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HYPERBARIC REVERSAL OF METHOHEXITAL-INDUCED ALTERATIONS OF MONOAMINE OXIDASE INHIBITION

DISSERTATION

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

By

James Donald Glenn, A.B., M.S.

* * * * *

The Ohio State University

1977

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INTRODUCTION

Literature Review

HISTORICAL CONCEPTS OF THE BIOLOGICAL MEMBRANE

Morphology, A Historical Perspective:

Concepts of the Membrane Before the Electron Microscope: The concept of the cell membrane was born as a functional necessity in the second half of the Nineteenth Century. Some of the early pioneers such as Nagelli and Cramer (1855), Pfeffer (1877), Dvries (1884; 1855) and Hedin (1897; 1898) observed that some substances entered the cell while others could not. They also observed that in hypotonic solutions the cells swell and in hypertonic solutions they shrink. The cell membrane theory was accepted reluctantly, if at all, by these early pioneers simply because it could not be seen (Cereijido and Rotunno, 1970). Overton (1899) pointed out that the cell was more permeable to substances which dissolved easily in lipids than to substances which have poor lipid solubility. From this principle arose the opinion that the cell membrane was a lipid film through which substances must traverse in order to get into the cell. Collander (1949) confirmed the role of lipids in the cell membrane by showing that the penetration of a substance into the cell (expressed as permeability times the square
root of the molecular weight) is roughly proportional to its olive-oil:water partition coefficient.

Gorter and Grendel (1925) extracted the lipids from erythrocytes, spread them as a monomolecular film on a water subphase and observed that the ratio of film area to erythrocyte area from which the lipids were extracted was 2:1. The molecules were presumably packed together in the monomolecular film with the polar, hydrophilic ends all submerged in the water and the nonpolar hydrophobic ends apposed upward in the air (Dowben, 1969). The erythrocytes have sufficient lipid to form two monomolecular layers suggesting a bi-molecular layer of lipids with the hydrophobic ends apposed at the center and the hydrophilic ends facing inward and outward.

Until the early thirties, the cell membrane was generally believed to consist only of lipid. Danielli and Harvey (1935) using mackerel egg oil found that the interfacial tension between oil and water could be lowered by adding factors derived from mackerel eggs to the aqueous phase. Davson and Danielli (1952) postulated the presence of a protein layer attached to the polar head groups on either side of Gorter and Grendel's bi-molecular lipid leaflet, in order to explain the low surface tension of the interface and satisfy the requirements of mechanical strength. Later studies on protein interactions with the lipid bilayer led Davson and Danielli to suggest that some globular proteins lose their native conformation when they come into contact with
the lipid layer. The presence of these layers of "unrolled" protein contribute to the stability and strength of the lipid film comprising the membrane (Dowben, 1969).

**Concepts of the Cell Membrane Based on the Electron Microscope:** Using thin-section electron microscopy, Robertson (1964) was able to observe, in myelin, the protein phospholipid "sandwich" Davson and Danielli has postulated. Robertson applied the term "unit membrane" to structures that appeared in electron micrographs as two dark lines separated by a lighter region. The thickness of the unit measure was 75Å, each dark band being approximately 20Å and the lighter region 35Å (Robertson, 1964). The micrographs gave support to the Danielli model by depicting the electron-dense dark lines as the protein layers and the lighter regions as bi-molecular lipid regions. Green (1970) on the basis of work with mitochondrial membranes argued that not all membranes are of the same function and therefore not all have the same structure. He postulated a discontinuous protein layer made of globular subunits in an α-helical conformation.

Ponder (1948) proposed a model based on studies of the erythrocyte membrane. He suggested that the membrane consists of a discontinuous phospholipid matrix intercalated with proteins. The membrane surface would appear as a complex mosaic structure -- existing in a dynamic state and influenced by exterior as well as intramembranous
interactions. This theory was subsequently restated by Singer and Nicolson (1972) as the Fluid-Lipid Globular Protein Mosaic Model diagrammatically drawn in Figure 1.

On the basis of electron micrographs obtained using the freeze fracture technique, it has become apparent that numerous particles, 80-120Å in diameter, are associated with various faces of the erythrocyte membrane and most other membranes studied (Douglas and Zuckerman, 1976). These intramembranous particles have been reported to exist either in aggregated form or in an even distribution on the inner and outer fracture surfaces of the membrane. Whether the aggregates represent foci for cell-cell interactions or are artifacts of freeze-etching is in question. According to Dempsey, et al. (1973) the intramembranous particles correspond to pinocytotic vesicles. Hackenbrock (1972) suggested they have an important role in oxidative phosphorylation in mitochondria. Tillack and Marchesi (1970) have proposed that these particles lie within the membrane and therefore have a support rather than a receptor function. Pinto da Silva, Douglas and Branton (1971) have related these particles to the ABO blood group antigens on the cell membrane. They also may be related to sites of active transport.
FIGURE 1

THE FLUID MOSAIC MODEL OF THE MEMBRANE

The lipid-globular protein mosaic model with a lipid matrix (the fluid mosaic model): schematic three-dimensional, cross-sectional view. The solid bodies with stippled surfaces represent the globular integral proteins, which at long range are randomly distributed in the phospholipid bilayer. At short range, some may form specific aggregates, as shown. (Singer and Nicolson, 1972)
Membrane Composition:

Much was known about the membrane composition even before electron micrographs were available. The chemical analysis of the plasma membrane has shown that the two major constituents of biological membranes are proteins and lipids, some of which are derivitized by chains of sugar residues. The relative proportion of these two molecular classes vary depending on the source of the membrane (Saier and Stiles, 1975). The recent studies detailed below have not only shown the percentage of chemical constituents in the cell membrane, but also have revealed something about whether they are on the inside or outer side of the cell membrane.

Lipids: Lipid content varies widely from membrane to membrane. It constitutes up to 80 percent of myelin, but only 15 percent of the skeletal muscle membrane. The chemical nature of the lipid also varies. The charges of the polar groups and the length and saturation of the chains have a profound influence on the permeability and other properties of the membranes (Cereijido and Rotunno, 1970).

Glycerophospholipids and sphingolipids comprise the polar lipids of animal cell membranes, whereas the neutral sterols comprise the principal nonpolar membrane lipids. Most phospholipids possess either anionic or zwitterionic polar head groups and have two fatty acids esterified to the glycerol moiety. One of these long chain acids usually possesses one or more cis-alkene double bonds, which confer
upon the membrane a certain degree of fluidity or reduction in membrane viscosity (Seeman and Roth, 1972). Plasma membranes are far richer in cholesterol than the intracytoplasmic membranes (Saier and Stiles, 1975). Approximately 99 percent of the neutral lipid in the cell membrane is cholesterol (Nelson, et al., 1967). Casper and Kirschner (1971) have indicated that cholesterol may be localized more in the outer half of the bilayer than in the inner half. Chapman and Salsbury (1970) reported that the presence of cholesterol within the bilayer tends to reduce membrane fluidity by restricting the random motion of the hydrocarbon chains of the phospholipids.

Lipid Asymmetry: The concept of asymmetry in the membrane refers to a difference in the distribution of a given membrane constituent between the inside and outside of the membrane. There is evidence that the lipid distribution of the cell membrane is asymmetric. For example, lipid asymmetry is not absolute since almost every type of lipid is present on both sides of the membrane bilayer. However, the quantitative distribution is asymmetric (Rothman and Lenard, 1977). Bretscher (1972) recognized that different lipids comprise the two monolayers of the lipid bilayer. Bretscher proposed that the amino-phosphatides, phosphatidylethanolamine and phosphatidylserine are located mainly in the cytoplasmic monolayer of the bilayer and suggested that the choline derivatives, phosphatidylcholine and sphingomyelin, the remaining are found in the external monolayer. Verkleij, et al. (1973) found that 48 percent of the total phospholipid of red cells
could be hydrolyzed without hemolysis. Specifically 76 percent of phosphatidylcholine, 82 percent of sphingomyelin, 20 percent of phosphatidylethanolamine, and none of the phosphatidylserine were degraded by hydrolysis, confirming the external localization of choline phosphatides (Rothman and Lenard, 1977).

