basal activity*	14.5 ± 1	14.8 ± 0.8	13.4 ± 0.6	13.5 ± 0.3	13 ± 0.5	12.4 ± 0.4
% over basal	135 ± 11	87.4 ± 4†	126 ± 10	80.7 ± 6†	120 ± 8	77 ± 3††

Values represent the means ± S.E.M. at various postnatal ages for the number of preparations indicated inside the parentheses.

*All activities are expressed as nmoles Glu-1-Pi/min/mg.
†Significantly different from the control ($P < 0.01$).
††Significantly different from the control ($P < 0.001$).

In normal rat liver catecholamine-induced glycogenolysis is mediated mainly by α-adrenergic receptors of the α_1-subtype (2,6,10). Since these receptors mediate the activation of glycogen phosphorylase a (3), it is very likely that the decreased α_1-adrenergic receptor density previously observed in the plasma membranes of the alcoholic progeny (26) may be partially responsible for the diminished hormonal sensitivity of the livers from the alcoholic progeny.

Because glycogen phosphorylase a is believed to be the primary regulator of glycogen metabolism in the liver (15) and since glucose is the main oxidative fuel in the newborn (28,29), our studies suggest that chronic maternal ethanol consumption may affect glycogen metabolism and glucose homeostasis in the newborn. Further, because (-) epinephrine stimulates liver glycogen phosphorylase a through a membrane-bound hormone receptor (the α_1-adrenergic receptor), our results also suggest a plausible membrane involvement in some of the physical (26) and metabolic anomalies observed in the alcoholic progeny. In fact, previous work (11,18,23,25,27) has shown that chronic administration of ethanol to various animal tissues lead to structural and functional changes in cell membranes of different tissues. These have been generally interpreted to reflect an adaptation to the presence of ethanol, partially mediated through modifications of the lipid composition and "fluidity" of the cell membranes (5,14,27). Moreover, changes in lipid composition have been shown to induce alterations in membrane-associated biochemical phenomena such as regulation of hormone receptor interactions (9,32), modulation of the activities of membrane-bound enzymes (21,31) and membrane phospholipid interactions with abused drugs (1). Changes in the lipid composition of rat liver plasma membrane during postnatal development sub-

sequent to chronic maternal ethanol ingestion are presently being investigated.

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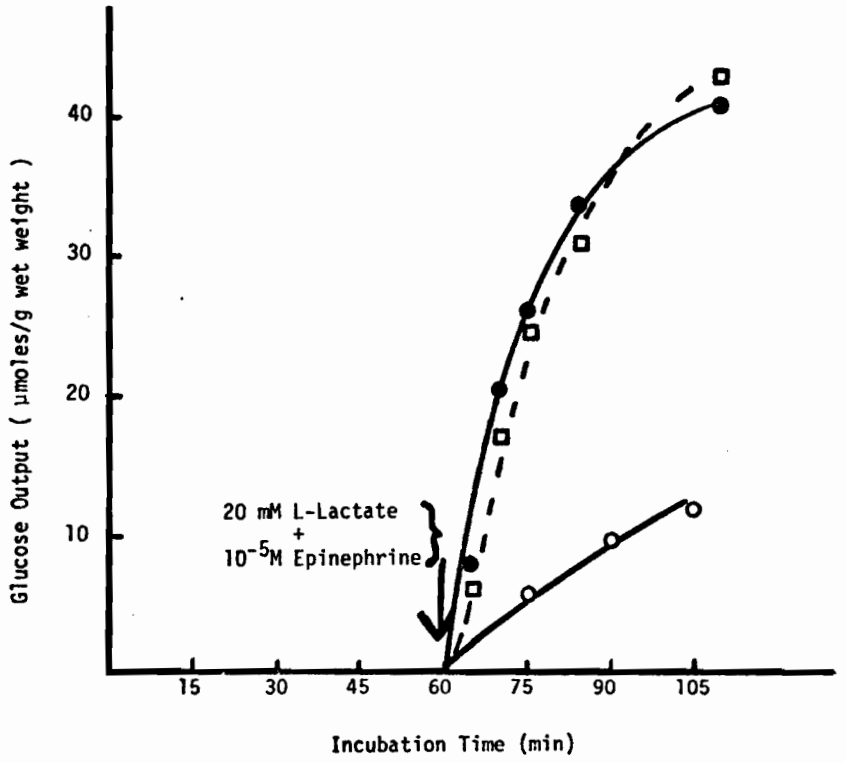
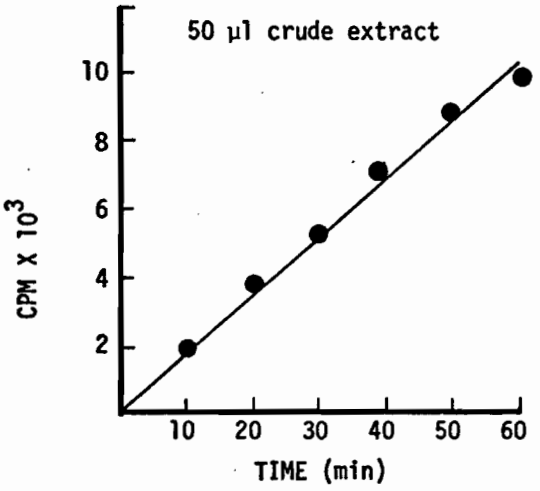
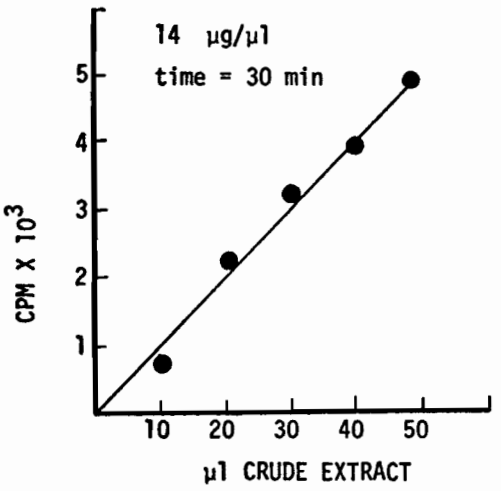

Glucose Output (µmoles/g wet weight)
40
30
20
10
20 mM L-Lactate
+
10⁻⁵M Epinephrine
15
30
45
60
75
90
105
Incubation Time (min)

APPENDIX 1

Glucose output in rat liver tissue slices from newborn pups. Rat liver slices from 15-day-old control (□- - -□) and alcoholic (●——●) pups were incubated in Krebs-Henseleit Buffer for a 60 min recovery period at 37°C. Following recovery, 20 mM L-Lactate + 10^{-5} M (-) Epinephrine were added to the medium, and glucose output was measured. Basal levels (○——○) were similar in both animal groups.


50 µl crude extract
CPM X 10^3
10
8
6
4
2
10
20
30
40
50
60
TIME (min)


14 µg/µl
time = 30 min
CPM X 10^3
5
4
3
2
1
10
20
30
40
50
µl CRUDE EXTRACT

APPENDIX 2

Time course and protein dependence of the stimulation by (-) epinephrine of glycogen phosphorylase a in liver tissue slices from the 15-day-old progeny of control rats. Values represent the means of three experiments.

