Influence of Chronic Alcohol Feeding to Pregnant Rats on the Teratogenicity of Various Membranes as Studied by Biophysical Methods

by

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ABSTRACT

Maternal alcohol consumption during pregnancy and lactation has long been thought to have deleterious effects on the progeny's growth and development. In the present study, the effects of chronic alcohol intake on biological membranes from the brain were investigated in an attempt to elucidate membrane perturbation by ethanol and the adaptative response after long term exposure to the drug.

A biophysical study of synaptosomal and mitochondrial membranes was performed using the pyrene excimer-formation technique. It was observed that only synaptosomal membranes from 10 day-old progeny were cross-tolerant to the *in vitro* fluidifying effects of pentanol. The lipid fractions of synaptic membranes were further analysed by this fluorescent method. The total lipid fraction from the alcohol-fed group unlike the phospholipid one was shown to be cross-tolerant to fluidization by pentanol. Cholesterol levels were slightly but not significantly elevated in the alcoholic group. Studies on reconstituted total lipids with equalized cholesterol molar fraction revealed a similar tolerance phenomenon. Reconstituted total lipid extracts with varying cholesterol showed that the sterol rigidifies lipid bilayers but also sensitizes them to fluidization by alkanols.

The Na,K-ATPase was then assayed in brain homogenates and it also revealed a component of tolerance to in vitro inhibition by ethanol. This was shown to be the case for both the basal and dopamine stimulated enzyme.

These findings suggest that cholesterol by itself is not responsible for acquired tolerance but is essential in mediating membrane perturbation by alkanols and is required in the expression of membrane adaptation to alcohol. This in turn can be correlated with membrane-associated functions such as ATPase activity.

RESUME

On a longtemps cru que la consommation maternelle d'alcool durant la grossesse et l'allaitement était dangereuse pour le developpement et la croissance du foetus. Dans cette étude, les effets de l'ingestion chronique d'alcool sur des membranes isolées du cerveau sont determinés dans le but d'elucider la perturbation causée par l'alcool et l'adaptation envers cette drogue.

Une étude biophysique des membranes synaptosomales et mitochondriales est faite utilisant une technique de fluorescence avec le pyrene. Seules les membranes synaptosomales se revèlent tolerantes aux effets fluidisant de l'addition in vitro de pentanol. Les fractions lipidiques des synaptosomes sont alors soumises a la même étude fluorescente. Contrairement aux phospholipides, la fraction de la totalité des lipides provenant du groupe alcoolique est observée comme étant tolerante à la fluidisation par le pentanol. Les niveaux de cholesterol sont legèrement plus elevés chez les alcooliques. Des études sur des fractions de la totalité des lipides reconstitués avec un contenu égal en cholesterol revèlent un phenomène similaire de tolerance. En variant les niveaux de cholesterol des fractions reconstituées, on a observé que ce sterol rend les membranes plus rigides mais les rend aussi plus sensibles à la fluidisation par des alkanols.

La Na,K-ATPase obtenue du cerveau est ensuite étudiée et re-

vèle une tolerance envers l'inhibition par l'ethanol *in vitro*. Ceci fut le cas pour l'activité de base et celle stimulée par la dopamine.

Ces résultats suggèrent que le cholesterol par lui-même n'est pas responsable du phenomène de tolerance mais est requis pour l'expression de l'adaptation membranaire à l'alcool. Ceci, en retour, peut être relié avec les fonctions associées aux membranes comme l'ATPase.

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INTRODUCTION

A. Fetal alcohol syndrome and teratogenicity of alcohol

1. Historical. The belief that maternal consumption of alcohol during pregnancy and lactation could have deleterious effects on the growth and normal development of offspring has a long and varied history (1). Throughout the 19th century, children of alcoholic parents were observed to have significantly higher frequencies of mental retardation, epilepsy, stillbirths and neonatal deaths (2). Early in this century, Stockard et al studied the effects of alcohol consumption on the development of chicks, minnows and guinea pigs (3,4). However, such studies along with others (5) performed at about the same time were fraught with methodological difficulties such as lack of adequate nutritional control (6). This made the assessment of the teratogenic potential of alcohol difficult. In the following 3 or 4 decades only a few scattered reports appeared in the clinical or experimental literature describing the effects of parental alcoholism on the offspring (6).

More recently, Jones et al recognized a specific pattern of malformations in children of alcoholic mothers (7). The term Fetal Alcohol Syndrome (or FAS) was first used to describe this pattern of prenatal and postnatal growth deficiency, developmental delay, mental deficiency, microcephaly, fine motor dysfunction and facial dysmorphology (2,7). All such anoma-

lies are now diagnostic of FAS. As a result of the increasing body of information regarding the FAS, researchers tried to develop suitable animal models to study the syndrome (6). Such animal studies were of great importance as they accomodated the possibility of controlling the numerous confounding variables inherent to human research. For example, the control of the amount of alcohol administered daily could be maintained. Furthermore, genetic variables and polydrug use were no longer confounding factors as they were in the human studies (6).

1.2 Teratology. Teratology is the field of basic research which endeavours to determine or elucidate the various causes of birth defects (2). Originally, only gross structural defects in offsprings were described by teratologists. Nowadays, such studies include microscopic abnormalities, behavioural disorders and biochemical consequences. Teratogenic agents seem to act primarily on a particular aspect of cell metabolism and accentuate the incidence of defects and malformations as a result of cellular and genetic instabilities. Teratogenic agents are widespread in nature and they include exposure to radiaton such as X-rays and radium sources; mercury, pesticides and a variety of drugs (2). For example, anticonvulsant drugs like phenobarbital or hydantoins are associated with a greater incidence of major malformations similar to those seen in FAS (2). Administration of hormones such as cortisone and some

synthetic progestins can also increase the incidence of anomalies (2). Deficiencies in vitamins and trace minerals like zinc and magnesium have also been shown in animal studies to be teratogenic (8). In fact, all chemicals administered experimentally in sufficiently high doses retard embryonic growth or kill them (9). The timing of exposure to the drug or chemical can also affect the progeny (2). In general, exposure early in gestational phases causes morphologic damage or death whereas exposure late in gestation causes functional decifits and growth retardations (10).

Although our understanding of multiple potential teratogens has increased, the origin of most developmental defects still remains unclear (2).

B. Pharmacology and biochemistry of alcohol

1. Source and chemistry. Beverage alcohol is a simple molecule, CH_3CH_2OH, of molecular weight 46.07. Ethanol may be produced either from bacterial fermentation of carbohydrates or synthesized by catalyzed hydration of ethylene (11). When yeast is grown under anaerobic conditions in a carbohydrate containing solution, sugars are fermented to alcohol and carbon dioxide. When the concentration of alcohol reaches about 12%, ethanol itself inhibits further growth of the microbial organisms and the process stops (2).

Ethyl alcohol by virtue of its polar hydroxy group can form

hydrogen bonds and thus exhibits intermolecular association in the same manner as water. However, the pK_a of the -OH group is about 20 making the terminal group virtually undissociated under physiological conditions (12). Furthermore, the ethyl moiety of the molecule is non polar and associates through Van der Waals forces (12). Therefore, many chemical properties of ethanol are a balance between the polar but uncharged -OH group and the non polar ethyl carbons.

2. Alcohol metabolism

2.1 Absorption. Following ingestion of alcohol, some 20 to 30% of the ethanol is rapidly absorbed through the stomach wall (2). At first absorption is rapid but then it decreases to a very slow rate even though gastric concentrations remain high (13). Absorption from the small intestine is extremely rapid so that the rate of absorption of alcohol in a given subject will depend upon the rate at which the ingested alcoholic beverage will flow from the stomach into the duodenum (13). Major factors influencing the rate of gastric emptying include the volume, character and dilution of the alcohol beverage together with the presence of food, the period of time taken to ingest the drink and individual peculiarities (13,14).

2.2 Distribution. After absorption, alcohol diffuses throughout the body and is fairly uniformly distributed into all tissues and

fluids (13). In fact, except for intact skin tissue; there appears to be no barrier for alcohol diffusion in the body (12). This is as a consequence of the solubility of ethanol in both aqueous and lipid compartments of tissues and organs. Since the placenta is permeable to alcohol; it gains free access to fetal circulation and therefore can have adverse effects on fetal development. Ethanol also crosses the blood brain barrier so that the central nervous system becomes an important target during alcohol intoxication. Moreover, as a result of the large blood supply to the brain, CNS alcohol concentration quickly approaches that of the blood (13). In general, during the course of its elimination alcohol can affect many functions and indeed there has been literature on pathological conditions brought about by chronic ethanol consumption on most of the body organs (2,13).

2.3 Elimination. Ninety to 98% of the ingested drug is completely oxidized (12,13). The small amounts remaining are excreted unchanged in the breath, urine and sweat (13). The rate of metabolic oxidation of alcohol is governed by zero order kinetics, that is, blood alcohol levels fall some 15 mg% per hour regardless of the initial alcohol levels in the blood (13). The rate-limiting step is the oxidation of ethanol to acetaldehyde (2). This initial oxidation of ethanol is accomplished mainly in the liver (14) although kidney (15), muscle (2), lung (2) and intestine (2,17) have been shown to metabolize small quantities. The hepatic enzyme responsible for the conversion of ethanol to acetal-

dehyde is alcohol dehydrogenase (ADH), a zinc dependent 80K polypeptide which utilizes NAD as hydrogen acceptor (18). The Km of ADH for ethanol lies in the order of 0.5 to 2.0 mM (14,19), ADH also has a broad substrate specificity and can be inhibited non competitively by pyrazole (14), a compound freqently used in animal studies to maintain steady blood alcohol levels. ADH functions normally in the liver to oxidize the ethanol that is spontaneously being produced within the intestine by the microbial flora which ferment carbohydrates (20). Lester et al estimated that humans and most mammals endogenously produce some 12 to 40 grams of alcohol per day (21,22). The production of acetaldehyde by ADH may also play a role in alcohol toxicity as the aldehyde is a reactive molecule which is also lipid soluble (2). For example, binding of acetaldehyde to proteins was demonstrated in liver microsomes of rats chronically fed alcohol (23). However, acetaldehyde can be further oxidized to acetate by the enzyme aldehyde dehydrogenase in a reaction coupled to the reduction of the coenzyme NAD (2,19). The acetate produced is ultimately oxidized to H_2O and CO_2 through the Krebs cycle of intermediate metabolism.

The acquired ability to consume large quantities of alcohol common to alcoholics results from CNS tolerance to higher blood alcohol levels as well as induction of additional oxidizing capacity (18,24). The hepatic microsomal ethanol oxidizing system (MEOS) which was recently discovered (25), has been shown to be of importance in the metabolism of alcohol. The hepatic

MEOS catalyzes the oxidation of ethanol to acetaldehyde using molecular oxygen and NADPH as substrates (24). The Km of MEOS for ethyl alcohol is in the order of 8-9 mM (14). The purified components of this system include cytochrome P450, NADPH cytochrome C reductase and require phospholipids for biological activity (26). In heavy drinkers MEOS can contribute as much 50% of the total alcohol metabolism (2). MEOS has been shown to be induced in chronic alcoholism and also oxidizes other sedative drugs like barbiturates (2,13). Thus, a cross-tolerance develops simultaneously between alcohol and various hypnotics.

2.4 Nutritional aspects. Alcohol is both a drug and a food. The metabolism of ethanol can produce some 7 calories per gram which amounts to about 105 calories per standard drink (2). Taking only these considerations into account, alcohol may provide more than half of the daily required calories in some heavy drinkers. However, it cannot provide the vitamins, minerals and essential amino acids normally present in an adequate diet. As a consequence, ethanol has often been referred to as providing "empty calories" (27). Furthermore, ethanol may interfere in gastrointestinal absorption of various nutrients and also increase their loss through vomiting, diarrhea and increased urinary excretion (2,27,28). For example, Flink et al showed that magnesium, zinc and folic acid are depleted as a result of increased urinary excretion and inadequate intake during chronic alcoholism (29). In terms of the teratogenic effects of ethanol,

magnesium has important roles in fetal development since it stabilizes DNA, RNA, binds SRNA to ribosomes and is involved in the activation and transfer systems of all amino acids (30). Zinc is required for RNA and DNA synthesis and for a number of metallo-enzymes including alcohol dehydrogenase (19). Zinc deficiencies have been associated with high rates of stillbirth, neonatal mortality, congenital abnomalies, low birthweight and growth retardation (2). Tanaka et al have demonstrated in rat model systems that diets rich in zinc can alleviate some of the teratogenic effects of alcohol (31). Ethanol also impairs intestinal absorption of folate. Sullivant and Herbert showed that hematologic response to folic acid therapy was repeatedly prevented by the administration of liquors or laboratory ethanol (32). Ethanol may also act as a weak folate antagonist when body stores are decreased and dietary intake is poor (2).

Folic acid antagonists have been shown to cause fetal resorption, stillbirths and congenital malformations in the rat (33).

Metabolism of large amounts of ethanol within liver cells causes fat accumulation and can predispose to alcoholic hepatitis and cirrhosis. These in turn may result in portal hypertension and repeated attacks of pancreatitis with subsequent nutritional deficiencies (2).

C. General Anesthetics.

General anesthetics comprise a wide variety of structurally unrelated compounds including alkanols (up to 9 carbon long), gaseous and volatile agents, halocarbons, steroids, amines and barbiturates. All such chemicals have been shown to perturb lipid bilayers and biological membranes (34,38). Although the molecular mechanisms of general anesthesia remain to be fully elucidated, current theories are roughly divided into 2 categories. Either general anesthetics bind directly on apolar sites of sensitive proteins or they partition within the lipid fraction of biomembranes and perturb membrane - associated functions.

1. Protein hypothesis. A major experimental problem arises when working with membrane-bound proteins: differentiation of direct effects on protein from those on the neighbouring lipids. There have been studies using simple, relatively pure protein systems where the observed effects can be unambiguously interpreted in terms of protein - anesthetic interactions. For example, some alkanols and anesthetic alkanes have been shown to bind to bovine serum albumin (39,40). Hansch et al also accumulated data on the binding of small molecules, including anesthetics, to bovine serum albumin and haemoglobin (41,42), where it was shown that binding of the anesthetics to protein surfaces could be correlated with the octanol-water partition coefficient of the

molecule. Although such information is irrelevant to general anesthesia, it serves to illustrate that a large range of molecules may bind to proteins in a fashion related to their solubility in organic solvent. Eventhough general anesthetic-protein interactions are a possibility in the mechanisms of general anesthesia, circumstantial support for the lipid theories of general anesthetic action has remained strong since the turn of the century (43,44).

2. Disordered lipid hypothesis. The disordered lipid hypothesis, on the other hand, is unitary since it correlates anesthetic potency with lipid solubility (45,46). In such a model, the anesthetic molecule is thought to partition within the lipid fraction of biomembranes and indirectly to affect membrane-associated functions (47).

A variety of experimental data have supported the lipid hypothesis since its conception. First, anesthesia in vivo and anesthetic - induced disordering of biological membranes and lipid bilayers can be reversed by an increase in hydrostatic pressure (48,49,50).

Also, the concentrations of anesthetic agents (like alkanols) required to induce physiological effects are much greater than those required for the pharmacological activity of drugs known to interact specifically with protein receptors (51). In addition, the high degree of chemical diversity found in the

family of general anesthetics (52) would seem to preclude any specific chemical interaction at the basis of general anesthesia. Further evidence for the non-specific interaction between alcohol and biological membranes comes from the development of tolerance to anesthetics. It is known that humans and rodents chronically fed with alcohol will have an increased requirement for the drug to still produce anesthetic effects (53,54).

In some cases organisms made tolerant to a given anesthetic drug also display tolerance to another general anesthetic. For instance, mice made tolerant to nitrous oxide were also tolerant to alcohol (55). This phenomenon, known as cross-tolerance, has been observed between many general anesthetics including barbiturates, alkanols, halocarbons and rare gases (55,56). Such observations would seem to exclude any specific membrane anesthetic interaction. Of great importance in the disordered lipid hypothesis is the correlation between anesthetic potency and lipid solubility of the drug molecule. In the early 1900's, Meyer and Overton simultaneously but independently noted a correlation between anesthetic potency and olive oil solubility (46,51,52,57). Since then, the solubility in olive oil has been shown to be inadequate in correlating anesthesia with lipid solubility. For example, it was observed that n-alkanols systematically deviated from other anesthetic agents in the olive oil correlation but fitted a correlation using the octanol partition coefficient (58). It became clear that a better model for

biological membranes had to be used in the lipid solubility correlation hypothesis. Since biomembranes are made of amphipathic components (59); octanol partitioning serves as a more precise figure than the olive oil solubility (58). More recently, it was shown that the original Meyer and Overton hypothesis accomodates a wide range of anesthetics including the alkanol series, volatile and gaseous agents and barbiturates, when partition into phosphotidylcholine vesicles is substituted for olive oil solubility (51).

D. Membrane structure and function

Membranes are ubiquitous to all living systems. In prokaryotic cells, the plasma membrane is the only membranous component of the cell. In Eukaryotes, biomembranes also constitute a large number of intracellular compartments including nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria and chloroplasts, lysosomes, etc.(60). It is evident that since all these organelles are involved in key cellular processes, membrane systems are vital to cell integrity.

1. Composition. Biomembranes are primarily composed of lipids and proteins with small contributions from carbohydrates (61,62,63). These components are held together mostly by hydrophobic interactions (64). Lipids constitute the major molecular

species present in biological membranes. Phospholipids can be subdivided following their polar head-group structure into 5 major classes including phosphatidylcholine (PC), phosphatidyl ethanolamine (PE), phosphatidic acid (PA), phosphatidyl inositol (PI) and phosphatidyl serine (PS). All the head-groups, except PA, are zwitterionic in nature but only PC and PE are electroneutral while the others carry a net negative charge (59). Phospholipids can also vary in their acyl chains both in length and unsaturation. Unsaturation is usually cis in conformation and is non conjugated in polyunsaturated fatty acids (59,61). All fatty acids in normal mammalian cell membranes possess an even number of carbon atoms (61,65). Essential fatty acids of the n-6 series are important constituents of all tissues and the n-3 series is important in many organs (65). Some of the more abundant fatty acids include palmitic, stearic, linoleic, linolenic, oleic and arachidonic acids. Other lipids include sphingomyelin, cerebrosides, gangliosides and sterols which comprise cholesterol and its esters, desmosterol, etc.