The distribution of cholesterol, the major lipid of the red blood cells, has not been established with certainty, although it has been reported that cholesterol is present on both surfaces in large amounts (Fisher, 1976). It has been suggested that this asymmetry, in conjunction with variations in the fatty acid constituents among lipid classes, results in different fluidities for the two monolayers (Bretchser, 1972). Rottem (1975) suggests this is not likely for plasma membranes where the cholesterol levels are high, however, where cholesterol is absent or present in small quantities, e.g., organelle membranes, differences in fluidity may be a real possibility (Rottem, 1975).

**Proteins:** Proteins may constitute up to 80 percent of the erythrocyte cell membrane (RBC) or as little as 18 percent nerve myelin membrane. The protein constituents of a membrane are of particular interest because they confer upon the structure its catalytic activities. On the basis of solubility and other physical factors, membrane proteins have been subdivided by Green (1972) into two distinct groups. Intrinsic proteins or integral proteins include those found within the
membrane continuum; extrinsic proteins or peripheral membrane proteins, which are water soluble, are associated with the intrinsic proteins or lipids of the membrane and are located on the aqueous surfaces of the membrane (Singer and Nicolson, 1972). Intrinsic proteins are oriented in a manner in which approximately one-half of their total surface area is exposed to the hydrocarbon portion of the lipid phase and the other half is exposed to the external aqueous phase. A protein in which one surface is predominantly polar (hydrophilic) and the other surface is nonpolar (hydrophobic) is bimodal in nature. The bimodal or amphoteric properties of intrinsic proteins make possible a correlation between the thermodynamic conditions that influence membrane stability and membrane structure (Saier and Stiles, 1975). Intrinsic proteins are characterized by hydrophilic and hydrophobic regions and can exist in either a dispersed or an aggregated state in the membrane. The quaternary structure of proteins, determined by interaction between the subunits of a given protein will be dependent upon hydrophobic and hydrophilic interactions both at the nonpolar hydrocarbon interface and with the aqueous environment. Most extrinsic proteins are considered to be water soluble and more polar than the intrinsic proteins (Rothman and Lenard, 1977). Two examples are cytochrome C of the inner mitochondrial membranes and acetylcholinesterase. The nature of the interaction of these extrinsic proteins with the membrane is unknown, although electrostatic or hydrogen bond interactions with polar regions of the phospholipids seem likely. Such interactions, involving noncovalent linkage of the extrinsic protein to a macromolecular
complex, may be strong enough to require lipid extractions for the detachment of proteins from the membrane surface (Douglas and Zuckerman, 1976).

**Protein Asymmetry:** Rothman and Lenard (1977) have postulated two types of intrinsic proteins "ectoproteins" and "endoproteins". Ectoproteins have substantial hydrophilic mass which projects beyond the extracytoplasmic surface of the lipid bilayer. Examples of ectoproteins are glycoproteins, histocompatibility antigens (Henning, et al., 1976), glycophorin (Segrest, et al., 1973) and intestinal brush border hydrolases (Henning, et al., 1976). Endoproteins do not project beyond the extracytoplasmic surface of the bilayer, and may have most of their mass associated with the cytoplasmic side of the membrane. Possible endoproteins include cytochrome b₅ reductase associated with the cytoplasmic side of the endoplasmic reticulum (De Pierre and Dallner, 1975) -- Figure 2.

All but two of the major proteins of the erythrocyte membrane are exposed only at the cytoplasmic surface (Steck, 1974). These two proteins, glycophorin and band 3 (Compound A), are exposed at both surfaces. The amino terminal portion of these glycoproteins is outside, their carboxy terminal portion is inside, and the segment that passes through the lipid bilayer is hydrophobic. The major red cell membrane proteins either span the membrane asymmetrically or are exposed only at the cytoplasmic surface. No protein has been found to be symmetrically
Arrangement of hypothetical ectoproteins and endoproteins in a membrane (Rothman and Lenard, 1977). Small black and white balls depict the different hydrophilic portions of the lipid matrix whereas the tails are the hydrophobic portions. Carbohydrates project from both the proteins and phospholipids on the extracytoplasmic side.
FIGURE 2
distributed or to be unexposed on either surface (Steck, 1974). Schneider, et al., (1972) indicated that mitochondrial membranes have an absolute asymmetry in the orientation of proteins in these membranes. Singer (1974) postulates that protein asymmetry is maintained by the exceedingly low rate at which the protein can decay. For a large membrane protein to invert its orientation, many polar and charged groups would be forced to pass through the lipid bilayer. Consistent with this finding, membrane transport proteins do not function by transmembrane rotations (Singer, 1974). Kornberg (1971) has indicated a "flip-flop"-membrane component that had been facing the external surface now faces the interior surface at a half time several orders of magnitude faster than the rate of lateral diffusion of the phospholipid. Isotopic phospholipid asymmetries can be created by replacing radioactive phospholipids of the external monolayer with unlabeled phospholipids, using phospholipid exchange proteins. This has been achieved with artificial phospholipid vesicles and with an influenza virion (Rothman and Lenard, 1977). In neither case could flip-flop be detected; only lower limits processes could be estimated. The lack of detectable flip-flop of phospholipids can be explained, in part, by the high activation energy that would be required to bring the polar group through the hydrocarbon core of the bilayer (Singer and Nicolson, 1972).

Carbohydrates: The observation that secreted proteins are generally glycosylated while cytoplasmic proteins are not, led to the expectation and subsequent discovery that plasma membrane carbohydrates
are associated exclusively with extracellular portions of membrane components (Eylar, 1965). It was also found that all of the sialic acid of red cells could be removed with neuraminidase, showing that most red cell sialic acid is linked to the external amino terminal portion of glycophorin (Whinzler, 1969). Experiments of Singer (1974) who used ferritin derivatives of lectins (plant proteins which bind to specific sugars) to stain the surface carbohydrate of red cells gave supportive evidence for the external localization of membrane oligosaccharides. Their results indicate that the outside surface of plasma membranes and the inner, extracytoplasmic surface of intracellular membranes were stained. Bretscher (1973) also indicated that glycolipids are external, the asymmetry of protein carbohydrate is maintained by the protein asymmetry (Rothman and Lenard, 1977).

INFLUENCE OF ANESTHETICS ON MEMBRANES

The early study of Bangham, et al. (1965) on the effect of anesthetics on model membranes indicated an appreciative change in permeability to cations at (2-10 percent) concentrations of some alcohols. General anesthetics such as ether, chloroform and a range of higher weight alcohols also increased the diffusion rate to cations. The amount of increase seemed to be correlated to the narcotic activity of each agent. In contrast, the same authors found that local anesthetics produced a decrease in the permeability of the phospholipid vesicles. Johnson and Bangham (1969) used the same anesthetics (diethyl
ether, chloroform and butanol) and studied the temperature dependence of the cation permeability increase. They reported that the enthalpy of activation for $K^+$ diffusion was the same in the presence or absence of anesthetics in spite of the increase in permeability.