CHAPTER 7

Adaptive Changes in Lipid Composition of Rat Liver Plasma Membrane During Postnatal Development Following Maternal Ethanol Ingestion

ABSTRACT

The fatty acid composition of constituent phospholipids and the cholesterol content of rat liver plasma membranes were determined subsequent to maternal alcohol ingestion during pregnancy and lactation. The alcoholic group was given a liquid Metrecal diet containing 37% ethanol-derived calories. The control group was pair-fed an isocaloric sucrose-Metrecal diet. Litters were killed for lipid analyses at days 5, 15 and 25 after birth. These studies revealed that the total phospholipid phosphorus was similar and increased significantly with age in both groups. Cholesterol (Chol) also increased significantly with age in both groups but was greater in the alcoholic pups, resulting in a higher Chol/phospholipid molar ratio. While the phosphatidylethanolamine (PE) content increased with age in both groups, that of sphingomyelin decreased. Phosphatidylserine + phosphatidylinositol (PS+PI) was significantly higher in the control group at all ages studied. A consistent increase of C22:6 in phosphatidylcholine (PC), sphingomyelin, PS+PI and in the total phospholipid fraction from alcoholic pups was observed. Although other fatty acid changes were found in PC, PS+PI and sphingomyelin, PE was not affected. These results suggest that specific adaptive changes were induced in the liver plasma membrane lipids of the progeny from alcoholic rats.

ABBREVIATIONS

Chol, cholesterol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol.

INTRODUCTION

Ethanol, like other general anesthetics, is known to act primarily within the lipid bilayer of biological membranes [1,2], and cellular adaptation to its chronic presence is believed to be mediated by alterations in the physical properties of the lipid bilayer, which can presumably be determined, at least in part, from the membrane lipid composition [3-6].

A variety of studies from this [7,8] and other laboratories [9-12] have demonstrated that chronic ethanol consumption may lead to functional and structural alterations of rat liver cellular organelles. These have been generally interpreted to reflect an adaptation to the presence of ethanol, partially mediated through modifications of the physical and chemical properties of the cell membranes. In fact, several authors have shown that the lipid composition of cellular membranes from microorganisms [13,14] and different animal tissues [15-21] is altered in response to prolonged exposure to ethanol.

We [22] have recently observed that chronic maternal ethanol ingestion has a detrimental effect on the postnatal development of rat liver plasma membrane α_1-adrenergic receptors. Results obtained with the alcohol-fed pups showed a significant decrease (30-70%) in receptor density (B_{max}) but no changes in the binding affinity (K_D) throughout postnatal development. Based on the above observations, these abnormalities can be envisioned as being partially modulated by changes in the lipid composition of the plasma membranes. We have, therefore, determined the effects of maternal ethanol ingestion on the fatty acid composition of individual liver plasma membrane phospholipids from newborn rats.

MATERIALS AND METHODS

Materials

Metrecal was prepared by Mead Johnson, Canada. Vitamin Diet Fortification Mixture was obtained from ICN Nutritional Biochemicals. Sucrose, phenol reagent and all chromatography grade organic solvents were purchased from Fisher Scientific Inc. Boron trifluoride-methanol was from Chromatography Specialties. Silicic acid and lipid mixtures for thin-layer chromatography standards were obtained from Sigma Chemicals. Fatty acid methyl esters for gas chromatography reference standards were from Applied Sciences. Ethanol (100%) was purchased from Consolidated Alcohol Co. Ltd., Toronto, Ontario.

Animal Treatment

Virgin female Sprague-Dawley rats weighing 200-225 g were purchased from Canadian Breeding Farms (St. Constance, Quebec) and given water and Purina Rat Chow ad libitum for 1 week. Rats were then mated overnight and the appearance of sperm in the vaginal washings established day 1 of pregnancy. The pregnant rats were randomly divided into two groups and had their diets changed to a nutritionally adequate liquid low-fat (5%) Metrecal diet providing 1 kcal/ml and supplemented with 3 g/l Vitamin Diet Fortification Mixture. The diet from the designated alcoholic group contained ethanol, which provided 37% of the total calories. The control group was pair-fed with the same diet, but with sucrose isocalorically substituted for ethanol. Both groups of rats were maintained on their respective diets throughout pregnancy and lactation. All pups were weaned at 21 days postnatally. Litters were sacrificed between 8 and 10 a.m. on days 5, 15 and 25 after birth, and liver plasma membranes were then isolated for lipid composition analyses.

Plasma Membrane Preparation

Livers were rapidly excised and the hepatic plasma membranes were isolated as previously described [22], using essentially the fractionation scheme of Neville [23] described by Wolfe et al. [24]. Experiments measuring marker enzymes (Table I; [25,26]) demonstrated that the highly enriched plasma membranes were only minimally contaminated with other cell organelles.

Lipid Separation and Phospholipid Analyses

Lipids were extracted from plasma membranes immediately after their preparation, using the procedure of Bligh and Dyer [27], dried under nitrogen and redissolved in chloroform. Neutral lipids and phospholipids were separated by silicic acid column chromatography (Sil-LC, 325 mesh, 5 cm X 5.5 mm internal diameter). Neutral lipids were eluted with 6 column vol. of chloroform, and phospholipids were eluted with 3 column vol. of chloroform/methanol (1:2) followed by 3 column vol. of methanol. The neutral lipids were dried under nitrogen, redissolved in chloroform and analyzed for cholesterol content by the method of Zlatkis et al. [68] as described by Kates [28]. An aliquot of the total phospholipid fraction was digested according to Duck-Chong [29] and the inorganic phosphorus assayed according to Chen et al. [30]. Individual phospholipids were separated from a second aliquot fraction by two-dimensional thin-layer chromatography on Merck Silica Gel G plates (250 μm thick). The solvents used were chloroform/methanol/7M ammonium hydroxide (65:35:5, v/v) and chloroform/methanol/acetone/acetic acid/water (50:10:20:10:5, v/v) for the first and second directions, respectively [31]. The separated phospholipids were visualized by exposure to iodine vapor, scraped from the plate and eluted with chloroform/methanol (1:1).

Quantitation of each phospholipid class was done by determination of phospholipid phosphate using the same method described for the quantitation of the total phospholipid fraction.

Gas Liquid Chromatography

Methyl esters of fatty acids from total and individual phospholipids were prepared with boron trifluoride-methanol reagent by the method of Morrison and Smith [32]. The fatty acid methyl esters were analyzed on a F&M model 402 gas liquid chromatograph equipped with a flame-ionization detector (detector temperature, 280°C) and a glass column (1.83 m X 4 mm internal diameter) packed with 3% OV-1 on 100-200 mesh Gas Chrome Q. The injector port temperature was 210°C while the initial column temperature was 150°C. The temperature of the oven was programmed from 150 to 250°C at a rate of 10°C/min. The flow rate of the carrier gas helium was adjusted to 40 ml/min under a head pressure of 40 lb/inch^2. Peak areas and retentions times were recorded with a Hewlett-Packard Model 3390A Integrator. Retention times were compared to those of known standards. Under these conditions in the lipid fraction of the Metrecal diet, 63% of the material present was identified as fatty acids. 25% of the unidentifiable material(s) was eluted after C22:6. Of the fatty acids identified in the diet, there were 22% of C16:0; 15% of C18:1,18:2; 10% of C18:0; 6% of C14:1; 4% of C14:0; and 3% each of C20:4 and C22:6.