There exists a large variety of membrane-bound proteins. They may largely be classified as peripheral (extrinsic) i.e. relatively water soluble or integral (intrinsic) i.e. lipid soluble and in strong association with membrane lipids (66,67).

Membrane oligosaccharides are built from 9 different residues (61). Six are simple sugars, while the others may contain an acetyl amino group (61).

2. Membrane architecture. Although there is no universally accepted model for membrane architecture much evidence stands to show that many membranal properties can be accounted for by a dynamic, fluid lipid bilayer structure (59,62,64,66,68). It had been acknowledged much earlier that biological lipids are amphipathic in nature (59) and that their tendency to form a polar/nonpolar interface was the key to membrane models (59,69). However, the earlier models suffered from a static design where diffusion of the various components could not be accounted for (70).

2.1 Organization of membrane lipids. It is now generally accepted that the phospholipid bilayer model can account for both structural and functional features of all biomembranes (66,68). In such a bilayer the polar phosphate head-groups face the aqueous milieu on both sides of the bilayer plane while the methyl end-groups of the fatty acyl chains face eachother at the hydrophobic core of the membrane (66,68,70). Cholesterol is intercalated between phospholipid molecules with its hydrophylic hydroxy group (OH) facing the aqueous surface of the bilayer and with the bulky hydrocarbon rings extending inside the hydrophobic domain (70,67). Such a bilayer configuration is of high stability; furthermore, the energy of the system can be lowered by folding over of the bilayer into a closed vesicle shape (64,67). This compartimentation effectively reduces interactions between the water phase and the fatty acids.

2.2 Organization of membrane proteins. Proteins constitute 40 to 50% of the total dry weight of biomembranes (63,64) and occupy half or more of the membrane surface (71). Membrane-bound proteins are roughly divided into 2 major classes: intrinsic and extrinsic. Extrinsic (or peripheral) membrane proteins are located on the surface of biological membranes and are in limited interaction with the hydrophobic milieu inside the bilayer (66). Such proteins bind to membranes mainly via electrostatic interactions and they can be extracted using mild conditions like alterations in ionic strength (66).

Intrinsic (or integral) membrane proteins are inserted more deeply within the lipid bilayer. They interact hydrophobically with either one phospholipid monolayer (leaflet) or traverse completely the bilayer spanning both sides of the membrane surface (59,64). Examples of integral membrane proteins are Na,K-ATP ase, adenylate cyclase, cytochrome C, etc. This class of proteins can only be removed from the membrane using relatively harsh methods such as detergent or organic solvent extraction (63,66,67). The long sequences in hydrophobic amino acid residues present in some intrinsic membrane proteins favor "solvation" of these parts of the polypeptide chain within the acyl chain domain of the bilayer (64). Membrane proteins may be topographically organized so that substrates or reaction intermediates can be channeled through some biological pathway. A good example of this is the electron

transport chain within the inner membrane of mitochondria where electrons are channeled to ultimately reduce molecular oxygen to water (30).

2.3 Asymmetrical distribution of membrane components.

Biological membranes display a fairly high degree of asymmetry in the distribution of their structural components (64, 72). The arrangement of polar head-groups in the erythrocyte membrane was shown to be highly asymmetric with PC and Sphingomyelin largely situated on the outer (external) leaflet and with PE and PS located mainly within the inner monolayer (62,73). Asymmetry extends also to glycosylation of membrane components where carbohydrates covalently linked to proteins or lipids are located exclusively on the external face of plasma membranes (72). The reverse holds true for intracellular membrane compartments (74).

3. Membrane dynamics

3.1 Molecular motions within the bilayer.

The lipid bilayer can be pictured as a 2 dimensional fluid (58,59). Using techniques like electron spin resonance (ESR), nuclear magnetic resonance (NMR), fluorescence polarization, X-ray crystallography and others it is possible to study the various

motions occurring within the lipid bilayer. Lateral diffusion is the term used to describe random translational diffusion of lipids within their own monolayer (61). The experimentally determined range of values for translational diffusion coefficient (D_T) of lipids is $10^{-8} - 10^{-7}$ $cm^2 s^{-1}$ (61,63). Another relatively fast motion occurring within lipid bilayers is rotation of a lipid molecule about its long axis; perpendicular to the plane of the bilayer. Experimentally observed values for rotational diffusion coefficients (D_R) of lipids lie in the order of 10^8 S^{-1} (75). Measurements of acyl chain rotation have shown an increasing gradient in molecular motion from polar head-groups near the surface of the bilayers, to the methyl end-groups within the hydrophobic core. For example, nitroxide spin-labeled fatty acids or lecithins incorporated into liquid-crystalline egg lecithin bilayers were found to decrease in their order parameter (s) with the number of methylene carbons separating the label from the phosphate head-groups (76). Since the increased motion towards central regions of bilayers is not consistent with a model in which the acyl chains are in parallel array, it has been suggested using spin-labeled phospholipids that there is a tilt of about 30° in the head-group region of hydrated egg lecithin multilayers (77). This results in a carbon atom density some 12% higher than that observed near the terminal methyl groups (77). The data on D_R is also consistent with deuteron magnetic resonance where it was shown that CD_2 groups near the

polar/apolar interface are restricted in their motion compared to those deeply burried within the hydrophobic core (78). Transbilayer motion of phospholipids or "flip-flop" across the bilayer has been shown to be exceedingly slow relative to lateral or rotational diffusion. For example by using spin-labeled single bilayered phospholipid vesicles and following the selective destruction of label on the external side of vesicles by ascorbate, exchange rates in the order of $2 \times 10^{-5}\ S^{-1}$ were obtained (79). H.J. Galla et al using pyrene lecithin as fluorescent probe detected a long-term exchange process with rate constant $k = 4 \times 10^{-5}\ S^{-1}$ (or T 1/2 of hours) in dipalmitoyl lecithin membranes at 50° C (80). This exchange was attributed to the "flip-flop" process between 2 layers of a single bilayer vesicle. A thermodynamically unfavorable transbilayer motion is expected since it would imply disturbing considerable amounts of neighbouring lipid molecules and introducing polar head-groups within the internal hydrophobic environment (63,64). Cholesterol, the main sterol in most biomembranes, has also been shown to have a fairly rapid rate of lateral diffusion. Using fluorescent methods, NBD-cholesterol had a $D_T = 1.6 \times 10^{-8}\ cm^2\ s^{-1}$ above the lipid phase transition in dimyristoylphosphatidyl choline liposomes (81). Again here "flip-flop" or transbilayer migration was slow, with a half-life of 3 days as obtained using tritiated cholesterol in liposomes at 37°C (82).

Since diffusion coefficients are inversely related to

molecular weight, proteins are expected to have lower mobilities than their lipid counterparts. The experimentally determined range of values for D_T is 10^{-9} - 10^{-8} $cm^2 s^{-1}$ and for $D_R = 10^3$ - 10^5 s^{-1} (83). Transbilayer migration of membrane-bound proteins has been shown to be virtually non-existent (84,85). Slow transbilayer migration rates are believed to be important in maintaining the high degree of asymmetrical distribution of components in biomembranes (64,72).

3.2 Lipid composition and fluidity. The recognition of all the molecular motions taking place in bilayers suggested that the 2 dimensional medium of a biological membrane has a characteristic viscosity (inversely related to fluidity) which is of importance in structure and function (86). Using biophysical methods the effects of lipid composition on bilayer fluidity can be determined. Sackmann and co-workers using the pyrene-excimer formation technique have shown that the jump frequency of the probe was two times greater in dioleyl lecithin than in the saturated analogue and that cholesterol enrichment of bilayers markedly reduces translational diffusion within fluid bilayers at physiological temperature (71). ESR and fluorescence polarization studies are in good qualitative agreement with such results (78). The study of thermotropic phase transitions which are characteristic of phospholipid-water systems like lipid dispersions, black lipid films and monolayers (87,88) has yielded

much useful information on membrane fluidity. Using differential scanning calorimetry (DSC) it was demonstrated that for the same head-group and extent of hydration, lipids with more unsaturated acyl chains have lower transition temperatures (Tm) than more saturated ones (89), and cis-unsaturated chains lower Tm than trans-unsaturated ones (91). The effects of cholesterol on the gel to liquid crystalline transition have been studied for several lipid classes (78). It was shown that cholesterol addition broadens melting endotherms of phospholipid dispersions over a wide temperature range and decreases heat of transitions. This suggests an "intermediate fluid" condition where cooperativity in the phase transition is lowered by cholesterol enrichment (89). The "dual" role of cholesterol in formation of an "intermediate fluid" state was demonstrated in many types of lipids using electron paramagnetic resonance (EPR) methods. For instance, using labeled stearate (4-NS) it was observed that cholesterol addition causes fluidization of dipalmitoyl phosphotidyl choline at 20°C (below T_m) and immobilisation of egg-yolk phosphotidyl choline in its liquid-crystalline state (92). X-ray crystallography of dipalmitoyl lecithin: cholesterol: H_2O systems below T_m (25 ° C) also demonstrated fluidization of the hydrocarbon chains between 7.5 and 50 mole% cholesterol as indicated by an increase in high angle spacing together with a decrease in the long spacing due to increased lateral motions of the chains (78,89).

3.3 Lipid-protein interactions. Since intrinsic membrane proteins have a large part of their amino acid residues "solvated" within the hydrophobic milieu of the bilayer (64) it is expected that lipid-protein interactions will play an important role in their activity. The dependence of membrane-associated enzyme activity on phospholipids was originally demonstrated with beef heart mitochondrial β-hydroxybutyrate dehydrogenase (93). Detergent extraction of the enzyme destroyed activity which was then gradually restored by addition of unsaturated lecithins. Since then cell membrane lipids have been shown to play a vital role in regulating the activities of several membrane-bound enzymes (61,66,94). Arrhenius plots which relate membrane-bound enzyme function to temperature provide other evidence for lipid involvment in the regulation of membrane-associated protein activity. Discontinuities in these plots are generally interpreted in terms of the gel to liquid-crystalline phase transition of membrane lipids (95). Although there remains some controversy about the direct relation between Arrhenius breaks and thermotropic phase transitions (61,86,96) it has been clearly shown that membrane lipid composition can modulate both temperature breaks and activation energies (61,86,97,98). There is now little doubt that membrane lipids interact with intrinsic proteins and that furthermore these proteins are surrounded by a rigid lipid annulus tightly bound to their hydrophobic domains (61,94). This lipid portion which is distinct from the bulk, structural

lipid fraction is termed boundary lipid. The existence of 2 motionally different lipid populations in biomembranes was first demonstrated using ESR (99). The two distinct spectral components observed were interpreted as bulk and boundary lipids. Boundary lipid cannot always be easily extracted (61,85) and for some membrane-bound proteins will not readily exchange with the more fluid bulk lipids in membranes (100). Boundary lipids are more irregularly packed than bulk lipids yet they experience a more severe restriction in motional freedom (61).

The existence of boundary lipid suggests fairly specific interactions between protein and lipid components within biological membranes. An example of this specificity is the preferential reactivation of detergent-extracted Na,K-ATPase by the anionic phospholipids PS and PI (101). The specificity of proteins for boundary lipids implies that structurally unimportant phospholipid species may have great functional importance. Furthermore, various lipophilic drugs may exert differential effects on boundary domains and thus selectively affect membrane-associated function (61).

The presence of boundary lipid in tight interaction with integral proteins renders membrane transport more effective since such transport processes will be restricted to the streamlets of fluid lipid spreading between these rigid halos of bound phospholipid molecules (71).

E. Ethanol Induced Membrane Perturbation

Current views on alcohol induced anesthesia suggest partitioning of ethanol into biomembranes (46). The alcohol molecules would presumably intercalate between the fatty acyl chains of phospholipids (47) and non-specifically disturb cell membrane structure and function (12,46,47).

1. Membrane expansion and fluidization. Seeman et al working on intact erythrocytes demonstrated that a wide range of anesthetics added in vitro could protect the cells against osmotic rupture in a hypotonic medium (102). In the case of alcohol a biphasic effect was observed; concentrations in the order of 100 mM displayed antihemolytic effects whereas higher ethanol concentrations caused a rapid lysis of the erythrocytes (103). Later on Seeman and Weinstein went on to interpret the antihemolytic effects of alcohol in terms of membrane expansion caused by tranquilizers and other general anesthetics (104). In this hypothesis, the binding of anesthetic molecules to biomembranes physically increases the membrane so that the cell can now hold a larger volume prior to osmotic lysis. In 1969, Seeman et al using a Coulter counter and a mean cell computer measured the cell volume of vesicular erythrocyte ghosts. This was found to be in the order of 150 μ^3 with a corresponding membrane surface area in the range of 135 μ^2 (105). When the

cells were hemolyzed in presence of low concentrations of anesthetic drugs like pentanol, nonanol and benzyl alcohol, the mean cell volume of the ghosts was larger than the control value. Erythrocyte ghost membrane area simultaneously increased by 1.3 to 1.6% and achieved a maximum of 5% in area expansion at high but sublytic drug concentrations (105).

1.1 Acute effects of alcohol

It is now widely accepted that anesthetic agents expand membranes (104,100) and increase the fluidity of both biological and model membrane systems (107,108). Although the effects on membrane osmotic stability and expansion were only observed at fairly high ethanol concentrations, recent work using sensitive biophysical techniques demonstrated disordering of membranes and lipid bilayers at physiologically relevant alcohol concentrations. For instance, S.J. Paterson et al using the steroid spin probe 3-spiro-cholestane observbed that the onset of perceptible changes in the order paramater correlated well with those causing anesthesia in vivo (109). Chin and Goldstein, using a sensitive electron paramagnetic resonance technique showed a decrease in order paramater of the nitroxide probe 5-doxyl stearic acid at ethanol concentrations as low as 20 mM (110). Hence, fluidization of biomembranes and lipid bilayers by physiological concentrations of ethanol could well be related to in vivo anesthesia. In later work, Chin and Goldstein probed mouse synaptosomal membranes at

different depths by incorporating stearic acid labeled at either the carbon 5, C 12 or C 16 position into liposomes (108). It was observed that ethanol's disordering effect was greater in the middle of the bilayer than near the water interface. The perturbation of egg lecithin vesicles by in vitro ethanol was potentiated by temperature and progressively reduced by cholesterol enrichment of the liposomes. In contrast, Jane M. Vanderkooi, using pyrene fluorescent probes observed that physiological concentrations of ethanol have little effect on the fluidity of the hydrocarbon core of membranes, while the interface, as probed by 1-aminopyrene, was fluidized by similar alcohol concentrations (111).

However, the reason alcohol affects the fluorescence of 1-aminopyrene is not yet fully understood and could result in part from excited state protonation reactions (111). Johnson et al working with diphenylhexatriene polarization also demonstrated the fluidifying effects of ethanol (112). It was observed here that addition of cholesterol to phospholipid fractions isolated from synaptosomal membranes resulted in enhanced disordering by the alkanol. Other workers like Pang et al also found that general anesthetics like amines and barbiturates induce disorder in lipid bilayers only if certain proportions of cholesterol are included in the phospholipid fraction (37).

1.2 Membrane adaptations in chronic alcohol consumption

Ethanol, like other anesthetic agents, seems to act non-specifically within the hydrophobic domains of biological membranes (105,106) and results in physical disordering of the bilayer (108,109). In chronic alcoholism, tolerance and dependence to the effects of alcohol probably arise from long-term induced alterations in membrane properties of the nervous system (113,114). To compensate for the fluidifying effects of alcohol, membranes could adapt by restoring their rigidity. Therefore, a decrease in membrane disordering by alcohol would be indicative of a tolerant state while dependence would be displayed in the fact that upon removal of alcohol, the membranes would be in a more rigid, "non-functional" state (115).

2. Tolerance and dependence. Lyon and Goldstein using 12-doxyl stearic acid as an electron paramagnetic resonance probe observed an increase in the order parameter of synaptosomal membranes isolated from ethanol-treated mice (115). Other workers using DPH fluorecence polarization detected a decrease in the intrinsic fluidity of synaptosomal membranes from alcohol-treated mice (112). On the other hand, Chin and Goldstein using EPR methods and labelling with 5-doxyl stearate found that synaptosomal and erythrocyte membranes from mice continuously exposed to ethanol were as fluid as control membranes (113). Such discrepancies may well result from differences in the location of the probes within

the lipid bilayer as this was shown to affect their sensitivity to ethanol and to the changes in lipid bilayers expected after prolonged intoxication with alcohol. Tolerance to the disordering effects of ethanol has been observed in membranes isolated from rodents after long-term exposure to the alkanol. For example, using DPH it was observed that reconstituted membranes formed from the lipid extracts of crude synaptosomal membranes from alcohol treated mice were less fluidizable by ethanol in a dose related manner than those from controls (112). Using other biophysical methods, similar results were reported for synaptosomal, erythrocyte and mitochondrial membranes (113, 115). The increase in molecular order observed in some biomembranes as a consequence of chronic ethanol consumption has been shown to be negatively correlated with the membrane partition of many lipophilic and amphiphilic compounds including ethanol, halothane and phenobarbitol . Kelly-Murphy et al have reported decreased partition of radiolabeled halothane into erythrocyte ghost membranes isolated from alcohol-fed rats (116). They obtained similar findings using 5-nitroxydecane (5N10) and EPR spectroscopy to determine the partition parameter of the spin probe. Rottenberg et al also demonstrated reduced membrane binding of ethanol and other anesthetics in brain synaptosomes obtained from alcohol-treated rats (117). The decreased anesthetic binding to membranes was correlated with the resistance of the bilayers to the structural disordering caused by in vitro ethanol

and halothane. The changes in physical properties of biological membranes occuring after prolonged intake of alcohol could result from modifications in the lipid composition (118,119).

2.1 Changes in membrane composition. Factors which influence membrane viscosity include the cholesterol to phospholipid ratio, protein to lipid ratio, sphingomyelin content, phospholipid methylation and the saturation degree of the fatty acyl chains (61,85,120). Since all these paramaters are related to membrane order, they would be expected to change following prolonged consumption of alcohol. However, experimentally the results together with their significance remain controversial.