It was proposed (Johnson and Bangham, 1969; Johnson and Miller, 1970) that disordering or distortion caused by anesthetics could inhibit normal membrane control mechanisms or alter the permeability and function of cellular membranes. The application of pressure could restore their functions and thus reverse the anesthetic state (Lever, et al., 1971). Johnson and Bangham (1969) showed an increase in cation permeability in liposomes using labeled ionophore studies. They proposed that this occurs by increasing the freedom of motion of the lipid molecules, especially at the aqueous-lipid interface. The application of pressure to this system caused a decrease in cation permeability (Johnson, et al., 1973; Johnson and Miller, 1975).

Although Lillie (1923) postulated that general anesthesia resulted from decreased membrane permeability, later investigators found that general anesthetics such as halothane increased mitochondrial permeability to sucrose-tris and chloride, causing an energy independent swelling to occur (Miller and Hunter, 1970).

There is an excellent correlation between anesthetic potency of a wide variety of inhalation anesthetics and their oil: gas partition coefficients (Eger, et al., 1969). Miller (1974a) stated that although
it seems clear that anesthetics act at some nonpolar site, there is
some question as to whether that site is protein-like or lipid-like.
He further suggested that since chemically inert substances such as argon
and xenon are known to exhibit anesthetic properties, and therefore
is the result of a physical rather than a chemical interaction.
Depending on the intermolecular forces of the anesthetic, xenon and
argon can be predicted to be anesthetics, but helium, because of its
extremely weak forces, can be predicted to be a non-anesthetic.

Seeman and Roth (1972) observed membrane expansion using the
erthrocyte as a model. They exposed human erythrocytes to clinical
concentrations of halothane, chloroform, ether and methoxyflurane. The
cells were protected from osmotic lysis by a factor of eight percent
when exposed to one minimum alveolar concentration (MAC) of the
anesthetic agents. This eight percent reduction in hemolysis was
accounted for by a 0.4 percent expansion in surface area.

Boggs, et al., (1976) showed that concentrations of anesthetic
which are sufficient to cause general anesthesia or which block the
conduction of impulses along a nerve in the case of a local anesthetic
("Local Anesthetic") have little or no effect on the fluidity of
artificial membranes. This led Richards (1977) to state that: "Although
such observations cast doubt on the view that anesthetics impair
membrane function through a generalized perturbation of the structure
of the lipid membrane they are consistent with the view that the
interface between lipid and protein may be the site of anesthetic action."
Some membrane-bound proteins require the presence of a layer of tightly bound lipid to stabilize them in their lattice form. This has been shown for cytochrome oxidase (Jost, et al., 1973) and for the calcium transport protein of the sarcoplasmic reticulum (Warren, et al., 1975). By studying the effects of temperature on the calcium transport protein reconstituted in a defined lipid mixture, Warren, et al. (1974) showed that the enzymatic activity of the complex decreased as the lipid became more crystalline. This may indicate that the activity of this protein is governed by the fluidity of the lipid in which it is embedded. Lee (1976) proposed that the sodium channel in nerve membranes may be surrounded by a ring of rigid molecules that serves to keep it open. He suggested that anesthetics block nerve impulse conduction by fluidizing this boundary lipid thus causing the sodium channel to collapse inwards. One consequence of this model is that a nerve blocked by an anesthetic should have its conduction restored when the temperature is lowered, thus "ordering" the lipid and restoring the channel. This has indeed been shown for the action of ethanol on the squid axon (Spyropoulos, 1957).

THE USE OF MEMBRANE MARKERS TO STUDY MEMBRANE PROPERTIES

Many investigative efforts have used enzymes as membrane markers. The validity of such studies is dependent on the specificity of the enzymatic marker for a particular local in the cell.
There are many enzymes available that can be used as membrane markers. In the mitochondria alone, differentiation between the outer membrane, intermembrane space, inner membrane and matrix can be accomplished by using specifically located enzymes. "Rotenone-insensitive" NADH-cytochrome C reductase, monoamine oxidase, kynurenine hydroxylase and at least ten more known enzymes are located in the outer mitochondrial membrane. Adenylate kinase, nucleoside diphosphokinase, xylitol dehydrogenase are located in the intermembrane space while cytochromes b, c₁, c, a, a₃ and a variety of other enzymes are potential markers for the inner membrane, whereas malate dehydrogenase, isocitrate dehydrogenase and some 13 plus enzymes are in the matrix alone (Tedeschi, 1976). Monoamine oxidase has been shown to be a reliable marker for mitochondria (DeChamplain, et al., 1969), especially in isolated outer membrane preparations (Schnaitman, et al., 1967; Schnaitman and Greenawalt, 1968; Greenwalt and Schnaitman, 1970).

INFLUENCE OF ANESTHETICS ON MAO

Schneider and Gardier (1969) subjected rat liver outer mitochondrial membrane monoamine oxidase to anesthetics in vitro and indicated a low order of inhibition for cyclopropane and diethyl ether, but a high order of inhibition for methoxyflurane, halothane and chloroform. However, in crude homogenate preparations (Schneider, et al., 1974), cardiac monoamine oxidase activity (MAO) was significantly increased both in cats ventilated with halothane, methoxyflurane, and
cyclopropane and in isolated guinea pig hearts perfused with a halothane-containing solution. These authors believed that the direct interaction between the anesthetic agents and the MAO enzyme was one of inhibition, which was exemplified in their isolated outer mitochondrial preparation. They explained the increased monoamine oxidase activity in the crude homogenate preparation may be due to the increased accessibility of substrate to the enzyme in the presence of possible anesthetic-induced alterations of membrane permeability. Izumi, et al., 1969; 1976) and Youdim and Sandler (1968) suggested a similar mechanism to explain increases in MAO activity with reserpine as did Yamamoto and co-workers (1970) with a series of imidazole carboxamides.

Muldoon, et al., (1975) reported that halothane did not inhibit the development of isometric tension of isolated cutaneous canine vein preparations to exogenous catecholamines. Halothane produced a decrease in response to electrical nerve stimulation and an increase in response to tyramine, a releaser of intra-neuronal norepinephrine. These authors suggested that halothane acts on the nerve terminal to inhibit the release of norepinephrine, possibly by reducing the influx of calcium ions which are necessary for nerve-stimulated release of norepinephrine but not for tyramine-stimulated release. Alternatively, they suggested that halothane may inhibit intra-neuronal monoamine oxidase, which would account for the increase in response to tyramine and the lack of change in response to exogenous norepinephrine. This
prophecy was supported by their observation that halothane did not increase the response to tyramine when monoamine oxidase had already been blocked by pargyline.

Costa and Greengard (1972a) showed evidence to support the idea that there are multiple forms of monoamine oxidase. Various forms had different substrate affinities and were inhibited to various degrees by some drugs. Two of the major forms of monoamine oxidase are classified as type "A" and type "B". Type "A" monoamine oxidase has a preferential substrate serotonin, whereas benzylamine is the preferred substrate of type "B" monoamine oxidase (MAO). They also stated that monoamine oxidase(s) is probably synthesized in cytoplasmic ribosomes and not by the mitochondria. The mitochondrion can be used as a "model" membrane to study drug-membrane interactions (Sordahl, et al., 1971). The use of the mitochondrion as an anesthetic receptor model, rather than the oil/gas or vapor pressure models, has been suggested (Nahrwold, et al., 1974). It is known that general anesthetics such as halothane inhibit ADP-stimulated respiration of liver and brain mitochondria (Rosenberg and Haugaard, 1973). Halothane is an inhibitor of oxidative phosphorylation and not normally an uncoupling agent. It was also shown that the depressant effect of nitrous oxide in combination with halothane on mitochondrial state 3 glutamate oxidation is additive (Nahrwold and Cohen, 1973). However, some investigators believe that depression of respiration does not predict in vivo anesthetic activity (Nahrwold, et al., 1974).
THEORIES OF GENERAL ANESTHESIA

Anesthetics affect every variety of living system, from microorganisms to mammals. They are nonspecific drugs which depress a multitude of cell functions such as cell motility, cell division, photosynthesis, oxidative metabolism and electrical excitability (Richards, 1976). Although the state of general anesthesia has been recognized for well over a century, the fundamental mechanism involved in its production is still unknown (Douglas and Zuckerman, 1976).