Protein Determination

Protein was determined by the method of Lowry et al. [33] using recrystallized bovine serum albumin as standard.

Statistical Analysis

All results are expressed as means ± S.E.M.; the level of significance of the difference between mean values was assessed by the two-tailed Student's t-test.

RESULTS

Chronic Maternal Ethanol Administration

The intake of total nutrients and calories for both the maternal alcoholic and pair-fed control groups fulfill the nutritional requirements of laboratory rats [34]. The average food intake for animals in the maternal alcoholic group was 247 ± 17 (S.E.M., n = 5) kcal/kg body weight/day, and the average ethanol intake ranged from 10 to 15.7 g/kg body weight/day. Although the weight gain during pregnancy was similar in both maternal groups, the postnatal weight gain over the period studied of the newborn pups of alcohol-fed mothers was reduced by 33% when compared to the corresponding control pups. Blood ethanol concentrations (measured between 8 and 10 a.m.) in newborn pups of alcohol-fed mothers ranged between 4 mM at day 5 to 40 mM at day 25. In pregnant and lactating mothers, blood alcohol levels varied between 35 and 40 mM.

Isolation of Rat Liver Plasma Membranes

The yield of hepatic plasma membrane protein per g wet liver weight was constant, being independent of either age or diet treatment and close to 2.5 mg/g. The specific activities of marker enzymes in liver plasma membranes from rats of different ages is shown in Table I. The relative specific activity of an enzyme, i.e., the ratio of the specific enzyme activity in the isolated membrane fraction to that in the homogenate, is an indication of the degree of purification. As shown in Table I, the isolated membrane fractions are highly enriched in 5'-nucleotidase, a specific plasma membrane marker. The extent of microsomal and mitochondrial contamination was assessed with the measurement of the relative specific activities of glucose-6-phosphatase and monoamine oxidase, respectively. Results shown demonstrate that the extent of such contami-

TABLE I

SPECIFIC ACTIVITIES OF MARKER ENZYMES IN LIVER PLASMA MEMBRANES FROM RATS OF DIFFERENT AGES

Specific enzyme activities are expressed in μmol Pi/mg protein per h (a), cpm X 10^5/mg protein per 15 min (b). The relative specific activity (in brackets) is the ratio of the enzyme activity in the isolated membrane fraction to that in the homogenate. Values represent the means ± S.E.M. for the number of preparations indicated in parentheses.

Enzymes	5 days (7)		15 days (5)		25 days (8)	
	Control	Alcoholic	Control	Alcoholic	Control	Alcoholic
5'-Nucleotidase[a]						
Liver homogenate	4.0 ± 0.8	3.5 ± 0.4	3.7 ± 0.5	4.2 ± 1.0	3.8 ± 0.5	4.0 ± 0.7
Plasma membrane	34.2 ± 5.0	33.0 ± 2.7	34.0 ± 4.1	32.0 ± 6.0	31.0 ± 3.0	35.0 ± 4.0
	[8.5 ± 2.0]	[9.2 ± 1.5]	[9.2 ± 2.4]	[7.6 ± 3.0]	[8.1 ± 2.0]	[8.7 ± 2.3]
Glucose-6-phosphatase[a]						
Liver homogenate	1.8 ± 0.3	1.6 ± 0.5	1.7 ± 0.5	1.6 ± 0.2	1.9 ± 0.7	1.8 ± 0.3
Plasma membrane	1.1 ± 0.2	1.2 ± 0.4	1.4 ± 0.2	1.4 ± 0.3	2.0 ± 0.5	1.4 ± 0.5
	[0.6 ± 0.2]	[0.7 ± 0.1]	[0.8 ± 0.2]	[0.9 ± 0.2]	[1.0 ± 0.1]	[0.7 ± 0.2]
Monoamine oxidase[b]						
Liver homogenate	28.3 ± 1.7	22.0 ± 1.1	21.0 ± 6.3	25.0 ± 3.0	22.3 ± 5.0	31.0 ± 4.3
Plasma membrane	59.3 ± 3.1	41.0 ± 5.0	41.0 ± 5.4	40.0 ± 5.0	45.0 ± 8.3	58.0 ± 5.1
	[2.1 ± 0.3]	[1.9 ± 0.2]	[1.9 ± 0.3]	[1.6 ± 0.1]	[2.0 ± 0.3]	[1.9 ± 0.2]

nation is minimal. It is interesting to note that the relative specific activities of the marker enzymes are independent of either age or diet treatment, and the values obtained are in agreement with others [35,36].

Total Phospholipid and Cholesterol Content of Plasma Membranes

As indicated in Table II, total phospholipid content significantly ($P < 0.01$) increased from day 5 to day 25 after birth for both alcoholic and control pups. Cholesterol also increased significantly ($P < 0.01$) with age in both groups. Although total phospholipid content is independent of diet treatment, cholesterol was greater in the alcoholic pups ($P < 0.05$ at days 5 and 15; $P < 0.01$ at day 25) resulting in a significantly higher ($P < 0.05$) Chol/phospholipid molar ratio. The values obtained for the phospholipid and cholesterol content of hepatic plasma membranes from 25-day-old pair-fed control pups are similar to those reported by other investigators [37-39].

Phospholipid Distribution in Plasma Membranes

As shown in Table III, the major individual constituent phospholipids in rat liver plasma membranes isolated from both alcoholic and control pups are PC, sphingomyelin, and PE. These results are in agreement with those obtained by other investigators [35,37,38]. Moreover, the phospholipid distribution in plasma membranes from 25-day-old pair-fed control pups was quite similar to that found in adult rat liver plasma membranes in other studies [35,39-44].

The developmental pattern of the phospholipid distribution was characterized by an increase in PE and a decrease in sphingomyelin for both animal groups. The content of PC did not vary with age or diet treatment. Although the content of PS+PI was independent of age for both

TABLE II

LIPID COMPOSITION OF LIVER PLASMA MEMBRANES FROM RATS OF DIFFERENT AGES

Plasma membranes were extracted according to Bligh and Dyer [27]. Polar and non-polar lipids were fractionated by silicic acid column chromatography as described in Materials and Methods. The cholesterol content was measured by the method of Zlatkis et al. [68] as described by Kates [28]. Phospholipids were digested according to Duck-Chong [29] and the inorganic phosphorus assayed according to Chen et al. [30]. Values represent the means ± S.E.M. for the number of preparations indicated in parentheses.

Lipid Class	5 days (8)		15 days (6)		25 days (10)	
	Control	Alcoholic	Control	Alcoholic	Control	Alcoholic
Phospholipid Phosphorus (μmol/mg protein)	0.33 ± 0.02[a]	0.31 ± 0.01[a]	0.39 ± 0.01	0.42 ± 0.02	0.41 ± 0.01	0.44 ± 0.02
Cholesterol (μmol/mg protein)	0.19 ± 0.02[a]	0.25 ± 0.02[a,c]	0.32 ± 0.02	0.38 ± 0.02[c]	0.34 ± 0.03	0.41 ± 0.03[c]
Molar Ratio (Chol/phospholipid)	0.64 ± 0.03[a]	0.74 ± 0.02[a]	0.81 ± 0.03	0.93 ± 0.04[c]	0.85 ± 0.03	0.92 ± 0.03[b]

[a]Significantly different from the same animal group at 15 and 25 days after birth ($0.001 < P < 0.01$).
[b]Significantly different from the control ($0.02 < P < 0.05$).
[c]Significantly different from the control ($0.001 < P < 0.01$).