(i) Fatty acid compositional changes. Morrisson et al working on mice embryo neuronal cultures have reported an increase in the double bond index to saturated fatty acid ratio (DBI/SFA) following growth in sublethal ethanol concentrations (121). Although, the time course showed some oscillations, they observed an increase in palmitoleic acid (16:1) and a decrease in both palmitic (16:0) and stearic (18:0) acids. LaDroitte et al detected significant changes in saturated and unsaturated fatty acids in rat and mice erythrocyte membranes following chronic intraperitoneal ethanol injections (122). The changes in fatty acid composition correlated well with the development of tolerance

to the effects of alcohol. Littleton and John found that inhalation of alcohol vapor for a 10 day period produced a decrease in the DBI/SFA of mouse synaptosomal membranes (123). On the other hand, Sun and Sun detected an increase in unsaturated fatty acids in guinea pig synaptic membranes after 3 weeks of feeding with an alcohol containing liquid diet (124). Wing and co-workers detected no changes in fatty acid composition of rat synaptosomal membranes after continuous intake of ethanol (125). Ingram et al using E.Coli cultured in alcohol containing media reported an increase in unsaturated fatty acids and a decrease in 18:0 levels (126). Therefore, it would seem that fatty acyl chains undergo delicate and complex changes which vary both in magnitude and direction. Studies which reported increases in the unsaturation levels cannot explain the increases rigidity of membranes from alcohol fed animals. Furthermore, the controversial results obtained on fatty acid compositional changes may arise from the type of diet given throughout the ethanol treatment. Other factors which could explain such discrepancies include duration of the alcohol treatment, the route of administration of the drug and the various membrane tissues examined (127).

(ii) Cholesterol levels. There have been reports on changes in membrane cholesterol levels after chronic treatment with ethanol. For instance, Chin et al reported an increase in the cholesterol to phospholipid ratio of both synaptic and erythrocyte

membranes from mice (128). Others studies on synaptic membranes have reported no significant changes in the cholesterol levels of these membranes following chronic exposure to alcohol (112,129). The requirement for cholesterol in the expression of tolerance to alcohol has been claimed by some workers (112,128). Indeed, cholesterol enrichment of biomembranes and lipid bilayers leads to a rigidification of the phospholipid matrix and could explain how membranes increase their rigidity in response to a continuous exposure to a disordering agent like alcohol (128). However, parallel studies also revealed that increased cholesterol levels resulted in potentiation of the fluidifying effects of alkanols and other general anesthetics (37,112). Furthermore, organelles such as mitochondria which have been shown to develop tolerance to alcohol (117,130) do not contain any appreciable amounts of the sterol (30). Thus, the true significance of cholesterol in membrane adaptation remains to be elucidated.

(iii) Membrane-bound proteins. As far as protein density is concerned, there have been some reports on changes brought on by a long-term treatment with alcohol. For instance, Lee and Hosein working on rat liver plasma membranes observed an increase in buoyant density following chronic ethanol consumption (131). Although changes in lipid composition were detected, they could not explain this difference since after 48h. withdrawal most of the lipids had reverted to normal but membrane peak density still

remained higher. It is likely that membrane proteins play an important role in such longer lasting adaptive changes (131). Other work in our laboratory showed an increase in synaptosomal membrane protein to lipid ratio from day-6 rat neonates following chronic exposure to alcohol (unpublished).

An increase in the protein to lipid ratio generally leads to membrane ordering as it augments the fraction of lipid molecules found in boundary regions (94,100). Therefore, this could explain in part the phenomenon of membrane homeoviscous adaption. It should be noted that although the changes in cell membrane lipid composition described above are small in magnitude, they may still have great functional importance. Since boundary lipids have been shown to be involved in regulating the activity of membrane-bound enzymes (97,98), slight changes in such phospholipid species may result in alteration of biological activity. For instance, increases in acidic phospholipids like phosphotidyl serine and phosphotidyl inositol have been reported in membranes isolated from animals following chronic ethanol intoxication (132). This could be correlated with an increased activity of the sodium, potassium adenosine triphosphatase (Na,K-ATPase), an enzyme known to require anionic phospholipids for biological function (132).

F. (Na+K) stimulated ATP-ase and its interaction with ethanol.

The behavioural manifestations of ethanol intoxication in humans include loss of inhibition, euphoria, diminished motor control and with increasing doses loss of consciousness and death (133). The interference of ethanol with key membranal processes of the central nervous system such as cation transport may be of importance in the production of central nervous depression by alcohol and other general anesthetics (133).

1. Properties of the Na,K-ATPase. The sodium-potassium adenosine triphosphatase is an integral membrane protein with exposed sites on both faces of the membrane (134). The protein is in tight interaction with the surrounding membrane and the delipidated enzyme is totally inactive (134). Furthermore, the enzyme shows a specific requirement for phosphotidylserine (PS). For example, using rat brain ATPase it was shown that addition of PS to the detergent extracted protein is far more efficient in restoring enzyme activity than any other phospholipid (135). The requirement for anionic phospholipids could reflect the involvment of the enzyme in cation transport as this offers a suitable charged environment for function.

The purified protein contains 2 types of polypeptide chains α and β probably in a 1:1 stoichiometry. The enzyme may either be of $\alpha\beta$ or $(\alpha\beta)_2$ configuration, with the latter seeming most probable (136). The molecular weight of α is in the order of 105 K and that of β is about 40 K (137). The β polypeptide is covalently coupled to carbohydrate, about 10% by weight (134). The cardiac glycoside ouabain is a potent and specific inhibitor of the enzyme ($K_J=10^{-7}$ M) and the photo affinity label 2-nitro -5 azido-benzoylouabain can be covalently linked to the α polypeptide (138).

Vanadate ion is a specific and very strong inhibitor of the enzyme with a K_J in the range of 4 nM (139). Since the catalytic site for ATP hydrolysis involves an aspartate residue on the α chain (140), vanadate ion has been postulated to be a transition state analogue as it resembles the trigonal bipyramid configuration expected for the hydrolysis of the phosphaste aspartate anhydride bond. The number of monovalent cation sites per functional unit is of considerable importance.

Equilibrium binding studies suggest 3 sodium and 2 potassium sites per phosphorylation site (141,142). Therefore, for each ATP hydrolyzed 3 sodium ions would move out of the cell and 2 potassium ions would move inwards. In the brain, the electrochemical gradient provided by (Na+K) activated ATPase is essential for neuronal cell excitability as well as the uptake processes for amino acids and neurotransmitters (13,30). The enzyme is also known to be stimulated by addition of catecholamines (143), however

the molecular mechanisms underlying this stimulation still remain controversial. For example, many authors have reported that stimulation occurs through chelation by the catecholamines of a metallic ion inhibitor (144). In contrast, Wu and Phillis (145) have shown that noradrenaline stimulates enzymatic activity via a receptor-specific mechanism.

2. Effects of ethanol on brain ATPases. Although the molecular mechanisms underlying the depressant effect of ethanol remain to be fully understood, a few neural processes have been shown to be inhibited at concentrations below those that are lethal. One of them is the activity of the (Na+K) stimulated adenosine triphosphatase (146,147). Since then, this enzyme has been extensively used as a useful and sensitive "probe" in the assessment of membrane perturbation by ethanol.

2.1 Acute effects. Ethanol in vitro has been shown in many instances to alter the activity of several brain ATPases. For example, rat brain microsomal Na,K-ATPase was shown to be stimulated by low concentrations of ethanol (<100mM) and inhibited by higher ones (148). While ethanol intoxication has been associated with an inhibition of the microsomal ATPases of brain (147,149), interference with the synaptosomal (Na+K)-ATPase may also be involved as this enzyme participates in ion transport and regulates polarization of neuronal membranes (133,150). A.Y.Sun

et al have shown a dose dependent inhibition of the (Na+K) stimulated ATPase in nerve ending particles by short chain aliphatic alcohols (133). In this study ethanol was shown to be a non-competitive inhibitor of the enzyme with respect to the rate of hydrolysis of ATP (133). Furthermore, the degree of inhibition increased with increasing chain length of the alcohols studied and correlated well with their oil/water partition coefficients. Thus, aliphatic alcohols seem to exhibit their anesthetic effects primarily through perturbation of the lipid component of biological membranes (46,47).

Potassium has been shown to competitively antagonize the inhibitory effects of ethanol. For instance, Goldstein and Israel have shown that 5mM K^+ abolishes the inhibition of 1% (w/v) ethanol on mouse brain (Na+K) activated ATPase (151). It has been suggested that ethanol in higher concentrations lowers the affinity of the outward-facing form of the enzyme for potassium ions thereby preventing subsequent dephosphorylation and conversion to the inward-facing form (143).

Stimulation of the (Na+K)-ATPase by catecholamines can provide further insight on how ethanol perturbs membranes associated function. Kalant and Rangaraj have shown that the combination of 0.05M ethanol plus 0.1mM noradrenaline (or dopamine) increased the Km of the enzyme for potassium by more than two fold (143). In contrast, ethanol alone in similar concentrations was shown to have no significant effect on the ATPase. Therefore, these findings indicate that the effect of

ethanol plus catecholamine is the same as that of a higher concentration of ethanol alone.

2.2 Chronic effects. Most of the accumulated evidence seems to indicate that the activity of (Na+K)-ATPase in brain homogenates is increased following chronic exposure to alcohol. Using rats as a model system, Israel and co-workers observed an increase in the activity of Na,K-ATPase in whole brain homogenates after chronic administration of ethanol (152). Furthermore, reversal of Na,K-ATPase activity to normal values after cessation of the alcohol treatment correlated well with disappearance of acquired tolerance. Thus, the metabolic properties of Na,K-ATPase apparently fit the "derepression" theory of tolerance and dependence proposed by Goldstein and Goldstein (153,154) and Shuster (155). On the other hand, (Na+K) activated ATPase in mouse whole brain homogenates does not respond similarily to chronic ethanol treatment. For example, Goldstein et al showed that (Na+K)-ATPase activity was not modified by chronic exposure to ethanol despite the fact that the animals were shown to be dependent on the drug (151). Besides differences in the species used, other factors may explain the discrepancies mentioned above. First, it is possible that the ATPase activity does not respond equally in different parts of the brain. For instance, in cats chronically treated with alcohol, (Na+K)-ATPase activity

increased in the cortex and hippocampus but not in other areas of the brain (156). Another possible factor is suggested by the work of Ebel et al which points out to the existence of 2 different (Na+K)-ATPase in synaptosomes and microsomes (157). In that study, it was shown that adrenalectomy markedly reduced the brain synaptosomal (Na+K)-ATPase activity while it increased it in microsomes. Thus, the ATPases from these two subcellular fractions appear to be under different control mechanisms. Such observations suggest that results obtained using whole brain can perhaps fail to detect a localized change in ATPase activity.

MATERIALS AND METHODS

A. MATERIALS

All solutions for the experiments were prepared with deionized distilled water and all chemicals were at least of reagent grade.

Pyrene, bovine serum albumin (BSA), cholesterol, adenosine 5'-triphosphate (ATP), tris-(hydroxymethyl)-aminomethane (Tris), imidazole, ouabain, ethylenediamine tetraacetic acid (EDTA), Ficoll, dithiothreitol (DTT), ascorbic acid and dopamine (DA) were purchased from Sigma Chemical Co., St. Louis, Missouri. Methanol (HPLC grade), sucrose, pentanol, magnesium chloride ($MgCl_2$). sodium chloride (NaCl), potassium chloride (KCl), sulfuric acid (H_2SO_4) and trichloroacetic acid (TCA) were obtained from Fisher Scientific Co., MTL, Quebec. Ammonium molybdate was purchased from Anachemia Chemicals Ltd. and ethanol (absolute) was obtained from Consolidated Alcohols Ltd., Toronto, Ontario. Chloroform was purchased from American Chemical Ltd. Sustacal was prepared by Mead Johnson, Canada.

B. METHODS

1. Animals

Female Spraque-Dawley rats obtained from breeding laboratories (St-Constant, Quebec) weighing between 200-230 gms were used. The females were caged with breeders and day-1 of pregnancy was determined from a positive sperm test. Once pregnant the females were housed individually and fed a totally liquid diet of Sustacal supplemented with zinc. The alcohol-fed group was slowly phased in to achieve a final 37% of calorie intake from ethanol while controls received diets isocalorically balanced with sucrose.

The birthdate of the pups was determined and 10-day old progeny of either control or experimental groups was used in the following experiments.

2. Preparation of brain synaptosomal and mitochondrial membranes.
(Modified method of Cotman and Matthews (158)).

The control and/or alcohol-treated progeny was decapitated and the brain was rapidly excised and placed in 9 volumes of cold sucrose tris (ST) buffer containing 320mM sucrose and 5mM Tris-HCL at pH 7.4.

All remaining steps were carried out at 0-4°C. The brains were homogenized with 9 up and down strokes in a Tri-R-glass-teflon homogenizer. The homogenate was then centrifuged at 2500

rpm for 10 minutes in a refrigerated Sorvall RC-2B centrifuge. The supernatant was centrifuged at 12000 rpm for 10 minutes. The pellet was resuspended in 9 volumes of ST buffer and the above procedure was repeated. The pellet obtained was resuspended in a small volume (about 0.3ml/2g brain wt.) of ST and layered on a discontinuous Ficoll gradient (7.5/13% w/v in sucrose-Tris buffer) to be centrifuged at 22500 rpm for 45 minutes in a sw27.1 rotor with a Beckman L5-50 ultracentrifuge. The synaptosomes were collected at the interphase of the gradient while the mitochondrial fraction was at the bottom. Both fractions were suspended in cold distilled water (2ml/g.brain weight) for 30 minutes. The purified membranes were then centrifuged at 17500 rpm for 15 minutes and the pellets resuspended in 50mM Tris-HCL pH 7.5. This was centrifuged at 15000rpm for 10 minutes and the pellets were again resuspended in about 2 mls of Tris-HCL pH 7.5 and used later for lipid extraction and pyrene labelling.

3. Lipid extraction.

Total lipid extracts were obtained from the purified synaptosomal and mitochondrial membranes according to the method of Bligh and Dyer as described by Kates (159).

4. Lipid resolution.

Polar and non-polar lipids were fractionated by silicic acid

column chromatography (SIL-LC, 325 mesh). The column was initially washed with 1 volume of anhydrous ether and 3 column volumes of distilled chloroform. Total lipid extracts were then applied and the non-polar fraction eluted with 5 volumes of chloroform. The polar fraction was obtained by elution with 3 column volumes of methanol: chloroform (2:1 v/v) followed by 2 volumes of methanol. Eluates were placed under a nitrogen atmosphere.

5. Chemical analysis.

Cholesterol was measured by the method of Zlatkis et al as described by Kates (159). Phospholipids were digested according to Duck-Chong (160) and the inorganic phosphorus assayed by the procedure of Chen et al (161). Protein was determined by the method of Lowry et al (162) using recrystallized bovine serum albumin as standard.

6. Pyrene labelling and vesicle preparation.

In all the samples studies, the pyrene to lipid weighted ratio was fixed at 0.0093mg probe/mg lipid (or a molar ratio of about 0.037 assuming molecular wt. of a phospholipid 794). This

showed to be in the linear range for fluorescence versus concentration of excimer forming probe. Lipids were weighed after extensive solvent evaporation using a Metler AE-163 analytical balance accurate to 20μg.

(i) Biomembranes:

Pyrene dissolved in absolute ethanol was added to the membrane suspensions in 50mM Tris-HCL pH 7.5. After a 15 minute incubation at 0-4°C the suspensions were centrifuged at 15000 rpm for 15 minutes. The pellets were resuspended in Tris-HCL buffer to afford a final lipid concentration of 0.13mg/ml (in presence or absence of added n-pentanol). With this procedure no pyrene fluorescence could be detected in the supernatants following centrifugation.

(ii) Liposomes:

Pyrene dissolved in chloroform was added to weighed lipid fractions and the organic solvent was evaporated by extensively flushing nitrogen gas onto samples places in a warm water bath.

After solvent evaporation, liposomes were prepared by incubating the dopped lipid films in a 2mM CsCl buffer, pH 7.1 for 45 minutes at about 45°C (above lipid phase transition) with vigorous vortexing. This method is similar to Galla and Sackmann (71) and removes at least 96% of the lipid from the glass walls. The liposomes were then suspended (in CsCl buffer) to a final concentration of 0.13mg lipid/ml (with or without in vitro

pentanol). All buffers used were freed from oxygen by bubbling nitrogen. The experiments were also performed under an atmosphere of nitrogen.

7. Fluorescence measurements:

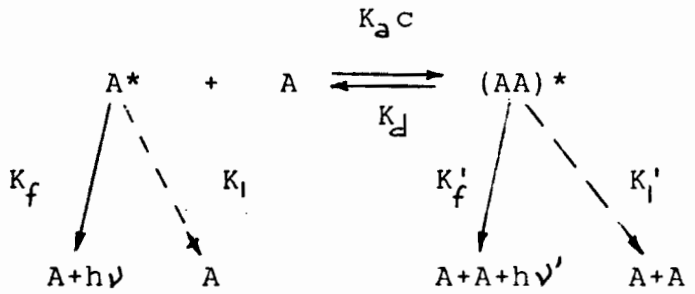
Pyrene fluorescence was read in a Turner Fluorometer Model 430, equipped with a circulating water jacket regulated at 37°C. The excitation monochromator was set at 340nm and emission was recorded at 373, 392 (monomer) and 470nm (excimer). All readings were corrected for light scattering and excimer to monomer ratios (related to fluidity) were calculated from F(470)/F(392)=R 470/392.
Increases in R 470/392 upon in vitro n-pentanol addition were also measured.

8. Principle of method:

The diffusion coefficient, D_T, of molecules performing a lateral diffusion parallel to the membrane surface may be related to the number of collisions per unit time. In principle the frequency of collisions can be determined if the diffusing particles undergo a reversible physical or chemical reaction upon the encounters. In the case of aromatic molecules, formation of short lived excited complexes between a ground state (A) and an excited state molecule (A*) provides a rever-

sible physical reaction which may be used to measure D_T.

- Excimer formation process first reported by Foster and Kasper (163):



Excimer formation is characterized by the second order rate constant K_a . K_f and K_f' are the transition probabilities (in s^{-1}) for the radiative decay of the excited monomer and excimer respectively. K_l and K_l' are the corresponding non radiative transition probabilities. K_d is the rate constant for dissociation of the excited dimer into A and A*.

For pyrene, the fluorescence ratio R 470/392 is directly related to K_a by (164,165):

$$R\ 470/392 = \frac{F(470)}{F(392)} = \frac{K_f'\ \tau_o'\ K_a c}{K_f\ (1+K_d\ \tau_o')}$$

Thus, excimer formation in fluid media is a diffusion controlled process where the rate of excited dimer formation, $K_a c$, is proportional to the collision rate. In 2-dimensional fluid bilayers, c(pyrene concentration) refers to the number of probe molecules per unit area of lipid.