General and Chemical Theories:

Claude Bernard (1875) proposed a colloid theory to explain production of anesthesia. He proposed that a reversible aggregation of cell colloids causes or accompanies anesthesia. Meyer (1899) and Overton (1901) developed a Lipid Theory and anesthesia. They observed a direct parallel between affinity of an anesthetic for lipid and its depressant action. Supportive work of Collander (1949) further extended the number of anesthetics studied by Meyer and Overton. Traube (1904) and Lillie (1909) related the potency of anesthetics to their ability to lower surface tension. Clements and Wilson (1962) have extended the theory of surface tension or adsorption by demonstrating that nitrous oxide, cyclopropane and chloroform lower the surface tension at a fat-water interface. They indicate the adsorption
of anesthetic agents may change the effective dielectric constant and permeability and may also alter critical structure relationships between those enzymes supporting oxidative phosphorylation and electron transport. Yamaguchi and Okumura (1963) have shown that ether, chloroform and urethan partially prevent the decrease in membrane resistance that normally occurs during excitation.

Allison and Nunn (1968) ascribed production of narcosis to depolymerization of microtubule structures that normally give rigidity to cytoplasm, which may indeed be the reversible aggregation of cell colloids in Claude Bernard's Colloid Theory (1875). Schoenborn's (1967) work depicting that anesthetics may interact with proteins has cast doubt on the Lipid Theory of Meyer (1899) and Overton (1901) as being the explanation of anesthesia. The surface tension or adsorption theory of Traube (1904) and Lillie (1909) relating the potency of anesthetics to their ability to lower surface tension as well as Winterstein's (1926) proposal that anesthetics cause a change in permeability of the cells of the central nervous system, all have interesting supportive evidence. These descriptions of alterations in surface tension or cell membrane permeability provide valuable insights, however, the relationship between these changes and anesthesia and the mechanism by which these changes are produced remain questions to be answered (Cohen, 1975).
The neurophysiological theories initially reported by Larabee and Posternak (1952) showing decreased synaptic transmission in the superior cervical ganglia caused by anesthetics have given important information regarding effects on neuronal function by anesthetics, but does not propose a fundamental mechanism by which these are produced (Cohen, 1975).

Biochemical Theories:

Because functional energy of the central nervous system (CNS) is derived mainly from metabolism of carbohydrate, oxygen consumption has been measured and correlated with the ability of drugs to produce anesthesia. Quastel (1952) has demonstrated the in vitro inhibition of oxygen uptake by the brain after exposure to barbiturates, chloralhydrate or urethan. Smith and Wollman (1972) also showed decrease in oxygen consumption in man with halothane. Biochemical theories based on overall oxygen consumption by anesthetics are not satisfactory because there is poor correlation between the ability of various compounds to depress oxygen consumption of the brain and their anesthetic potency (Goth, 1974). Eventhough the reports on cellular uptake of glucose and glucose metabolism are not congruent, Greene and Cervenko (1967) proposed the variation may involve the concentration of the anesthetics used. They found that low anesthetic concentrations increase and high concentrations decrease glucose entry into erythrocytes.
Decrease in synaptic transmission is the most rational explanation for finding an increase in brain acetylcholine content during anesthesia (Eiler and McEwen, 1949). Larabee and Holaday (1952) found a decreased release and increased brain content of acetylcholine occurring with barbiturate, ether and chloralose anesthesia. The difficulty in accepting this theory is related to cause and effect since it can be equally argued that direct depression of cell bodies is responsible for the reduced release of acetylcholine. Therefore the biochemical theories may only explain events that accompany anesthesia rather than cause it. A decreased CNS activity that occurs during anesthesia may, in turn, result in diminished need for oxygen, since cellular energy stores remain normal during anesthesia (Nilsson and Siesjo, 1970).

**Physico-Chemical Theories:**

Four observations suggest that anesthesia is the result of some physical or physico-chemical interaction of the anesthetic with certain constituents of the cell. 1) The facility of general anesthetic usage resides in the ability to control this state because the drug effects are freely and rapidly reversible (Goth, 1974); 2) Collander (1949) indicated that a potency of an anesthetic is directly related to its lipid solubility and easy tissue diffusibility; 3) Anesthetic properties are shown by a wide variety of chemically unrelated substances. Cullen and Gross (1951) even showed that the chemically inert gas, xenon, was an anesthetic agent; 4) Anesthesia can be reversed
by physical means, i.e., high pressures. An early reporting of this phenomenon was that inhibition of bacterial luminescence by alcohol and urethan could be reversed by a high hydrostatic pressure applied to the system (Johnson, et al., 1942a; 1942b).

The physico-chemical theories of general anesthesia can be generally classified into two groups, those that involve events taking place in the aqueous regions of tissue and those in the lipid regions.

**Theories of Aqueous Interactions:** Miller (1961) and Pauling (1961; 1964) working independently proposed a theory that some hydrate microcrystals appearing in the aqueous layers of nervous tissue were responsible for general anesthesia. Contrawise, there is no correlation between the solubility of gases in water and their anesthetic potency (Miller, et al., 1965). The initial phase for anesthetic action appears to be not the aqueous but rather the non-aqueous region of the tissue (Miller, et al., 1965; Miller, et al., 1967; Eger, et al., 1969).

**Theories of Lipid Interactions:** Meyer (1899) and Overton (1901) proposed the fat solubility hypothesis, based on the correlation between anesthetic potency and anesthetic solubility in olive oil or other lipids. Meyer (1937) suggested that anesthesia is induced when the anesthetic substance has attained a certain molar concentration in the lipids of the cell. He postulated that anesthetic potency
depends not only on the solubility of the chemical in the lipid phase, but also on the molecular dimensions of the drug. This theory is dependent on a critical fraction of anesthetic being localized in the membranes of the cell. This critical amount or volume is held to be dependent on the species under study but independent of the nature of the anesthetic.

More recent attempts to explain anesthesia on the basis of lipid interactions have led to the critical volume hypothesis, as it is presently named (Miller, et al., 1973) which proposes that anesthesia occurs when the volume of the hydrophobic region is expanded beyond a definite critical volume by the absorption of inert molecules. This expansion of a biological membrane causes an increase in its fluidity, which subsequently may cause some permeability change to result in interference with normal physiological processes. Thus, the state of general anesthesia is achieved (Miller, et al., 1973). The melting transition of the lipids are within normal physiologic temperature ranges, suggesting that membrane lipids are capable of existing in a fluid phase (Douglas and Zuckerman, 1976) and lends support to the critical volume-membrane fluidity model of Miller, et al. (1973). From the amount of pressure required to reverse the effects of various doses of anesthetic, Miller calculated that during general anesthesia the hydrophobic region should expand by about 0.4 percent (v/v) (Lever, et al., 1971; Miller, et al., 1973).
By means of the technique of critical hemolytic volumes, it is possible to calculate the percent membrane expansion for different amounts of antihemolysis (Seeman, et al., 1969a; 1969b). A drug that will allow a cell to take in an amount of fluid greater than would normally lyse the cell, is defined as having antihemolytic properties. Anesthetic concentrations which cause 50 percent antihemolysis are virtually identical to those required for conduction block of peripheral nerve fibers. Approximately a three percent membrane expansion occurs at drug concentrations that cause 50 percent antihemolysis. A membrane expansion of three percent, therefore, could be expected to occur in nerve cells in the presence of conduction-blocking concentrations of local anesthetics (Seeman and Roth, 1972).