TABLE III

PHOSPHOLIPID DISTRIBUTION IN LIVER PLASMA MEMBRANES FROM RATS OF DIFFERENT AGES

Plasma membrane phospholipids were extracted as in Table II. The different classes of phospholipids were separated by two-dimensional thin-layer chromatography using the solvents described by Kates [31]. The separated phospholipids were quantitated as μmol of phospholipid phosphate. The data are expressed as % of total phospholipids. Values represent the means ± S.E.M. for the number of preparations indicated in parentheses.

Phospholipids		5 days (6)	15 days (5)	25 days (6)
		%	%	%
Phosphatidylethanolamine	Control	13.9 ± 1.1***	23.2 ± 2.7	24.0 ± 3.7
	Alcoholic	15.8 ± 1.5***	27.0 ± 1.7	27.5 ± 2.1
Phosphatidylcholine	Control	36.1 ± 3.4	34.7 ± 2.0	37.9 ± 3.8
	Alcoholic	40.2 ± 2.1	37.0 ± 2.5	42.0 ± 2.6
Sphingomyelin	Control	31.6 ± 2.0***	24.8 ± 2.3	21.4 ± 4.0
	Alcoholic	30.0 ± 2.4***	22.2 ± 2.1	17.1 ± 2.3
Phosphatidylserine + Phosphatidylinositol	Control	18.4 ± 0.7	17.3 ± 1.0	16.7 ± 0.8
	Alcoholic	14.0 ± 1.0**	13.8 ± 0.9**	13.0 ± 1.1**

** Significantly different from the control ($0.001 < P < 0.01$).
*** Significantly different from the same animal group at 15 and 25 days after birth ($0.001 < P < 0.01$).

groups, it was significantly ($P < 0.01$) reduced in the plasma membranes from alcoholic pups at all ages studied.

Fatty Acyl Composition of Total Phospholipids

The fatty acid composition of liver plasma membrane total phospholipids from rats of different ages is shown in Table IV. From a developmental point of view, only one significant change was observed in the fatty acid content of total phospholipids. There was an increase of 39% in the level of C14:0 for both animal groups between days 5 and 15. The levels of the other fatty acid components of total phospholipids remained quite stable between days 5 and 25. There was also a significant increase ($P < 0.05$) in the level of C22:6 as a result of ethanol feeding at all ages studied. However, the level of unsaturation was not significantly modified by these changes.

Fatty Acyl Composition of Liver Plasma Membrane Individual Phospholipids

The fatty acid composition of liver plasma membrane PE, PC, sphingomyelin, and PS+PI, is reported in Tables V-VIII. Fatty acids C16:0, C18:1,18:2 and C18:0 are the major components of individual plasma membrane phospholipids at all ages studied.

Significant changes during development can be observed in the fatty acid composition of PC and sphingomyelin. As shown in Table VI, the level of C14:0 in PC increased 38% by day 15 and was up 55% by day 25 in both groups of animals. A slight decrease in the levels of C18:1,18:2 in PC was also observed in the control group. The changes in sphingomyelin during development are summarized in Table VII. A significant increase ($P < 0.01$) in the levels of C18:1,18:2 in both animal groups can be observed between day 5 and 25. This change was responsible for the significant increase in unsaturated fatty acids in the control and

TABLE IV

FATTY ACID COMPOSITION OF LIVER PLASMA MEMBRANE TOTAL PHOSPHOLIPIDS FROM RATS OF DIFFERENT AGES

Plasma membrane phospholipids were extracted as described in Table II. Samples were then transmethylated using the boron trifluoride-methanol reagent as described by Morrison and Smith [32]. The methylated fatty acid esters were separated by gas liquid chromatography as described in Materials and Methods. Peak areas and retention times were recorded on a Hewlet-Packard Model No. 3390A Integrator. Data are presented as percent of total phospholipid fatty acid, and values represent the means ± S.E.M. for the number of preparations indicated in parentheses.

Fatty Acids	5 days (6)		15 days (4)		25 days (7)	
	Control	Alcoholic	Control	Alcoholic	Control	Alcoholic
14:0	4.2 ± 0.7***	3.4 ± 0.5***	6.5 ± 0.6	6.1 ± 1.0	6.9 ± 1.0	5.5 ± 0.8
16:1	6.0 ± 0.9	6.4 ± 1.1	6.0 ± 0.8	7.0 ± 0.8	5.0 ± 0.8	5.6 ± 0.9
16:0	22.0 ± 1.3	21.2 ± 1.0	21.5 ± 1.1	18.5 ± 1.3	22.9 ± 0.9	22.5 ± 1.4
18:1, 18:2	18.0 ± 1.2	17.7 ± 1.0	19.3 ± 1.0	16.6 ± 2.0	16.6 ± 1.2	15.7 ± 0.8
18:0	24.0 ± 1.5	22.8 ± 1.3	21.2 ± 1.1	23.3 ± 1.6	24.4 ± 1.1	24.0 ± 1.4
20:4	14.1 ± 0.8	13.6 ± 1.3	13.5 ± 0.9	13.2 ± 1.3	13.9 ± 1.1	13.0 ± 0.5
22:6	11.7 ± 1.0	14.9 ± 1.1*	12.0 ± 0.8	15.3 ± 0.5*	10.3 ± 0.9	13.7 ± 0.8*
Unsaturated	49.8 ± 0.9	52.6 ± 1.3	50.8 ± 1.1	52.1 ± 0.9	45.8 ± 1.1	48.0 ± 1.2

* Significantly different from the control ($0.02 < P < 0.05$).
*** Significantly different from the same animal group at 15 and 25 days after birth ($0.001 < P < 0.01$).

TABLE V

FATTY ACID COMPOSITION OF LIVER PLASMA MEMBRANE PHOSPHATIDYLETHANOLAMINE (PE) FROM RATS OF DIFFERENT AGES

Phosphatidylethanolamine was separated from other plasma membrane phospholipids by two-dimensional thin-layer chromatography as described by Kates [31]. After elution from the silica gel, the phosphatidylethanolamine was transmethylated and analysed for fatty acyl composition as described in Table IV. Data are presented as percent of total phospholipid fatty acid, and values represent the means ± S.E.M. for the number of preparations indicated in parentheses.

Fatty Acids	5 days (6)		15 days (4)		25 days (7)	
	Control	Alcoholic	Control	Alcoholic	Control	Alcoholic
14:0	11.2 ± 1.4	11.7 ± 1.0	10.7 ± 0.9	11.7 ± 1.1	9.7 ± 0.7	9.0 ± 0.9
16:1	11.0 ± 1.4	13.5 ± 1.1	10.0 ± 1.3	12.0 ± 1.5	10.8 ± 1.4	12.8 ± 1.1
16:0	18.9 ± 1.3	17.6 ± 1.3	16.7 ± 0.8	17.5 ± 1.5	20.4 ± 0.8	18.3 ± 1.0
18:1, 18:2	15.7 ± 1.0	14.0 ± 1.1	16.6 ± 1.6	15.3 ± 1.1	17.4 ± 1.3	16.0 ± 1.0
18:0	18.6 ± 1.3	16.8 ± 1.4	19.6 ± 1.5	17.6 ± 1.2	18.8 ± 1.3	18.6 ± 1.3
20:4	11.0 ± 1.1	13.2 ± 1.3	11.2 ± 0.7	11.4 ± 1.4	10.3 ± 0.9	11.1 ± 1.0
22:6	13.6 ± 0.9	13.2 ± 1.0	15.2 ± 1.0	14.5 ± 0.8	12.6 ± 1.2	14.2 ± 1.5
Unsaturated	51.3 ± 1.4	53.9 ± 1.1	53.0 ± 1.6	53.2 ± 1.1	51.1 ± 1.4	54.1 ± 1.2

TABLE VI

FATTY ACID COMPOSITION OF LIVER PLASMA MEMBRANE PHOSPHATIDYLCHOLINE (PC) FROM RATS OF DIFFERENT AGES

All conditions were the same as in Table V.