9. Na,K-ATPase activity in brain homogenates:

(i) Homogenates - Brain homogenates were obtained as described earlier in this section. The iso-osmolar homogenates (in sucrose) were then diluted in about 30 times their volume with cold distilled water. Such a hypotonic shock fragments vesicular synaptosomes into open forms and dilutes the various salts and effectors endogenously found in brain tissue.

(ii) Na,K-ATPase assay (166) - The sample incubation mixture contained 50 mM imidazole-HCL, pH 7.4, 4 mM MgCl , 120 mM NaCl, 1 mM KCl and ethanol 1 to 2% w/v. In the control cuvettes Na^+ and K^+ were omitted from the incubation mixture and 1.5 mM ouabain was added. For the dopamine (DA) stimulated ATPase activity 0.1 ml (0.1 μmole) of DA solution* was added to sample and control incubation mixture. To all cuvettes, 0.4 ml of diluted brain homogenate (90-110μg protein) was added and the mixtures were allowed to equilibrate to 37°C for 4 minutes in a preset water incubator. The reaction was then initiated by addition of 0.1 ml (3 μmoles) of ATP and all the cuvettes now contained 1 ml as final volume. The reaction was terminated after 20 minutes with 1 ml of add 10% TCA. The solutions were centrifuged at 2000g for 4 minutes in an IEC table-top centrifuge. The amount of inorganic phosphate present in the supernatant was determined by the method of Chen et al (161). The Na,K-ATPase activity was estimated by the difference between sample and control values. Thus, it refers to the ouabain-sensitive, (Na+K)-stimulated, Mg-dependent ATPase activity.

* DA was initialy dissolved in 50 mM Imidazole-HCL, pH 7.4 containing 0.2 mM DTT. The solution was made up fresh each day.

10. Statistical analysis:

All values are expressed as means ± S.E.M. Two-tailed student's t-test was used to assess the level of significance of differences between mean values. $P<0.05$ was taken as being significantly different. For pyrene fluorescence, two-way analysis of variance (ANOVA) was performed and the F values were used to determine statistical differences (167).

RESULTS

The pyrene treated mitochondrial and synaptosomal membranes were analysed for their excimer-formation efficiency (R 470/392; related to fluidity) and for the dose-dependent increase in the R 470/392 values induced by *in vitro* pentanol. Total and polar lipid extracts of synaptosomes were also analysed by the pyrene excimer technique.

A - Mitochondrial membranes

The fluidizing effects of pentanol on the purified mitochondrial fractions at 37°C are shown in Figure 1. The increase in the basal fluidity of mitochondrial membranes obtained from alcohol-fed pups in presence of the alkanol *in vitro* was not significantly different from the control ($F=0.39$, d.f.$=1,31$, $p>0.05$). The basal fluidity values for control and alcohol-fed groups were 0.528 and 0.515 respectively (Table 1).

B - Synaptosomal membranes

The dose-related increase in dimer formation was significantly lower in the alcohol-fed synaptosomal fraction; $F=16.4$, d.f.$=1,34$, $p<0.01$ (Figure 2). Basal fluidities were similar in the alcohol-fed and control groups and were also in the same range as the mitochondrial values (Table 1). As shown in Table 2, the

synaptosomal membrane lipid to protein ratio was significantly higher in the alcohol-fed group (1.75 and 1.61 for control; $p<0.01$).

To determine whether protein density alone is responsible for the long term adaptations to chronic maternal ethanol ingestion, lipid extracts were obtained from the synaptosomal membrane fractions as described under methods p.40. The results on the liposomes total lipid extracts treated with pyrene are presented in Figure 3. The alcohol-fed group was significantly less fluidized than the corresponding control ($F=37.1$, d.f.=1,30 , $p<0.01$).

This indicated that tolerance to in vitro alkanols persists in the lipid fraction of tolerant membranes. To elucidate the effects of cholesterol on the fluidizability (and fluidity) of synaptosomal lipid extracts, pyrene labeled liposomes made of polar (or phospho) lipid were analysed. As shown Figure 4, the alcohol-fed and control polar lipid fractions were equally perturbed by in vitro pentanol ($F=0.38$, d.f.=1,30 , $p>0.05$) suggesting the requirement for cholesterol in the expression of tolerance. However, the cholesterol to phospholipid molar ratio of the alcohol-fed progeny was not significantly different from the controls; 0.41 versus 0.37 respectively, $p>0.1$ (Table 2). Also, equalization of the cholesterol molar fraction (at the control value 0.37) in reconstituted total lipid extracts from the polar fractions of alcohol-fed and control groups restored "tolerance" to the alcohol-fed group to in vitro

pentanol ($F = 10.1$, d.f.=1,18 , $p<0.01$, Figure 5). This indicated that some difference in the polar fractions between control and alcoholics must exist, but it is only expressed in presence of cholesterol.

The rigidifying effects of cholesterol in bilayers are shown in Figure 6A. The excimer-formation efficiency decreases with increasing cholesterol molar fraction in control samples.

Increasing the cholesterol to phospholipid ratios enhanced the ability of pentanol to disorder the reconstituted liposomes from the control polar fractions (Figure 6B).

C - "Polarity" within biomembranes and liposomes.

It is known that the ratio of fluorescent intensities of 392 to 373 nm (R 392/373) is inversely related to the "polarity" of the probe's microenvironment. As shown in Table 3, mitochondrial and synaptosomal membranes seem to afford a more hydrophobic (or apolar) hydrocarbon interior than liposomes of total or phospholipids.

D - Experimental design.

In the present study, pentanol was used to perturb biomem-

branes and lipid bilayers as ethyl alcohol in lower concentrations did not consistently alter the excimer to monomer ratio of pyrene. In preliminary studies it was found that although ethanol produced slight increases in the excited dimer fluorescent intensity it often causes the monomer intensity to fluctuate with no clear cut pattern. Only at concentrations in the order of 6% (v/v) did ethanol achieve a consistent and appreciable decrease in the fluorescent intensity of the monomeric species. On the other hand, more lipophilic alkanols (butanol, pentanol, isopentanol) or chloroform noticeably increased the excimer to monomer ratio in a dose-dependent manner, pentanol yielding a practically linear response with concentration.

E - Na,K-ATPase assays.

The inhibitory effects of in vitro ethanol on Na,K-ATPase activity from brain homogenates of day-10 pups are shown in Figure 8A. The basal activity of the ATPase from the alcohol-fed group was resistant to inhibition by 1% and 2% (w/v) ethanol relative to controls ($p<0.01$).

Inhibition of dopamine-stimulated Na,K-ATPase activity by in vitro ethanol is depicted in Figure 8B. The dopamine stimulated component of the enzyme from alcohol-fed neonates was also resistant to inhibition by similar alcohol concentrations ($p<0.01$ at 1% and $0.02<p<0.05$ at 2% (w/v) ethanol). Furthermore, chronic exposure to alcohol resulted in increasing the DA-stimulated

Na,K-ATPase activity even in absence of added reagent.

The percent stimulation of Na,K-ATPase activity by 0.1mM DA as a function of *in vitro* ethanol is shown in Figure 9. Ethanol *in vitro* resulted in a biphasic effect on the control group with enhancement of the stimulatory effect of DA at the intermediate alcohol concentration. In contrast, added ethanol did not yield much response on the alcohol-treated group.

It can be seen from Table 4 that prolonged treatment with alcohol did not result in altering basal Na,K-ATPase activity (at 0% *in vitro* ethanol) from 10 day-old rat neonates.

Figure 1: Effect of chronic maternal ethanol ingestion on the fluidizability of brain mitochondrial membranes from 10 day-old progeny. Control (—□—), alcohol-fed (- -○- -).

$$\Delta R470/392 = R470/392(+\text{pentanol}) - R470/392(\text{basal})$$
$$= R_A - R_O$$

$\bar{X} \pm$ S.E.M. of 5 to 6 experiments

$F = 0.39$, d.f. = 1, 31, $p > 0.05$

Figure 1

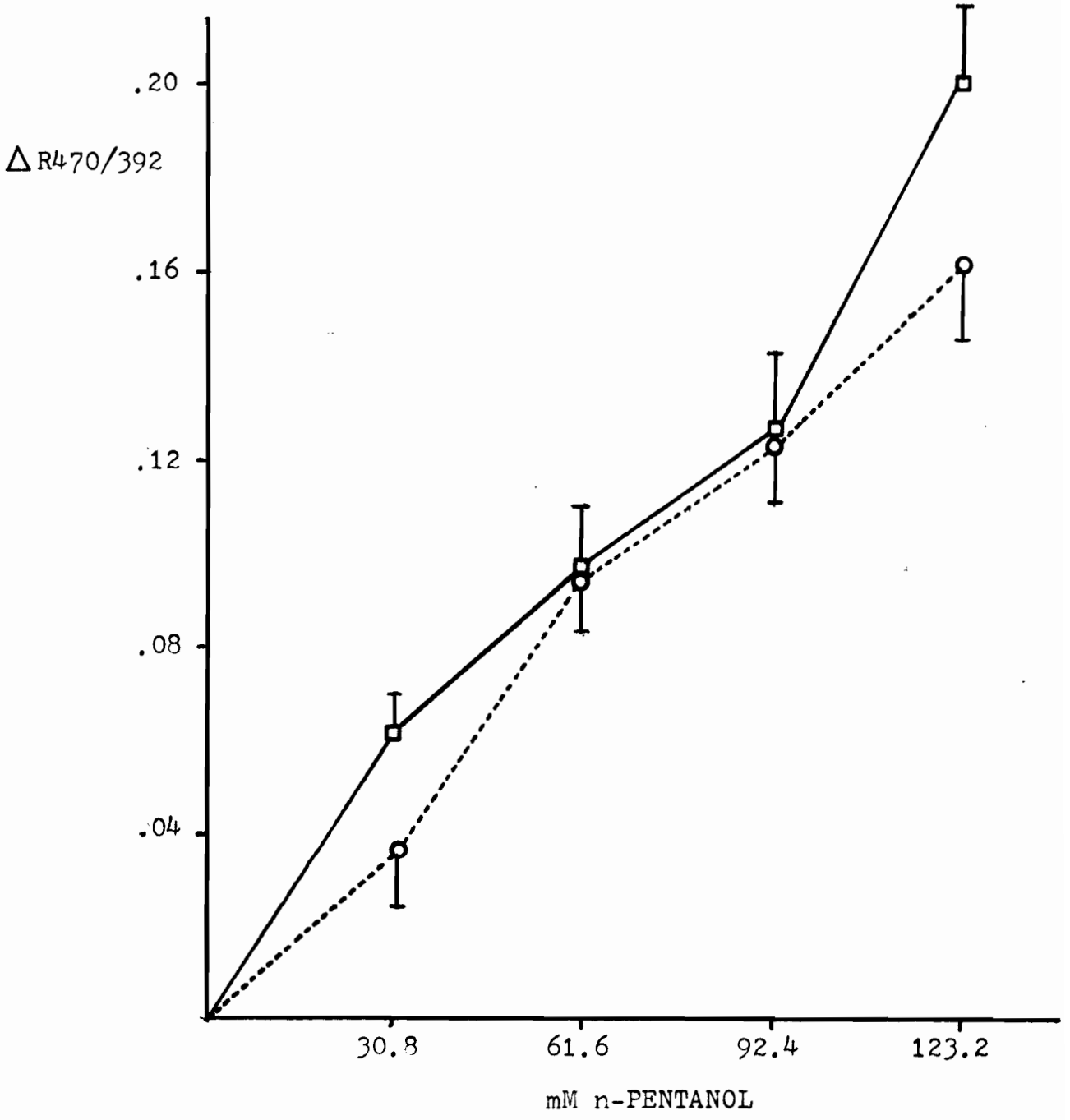


Figure 2: Effect of chronic maternal ethanol consumption on the fluidizability of rat brain synaptosomal membranes from 10 day-old progeny. Control (—□—), alcohol-fed (--o--).

$\bar{X} \pm$ S.E.M. of 6 experiments

$F = 16.4$, d.f. = 1, 34, $p < 0.01$

Figure 2

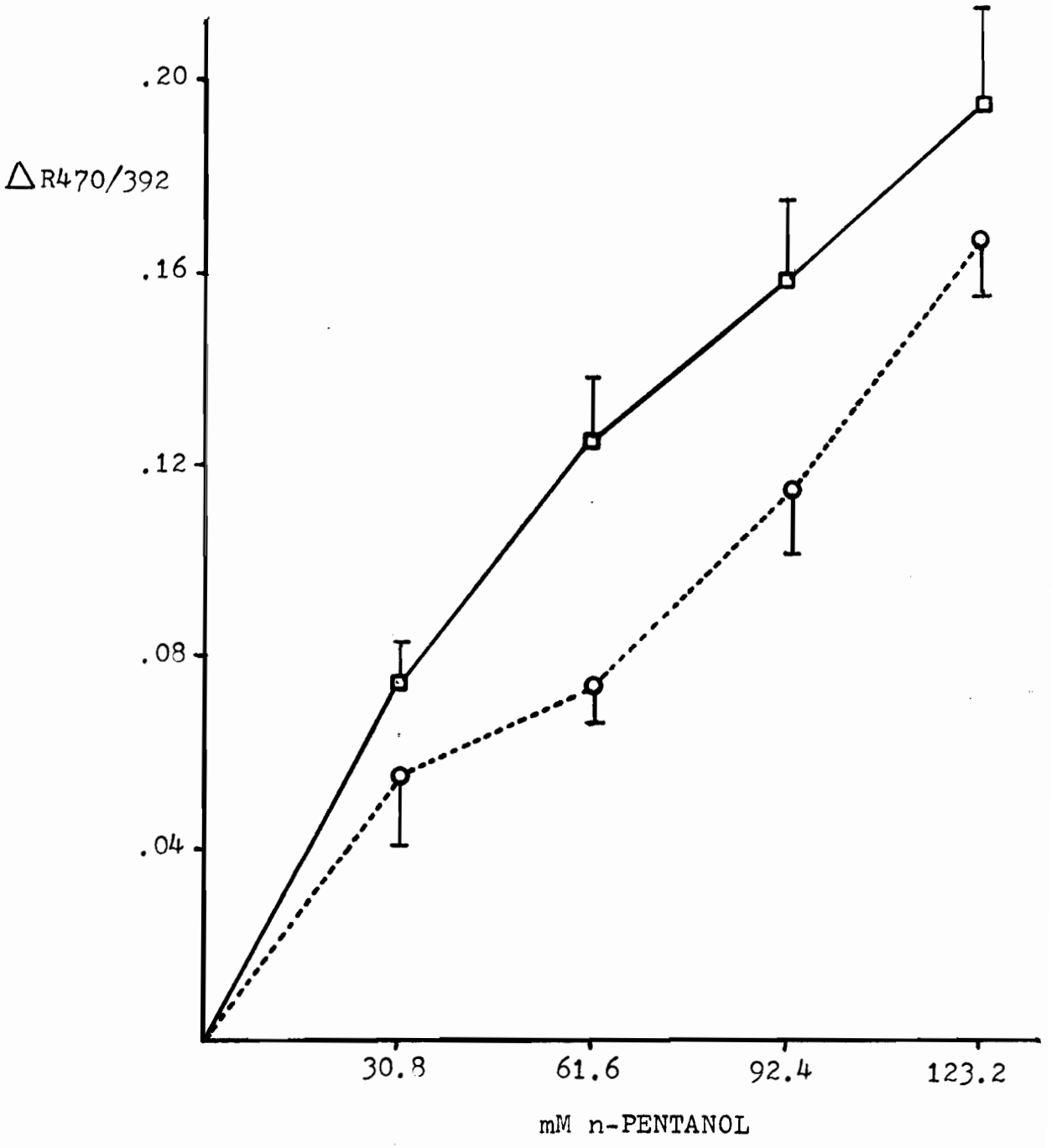


Figure 3: Pentanol induced fluidization on the liposomes of total lipid extracts from rat brain synaptosomal membranes of day-10 progeny from alcohol-fed or control mothers. Control (—□—), alcohol-fed (- -○- -).

$\bar{X} \pm$ S.E.M. of 5 to 6 experiments

$F = 37.1$, d.f. = 1, 30, $p < 0.01$

Figure 3

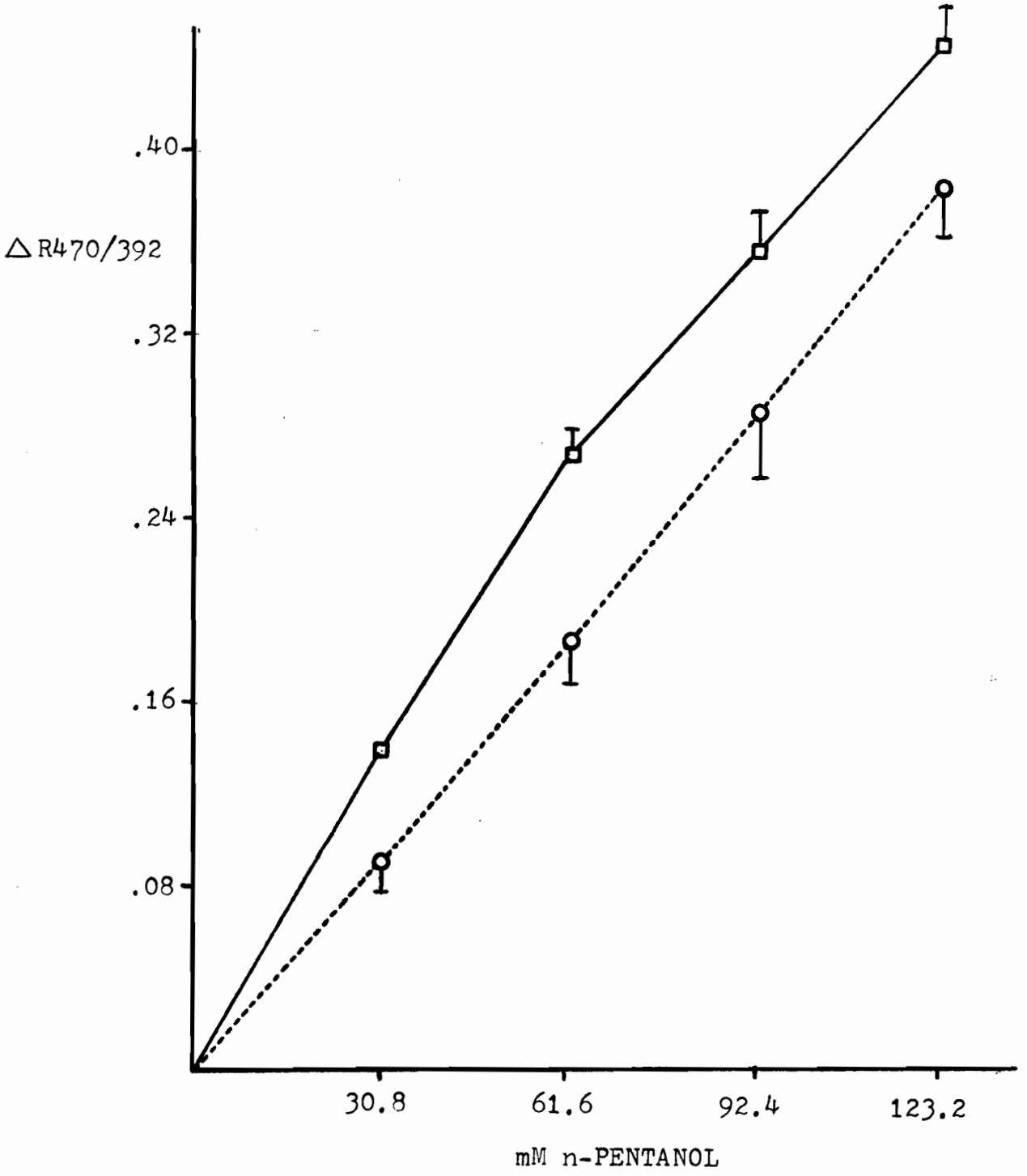


Figure 4: In vitro pentanol induced fluidization on liposomes of phospholipid extracts from rat brain synaptosomal membranes of day-10 progeny from alcohol-fed or control mothers. Control (—□—), alcohol-fed (- -○- -).