Seeman (1973) has proposed several mechanisms by which anesthetics could expand membranes.

First, the anesthetic molecules could dissolve in the membrane and cause membrane expansion by virtue of their bulk volume. Sears and Fuller (1968) have shown swelling of olive oil and hexane by anesthetic gases. Also monolayers of lipid, protein or lipo-protein spread on a Langmuir trough can be penetrated and expanded by drugs added to a subadjacent bulk phase (Clements and Wilson, 1963; Sears and Brandes, 1969). Anesthesia induced expansion is about three or four times greater than can be accounted for by the bulk volume of the absorbed anesthetic molecules (Seeman, 1973).
Second, anesthetics might only adsorb to the membrane (without necessarily entering or dissolving in the membrane), thereby altering the amount of water exposed to the membrane, and consequently decreasing the interfacial tension at the membrane-water interface. One resulting effect would be expansion of the membrane. This is the "adsorption-extension" hypothesis of Schneider (1968). Schneider's studies on entropy adsorption of the alcohols to the erythrocyte membrane indicate that the anesthetics lodge in the nonpolar portions of lipid molecules, the nonpolar interfaces between lipid and protein molecules or the hydrophobic regions of protein molecules.

Third, the anesthetic could displace some membrane-associated component which normally might keep the membrane in a condensed state. Calcium ion (Ca\(^{2+}\)) condenses lipid monolayers spread on a Langmuir trough (Shah and Schulman, 1967; Hauser and Dawson, 1968). Chlorpromazine, a potent local anesthetic, displaces membrane bound Ca\(^{2+}\) and could, therefore, readily expand the membrane in this way (Kwant and Seeman, 1969).

Fourth, the membrane might enlarge as a result of distortion or expansion of the membrane proteins. Balasubramanian and Wethauer (1966) showed that anesthetics can produce denaturation of proteins. Johnson and Flagler (1951), published results dealing with tadpoles and salamanders immobilized in three percent ethanol. When exposed to 300 p.s.i. of hydrostatic pressure their anesthesia was reversed. Similarly, Eyring (1966) showed congruent results with bacterial
luminescence. Eyring expressed the view that an active state of protein has a certain optimal volume for activity. If the volume of this protein is made greater or smaller than its optimal volume then the activity of the protein is inhibited. His results are in good agreement with those of Miller, et al. (1973).

Fifth, the anesthetic might increase membrane hydration. Seeman, et al. (1970) showed that erythrocyte permeability to water (hydraulic permeability coefficient) is increased by chlorpromazine and ethanol. Membrane expansion may then result from a small increase in membrane water.

Sixth, the membrane might become thinner and extended by anesthetics because of reduction in membrane viscosity or "fluidize" the membrane (Metcalfes and Burgen, 1968; Metcalfe, et al., 1968).

All of these postulates ignore the possibility that membrane associated enzymes do play a role in the development of membrane anesthesia. Anesthetics such as chlorpromazine or procaine can abolish action-potentials in excitable lipid bilayers where no enzymes exist (Mueller and Rudin, 1967). Metcalfe, et al. (1968) discovered that benzyl alcohol, which can act as an anesthetic, increased the fluidity of biological membranes reflected by relaxation of membrane proteins. Trudell, et al. (1973a) gave further supportive evidence by including clinically important anesthetics, such as halothane. It was subsequently proposed that anesthetics cause membrane expansion by
increasing the disorder of the fatty acid chains of the phospholipids of the membrane bilayer. During anesthesia, general anesthetics occupy only 0.2 percent (v/v) of the membrane whereas membrane expansion is of the order of 0.4 percent (Metcalfe, et al., 1968). Seeman (1973) has shown, however, that the artificial phospholipid bilayers only expanded by 0.2 percent and so he proposed that changes in the conformation of the membrane proteins are the cause of the large expansion of natural membranes.

_Influence of Hyperbaric Conditions on Anesthesia:_

**Biological Experiments:** Hydrostatic pressure between 100 and 200 atmospheres restores not only spontaneous activity in narcotized unicellular organisms and nerve preparations but also the righting reflex in anesthetized animals (Lever, et al., 1971). Johnson and Flagler (1950) reported that tadpoles of _Rana Olyvatica_, which were anesthetized in 3 to 6 percent alcohol at 22°C to 26°C and were lacking in both spontaneous activity and response to gentle mechanical stimulation, could be "awakened" while still in the alcohol solution by the application of hydrostatic pressures between 2000 and 5000 pounds (p.s.i.), or 130 and 300 atmospheres. Unanesthetized tadpoles used as controls appeared more active at pressures under 2000 p.s.i. but less active as pressure increased, and finally were motionless at 5000 p.s.i. Johnson and Eyring (1948) postulated that anesthetics cause a reversible
protein denaturation. This increased volume of the protein was proposed to be reversed by pressure.

Similar experiments were conducted with sulfur hexafluoride (SF₆), nitrous oxide, pentobarbital sodium, ether and halothane in Italian crested newts (Triturus cristatus carnifex) and mice (Lever, et al., 1971). Hydrostatic pressure for the newts and helium pressure for the mice reversed the previous anesthetic states. These researchers proposed that anesthetic potency is proportional to the product of the solubility of the anesthetic in lipid portions of the cell membranes and its partial molar volume. They believed that the change in the lipid portion of the membrane could be transmitted to a vital protein and reduce its function. The end result would be the state of general anesthesia. An expansion of the lipid in this instance would be approximately 0.4 percent. The pressure to reverse this anesthesia was found to be approximately 100 atmospheres which was in turn calculated to change the volume of the membrane about 0.4 percent.

Thermodynamic analysis showed that the inhibitory mechanism of anesthetics on firefly extract is identical to the reversible thermal inactivation of the luciferase enzyme. Thus, the originally folded luciferase becomes unfolded by the expansion caused by the anesthetic agent at the enzymes' hydrophobic sites (Ueda and Kamaya, 1973).
**Bilayer Experiments:** Experiments on spin-labeled phosphatidylcholine bilayers demonstrated that inhalation general anesthetic agents in clinical concentrations increased the internal motion within these bilayers (Trudell, *et al.*, 1973a). Subsequently the application of 100 atmospheres of pressure to the system decreased the internal motion in the bilayers (Trudell, *et al.*, 1973b). Further, this reversal was shown to have occurred from a re-ordering of fatty acid chains around the anesthetic molecule instead of from an exclusion of the anesthetic agent from the bilayer itself (Trudell, *et al.*, 1973c). This finding answered the question raised by Mullins (1973) concerning the possibility that pressure may merely squeeze the anesthetic molecules from the lipid phase of the membrane to explain pressure reversal of anesthesia.