Fatty Acids	5 days (6)		15 days (4)		25 days (7)	
	Control	Alcoholic	Control	Alcoholic	Control	Alcoholic
14:0	5.0 ± 0.4***	5.2 ± 0.7***	8.2 ± 0.7	8.0 ± 0.6	12.1 ± 0.9	10.0 ± 1.1
16:1	8.3 ± 0.9	9.5 ± 0.4	7.9 ± 1.3	9.3 ± 1.0	7.6 ± 0.9	9.1 ± 0.8
16:0	19.7 ± 0.7	17.8 ± 0.3	19.5 ± 0.5	17.1 ± 1.1	19.0 ± 0.4	17.7 ± 1.3
18:1, 18:2	20.7 ± 1.1	19.9 ± 0.7	19.0 ± 0.5	17.5 ± 1.0	16.7 ± 0.7	18.4 ± 0.8
18:0	23.1 ± 1.4	20.7 ± 0.7	21.9 ± 1.3	20.7 ± 0.9	21.5 ± 2.0	19.5 ± 1.3
20:4	11.0 ± 1.0	10.4 ± 1.0	12.4 ± 0.4	12.0 ± 0.3	12.0 ± 1.1	10.3 ± 0.7
22:6	12.2 ± 0.8	16.5 ± 0.6**	11.1 ± 0.5	15.4 ± 1.0**	11.1 ± 0.4	15.0 ± 0.6**
Unsaturated	52.2 ± 0.7	56.3 ± 1.0*	50.4 ± 1.0	54.2 ± 0.7*	48.4 ± 0.5	52.8 ± 0.6*

* Significantly different from the control ($0.02 < P < 0.05$).
** Significantly different from the control ($0.001 < P < 0.01$).
*** Significantly different from the same animal group at 15 and 25 days after birth ($0.001 < P < 0.01$).

TABLE VII

FATTY ACID COMPOSITION OF LIVER PLASMA MEMBRANE SPHINGOMYELIN FROM RATS OF DIFFERENT AGES

All conditions were the same as in Table V.

Fatty Acids	5 days (6)		15 days (4)		25 days (7)	
	Control	Alcoholic	Control	Alcoholic	Control	Alcoholic
14:0	7.4 ± 0.4	7.6 ± 0.8	9.6 ± 0.6	10.5 ± 1.1	7.5 ± 0.7	7.8 ± 1.1
16:1	8.2 ± 1.0	7.4 ± 0.7	10.2 ± 1.1	7.8 ± 0.8	8.8 ± 1.0	8.7 ± 1.0
16:0	23.1 ± 0.7	24.3 ± 0.9***	21.8 ± 0.7	19.8 ± 0.5	22.0 ± 1.4	20.0 ± 0.7
18:1, 18:2	15.5 ± 0.5***	14.6 ± 1.1***	19.2 ± 1.1	18.8 ± 0.7	21.5 ± 0.5	20.1 ± 0.9
18:0	21.8 ± 1.3	20.8 ± 0.8	16.4 ± 0.8	16.7 ± 1.1	18.9 ± 0.7	17.4 ± 1.0
20:4	12.4 ± 0.6	10.5 ± 1.0	11.1 ± 0.6	11.5 ± 1.2	10.0 ± 0.5	10.6 ± 0.5
22:6	11.6 ± 0.7	14.8 ± 0.8*	11.7 ± 1.0	14.9 ± 0.7*	11.3 ± 0.6	15.4 ± 0.9*
Unsaturated	47.7 ± 0.7***	47.3 ± 0.9***	52.2 ± 0.5	53.0 ± 0.8	51.6 ± 0.5	54.8 ± 1.1*

* Significantly different from the control ($0.02 < P < 0.05$).
*** Significantly different from the same animal group at 15 and 25 days after birth ($0.001 < P < 0.01$).

TABLE VIII

FATTY ACID COMPOSITION OF LIVER PLASMA MEMBRANE PHOSPHATIDYLSERINE + PHOSPHATIDYLINOSITOL (PS+PI) FROM RATS OF DIFFERENT AGES

All conditions were the same as in Table V.

Fatty Acids	5 days (6)		15 days (4)		25 days (7)	
	Control	Alcoholic	Control	Alcoholic	Control	Alcoholic
14:0	13.4 ± 0.7	12.0 ± 0.5	11.0 ± 0.7	10.0 ± 0.6	11.1 ± 0.2	10.7 ± 0.9
16:1	9.4 ± 0.5	9.1 ± 0.6	8.9 ± 0.9	9.7 ± 0.4	8.1 ± 0.8	9.2 ± 0.5
16:0	18.3 ± 0.7	16.4 ± 1.0	18.9 ± 1.0	17.8 ± 1.5	19.0 ± 1.1	16.9 ± 1.2
18:1, 18:2	17.1 ± 0.7	18.0 ± 1.3	19.4 ± 0.5	18.8 ± 0.7	17.4 ± 0.7	19.6 ± 1.0
18:0	19.0 ± 1.0	18.7 ± 0.2	19.1 ± 1.7	18.0 ± 1.1	21.4 ± 2.0	18.4 ± 1.3
20:4	11.4 ± 1.7	10.3 ± 0.7	11.7 ± 0.5	11.2 ± 0.4	10.9 ± 1.0	10.1 ± 0.9
22:6	11.4 ± 0.6	15.5 ± 0.5*	11.0 ± 0.4	14.5 ± 0.8*	12.1 ± 0.9	15.1 ± 1.1*
Unsaturated	50.3 ± 0.5	53.9 ± 0.8*	51.0 ± 0.7	54.2 ± 0.3*	48.5 ± 0.9	54.0 ± 1.0*

* Significantly different from the control ($0.02 < P < 0.05$).

alcoholic pups. A marked decrease in the level of C16:0 can also be observed in the alcoholic group. The fatty acid composition of PE and PS+PI in both animal groups did not change between days 5 and 25, as shown in Table V and VIII, respectively.

The changes in fatty acid composition brought about in individual plasma membrane phospholipids by the presence of ethanol in the diet are reported in Tables V-VIII. As shown in Table V, no major changes were induced by ethanol feeding in the fatty acid composition of PE. However, a consistent and significant increase ($P < 0.05$) in the level of C22:6 is observed in PC, sphingomyelin, and PS+PI in the plasma membranes from alcoholic pups. This ethanol-induced increase in C22:6 was found at all ages studied and it is responsible for the significant increase in the unsaturation levels at days 5, 15 and 25 in PC and PS+PI. An ethanol-induced increase in the unsaturation of sphingomyelin was only observed 25 days after birth.