$\bar{X} \pm$ S.E.M. of 5 to 6 experiments

$F = 0.38$, d.f. = 1, 30, $p > 0.05$

Figure 4

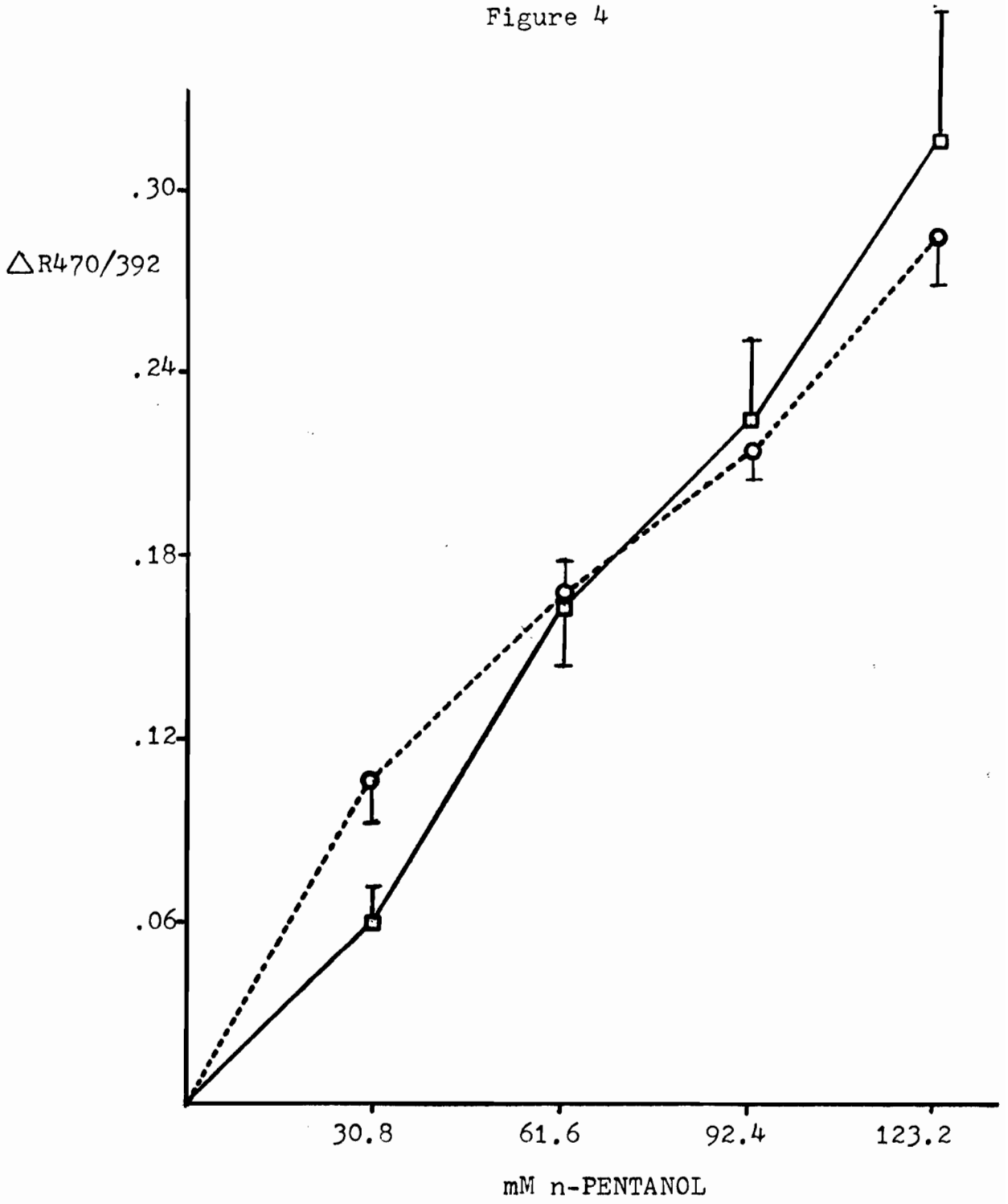


Figure 5: In vitro pentanol induced fluidization of reconstituted liposomes made of polar lipids with equalized cholesterol to phospholipid ratio (0.37) from control and alcohol-fed rat pups. Control (—□—), alcohol-fed (- -○- -).

$\bar{X} \pm$ S.E.M. of 3 to 4 experiments

$F = 10.1$, d.f.$= 1, 18$. $p < 0.01$

Figure 5

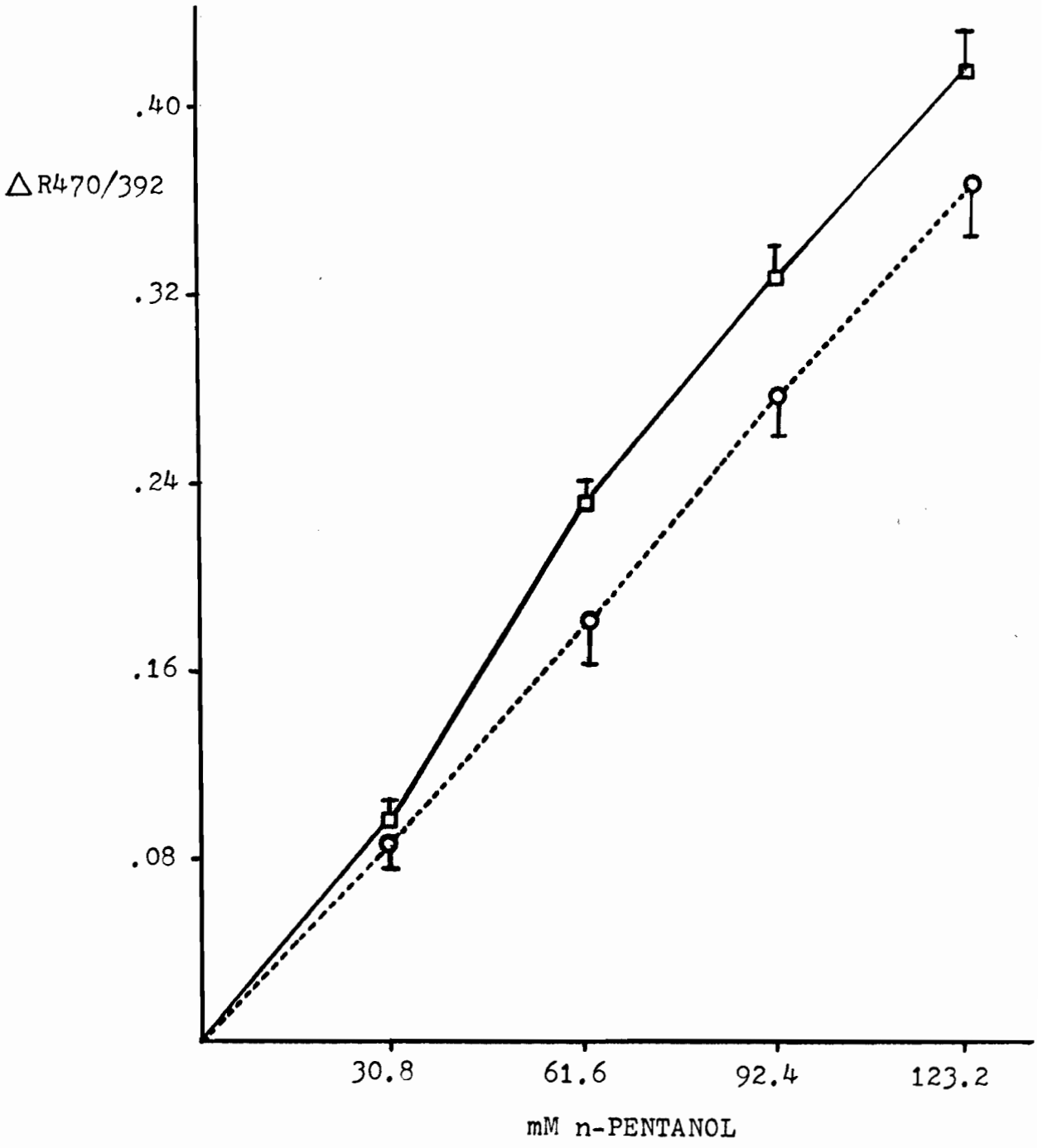
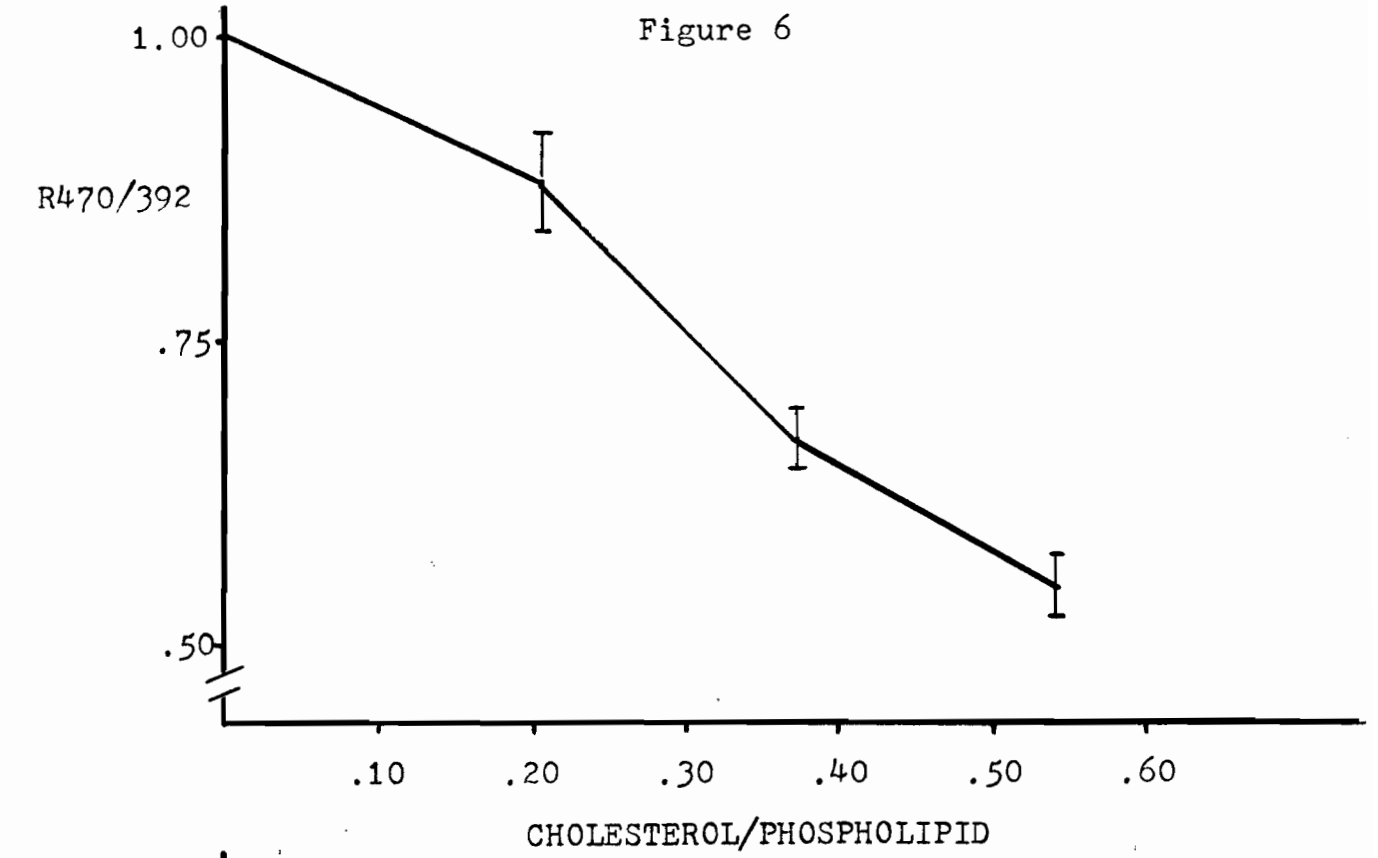


Figure 6A: Effects of cholesterol on excimer-formation efficiency of reconstituted liposomes from control phospholipid fractions from the brain of rat pups.

Figure 6B: Effects of cholesterol on pentanol-induced fluidization of reconstituted liposomes from the polar lipid fraction of the brain of 10 day-old control rat progeny.

$$\Delta R/R_O\ (\%) = \frac{R_A - R_O}{R_O} \times 100\%$$

Figure 6


1.00
R470/392
.75
.50
.10
.20
.30
.40
.50
.60
CHOLESTEROL/PHOSPHOLIPID

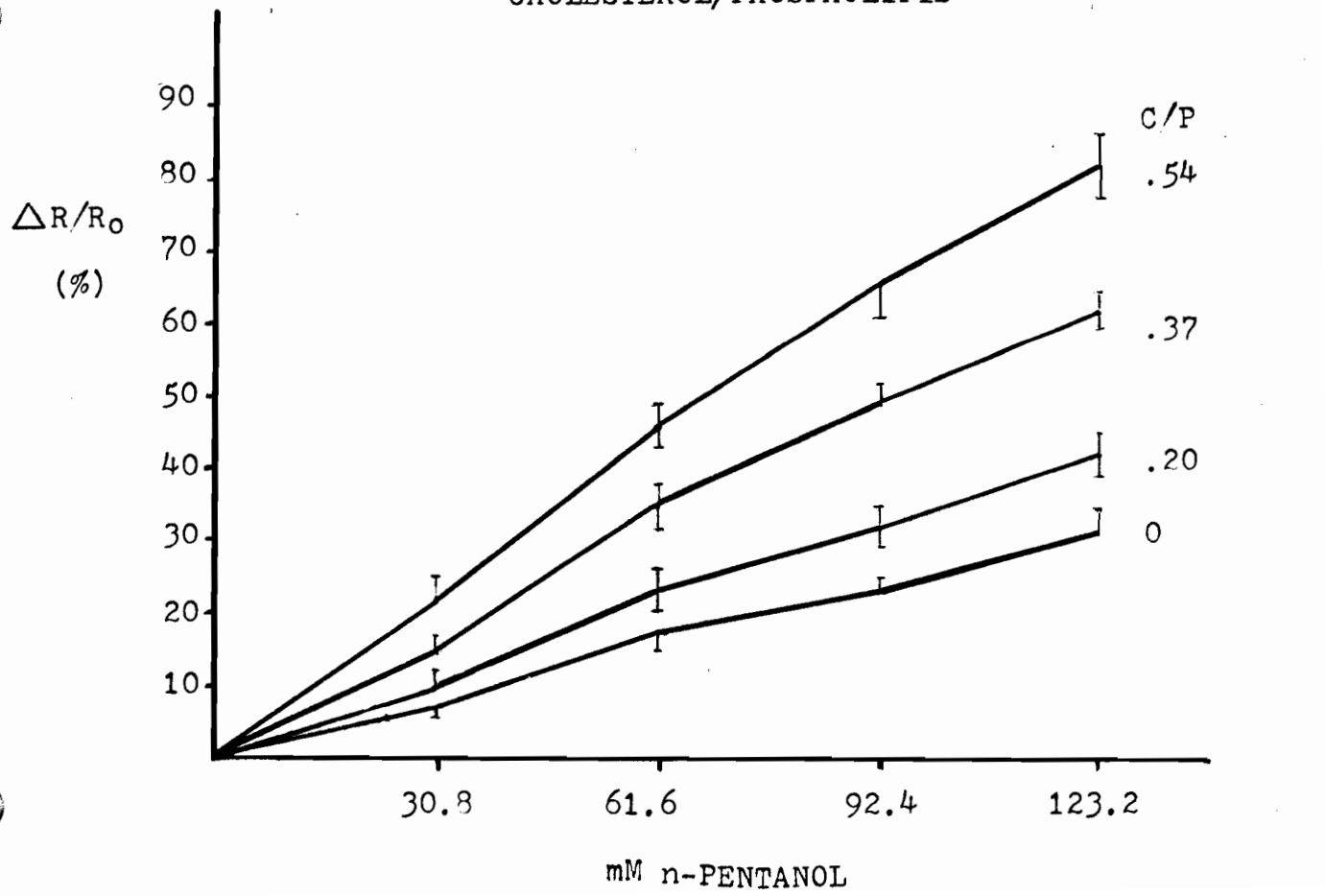
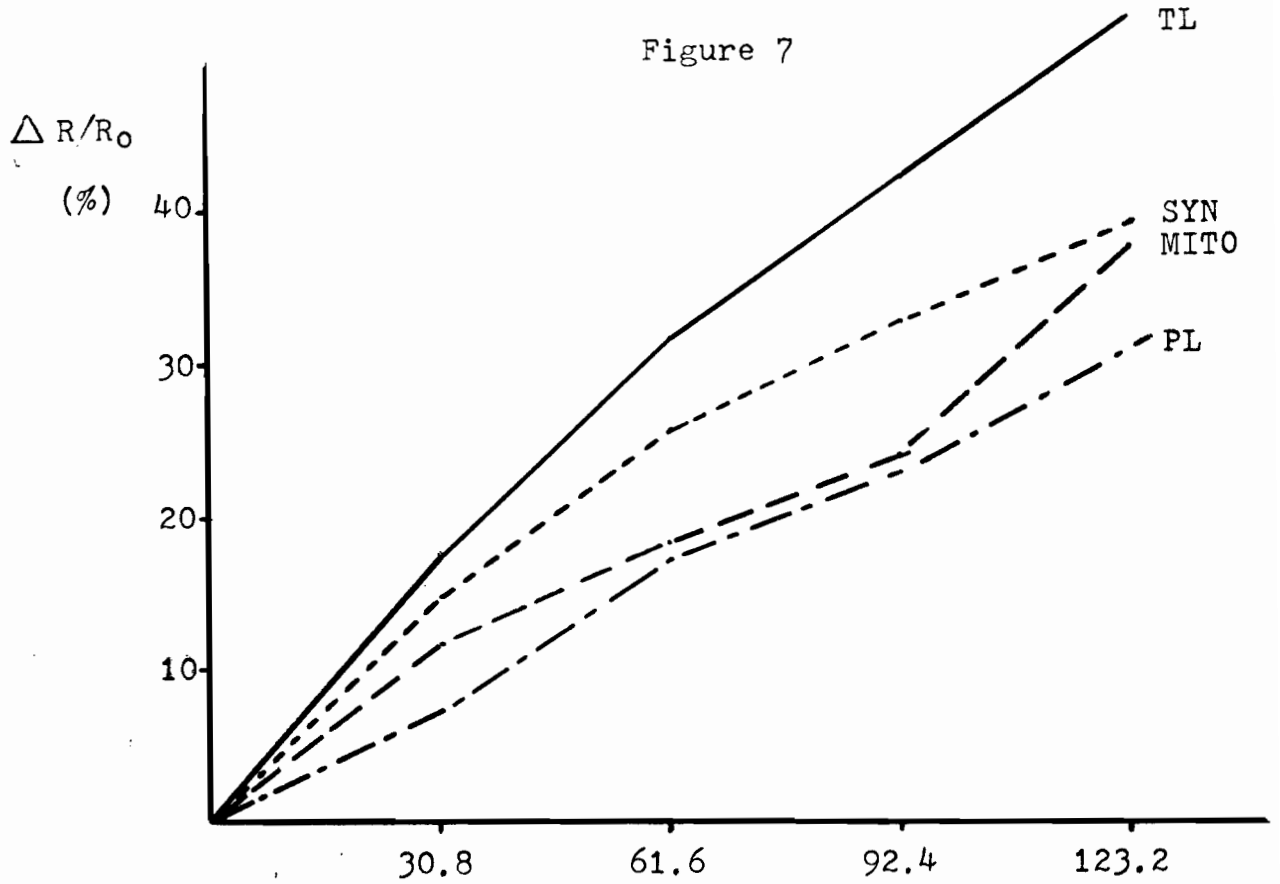