From studies on mixed di-palmitoyl-dimyristoyl-phosphatidylcholine bilayers, Trudell, *et al.* (1975) proposed that anesthetics may act within nerve membranes by altering neuronal solvated proteins necessary for nervous function. They also indicated that pressure is not a perfect antagonist of anesthetic-induced changes in the bilayer system. This evidence may help to account for the fact that pressure alone has been known to cause animals to lose control of some functions and to convulse (Regnard, 1891; Catell, 1936; Lever, *et al.*, 1971).
**Site of Anesthetic Action:**

A controversy has arisen concerning the anatomical location of the anesthetic-induced blockage of the neuron. Anesthetics have been shown to block conduction in electrically excitable axonal membrane, depress postsynaptic electrogenesis at chemical synapses (Thesleff, 1956; Bloom, *et al.*, 1965) and also alter release of transmitter from the presynaptic terminal (Matthews and Quilliam, 1964). More recent experiments using humans anesthetized with thiopental, nitrous oxide-oxygen and halothane showed no significant change in ulnar nerve conduction velocity before and during the anesthesia (Thornton, *et al.*, 1968). Synaptic transmission has been considered a more probable site for anesthesia than conduction on the basis of a greater sensitivity of this site to anesthetic gases (Larabee and Posternak, 1952). One hypothesis states that anesthetic actions inactivate lipoproteins essential for synaptic transmission (Eyring, *et al.*, 1973). Another hypothesis, however, suggests that the reversible anesthetic-induced blockage of the action potential in an axon is due to the presence of foreign molecules (anesthetic) which sterically impede the rearrangement of groups near the surface of the lipid, producing a sudden increase in $K^+$ permeability (Johnson and Bangham, 1969). Results of monosynaptically derived neural output patterns in snails have supported the theories which propose depression of overall neural activity during anesthesia (Parmentier and Wilson, 1974). However, only partial blockage of conduction is overcome by pressure and not the depression of anesthetics
on excitatory synaptic transmission in rat superior cervical ganglion (Kendig, 1975). He suggested that high pressure has antagonized the effect of the anesthetic involved in the production of the anesthetic state, but failed to antagonize effects unrelated to anesthesia.

STATEMENT OF THE PROBLEM

Membrane expansion brought about by the action of anesthetic agents has been proposed, as a possible mechanism of action of these drugs. Part of the proof for this action has been the demonstration of the reversal of the anesthetic effect in lower animals by pressures varying between 70 and 100 atmospheres. If alteration of membrane geometry is responsible for the anesthetic effect, and anesthesia is a universal membrane phenomena, then the drug action should be demonstrable at cellular and subcellular levels. In order to do this, an evaluation of anesthetic or hypnotic drug effects on the activity of a membrane dependent enzyme was considered to be a valid means of identifying a change in membrane function resulting from a reversible modification in structure. The enzyme chosen was monoamine oxidase, because it is an outer mitochondrial membrane-bound enzyme which can be studied in biological forms ranging from isolated mitochondrial membrane fragments to intact viable cells either freely moving or confined in a tissue matrix. The various cell types likewise added a further dimension in terms of membrane types to be investigated. Therefore the experiments, herein reported, concern the
effects of an ultrashort-acting barbiturate on monoamine oxidase activity in guinea pig (a) fragmented and (b) intact brain mitochondria (c) cerebral cortical slices and (d) peritoneal exudate cells. The early data are confined to dose-responses of this agent per se and as these might be influenced by hyperbaric conditions. Subsequently, the inhibition of monoamine oxidase activity is compared quantitatively and qualitatively with two new investigational compounds that selectively act on separate forms of the enzyme. This comparison was felt to be important in defining the site of barbiturate action.

The final experiments were directed specifically toward the question of the ability of methohexital to alter membrane permeability. This was approached by determining its effect, if any, on the early dynamics of monoamine oxidase inhibition by the new irreversible inhibitors as this might be reversed by hyperbaric conditions.

If general anesthetics increase the fluidity of the membrane to influence the diffusion of either the substrate and/or the inhibitors, then product formation will be altered. Conversely, if high pressure decreases membrane fluidity and restores permeability in spite of the presence of the anesthetic agent, the alteration of product formation so induced under normobaric conditions should be overcome.
The studies briefly described above will be presented in more depth in the succeeding sections on "Methods and Materials" and "Results". The data were analyzed statistically and are discussed as possible confirmation of the membrane expansion theory as the mechanism for anesthesia.
METHODS AND MATERIALS

INTACT BRAIN MITOCHONDRIAL PREPARATION

During preliminary experiments, whole brain mitochondria were prepared by the methods of Ozawa, et al. (1966) or Barberis and McIlwain (1976). Electron microscopy studies revealed that the latter method yielded mitochondria with less contamination by other subcellular organelles. The mitochondria prepared by the technique of Ozawa, et al. had higher respiratory quotients which was taken as an indication of intact membranes and therefore this method was selected for the intact and lysed mitochondrial experiments. The mitochondrial isolation procedure is diagramed in Figure 3.

Male guinea pigs weighing 500 to 700 grams were sacrificed by decapitation in all experiments. Their skulls were opened sagitally with sharp clean scissors, the brains removed from the calvarium and placed in a beaker containing chilled (4°C) medium (0.3M mannitol, 0.1mM EDTA, and 0.1 percent bovine albumin) which had been adjusted to a pH of 7.4 with KOH. The cerebellum and the brain stem were removed and the remaining cortex was weighed. The cerebral hemispheres were placed in medium ten times their weight and minced with scissors. Using a Dounce glass homogenizer with a teflon pestle, the tissue was homogenized with four gentle strokes. The suspension was centrifuged

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FIGURE 3

Flow diagram of mitochondrial isolation procedure based according to the technique of Ozawa, et al. (1966).
BRAIN HOMOGENATE
(9.0 cc. MANNITOL-MEDIUM PER GRAM OF TISSUE)

\[ \rightarrow \text{600 X g for 8 min.} \]

\[ \text{PELLET} \quad \text{SUPERNATANT} \]
\[ \text{(RBC'S, CELL WALLS, \textbf{N}UCLEI)} \quad \text{\textit{MITOCHONDRIA, MYELIN, FILAMENTS, SYNAPTOSOMES, MICROSOMES)}} \]

\[ \rightarrow \text{10,000 X g for 10 min.} \]

\[ \text{PELLET} \quad \text{SUPERNATANT} \]
\[ \text{\textit{MITOCHONDRIA, MYELIN, SYNAPTOSOMES}} \quad \text{\textit{MICROSOMES, SOME MYELIN}} \]

\[ \rightarrow \text{5,000 X g for 10 min.} \]

\[ \text{(F1) PELLET} \quad \text{SUPERNATANT} \]
\[ \text{\textit{MITOCHONDRIA, SYNAPTOSOMES}} \quad \text{\textit{MYELIN, SYNAPTOSOMES, SOME MITOCHONDRIA}} \]

\[ \text{REMOVE WHITE SEDIMENT BY S\textsc{w}IRLING, THEN COMBINE WITH SUPERNATANT} \]

\[ \rightarrow \text{5,000 X g for 10 min.} \]

\[ \text{(F2) PELLET} \quad \text{SUPERNATANT} \]
\[ \text{\textit{CONTAMINANTS}} \]

**Pellets F1 and F2 are combined, then resuspended in 1.0 ml of mannitol buffer medium.**

**FIGURE 3**
at 600 X g for eight minutes and the pellet was resuspended in 40ml of the same medium followed by another centrifugation at 5000 X g for ten minutes. After decanting the supernatant solution, the loosely packed white pellet was removed by shaking several times with approximately 0.3ml of the above medium. The remaining sediment was composed of mitochondria in a tightly packed brown pellet (F1). The F1 pellet was used after it was resuspended in 0.5ml of medium. The decanted supernatant solution and the washing solution from the F1 pellet were combined, mixed well and centrifuged at 5000 X g for ten minutes. The supernatant was removed and the pellet of tightly packed mitochondria (F2) was resuspended in the medium. The F1 and F2 pellets were then combined and the protein content was assayed by the Biuret method, similar to that of Jacobs, et al. (1956).