The postnatal developmental pattern of the fatty acids in rat liver plasma membrane phospholipids is, therefore, mainly characterized by significant changes in C14:0 and C18:1,18:2 of PC and sphingomyelin, respectively. Maternal ethanol administration induced a significant increase in C22:6 of PC, sphingomyelin and PS+PI, which resulted in an increase in the level of membrane fatty acid unsaturation. The composition of fatty acids in PE was not affected by the ethanol administration.

DISCUSSION

The overall focus of this investigation was to study the lipid composition of the rat liver plasma membrane during the postnatal development following maternal ethanol ingestion during pregnancy and lactation. The results reported suggest that specific adaptive changes were induced in the liver plasma membrane lipids of the progeny from alcoholic rats.

We have previously observed [22] that although the weight gain during pregnancy was similar in both the alcoholic and pair-fed control dams, the postnatal weight gain over the period studied of the newborn pups of alcohol-fed mothers was reduced by 33% compared with pair-fed controls. However, regardless of diet treatment or age, both the liver wet weight to body weight ratio and the yield of hepatic plasma membrane protein per wet liver weight in both pair-fed groups were constant. Moreover, results shown in Table I indicate that the liver plasma membranes isolated from both alcoholic and pair-fed control pups were equally enriched in 5'-nucleotidase and minimally contaminated with microsomes and mitochondria. Thus, chronic maternal ethanol ingestion does not affect either the yield or the degree of purification of the hepatic plasma membranes isolated from newborn pups.

The postnatal changes in the total phospholipid and cholesterol content of plasma membranes from alcoholic and pair-fed control pups are shown in Table II. Total phospholipid and cholesterol content increased by 30 and 70%, respectively, in both groups; adult values were attained 15 days after birth. Although the total phospholipid content was similar in both groups at all ages studied, the amount of cholesterol present in the hepatic plasma membranes from alcoholic pups was significantly

greater than that of the pair-fed controls, resulting in a higher Chol/phospholipid molar ratio. An increase in the Chol/phospholipid molar ratio may, therefore, represent an adaptive response of the plasma membrane to the chronic presence of ethanol. Chin et al. [4] reported an increase in the cholesterol content of erythrocyte and brain membranes from mice physically dependent on ethanol and they concluded that this alteration may be partially responsible for the development of tolerance to the presence of ethanol. Although we did not investigate whether the plasma membranes from the alcoholic pups were tolerant to ethanol, it is conceivable that an increase in the cholesterol content may well result in an enhancement of the degree of order in the membranes, which become more resistant to the fluidizing effects of ethanol [4].

While other investigators have reported an increase in the Chol/phospholipid ratio in synaptic plasma membranes from mice [19] and brain microsomal membranes from rats [45] physically dependent on ethanol, other studies using mice synaptosomal membranes [46] and rat liver plasma membranes, myelin and synaptosomes [47] have reported no changes in the Chol/phospholipid ratio subsequent to chronic ethanol administration. Such discrepancies might be due to a variety of reasons including the strains of animals used and the routes, dosages and duration of ethanol administration. Nevertheless, the increase in the Chol/phospholipid ratio observed in our study most probably represents adaptive changes at the cellular level following chronic maternal ethanol ingestion.

The phospholipid distribution in plasma membranes isolated from alcoholic and pair-fed control pups is shown in Table III. In agreement with other data [35,39-44], our study indicates that the major individual

constituent phospholipids in the plasma membrane are PE, PC and sphingomyelin. This pattern was independent of either age or the diet fed. Adult values for the phospholipid distribution in plasma membranes from both animal groups were seen in animals 15 days old, and it is interesting to point out that the phospholipid distribution in plasma membranes from 25-day-old pair-fed control pups was also quite similar to that found in adult rat liver plasma membranes [35,39-44].

The developmental pattern of the distribution of phospholipids was also characterized by an increase of 70% in PE and a concomitant decrease of 65% in sphingomyelin in both groups of animals. The content of PC did not vary with age or diet, and although that of PS+PI was independent of age in both animal groups, it was significantly ($P < 0.01$) reduced in the hepatic plasma membranes from alcoholic pups at all ages studied. Hence, chronic maternal ethanol ingestion led to a decrease in the hepatic plasma membrane content of PS+PI in newborn alcoholic pups. Crane et al. [48] studied the influence of ethanol on lipid metabolism in various mouse tissues and they showed that ethanol ingestion resulted in an increased degradation of phospholipid fractions, which was most evident in the PS+PI fraction. They ascribed their results as being due to a marked stimulation of peroxisomal β-oxidation induced by ethanol ingestion in the alcoholic livers. Since alcohol feeding induces lipid peroxidation [49], we cannot rule out that possibility as a plausible explanation of our results.

Analysis of the fatty acids in the total and individual phospholipids of liver plasma membranes (Tables IV-VIII) during the postnatal development showed that C16:0, C18:1,18:2 and C18:0 are the major components. These results are in agreement with those of Dobiasova et al.

[50], who studied the fatty acid composition of rat liver phospholipids during postnatal development. While the fatty acid composition of PE and PS+PI did not vary in either group over the period studied, significant developmental changes took place in the fatty acid composition of PC, sphingomyelin and total phospholipids. An increase of 39% in C14:0 was observed in the fatty acid content of total phospholipids in both animal groups between days 5 and 15, which was compensated for by slight changes in the level of other fatty acids. The amount of C14:0 in PC (Table VI) was also increased by 38% in the 15-day-olds and up to 55% in the 25-day-olds from both groups of animals. A concomitant slight decrease in C18:1,18:2 was observed in the 25-day-old animals. The developmental changes that took place in sphingomyelin (Table VII) between days 5 and 25 were characterized by a marked decrease in C16:0 in the alcoholic pups and a significant increase in C18:1,18:2 in both groups which was responsible for the age-related increase in lipid unsaturation. Although not significant, a slight increase in C14:0 was also observed in sphingomyelin from the 15-day-olds in both groups. The developmental changes taking place in the fatty acid composition of total phospholipids, PC and sphingomyelin were, therefore, independent of diet treatment in most cases and they revealed changes mainly in the levels of C14:0 and C18:1,18:2. Since the fatty acid composition of individual phospholipids in the plasma membranes from newborn pups fed both diets did not reflect the fatty acid content of the diet (refer to Materials and Methods), the increase in the relative amounts of C14:0 and C18:1,18:2 might be partially explained by the induction of lipogenesis in response to the suckling-weaning transitional period [51] rather than by a change in the suckling pattern during lactation.

The effect of ethanol administration on membrane fatty acid composition has been studied in unicellular organisms, mammalian cells and rodents given ethanol either in their drinking fluid, via intraperitoneal injection or through continuous inhalation of ethanol vapor. Ingram et al. [52] found that ethanol induced an increase in unsaturated fatty acids in Escherichia coli, and a decrease in C18:0, with a concomitant increase in C16:0 in Chinese hamster ovary cells. Thompson and Reitz [18] found the content of C18:1 and C18:2 to be significantly increased while that of C20:4 was decreased significantly in total mitochondrial fatty acids from male rats fed a low-fat diet as opposed to those fed a high-fat, ethanol containing diet. Cunningham et al. [20] reported major changes in C16:0 and C18:1 in microsomes, and in C16:0, C20:4, C18:1 and C18:2 in mitochondria from rats maintained on a liquid high-fat diet containing 36% of calories as ethanol. More recently, Shorey et al. [53] observed that muscle phospholipids from rats fed an ethanol-containing liquid diet did not appreciably differ in fatty acid composition from that in the pair-fed controls. Taken altogether, these results seem to indicate that no clear pattern was observed in the fatty acid composition changes in membranes from either cells or tissues chronically exposed to ethanol. This discrepancy may reflect not only variations in methodological approaches, but also different responses to ethanol in various membranes.