90
80
70
60
50
40
30
20
10
ΔR/Ro
(%)
C/P
.54
.37
.20
0
30.8
61.6
92.4
123.2
mM n-PENTANOL

Figure 7A: Pentanol induced fluidization on the various fractions of brain from 10 day-old control progeny.

Figure 7B: Pentanol induced fluidization on the various fractions of brain from the 10 day-old progeny of alcohol-fed mothers.

$$\triangle R/R_O\ (\%) = \frac{R_A - R_o}{R_o} \times 100\%$$

Figure 7


TL
SYN
MITO
PL
Δ R/Ro
(%)
40
30
20
10
30.8
61.6
92.4
123.2

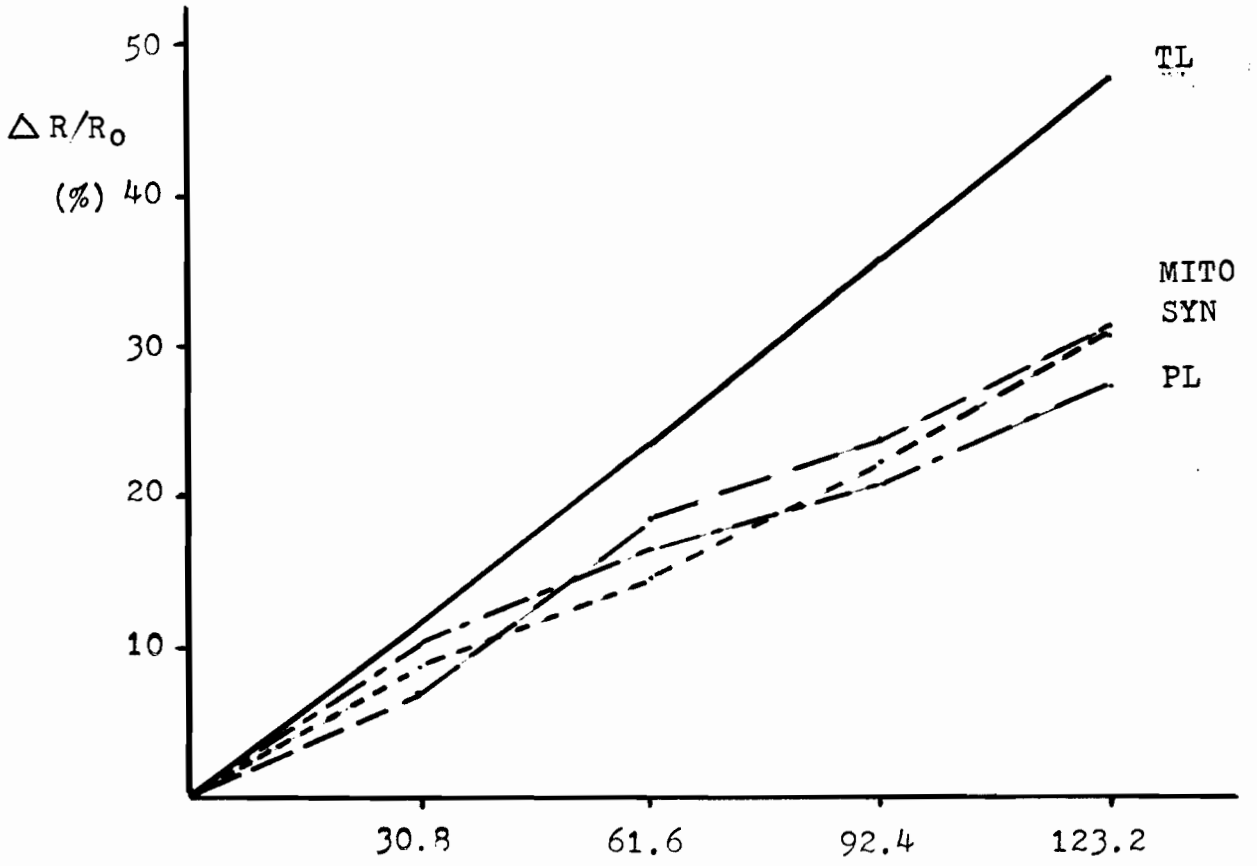

50
TL
Δ R/Ro
(%) 40
MITO
SYN
30
PL
20
10
30.8
61.6
92.4
123.2
mM n-PENTANOL

Figure 8: Effect of chronic ethanol intake on basal and dopamine-stimulated Na,K-ATPase. Control (—□—), alcohol-fed (--○--).

Figure 8

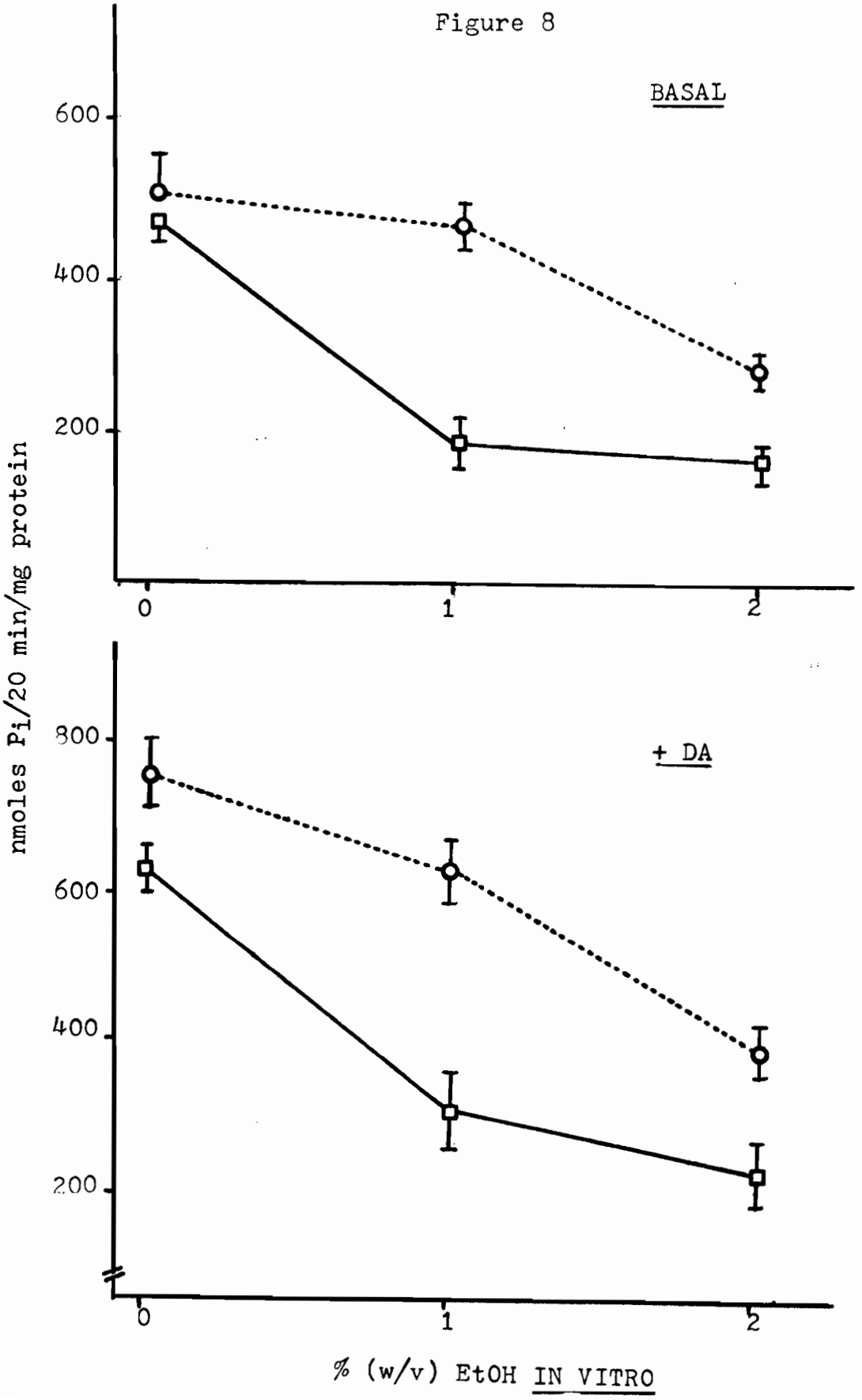

BASAL
600
400
200
0
1
2
+ DA
800
600
400
200
0
1
2
nmoles Pi/20 min/mg protein
% (w/v) EtOH IN VITRO

Figure 9: Percent stimulation of Na,K-ATPase activity from 10 day-old rat brain by dopamine as a function of *in vitro* ethanol. Control (—□—), alcohol-fed (– –○– –).

Figure 9

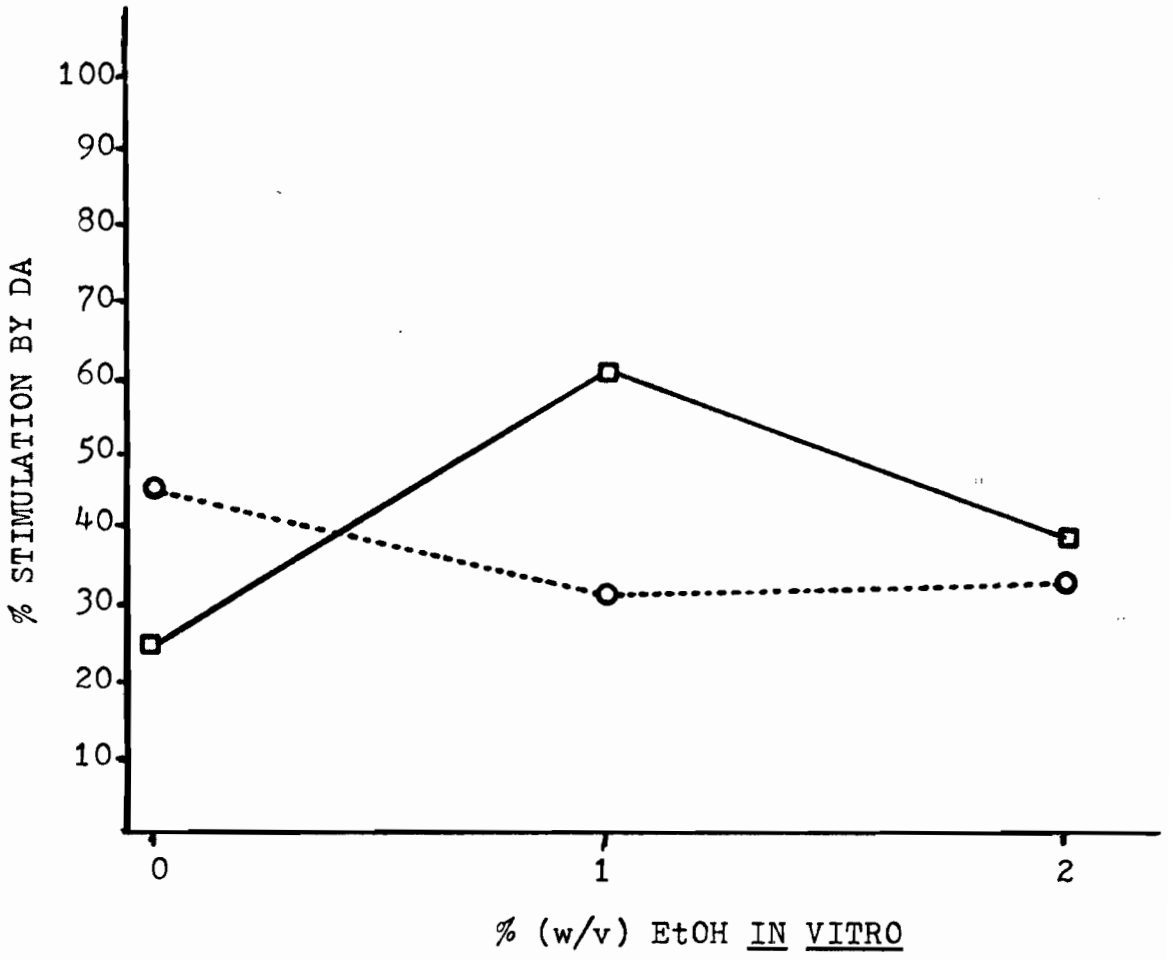

100
90
80
70
60
50
40
30
20
10
% STIMULATION BY DA
0
1
2
% (w/v) EtOH IN VITRO

Table 1.

- Pyrene excimer-formation efficiency in mitochondrial membranes, synaptosomal membranes, synaptosomal total and phospholipid fractions.

R470/392, 37°C

Fraction	alcohol-fed	n	control	n
Mitochondrial membranes	0.515 ± 0.010	5	0.528 ± 0.015	6
Synaptosomal membranes	0.513 ± 0.017	6	0.487 ± 0.016	6
Synaptosomal total lipid	0.792 ± 0.026	6	0.842 ± 0.027	5
Synaptosomal phospholipid	1.04 ± 0.04	6	1.00 ± 0.05	5
Syn. reconstituted total lipid	0.619 ± 0,035	3	0.671 ± 0.027	4

Table 2.

- Synaptosomal protein to lipid ratio and cholesterol to phospholipid ratio for the 10 day-old progeny of control and alcohol-fed groups.

	Fraction	Alcohol-fed	n	Control	n
Protein/total lipid (mg/mg)	mitochondrial membranes	2.19 ± 0.07	8	2.07 ± 0.06	9
	synaptosomal membranes	* 1.75 ± 0.03	5	1.61 ± 0.03	7
C/P molar ratio	synaptosomal total lipid	0.41 ± 0.03	3	0.37 ± 0.02	4

($\bar{X}$ ± S.E.M. of n experiments)

* $p<0.01$

Table 3.

- "Polarity" of the hydrocarbon interior of biomembranes and liposomes.

		Treatment	
	Fraction	Alcohol-fed	Control
R392/373 (37°C)	1.Mito. membrane	2.05 ± 0.06	1.97 ± 0.09
	2.Syn. membrane	1.96 ± 0.02	2.00 ± 0.05
	3.Syn.Total lipid	1.64 ± 0.03	1.64 ± 0.03
	4.Syn.Polar lipid	1.60 ± 0.03	1.59 ± 0.04

($\bar{X}$ ± S.E.M. of 5 experiments)

Table 4.

- Basal and dopamine stimulated Na,K-ATPase specific activity in brain homogenates from control and alcohol-fed rat neonates.

Basal	Controls	Alcohol-fed
0%	496 ± 16	518 ± 46
1% (w/v) EtOH	196 ± 31	* 480 ± 26
2%	168 ± 19	* 290 ± 20
DA-stimulated		
0%	626 ± 26	758 ± 47
1% (w/v) EtOH	316 ± 50	* 634 ± 43
2%	232 ± 43	** 388 ± 30

($\bar{X}$ ± S.E.M. of 4 experiments)

* $p < 0.01$

** $0.02 < p < 0.05$

DISCUSSION

I- Biophysical

In recent years, biophysical studies have provided us with a great deal of information concerning the properties of lipid bilayers and biological membranes (61,71,76,78). In particular, fluorescent probe analyses of membranes yielded much data on the physical behavior of bilayers and on the effects of ethanol on such systems (111,112). Pyrene is a fluorescent hydrocarbon ($C_{16}H_{10}$) being soluble in aqueous medium only in micromolar concentrations (168). By virtue of its lipophilic nature it is expected to favorably partition within the hydrophobic domains of lipid bilayers (71,111,169). Mitochondrial membranes isolated from the brains of day 10-old rat pups did not show any changes in baseline "order parameter" associated with prolonged maternal alcohol consumption (Table 1). These results are in agreement with other studies using EPR techniques. For instance, using 5-doxyl stearate it was shown that the baseline order parameter of brain mitochondrial membranes from mice was the same between alcohol and sucrose-fed animals (113). When the purified mitochondrial membranes were subjected to the in vitro addition of n-pentanol, the fractions from both control and alcohol-fed groups were fluidized to the same extent. This confirms the results of Chin and Goldstein where it was shown that no tolerance to the fluidifying effects of alcohol develops in the mitochon-

drial or myelin fractions isolated from mice brain (113). On the other hand, synaptosomal membranes from the day-10 pups revealed a component of cross-tolerance as n-pentanol was more efficient in fluidizing the control membranes than the ones isolated from the alcohol-fed group. An ANOVA using data from all 6 experiments confirmed the differences between the 2 groups.