**Protein Determination:**

The Biuret method of protein determination was carried out as follows: A 0.1ml aliquot of the resuspended whole mitochondrial pellet (F1 and F2) was added to each of three tubes. A like volume of buffer medium was added to each of two control blanks. To each tube was added 0.2ml of 20 percent Triton X-100 solution, followed by 1.2ml of water and 1.5ml of Biuret reagent (Jacobs, et al., 1956). All tubes were placed in boiling water for 20 seconds, then cooled in an ice water bath for one minute, and finally allowed to equilibrate at room temperature.
for ten minutes. Samples were read in a spectrophotometer at 540\text{nm}
and the protein content was calculated as follows:

$$\text{mg protein/ml} = \frac{\text{optical density}}{0.095} \times \frac{10}{1}$$

\textbf{Lysis of Mitochondrial Preparation:}

The concentration of whole mitochondria was adjusted to 1mg protein
per ml and sonicated for 30 seconds with a Bronwill Biosonic IV with
low probe followed by a 30 second cooling in an ice water bath. The
variable power control knob was set at 55 percent. On the basis of
respiratory studies and electron microscopy, described below, two
minutes of sonication were adequate.

\textbf{PREPARATION OF SYNAPTOSOMES}

Whole intact synaptosomes were prepared by the method of Barberis
and McIlwain (1976). The neocortex of the guinea pig was removed from
the animal within one minute after death. The cerebral hemispheres
were opened and the subcortical white matter removed, according to the
technique of Kuroda and McIlwain (1974). The cortex was dispersed using
a glass homogenizer with a Teflon pestle containing sufficient 0.32M
sucrose solution to make a ten percent w/v homogenate. The homogenate
fractions were washed and centrifuged twice at 1000 \times g for ten minutes
to remove the nuclear fraction. A second centrifugation of 11000 \times g
for 20 minutes was carried out with a Sorvall RC-2B centrifuge. The combined mitochondrial and synaptosomal fraction was resuspended gently in 0.32M sucrose (3ml/gm of original tissue) and placed in cellulose tubes above a sucrose gradient consisting of 15ml of 0.8M and 15ml of 1.2M sucrose (Gray and Whittaker, 1962). The gradients were set up one hour before use and kept in a cold room at 5°C. These tubes were centrifuged in a Beckman ultracentrifuge at 105000 X g for 45 minutes. The synaptosomal fractions between 0.8M and 1.2M sucrose were collected using Pasteur pipettes, gently stirred and gradually diluted 2.2 times with ice cold distilled water to make a sucrose concentration of 0.45M. This suspension was centrifuged at 17000 X g for 20 minutes. The supernatant fluid was carefully removed and the precipitate was suspended gently in sucrose-tris buffer at 0.25M, then assayed for protein content by the Biuret method.

PREPARATION OF BRAIN AND LIVER SLICE

The liver was excised from the sacrificed guinea pig used for the brain and immediately placed in ice cold 0.25M sucrose, 0.01M tris-HCl buffer. The peripheral edges of the small lobes were placed on a Sorvall TC-2 "Smith and Farquhar" tissue sectioner with the following settings: stroke control -- normal, tension control -- #5, section thickness at 225 microns, and speed control at 1. The sections were selected and weighed, then placed in the reaction tube with 100µl of the sucrose-Tris buffer.
The brain was removed from the calvarium and placed in the buffer used for isolated mitochondria. The cerebellum and brain stem were removed and the cerebral hemispheres then separated. A sagital cut was made in each hemisphere 1mm from the most rostral edge. These strips were placed on the sectioner and cut transversely. The same procedures were then followed as in the liver slices.

PERITONEAL EXUDATE CELLS

Obtaining the Cells:

The method of inducing peritoneal exudate cells (PEC) was that of Remold, et al. (1970). Male guinea pigs weighing between 500 to 700g were injected intraperitoneally with 30ml of mineral oil (Squibb light mineral oil). After five days they were sacrificed by decapitation and exsanguinated. The peritoneum was washed with 200ml of cold Hanks balanced salt solution (HBSS). The cell and oil suspension was washed and centrifuged twice at 500 X g for ten minutes, resuspended in 10ml of medium and finally counted using a hemocytometer. The viability of the preparation was determined by the trypan blue exclusion dye test (Pennant, 1964). Viability of the PEC averaged greater than 92 percent and was never lower than 87 percent.

After the PEC were adjusted in concentration they were washed once more and resuspended to the same volume in minimal essential medium containing 100 units/ml of penicillin, 100μg/ml of streptomycin (MEM-PS)
and 15 percent fresh guinea pig serum (GPS). The cells were drawn into 0.5mm capillary tubes and sealed at one end with clay (Seal Ease) from Clay Adams Co. followed by a centrifugation at 280 X g for ten minutes (Hughes, 1972). The capillary tubes were cut at the cell fluid interface and then mounted in Sykes-Moore chambers. After the chamber was sealed, it was filled with the MEM-GPS by inserting a 25 gauge needle through the silicon rubber ring via a side hole, used as the air vent. On the opposite side, the chamber was filled with MEM-PS-GPS using a 1ml tuberculin syringe containing MEM-PS-GPS along with the drugs used.

**Measurement of Cell Migration:**

The chambers were kept at 37°C for 20 to 24 hours. Quantitation of the degree of migration was made by measuring the area of the fan or mushroom made by the macrophages migrating from the capillary tube onto the bottom glass coverslip.

The migration area was traced on onion skin paper by projecting the image of the migration with a Nikon Comparator. Using a dark field, the leading edge of migrating macrophages was clearly seen. The tracing of the migration area was quantitated by cutting out and weighing the paper.
ELECTRON MICROSCOPY STUDIES

Brain mitochondria, lysed brain mitochondria, synaptosomes, as well as peritoneal exudate cells were subjected to fixation and embedding procedures developed by Clausen (1972). The appropriate medium was replaced with two percent glutaraldehyde for 30 minutes. The pellet was then rinsed in phosphate buffer and postfixed for 45 minutes in osmium tetroxide. Dehydration was carried out in 15 minute steps in consecutive ethanol concentrations of 50, 70, 95 and 100 percent. The pellet then was treated for 15 minutes in propylene oxide, followed by 45 minutes in an equal mixture of propylene oxide and Spurr low viscosity imbedding medium at 45°C, and finally for 90 minutes in Spurr medium alone at 37°C. Polymerization was allowed to take place for a minimum of eight hours in an oven at 70°C. The "blocks" were sectioned with a Dupont diamond knife in a Sorvall Portor-Blum MT-1 Microtome at 60 to 90 micron thickness. These sections were picked up on 300 mesh copper grid, stained with uranyl acetate and lead citrate, and viewed through a Phillips 300 electron microscope.

Migrating macrophages were fixed for one hour at room temperature in one percent paraformaldehyde, 1.25 percent glutaraldehyde, and 0.025 percent CaCl in 0.1M cacodylate buffer, pH 7.4. Cultures migrating from capillary tubes were fixed in situ on the glass coverslips to which they were attached. After fixation specimens were washed twice in cold 0.1M cacodylate buffer, pH 7.4, then postfixed for two hours in cold two percent collidine-buffered osmium tetroxide.
Subsequently dehydration was performed in a graded series of alcohols, and embedded in a propylene oxide-Spurr sequence. These migrating macrophages on glass coverslips were embedded *in situ* by inverting Spurr-filled BEEM capsules over the area of migration. Glass coverslips were removed after polymerization by successive application of liquid nitrogen to the glass surface. The blocks were sectioned, stained and viewed under the electron microscope as above (Dvorak, *et al.*, 1972).