Although several studies have been published on the fatty acid composition of rodent liver plasma membranes [37,43,54-57], there are no reports in the literature on the effects of ethanol administration on the fatty acid composition of such membranes. Results from our experiments indicate that, at all ages studied, chronic maternal ethanol administra-

tion induced a significant increase in the levels of C22:6 in total phospholipids, PC, sphingomyelin and PS+PI (Tables IV, VI-VIII), which resulted in an increase in the level of unsaturation of the hepatic plasma membranes from the alcoholic pups. PE (Table V), was not affected by the ethanol treatment.

The increase in the phospholipid content of C22:6 in the alcoholic pups was observed during lactation (days 1 to 21) and after the pups had been weaned (days 21 to 25). Inasmuch as this change in C22:6 persists throughout the suckling-weaning period and since the fatty acid composition of the hepatic plasma membranes does not reflect the content of the diet, our results cannot be attributable either i) to an indirect effect of ethanol via changes in the fatty acid content of the maternal milk which is delivered to the pups or ii) to an effect of ethanol on the fatty acid content of the liquid diet that will by itself affect the liver plasma membrane composition of the alcoholic pups. The latter possibility was also considered by Cunningham et al. [20], who found that the liquid diet alone had no significant effects on the fatty acid content of the mitochondria.

The elevated levels of C22:6 in the hepatic plasma membranes from alcoholic pups resulted in a significant increase in phospholipid unsaturation. Sun and Sun [5] also reported an increase in the polyunsaturated acids of ethanolamine phosphoglycerides from synaptic plasma membranes after chronic ethanol treatment. Since ethanol causes disruption of biological membranes [1-3], it is possible that the changes in the degree of unsaturation observed here may cause generalized changes in the intrinsic 'fluidity' of the hepatic plasma membranes from these newborn alcoholic pups [58,59]. Further, these changes may, through

adaptation, induce alterations in membrane-associated biochemical processes, such as modulation of the activities of membrane-bound enzymes [60,61] and transport systems [62,63], regulation of the binding properties of hormone receptors [64-66] and membrane phospholipid interactions with abused drugs [67]. Some of these possibilities are now being examined.

These results indicate that although chronic maternal ethanol administration did not appear to affect the postnatal development of the liver plasma membrane lipids, specific adaptive changes were induced in the membrane lipids of the progeny. Further work is being directed at elucidating i) the precise structural and functional implications of the changes observed and ii) the relative contribution of the in utero exposure to ethanol as opposed to its chronic administration during both pregnancy and lactation on these changes.

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CHAPTER 8

General Discussion and Conclusions

Animal models of maternal alcoholism have been developed during the past decade by several investigators who have conclusively demonstrated that maternal alcohol exposure can induce developmental malformations and biochemical anomalies in the fetus and the neonate. The molecular basis of the mechanism(s) of the deleterious action(s) of maternal alcohol consumption is however not known.

In view of the demonstration that ethanol causes disorder of cell membrane structure (Chapter 1, Section 1.7), it is possible that cellular adaptation to its continued presence may likely be involved in causing tissue damage. Since the liver is one of the primary targets of alcohol toxicity (Chapter 1, Section 1.5), it was felt that studies on the influence of maternal ethanol consumption on the developing rat liver and on the structure and function of the postnatal rat liver plasma membrane might contribute a better understanding of the molecular basis of the deleterious effects of maternal alcoholism on the progeny.

The general characteristics of the animal model under investigation were described in Chapters 2 and 5. The administration of alcohol (37% of the total calories) to pregnant and lactating rats in a nutritionally adequate liquid low-fat (5%) Metrecal diet was shown to produce intoxicating levels of alcohol in maternal blood which varied between 35 and 40 mM. Moreover, upon withdrawal from alcohol, animals exhibited tremor and hyperexcitability. Blood alcohol levels in newborn pups of alcohol-fed mothers ranged between 4 mM at day 5 to 40 mM at day 25. Although no significant differences were found in maternal weight gain, litter size, and average litter weight at birth between animals in the alcoholic and pair-fed sucrose control groups, the postnatal weight gain over the period studied of the newborn pups of alcohol-fed mothers was reduced by

33% when compared to the corresponding control pups (Chapter 5, Table 2). In general, maternal alcohol exposure was found to have a detrimental effect on the postnatal physical performance of the progeny.

Studies on the developing rat liver showed the emergence of distinct fatty infiltration in fetal and neonatal hepatic tissues following chronic maternal ethanol ingestion (Chapter 3, Figs. 2 and 3). Fatty infiltration first appeared in 19-day-old fetal liver tissues from the alcoholic progeny, and this hepatic lesion was found to persist throughout postnatal development. Although fatty liver has been shown to occur in humans (1) and laboratory animals (2,3) following prolonged alcohol exposure, our results demonstrated that this condition can also develop in the progeny subsequent to maternal alcohol ingestion.

The ontogenetic development of liver alcohol dehydrogenase (ADH) activity (Chapter 3, Fig. 1) revealed that ADH was first detectable at day 19 of gestation in the fetal livers of rats from both maternal groups. After birth, the enzyme activity increased almost linearly in both groups of animals up to 14 days of age. At this age, ADH activity reached a plateau and adult values were consistently obtained between 14 and 18 days postnatally. Although ADH activity and certain kinetic properties of enzyme preparations from the progeny of alcohol-fed and pair-fed mothers were similar, it is interesting to note that a positive correlation could be established between the emergence of fatty liver and the appearance of ADH in fetal tissue from the alcoholic progeny. Since metabolic derangements in the liver during ethanol administration are caused primarily by changes in the [NADH/NAD^+] ratio (Chapter 1, Section 1.3), one might expect that the appearance of ADH may alter the hepatic redox state in the alcoholic progeny and lead to hepatic lesions such as

fatty liver. Not being within the scope of this thesis, whether or not this is true in our animal model is still uncertain and remains to be elucidated.

Liver plasma membranes from alcoholic and pair-fed control groups were isolated at various postnatal ages. The yield of hepatic plasma membrane protein per wet liver weight was constant, independent of either age or diet, and close to 2.5 mg/g (Chapter 5, Fig. 3). Characterization of the membranes with electron microscopy (Chapter 4, Figs. 1 and 2), isopycnic centrifugation (Chapter 4, Table 1) and marker enzymes (Chapter 7, Table I) revealed no differences in ultrastructure, equilibrium density and extent of purification with respect to either age or alcohol treatment.