Again here, using pyrene fluorescence it was impossible to detect a difference in the baseline fluidity (E/M value; Table 1) of the synaptic fractions. As pointed out by other workers (113), methods sensitive enough to detect tolerance may not necessarily detect dependence which is associated to a change in the baseline order parameter. This is because tolerance is usually estimated by adding various concentrations of an alkanol to a single probe-labeled preparation, whereas dependence data arise from comparisons between different preparations which were separately labeled. The dependence data is thus expected to have greater variability than the tolerance one. Using the pyrene excimer-formation method mitochondrial and synaptosomal membranes were shown to have similar intrinsic fluidities. These present results contrast with studies performed on spin-labeled membranes where it was observed that mitochondrial membranes are usually more fluid than synaptosomal membranes (113). The data observed in such ESR studies is usually explained by the fact that mitochondrial membranes do not contain cholesterol which increases rigidity in biomembranes, whereas synaptic membranes may contain up to 25%

by weight of the sterol. However, as shown in Table 2 mitochondrial membranes have a much greater protein to lipid ratio and this may off-balance the cholesterol factor especially in methods which measure lateral diffusion. For instance, an increase in protein to lipid values not only increases the population of lipid in the boundary regions but also reduces the number of pathways by which an excited-state pyrene molecule may reach a ground-state molecule to form the excimer. Therefore, rotational diffusion may only sample the local lipid environment whereas translational motions sample more of the bulk lipid. It is possible that there is lack of relationship between the two methods and that this may explain the differences in membrane micro-viscosities obtained from both techniques. As shown in Table 2, maternal consumption of alcohol did not alter the protein to lipid ratio of brain mitochondrial membranes isolated from 10 day-old rat neonates. However, chronic exposure to alcohol did result in a signifiant increase in protein density of the synaptosomal membranes. The alcohol-fed group afforded a value of 1.75 mg prot./mg lipid versus 1.61 for the control rat neonates ($p<0.01$). Such results are in agreement with those obtained earlier in our laboratory which noted an increase in the protein to lipid values of day-6 rat pups after exposure to alcohol (unpublished).

Hitzemann and co-workers who studied developmental changes in synaptic membrane fluidity have shown that protein/lipid ratio increases with age and that such an increase can be correlated

with more ordered membranes (170,171). Since protein density regulates membrane viscosity, it becomes possible that in response to the continual presence of ethanol, membranes adapted or restored their rigidity so as to minimize perturbation by the alkanol. Thus, such adaptation could play an important role in the tolerance (or cross-tolerance) phenomenon displayed by the synaptosomal fractions (Figure 2).

When the protein/lipid ratios of mitochondrial membranes are compared with the synaptic membranes it is clear that protein density is much greater in the former fraction e.g. 2.1 versus 1.7. This is expected as the inner mitochondrial membrane is quite rich in membrane-bound enzymes such as Mg-ATPases, the ATP-proton regenerating system and the electron transport chain (30).

The above findings suggest that in the developing rat, the protein to lipid ratio is an important factor in regulating membrane perturbation by general anesthetics. However, since such disordering agents have been shown to partition and interact within the lipid fraction of biological membranes, an investigation of the lipid extracts from "tolerant" membranes becomes critical. Again in liposomes of total lipid from synaptic membranes, basal "fluidities" were similar between the control and alcohol-fed groups and ranged at about 0.8(=E/M). The total lipid extracts contain practically all of the lipid from the purified synaptosomal fraction. Therefore, by fixing the probe to

lipid weighted ratio the same concentration of fluorescent molecules (pyrene/unit area of lipid) should be achieved in both systems. It is thus likely that the increase in excimer to monomer (E/M) values observed upon removal of membrane-bound polypeptides reveals the rigidifying effects of protein incorporation into lipid bilayers. Cross-tolerance to the fluidizing effects of pentanol was also observed between control and alcohol-treated animals at identical in vitro concentrations (Figure 3). Two-tailed student's t-test and ANOVA performed on the total lipid data showed similar statistical significance as whole synaptic membranes in terms of resistance to pertubation by pentanol. Therefore, although proteins could be involved in "homeoviscous adaptation" to alcohol in the young animal, the lipid component alone may be responsible for the tolerance observed in such biomembranes. Many investigators have claimed the importance of cholesterol in "tolerance and dependence" to alcohol (108,112). Cholesterol in general reduces membrane fluidity by virtue of its condensing effect. Indeed, acyl chain mobility has been shown to decrease in a dose-dependent manner with increasing cholesterol molar fraction (78). Thus, after prolonged exposure to alcohol, synaptosomal membranes may increase in their cholesterol content and restore their rigidity so as to lower membrane fluidifying by the drug. However, some studies have detected no significant change in the cholesterol to phospholipid ratio following chronic treatment with alcohol (112,129). Furthermore, in some instances

cholesterol has been shown to enhance the disordering potential of alcohol and other general anesthetics on biological membranes (37,112). These results were confirmed in the present study. The cholesterol/phospholipid ratio was slightly but not significantly higher in the alcohol-fed group relative to the control (Table 2). As depicted in Figure 6B, increasing the cholesterol % molar fraction (by addition to synaptic polar lipid) resulted in an enhancement of pentanol's disturbing effects as assessed by the pyrene excimer-formation method. This potentiation was also more evident at the higher pentanol concentrations. It should be noted that Figure 6B plots $\Delta R/R_o$% rather than ΔR (R_A-R_o; absolute change in excimer to monomer when pentanol added in vitro). Since the R value (excimer/monomer) is a function of probe concentration times the second order rate constant K_a ;in comparing systems with different probe/unit area of lipid, one divides by the basal E/M value (or R_o) in order to observe a parameter proportional to fluidity rather than some concentration dependent phenomenon. Similarly, when ANOVAs were performed on Figures 1 to 5 but with $\Delta R/R_o$ % as y-axis parameter, the same statistical significances were obtained as with ΔR alone. This is to be expected since in all cases the bilayers from the controls or alcohol-fed were not significantly different in intrinsic fluidity (assessed by the R_o value). In order to determine the importance of cholesterol in fluidity and fluidizability of synaptosomal membranes, polar lipid extracts (with no chole-

sterol present) were obtained and then analysed by excimer formation. Liposomes of polar lipid were similar in fluidity between controls (E/M=1.00) and alcoholics (E/M=1.04;c.f. Table 1). In contrast, the polar lipid fraction from the alcohol-fed animals did not display resistance to the fluidizing effects of pentanol. These findings suggest that either subtle differences between alcohol-fed and control groups exist in the phospholipid fractions but are only expressed in presence of cholesterol or that the slight increase in cholesterol to phospholipid ratio observed in the alcoholic group explains "tolerance" in the total lipid fraction. Reconstituted total lipid extracts were then obtained by adding cholesterol to polar lipid extracts so that both alcohol and control-fed groups would be equal in their sterol content (with cholesterol/phospholipid equalized at 0.37, the control value). Interestingly, with equal cholesterol molar fraction there was a detectable difference in resistance to pentanol between the alcohol and control-fed groups (Figure 5). That is, cholesterol restored the differential efficacy of pentanol to disturb total lipid liposomes and thus the sterol is required in the expression of tolerance (or cross-tolerance) to alcohol. It is concluded that differences in phospholipids are present but only expressed whenever certain proportions of cholesterol are added back into the bilayers.

The reconstituted total lipid extracts did not have identical fluidities as their native total lipid counterparts. The exci-

mer to monomer values were decreased upon reconstitution of total lipid fractions with pure cholesterol. "Native" total lipid extracts are resolved by silicic-acid chromatography into polar and neutral lipid fractions. It has been shown in our laboratory that cholesterol constitutes about 80% by weight of the neutral lipid fraction of synaptosomal membranes. Thus, it is possible that the 20% remainder is of some importance in regulating membrane fluidity and that a more fluid hydrocarbon interior would result in its presence. It can be seen from Figure 6A that cholesterol enrichment of the synaptosomal phospholipid fraction leads to a pronounced reduction in excimer-formation efficiency. Film balance studies using mixed cholesterol/DPPC monolayers have shown that addition of cholesterol produces a decrease in the average area occupied per lipid molecule. It becomes possible that the phenomenon observed in Figure 6A is influenced by probe concentration, as in all samples the pyrene to lipid weighted ratio was fixed. However, using $c=R/(1-\alpha)F+\alpha F_c$ (where R is the molar ratio of pyrene to lipid, F is the average area of a phospholipid molecule =58 $\mathring{A}^2$, F_c the area of 1 cholesterol molecule =40 $\mathring{A}^2$(169), and α the molar fraction of cholesterol) it can be estimated that probe concentration only contributes about 10% of the decrease observed upon adding cholesterol to the phospholipid extracts.

Thus, it is concluded that cholesterol leads to a rigidification of the lipid matrix resulting in a decrease in excimer formation. As shown in Figures 7(A and B) the phospho-

lipid fractions from both alcohol-fed and controls are resistant to fluidization by pentanol. With the alcohol-fed, the total lipid extract remains the most fluidizable (or inducible) system. All 3 other fractions (i.e. mito.,syn.,PL) being similar in their fluidizability. In contrast, control mitochondrial and polar lipid fractions are resistant relative to synaptosomal and total lipid fractions; the latter still being the most fluidized. Such results suggest that cholesterol potentiates fluidization by the alkanol as total lipid and synaptic membranes are most sensitive to it. Mitochondrial membranes which do not contain cholesterol behave like phospholipids and are not greatly perturbed by pentanol. Interestingly, in the alcohol-fed group, the synaptosomal membranes adapted to the chronic effects of alcohol in vivo and now stand in a similar fluidizing range as polar lipids and mitochondrial membranes. These findings agree with those of Pang and co-workers where it was shown that in order for general anesthetics to perturb membrane order cholesterol must be present in lipid bilayers and phosphatidic acid must be low (37). Presumably at high PA and low cholesterol the surface charge density increases and the fluidizability is reduced.

Of importance is the fact that membrane systems which contain cholesterol could be more sensitive to alcohol in vivo and therefore would be expected to respond to chronic insult by the drug. This may explain why homeoviscous membrane adaptation was only observed in the synaptosomal fraction and not in the mitochondrial one from the alcohol-fed group.

The results on the "polarity" of the hydrocarbon interior of lipid bilayers and biological membranes are depicted in Table 3. From previous reports, it is known that the ratio of fluorescent intensities of 392 to 373 nm (or R 392/373) is inversely related to the "polarity" of the probe's microenvironment (168,172). This was also confirmed in preliminary studies using water/ethanol binary systems (data not shown). It appears from Table 3 that biomembranes (syn. and mito.) have a much greater R392/373 value than synaptosomal total and polar lipid liposomes. Although light scattering is increased at lower emission wavelengths, the scatter was similar in presence or absence of membrane-bound proteins. Therefore, the results are interpreted in terms of liposomes being more "polar" than corresponding biomembranes. A strong possibility is the fact that intact membranes with intrinsic proteins and cytoskeletal components are much less "leaky" to water and hydrated ions than just bilayers made of biological lipids. In general, the biophysical part of this study shows that there is no apparent relation between fluidity and fluidizability. For instance, although cholesterol effectively reduced membrane intrinsic fluidity it did not protect these membrane systems from perturbation by an alkanol. On the contrary, cholesterol potentiated the fluidifying effects of pentanol and thus would not seem to be responsible for the tolerance (or cross-tolerance) phenomenon observed after chronic treatment of rat pups with alcohol. The fact that lipid bilayers from control and alcoholic groups

with equalized cholesterol still remained different in fluidizability brings further proof that cholesterol is not responsible for acquired membrane adaptations but that rather phospholipid and fatty acid composition is of importance here. Nontheless, tolerance is only observed when the sterol is added back to lipid bilayers and may only develop *in vivo* in systems which contain cholesterol (*i.e.* maybe more sensitive to the effects of ethanol consumption).

II- Biochemical

In contrast to studies performed on adult rat brain, Na,K-ATPase activity was not significantly increased in brain homogenates of 10 day-old pups. Nontheless, a component of tolerance was revealed when ethanol was added *in vitro* at concentrations of 1%(w/v) (or 217mM) and 2%(w/v). Potassium was kept low in this study as this cation has been shown to be a competitive antagonist of alcohol *in vitro* (151). This would therefore sensitize the enzyme to inhibition by ethanol and bring out differences between the activities of Na,K-ATPase from control and alcohol-fed animals.

The effects of chronic alcohol ingestion on brain catecholaminergic systems are complex and delicate and have been subject of an extensive review by Hoffman and Tabakoff (173).In rats, "supersensitivity" of dopamine (DA) receptors was found following prolonged intoxication with alcohol (173). The results on DA-stimulated Na,K-ATPase from rat pups also agree with these

findings. As shown in Figures 8 and 9, the DA-stimulated enzyme activity was greater in the alcohol-fed group in absence of added alcohol ($p=0.05$). Here also, resistance to inhibition by in vitro ethanol was displayed in the alcohol-fed fraction.

From Figure 9 it can be seen that in vitro ethanol has a biphasic effect on DA-stimulation of control Na,K-ATPase; being stimulatory at the intermediate alcohol concentration. On the other hand, added ethanol did not have much effect on the DA-stimulated component of Na,K-ATPase from alcohol-fed pups. It is possible that since control synaptic membranes are more perturbed (or fluidized) by in vitro ethanol, receptor diffusion and coupling is enhanced. In contrast, membranes from the alcohol-adapted animals would not respond to perturbation by ethanol reflecting their "tolerant and dependent" state.

In this study, brain membranes isolated from developing rats have been shown to acquire tolerance to the fluidizing effects of alkanols following chronic exposure to alcohol. The results obtained using pyrene excimer formation can be correlated to membrane-associated activities which are of great functional importance. That is, the fact that synaptosomal lipids were resistant to disordering by an alkanol can explain tolerance of critical enzymes in intimate interaction with synaptic membranes like Na,K-ATPase.

In general, synaptosomal membranes are an important model system for elucidating alcohol perturbation as they contain cholesterol and they interact with various catecholamines. Both these factors have been shown to potentiate membrane alcohol effects and adaptations during alcoholism (112, 143).

REFERENCES

1. Warner R.H. and Roselt H.L.,
J.Stud. Alcohol 36, 1395-1420, 1975

2. Henry L. Roselt and Lyn Weiner : Alcohol and the fetus : A clinical perspective, 1984

3. Stockard C.R., Am. J. Anat. 10, 369-392, 1910

4. Stockard C.R. : The effects of alcohol in development and heredity. In: Alcohol and Man, edited by H.Emerson, New York: The MacMillan Co., 1932

5. Pearl R., J.exp.Zool. 22, 240, 1917

6. Randall C.L. and Riley E.P.,
Neurobehar. Toxicol. Teratol. 3 (2), 111-115, 1981

7. Jones K.L. and Smith D.W., Ulleland C.N. and Streissguth A.P. lancet 1, 1267-1271, 1973

8. Hurley L.S. : The Fetal Alcohol Syndrome: possible implications of nutrient deficiencies. In TK Li, S. Schenker, L. Lumeng, eds, Alcohol and Nutrition, Res Mon 2, NIAAA, Washington D.C., 367-379, 1979

9. Kalter H. and Warkany J. : Congenital malformations.
N. Engl. J. Med. 308, 491-497, 1983b

10. Wilson J.G. : Environment and Birth Defects.
Academie Press, New York, 1973

11. Hendrickson J.B., Cram D.J. and Hammond G.S.,
Organic Chemistry, McGraw-Hill Co., U.S.A., 1970

12. Kalant H., - Absorption, Diffusion, Distribution and Elimination of Ethanol: Effects on Biological Membranes. in The Biology of Alcoholism, Edited by B.Kissin and H.Begleiter, Plenum Press, New York, 1, 1-62, 1971

13. Murdoch Ritchie J., The aliphatic alcohols. In, The Pharmacological Basis of Therapeutics, Ed.: Goodman L.S. and Gilman A., 5th edition, 137-151, 1975

14. Lieber C.S., Metabolic and Hepatic Effects of Alcohol. In, Drug-Nutrient Interrelationships Proceedings of The Miles Symposium, Ed. by W.W. Hawkins, Nutrition Society of Canada, copyright by Miles Laboratories, Ltd., 43-69, 1975

15. Cherrick G.R. and Leevy C.M., Biochim. Biophys. Acta. 109, 29-37, 1965

16. Duncan R.J.S., Kline J.E. and Sokoloff L., Biochem J. 153, 561-566, 1976

17. Seitz H.K., Korsten M.A. and Lieber C.S., Life Sci. 25, 1443-1448, 1979

18. Wartburg J.P.von, The metabolism of alcohol in normals and alcoholics: enzymes. In, The Biology of Alcoholism., Vol.1, Biochemistry. (Kissin B. and Begleiter H. eds.), Plenum Press, New York, 63-102, 1971

19. Pietruzko R., Alcohol and Aldehyde Dehydrogenase Isozymes from Mammalian Liver Their Structural and Functional Differences. In, Isozymes: Current topics in biological and medical research, Alan R., Liss Inc. v.4, 107-130, 1980