The effect of chronic maternal ethanol feeding on the postnatal hepatic plasma membrane, as assessed with the development of alpha$_1$-adrenergic receptors, was subsequently investigated. Using [^{3}H]-prazosin as radioligand, equilibrium binding studies were carried out. Results indicated that, in general, the postnatal development of the hepatic alpha$_1$-adrenergic receptors was quite similar for the pair-fed sucrose control and alcoholic pups, both following a sigmoidal pattern (Chapter 5, Fig. 5). Between 6 and 30 days, receptor density (B_{max}) for pups in the alcoholic group was decreased significantly compared with the pair-fed sucrose controls. No difference was observed in the binding affinity (K_D) during the entire postnatal period for both groups, and this suggested that the fundamental functional characteristics of these receptors were not altered (Chapter 5, Table 3 and Fig. 5).

Although the precise mechanisms responsible for the diminished hepatic alpha$_1$-adrenergic receptor density are not known, it was suggested that this anomaly could represent the manifestation of some fundamental intrinsic difference between the control and alcoholic plasma membranes as was observed earlier in studies with adult male rats (4). Structurally, lipid compositional changes might have occurred in the alcoholic membranes (5), and these could conceivably have modified the dynamics of lipid-protein interactions (6) which could have altered the accessibility of the receptor to the ligand. On the other hand, functionally, the decreased receptor density may have seriously affected the capacity of the liver to respond to the regulatory actions of epinephrine in the alcoholic pups (7). Further studies were conducted to test these two possibilities.

Functional studies were carried out on the stimulation by (-) epinephrine of glycogen phosphorylase a in rat liver slices from the progeny during postnatal development. Results showed that neither the basal nor the stimulated enzyme activity varied with age during development in both groups of animals (Chapter 6, Table I). Also, the basal hepatic phosphorylase a activity was found to be identical between the control and experimental groups at 5, 15 and 25 days of age. However, stimulation with epinephrine (10 μM) demonstrated a significantly diminished capacity of the enzyme in the alcoholic liver to be activated by the hormone; in every instance, the livers from 5, 15 and 25-day-old pups from alcoholic mothers displayed diminished epinephrine-stimulated phosphorylase a activity of about 30%, compared with the controls (Chapter 6, Table I). These results indicated that chronic matenal ethanol feeding had a detrimental effect on the hormonal sensitivity of the postnatal liver.

Finally, we investigated whether the described membrane-associated abnormalities in the alcoholic progeny were partially modulated by changes in the lipid composition of the postnatal rat liver plasma membranes.

The fatty acid composition of constituent phospholipids and the cholesterol content of the postnatal hepatic plasma membranes were determined subsequent to maternal alcohol ingestion. Results revealed that although the developmental pattern of the phospholipid distribution and fatty acid content was similar in the plasma membranes from both groups of animals, specific changes were induced in the membrane lipids of the progeny from alcoholic rats. Cholesterol content was greater in the alcoholic pups, resulting in a higher cholesterol/phospholipid molar ratio (Chapter 7, Table II). Likewise, a consistent increase of fatty acid C22:6 in most of the constituent phospholipids from alcoholic pups was observed (Chapter 7, Tables VI-VIII). The increase in the cholesterol content may well represent an adaptive change because it may result in an enhancement of the degree of order in the alcoholic membranes, which become more resistant to the fluidizing effects of ethanol (5). On the other hand, the elevated levels of C22:6 in the hepatic plasma membranes from alcoholic pups resulted in a significant increase in phospholipid unsaturation which may induce generalized changes in the intrinsic 'fluidity' of the alcoholic membranes and affect cellular function.

In summary, all the results reported suggest that adaptive changes were induced in the structure and function of the postnatal rat liver plasma membrane following chronic maternal ethanol ingestion. Since ethanol seems to act by disordering biomembranes, it is possible that

these changes may represent homeoviscous adaptation of the membranes, defined as alterations in the physical properties of the membrane bilayer which tend to maintain normal cellular function in the presence of ethanol (Chapter 1, Section 1.7).

In general, cellular function is closely associated with the structural integrity of its membrane components, and membrane-associated cellular functions are in turn influenced by the physical state of the lipid bilayer. Moreover, it is known that various membrane bound components, such as enzymes, receptors and transport systems, are therefore highly sensitive to perturbations of the lipid bilayer caused by external agents that affect the composition and physical state of the membrane lipids. This is particularly important during gestation when maternally transferred perturbations may severely affect the normal development of cellular membranes from different fetal tissues. Since fetal rat liver alcohol dehydrogenase was shown to appear only a few days prior to birth, the prolonged exposure to unmetabolized alcohol may have produced adaptive alterations in the structure and function of cellular membranes from different fetal tissues, and these anomalies could have conceivably affected fetal development.

In view of the fact that chronic alcohol consumption causes disorder of cell membranes from many different tissues, it seems reasonable to suggest that the structural and functional changes in the postnatal rat liver plasma membrane described above, most probably represent a specific correlate of a general mechanism by which membrane alterations in the newborn contribute to ethanol-induced damage and thus may be partially responsible for the deleterious actions of maternal alcoholism at the molecular level. It is also likely that maternal ethanol exposure may

induce adaptive changes in other membrane components such as proteins, and these combined effects could represent a strong biochemical basis for the teratogenic effects of alcohol *in utero*.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. The study on the ontogenetic development of liver alcohol dehydrogenase activity and the emergence of fatty liver in rat offspring following chronic maternal ethanol ingestion demonstrated that:
   a) fatty liver developed in the alcoholic progeny before birth.
   b) a positive correlation could be established between the emergence of fatty liver and the appearance of liver alcohol dehydrogenase in fetal tissue from the alcoholic progeny.
   c) chronic maternal ethanol consumption during pregnancy and lactation did not affect the ontogenetic development of liver alcohol dehydrogenase in the progeny.

2. Characterization of postnatal rat liver plasma membranes with electron microscopy, isopycnic centrifugation and marker enzymes revealed no differences in ultrastructure, equilibrium density and extent of purification with respect to either age or alcohol treatment.

3. A study of the postnatal development of rat hepatic alpha$_1$-adrenergic receptors, as assessed with the binding of [^{3}H]-prazosin to liver plasma membranes from the progeny, demonstrated:
   a) a developmental sigmoidal pattern of the membrane-bound receptors which was not affected by maternal alcohol exposure.
   b) a significant decrease in receptor density (B_{max}) in the alcoholic progeny which ranged from 30 to 70% throughout

postnatal development, but no changes in binding affinity (K_D) with respect to either age or diet treatment.

4. Functional studies on the stimulation by (-) epinephrine of glycogen phosphorylase a in rat liver slices from the progeny showed that although basal levels of enzyme activity were independent of either age or diet treatment, alcoholic livers exhibited a significantly diminished sensitivity to hormonal stimulation throughout postnatal development.

5. A determination of the effects of maternal ethanol ingestion on the lipid composition of rat liver plasma membranes during postnatal development revealed that:
   a) the total phospholipid phosphorus was similar but increased significantly with age in both control and alcoholic pups.
   b) cholesterol also increased significantly with age in both groups but was greater in the alcoholic pups, resulting in a higher cholesterol/phospholipid molar ratio.
   c) while the phosphatidylethanolamine (PE) content increased with age in both groups, that of sphingomyelin decreased.
   d) phosphatidylserine + phosphatidylinositol (PS+PI) content was significantly decreased in the alcoholic progeny throughout postnatal development.
   e) there was a consistent increase of the fatty acid C22:6 in phosphatidylcholine, sphingomyelin, PS+PI and in the total phospholipid fraction from alcoholic pups.