20. Blomstrand R.,
Life Sci. 10, 575-582, 1971

21. Lester D., Quart.J. Stud. Alcohol 22, 554-574, 1961

22. Lester D., Quart.J. Stud. Alcohol 23, 17-25, 1962

23. Nomura F. and Lieber C.S.,
Biochem. Biophys. Res. Commun. 100, 131-137, 1981

24. Lieber C.S. and DeCarli L.M.,
J.Pharmacol. Exp. Ther. 181, 279-287, 1972

25. Orme-Johnson W.H. and Ziegler D.M.,
Biochem. Biophys. Res. Commun. 21, 78-82, 1965

26. Miwa G.T., Levin W., Thomas P.E. and Lu A.Y.H.,
Arch. Biochem. Biophys. 187, 464-475, 1978

27. Baghurst K.I., Med J.Australia 2, 177-180, 1980

28. Lieber C.S. and DeCarli L.M., Metabolic Effects of Alcohol on the Liver. In, Metabolic Aspects of Alcoholism, Ed. by Lieber C.S., University Park Press, Baltimore, 31-79, 1977

29. Flink E.B., Mineral Metabolism in Alcoholism. In, The Biology of Alcoholism, Ed.: B.Kissin and H.Begleiter, Plenum, New York, 1, 377-395, 1971

30. Lubert Stryer, Biochemistry, 2nd Edition, W.H. Freeman and company, USA, 1981

31. Tanaka H., Nakazawa K., Suzuki N. and Arima M.,
Brain. Dev. 4, 429-438, 1982

32. Sullivan L.W. and Herbert V.
J.Clin. Invest. 43, 2048-2062, 1964

33. Sullivan L.W., Folates in Human Nutrition. In, AA Albanese, ed., Newer methods of nutritional biochemistry, Vol.III, Academic Press, New York, 365-406, 1967

34. Trudell J.R., Hubbell W.L. and Cohen E.N., Biochim. Biophys. Acta. 291, 321-327, 1973

35. Lawrence D.K. and Gill E.W., Mol. Pharmacol. 11, 280-286, 1975

36. Boggs J.M., Yoong T. and Hsia J., Mol. Pharmacol. 12, 127-135, 1976

37. Miller K.W. and Pang K.Y.Y., Nature 263, 253-255, 1976

38. Paterson S.J., Butter K.W., Huang P., Labelle J., Smith I.C.P. and Schneider H., Biochim. Biophys. Acta. 266, 597-602, 1972

39. Wishnia A. and Pinder T., Biochemistry 3, 1377-1384, 1964

40. Ray A., Reynolds J.A., Polet H. and Steinhard J., Biochemistry 5, 2606-2616, 1966

41. Helmer F., Kiehs K. and Hansch C., Biochemistry 7, 2858-2863, 1968

42. Kiehs K., Hansch C. and Moore L., Biochemistry 5, 2602-2605, 1966

43. Meyer H., The theory of narcosis. In, The Harvey lectures, Lippincott, Philadelphia, 11-17, 1906

44. Pang K.Y.Y., Braswell L.M., Chang L., Sommer T. and Miller K.W., Mol. Pharmacol. 18, 84-90, 1980

45. Miller J.C. and Miller K.W., Approaches to the Mechanism of Action of General Anesthetics. In, MTP International Review of Science. Physiological and Pharmacological Series, Ed. H.Blaschko, University Park Press, Baltimore, 33-76, 1975

46. Seeman P., Pharmacol. Rev. 24, 583-655, 1972

47. Freund G., Cancer. Res. 39, 2899-2901, 1979

48. Johnson S.M. and Miller K.W., Nature 288, 75-76, 1970

49. Miller K.W., Paton W.D.M., Smith R.A. and Smith E.B., Molec. Pharmac. 9, 131-143, 1973

50. Halsey M.J. and Wardley-Smith B., Nature 257, 811-813, 1975

51. Janoff A.S., Pringle M.J. and Miller K.W., Biochim. Biophys. Acta. 649, 125-128, 1981

52. Labella F.S., Can. J. Physiol. Pharmacol. 59, 432-442, 1981

53. Han Y.H., Anesthesiol. 30, 341-342, 1969

54. Johnstone R.E., Kulp R.A. and Smith T.C., Anesth. Analg. 54, 277-281, 1975

55. Koblin D.D., Deady J.E., Dong D.E. and Eger E.I., J. Pharmacol. Exp. Therap. 213, 309-312, 1980

56. Kelly-Murphy S., Waring A.J., Rottenberg H. and Rubin E., Lab. Invest. 50, 2, 174-183, 1984

57. Meyer H., Arch. Exp. Pathol. Pharmacol. 42, 109-118, 1899

58. Franks N.P. and Lieb W.R., Nature 274, 339-342, 1978

59. Robertson J.D., A Review of Membrane Structure with Perspectives on Certain Transmembrane Channels. In, Demyelinating Disease: Basic and Clinical Electrophysiology, Ed. by S.G. Waxman and J.M. Ritchie, Raven Press, New York, 419-477, 1981

60. DePierre J.W. and Ernster L., Ann. Rev. Biochem. 46, 201-262, 1977

61. Jain M.K. and Wagner R.C., Introduction to Biological Membranes. John Wiley and Son, Inc. LISA, 1980

62. Bretscher M.S., Some Aspects of Membrane Structure. In, Perspective in Membrane Biology, Ed. by S. Estrada O. and C. Gitler, Academie Press, London, 3-24, 1974

63. Insel P.A., Membrane Active Hormones: Receptors and Receptor Regulation. In, Biochemistry and Mode of Action of hormones II, Ed. by H.Y. Rickenberg, University Park Press, Baltimore, 1-43, 1978

64. Rothman J.E., An Overview of Membrane Structure and Biosynthesis. In, Membrane-Membrane Interactions, Ed. by N.B. Gilula, Raven Press, New York, 1-9, 1980

65. Manku M.S., Horrobin D.F., Huang Y.S. and Morse N., Lipids 18 (12), 906-908, 1983

66. Jackson R.L., Current Views on the Organization of Lipids and Proteins in Plasma Membranes. In, Receptors and Hormone Action I, Ed. by B.W. O'Malley and L. Birnbaumer, Academic Press, London, 411-426, 1978

67. Lodish H.F. and Rothman J.E., Scientific America 240, 48-63, 1978

68. Singer S.J., The Fluid Mosaic Model of Membrane Structure. In, Structure of Biological Membranes, Ed. by S. Abrahamson and I. Pascher, Plenum Press, New York, 443-461, 1976

69. Jain M.K. and White H.B., Adv. Lipid. Res. 15, 1-60, 1977

70. Roman-Franco A.A., Med. Hypothesis 3, 235-240,1977

71. Galla H.J., Hartmann W., Theilen U. and Sackmann E., J. Membrane Biol. 48, 215-236, 1979

72. Rothman J.E. and Lenard J., Science 195, 743-753, 1977

73. Bretscher M.S., J. Mol. Biol. 71, 523-528, 1972

74. Hirono H., Parkhouse B., Nicolson G.L., Lennox E.S. and Singer S.J., Proc. Nat. Acad. Sci. 69, 2945-2949, 1972

75. Hughes B.D., Pailthorpe B.A., White L.R. and Sawyer W.H., Biophys J. 37, 673-676, 1982

76. Hubbell W.L. and McConnell H.M., J. Ainer. Chem. Soc. 93, 314, 1971

77. McFarland B.G. and Mcconnel H.M., Proc. Natl. Acad. Sci. U.S. 68, 1274, 1971

78. Oldfield E. and Chapman D., FEBS letters 23 (3), 285-296, 1972

79. Kornberg R.D. and McConnell H.M., Biochemistry 10, 1111, 1971

80. Galla H.J., Theilen L.I. and Hartmann W., Chem.Phys.Lipids 23, 239-251, 1979

81. Alecio M.R., Golan D.E., Veatch W.R. and Rando R.R., Proc. Nat. Acad. Sci. U.S.A. 79, 5171-5174, 1982

82. Poznansky M.J. and Lange Y., Nature 259, 420-421, 1976

83. Cherry R.J., Biochim. Biophys. Acta. 559, 289, 1979

84. Dragsten P.R., Blumenthal R. and Handler J.S., Nature 294, 718-722, 1981

85. Barnett R.E., Fluidity in membranes. In, Receptors and Hormone Action I, Ed. by B.W. O'Malley and L. Birnbaumer, Academic Press, London, 427-446, 1978

86. Melchoir D.L. and Steim J.M., Ann. Rev. Biophys. Bioenerg. 5, 205-237,1976

87. Krasne S., Eisenman G. and Szabo G., Science 174, 412, 1971

88. Stark G., Beng R., Pohl G.W. and Tanko K., Biochim. Biophys. Acta. 266, 603, 1972

89. Ladbrooke B.D., Williams R.M. and Chapman D., Biochim. Biophys. Acta. 150, 333, 1968

90. Chapman D., Williams R.M. and Ladbrooke B.D., Chem. Phys. Lipids 1, 445, 1967

91. Chapman D., Byrne P. and Shipley G.G., Proc. Roy. Soc. A. 290, 115, 1966

92. Oldfield E. and Chapman D., Biochem. Biophys. Res. Commun. 43, 610, 1971

93. Sekuzu I., Jurtshuk P. and Green D.E., Biochem. Biophys. Res. Commun. 6, 76-80, 1961

94. Sandermann H., Biochim. Biophys. Acta. 515, 209-237, 1978

95. Raison J.K., J.Bioenergetics 4, 285-309, 1973

96. Silvius J.R. and McElhaney R.N., J. Theor. Biol. 88, 135-152, 1981

97. DeKruyff B., van Dijck P.W.M., Goldback R.W., Demel R.A. and van Deenan L.L.M., Biochim. Biophys. Acta. 330, 269-282, 1973

98. Bloj B., Galo M.G., Morero R.D. and Farias R.N., J. Nutrition 109, 63-69, 1979

99. Jost P.C., Griffith O.H., Capaldi R.A. and Vanderkooi G., Proc. Nat. Acad. Sci. 70, 480-484, 1973

100. Dahlquist F.W., Muchmore D.C., Davis J.H. and Bloom M., Proc. Nat. Acad. Sci. 74, 5435-5439, 1977

101. Fenster L.J. and Copenhaver J.H., Biochim. Biophys. Acta. 137, 406-408, 1967

102. Seeman P., Biochem. Pharmacol. 15, 1632, 1966

103. Hunt W.A., The Effects of Aliphatic Alcohols on the Biophysical and Biochemical Correlates of Membrane Function. In, Biochemical Pharmacology of Ethanol, Ed. by E. Majchrowicj, 195-210, 1975

104. Seeman P. and Weinstein J., Biochem. Pharmacol. 15, 1767, 1966

105. Seeman P., Kwant W.O. and Sauks T., Biochim. Biophys. Acta. 183, 499-511, 1969

106. Seeman P., Experientia 30, 759-760, 1974

107. Lyon R.C., McComba J.A., Schreurs J. and Goldstein D.B., J. Pharmacol. Exp. Ther. 218, 669-675, 1981

108. Chin J.H. and Goldstein D.B., Mol. Pharmacol. 19, 425-431, 1981

109. Paterson S.J., Butter K.W., Huang P., Labelle J., Smith I.C.P. and Schneider H., Biochim. Biophys. Acta. 266, 597-602, 1972

110. Chin J.H. and Goldstein D.B., Mol. Pharmacol. 13, 435-441, 1977

111. Vanderkooi J.M., Alcoholism. Clin. Exp. Res. 3 (1), 60-63, 1979

112. Johnson D.A., Lee N.M., Cooke R. and Loh H.H., Mol. Pharmacol. 15, 739-746, 1978

113. Chin J.H. and Goldstein D.B., Science 196, 684, 1977

114. Traynor M.E., Woodson P.B.J., Schlapfer W.T. and Barondes S.H., ibid. 193, 510, 1976

115. Lyon R.C. and Goldstein D.B., Mol. Pharmacol. 23, 86-91, 1982

116. Kelly-Murphy S., Waring A.J., Rottenberg H. and Rubin E., Lab. Invest. 50 (2), 174-183, 1984

117. Rottenberg H., Waring A. and Rubin E., Biophys. J. 37, 14-16, 1982

118. Goldstein D.B., Speculations on Membrane Lipid Adaptation as a Mechanism for Drug Tolerance and Dependence. In,

Membrane Mechanisms of Drugs of Abuse, Ed. by O.W. Sharp and L.G. Abood, Alan R. Liss Inc., New York, 151-166, 1979

119. Hill M.W. and Bangham A.D., Adv. Exp. Med. Biol. 59, 1-9, 1975

120. Shinitzky M. and Henkart P., Intern. Rev. Cytol. 60, 121-147, 1979

121. Morrison M., Wilce P.A. and Shanley B.C., Biochem. Biophys.Res.Commun. 122 (2), 516-521, 1984

122. LaDroitte P., Lamboeuf Y. and de Saint Blanquet G., Life Sciences 35, 1221-1229, 1984

123. Littleton J.M. and John G., J. Pharm. pharmac. 29, 579-580, 1977

124. Sun G.Y. and Sun A.Y., Res. Commun. Chem. Path. Pharmac. 24, 405-408, 1979

125. Wing D.R., Harvey D.J., Hughes J., Dunbar P.G., McPherson K.A. and Paton W.D.M., Biochem. Pharmac. 31, 3431, 1982

126. Ingram L.O., J. Bacteriol. 125, 670-678, 1976

127. Lee H., Hepatotoxicity of Alcohol, Ph.D.thesis, 42, 1982

128. Chin J.H., Parson L.M. and Goldstein D.B., Biochim. Biophys. Acta. 513, 358-363, 1974

129. Rawat A.K., Res. Commun. Chem. Path. Pharmacol. 8, 461-469, 1974

130. Waring A.J., Rottenberg H., Ohnishi T. and Rubin E., Proc. Nat. Acad. Sci. 78, 2582-2586, 1981

131. Lee H. and Hosein E.A.,
Can. J. Physiol. Pharm. 64 (1), 85-92, 1986

132. Sun G.Y., Huang H-M., Lee D.Z. and Sun A.Y.,
Life Sci. 35, 2127-2133, 1984

133. Sun A.Y. and Samorajski T., J. Neurochem. 17,
1365-1372, 1970

134. Hammes G.G., Membrane-Bound Enzymes. In, Enzyme Catalysis and Regulation, Ed. by Horecker B., Kaplan O.N., Marmum J. and H.A. Scheraga, 226-246, 1982

135. Fenster L.J. and Copenhaver J.H., Biochim. Biophys. Acta. 137, 406-408, 1967

136. Cantley L.C., Curr. Top. Bioenerg. II, 201, 1981

137. Hastings D.F. and Reynolds J.A., Biochemistry 18,
817, 1979

138. Forbush B. and Hoffman J.F., Biochemistry 18, 2308,
1979

139. Cantley L.C., Cantley L.G. and Josephson L., J. Biol. Chem. 253, 7361, 1978

140. Post R.L. and Kume S., J. Biol. Chem. 248, 6993, 1973

141. Kaniike C., Lindenmeyer E., Wallick E., Lane L. and Schwartz A., J. Biol. Chem. 251, 4794, 1976

142. Matsui M., Hayashi Y., Homareda H. and Kimimura M.,
Biochem. Biophys. Res. Commun. 75, 373, 1977

143. Kalant H. and Rangaraj N., European J. Pharmacol. 70, 157-166, 1981

144. Cantley L.D., Josephson L., Warner R., Yanagisawa M., Lechene C. and Guidolti G., J. Biol. Chem. 252, 7421, 1977

145. Wu P.H. and Phillis J.W., Gen. Pharmacol. 10, 189, 1979

146. Jarnefelt J., Ann. Med. Exp. Fenn. 39, 267, 1961

147. Israel Y., Kalant H. and Laufer I., Biochem. Pharmacol. 14, 1803, 1965

148. Roland C.A., Paxton J., Daviau J.S., van Gelb O., Mlekusch W., Truppe W., Meyer J.A. and Brauer F.S., Life Sci. 36, 1003-1017, 1985

149. Israel Y. and Salazer I., Archs. Biochem. 122, 310, 1967

150. Skou J.C., Physiol. Rev. 45, 596, 1965

151. Goldstein D.B. and Israel Y., Life Sci. 11, 957-963, 1972

152. Israel Y., Kalant H., Leblanc E., Bernstein J.C. and Salazar I., J. Pharm. Exp. Ther. 174 (2), 330-336, 1972

153. Goldstein D.B. and Goldstein A., Biochem. Pharmacol. 8, 48, 1961

154. Goldstein A. and Goldstein D.B., Res. Publ. Ass. Res. Nerv. Ment. Dis. 46, 265, 1968

155. Shuster L., Nature 189, 314, 1961

156. Knox W.H., Perrin R.G. and Sen A.K., J. Neurochem. 19, 2881-2884, 1972

157. Von Ebel H., Wolff J.R. and Gunther T., Z. Klin. Chem. Klin. Biochem. 9, 249, 1971

158. Cotman C.W. and Mattews D.A., Biochim.Biophys. Acta. 249, 380-394, 1971

159. Kates M., Laboratory Techniques in Biochemistry and Molecular Biology, Ed. by Work T.S. and Work E. North Holland Publishing Co., Amsterdam Part II, 3, 351, 1972

160. Duck-Chong C.G., Lipids 14, 492-497, 1974

161. Chen P.S., Toribara T.Y. and Warner H., Anal.Chem. 28, 1756, 1976

162. Lowry O.H., Rosenbrough H.I., Farr A.L. and Randall R.J., J.Biol.Chem. 193, 265-275, 1951

163. Foster T. and Kasper K., Z.Phys.Chem.N.F. 1, 275-277, 1954

164. Foster T. and Seidel P., Z.Phys.Chem.N.F. 48, 58-71, 1965

165. Leonhardt H. and Weiler A., Z.Elektrochem. 67, 791-795, 1963

166. Lee S.L. and Phillis J.W., Can.J.Physiol.Pharmacol. 55, 961-964, 1977

167. Freund J.E. and Walpole R.E., Mathematical Statistics, 3rd edition, Prentice-Hall Inc., New Jersey, 465, 1980

168. Ohyashiki T. and Mohri T., Chem.Pharm.Bull. 26 (10), 3161-3166, 1978

169. Galla H-J. and Sackmann E., Biochim.Biophys.Acta. 339, 103-115, 1974

170. Hitzemann R.J. and Harris R.A., Develop.Brain Res. 14, 113-120, 1984

171. Hitzemann R.J. and Johnson D.A., Neurochem.Res. 8 (2), 121-131, 1983

172. Ohyashiki T. and Mohri T., Biochim.Biophys.Acta. 731, 312-317, 1984

173. Hoffman P.L. and Tabakoff B., Ethanol's Action on Brain Biochemistry. In, Alcohol and the Brain Chronic Effects, Eds. Tarter R.E. and D.H. Van Thiel, Plenum Publishing Corp., New York, 19-67, 1995