**MONOAMINE OXIDASE ASSAY**

The determination of the monoamine oxidase activity in the various biological preparations was done according to the method of Nagatsu (1973) — Figure 4. The following reagents were used.

- Tryptamine - $^{14}$C bisuccinate (0.05mCi/0.5ml)
- Tryptamine HCl solution with above $^{14}$C isotope (0.1μCi/50μl and 51mM/50μl)
- HCl (2N)
- Toluene scintillation fluid: PPO 2,5-di-(phenyloxazoyl) 4gm
- POPOP {1,4-di-2-(5-phenyloxazoly1) benzene}
  0.05gm dissolved in 1 liter of toluene
- Toluene
FIGURE 4

Monoamine oxidase assay reactions.
TRYPTAMINE

\[ \text{H}^*\text{CH}_2\text{CH}_2\text{NH}_2 \xrightarrow{\text{MAO}} \text{H}^{*}\text{CH}_2\text{CH} \]

Acetaldehyde

\[ \text{H}^*\text{CH}_2\text{Co} \]

Acetic acid

EXTRACTED AND COUNTED

EXTRACTED AND COUNTED
Buffers:

Brain and Liver Slice — 0.25M sucrose, 0.01M tris HCl adjusted to pH 7.4

Peritoneal Exudate Cells — Hanks Balanced Salt Solution (HBSS), pH 7.4

Brain Mitochondria — 0.3M mannitol, 0.1mM EDTA, 0.1 percent bovine albumin; medium adjusted to pH 7.4 with KOH

Synaptosomes — 0.32M sucrose, 0.01M tris-HCl

Suspensions of isolated intact brain mitochondria, synaptosomes and lysed brain mitochondria used for monoamine oxidase assays were adjusted to 1.0mg protein per ml by dilution with appropriate buffer solution. Peritoneal exudate cells employed $1.0 \times 10^8$ and $1 \times 10^9$ cells for the 20 minute and 6 minute monoamine oxidase assays respectively. The liver and brain slices used weighed from 3 to 8mg wet.

To each test tube containing 100μl of the enzyme preparation, one-half of the reaction tubes received appropriate buffer and the remaining received buffer with methohexital. All tubes were pre-incubated at $37^\circ C \pm 0.25^\circ C$ for 30 minutes. The reaction was started by adding 50μl stock radioactive tryptamine solution and 100μl additional buffer with or without methohexital and/or the new MAO inhibitors (Figure 4). Blank control tubes were used in the assay by
substitution of 100μl of enzyme preparation which had been inactivated previously by boiling for 50 minutes. Two procedural sequences were followed, one involving a 20 minute reaction period and the other, reaction times of 1.5, 3.0 and 6.0 minutes.

The 20 Minute Reaction:

The control experiment was preincubated for 30 minutes with buffer alone and then reacted for 20 minutes with substrate. Drug treatment involved a 30 minute preincubation in various concentrations of methohexital followed by a 20 minute reaction with substrate and methohexital. The Lilly inhibitors were reacted with substrate at Time zero (T₀) after preincubations in buffer alone. These reactions were carried out under normobaric conditions of one atmosphere of air and hyperbaric conditions of 100 atmospheres of helium pressure.

The 1.5, 3.0 and 6.0 Minute Reactions:

The control preparation was preincubated in buffer for 30 minutes and then reacted with substrate for 1.5, 3.0 and 6.0 minutes (Figure 5). The experimental preparations also were preincubated for 30 minutes followed by the addition of substrate and the reaction scheduled as above. When the Lilly compounds alone were the test drugs, these were added at T₀ in concentrations equivalent to the previously calculated 20 minute ID₅₀ and preincubation in these cases was with buffer alone. When methohexital was tested either alone or in combination with the
FIGURE 5

Procedural sequence for the preincubation of preparations with methohexitol and the subsequent 1.5, 3.0 and 6.0 minute reaction with Lilly inhibitors 51641 and 54761.
Procedural Sequence

Buffer

Buffer + Methohexital

Buffer + 54761 at 50% inhibitory dose
Buffer + 51641 at 50% inhibitory dose
Buffer + Methohexital
Buffer + " + 51641
Buffer + " + 54761

Preincubation

Reaction

FIGURE 5
Lilly compounds, the concentration used was $10^{-7}$M, which had been calculated as an in vivo anesthetic dose (Gardier, et al., 1963). Methohexital, when present, was always added to the 30 minute preincubation medium and maintained at the appropriate concentration through the subsequent reaction. When combined with methohexital, the Lilly compounds were added to the methohexital containing medium at $T_0$. The reactions were carried out under both normobaric and hyperbaric conditions.

The reactions were stopped by immediately placing the reaction tubes in an ice bath. Two hundred microliters of 2N HCl were added to acidify the reaction medium followed by addition of 6.0 cc of toluene and vortexing for 15 seconds. Each tube was centrifuged for ten minutes at 500 X g which produced an organic solvent-water separation. A 3.0 cc aliquot of the organic layer was pipetted into a vial containing 10 ml of scintillation fluid which in turn was placed in a Packard Tri-Carb scintillation spectrometer.

The counting efficiency of the Packard Tri-Carb was accomplished as follows: 0.1 ml of a standard toluene-$^{14}$C tryptamine solution and 2.9 cc of toluene were placed in a vial containing 10 ml of scintillation fluid. The counts per minute were determined and compared to the known disintegrations per minute of this standard. The calculated ratio indicated an efficiency of 87.5 percent. A pulse height spectrum along with a quench curve were conducted to determine the proper window settings.
on the spectrometer. The calculation of the amount of product formed was as follows:

\[
\text{nM of metabolites/min} = \frac{(\text{experimental-blank}) \text{c.p.m.}}{2.2 \times 10^5 \text{ d.p.m.}} \times \frac{100}{87.5} \times \frac{6 \text{cc}}{3 \text{cc}} \times \text{Tryptamine (51nM) nM} \times \frac{1 \text{ minute}}{20 \text{ minutes}}
\]

The product formed and extracted consisted of indolacetaldehyde and indolacetic acid. The concentration of the acid was dependent on the presence or absence of the aldehyde dehydrogenase. Both of these are extracted into toluene (Nagatsu, 1973). To discount the possibility of the substrate contamination of the product, blank extracts were subtracted from the reaction tubes. The products were identified by thin layer chromatography developed in two solvent systems which confirmed the lack of substrate contamination. Radiochromatographic scans of the plates verified the purity of the labeled substrate as well as reconfirming the negligible amount of non-metabolized tryptamine found with the products after extraction in toluene.

**THIN LAYER CHROMATOGRAPHY**

The labeled substrate $^{14}$C tryptamine-bisuccinate and each of the reaction products was identified using thin layer chromatography. The products of the reaction were extracted into toluene prior to counting in the scintillation spectrometer. Ten microliter aliquots of the
toluene extract, cold tryptamine, and the expected products were spotted on silica gel G plates with 0.25mm thickness and developed in either of two solvent systems: 25 parts butanol, 4 parts acetic acid, 10 parts water, or 4 parts ethanol, and 1 part ammonium hydroxide. The plates were dried, exposed to ultraviolet light and iodine vapors and the resulting spots were compared with known standards and identified.

Samples with radioactivity were scraped along the migration path, and these scrapings were placed in vials containing scintillation fluid. The results are presented in Figure 6. They indicated that the radioactive products were indoleacetaldehyde and indoleacetic acid, the metabolites of monoamine oxidase metabolism of tryptamine. Indoleacetaldehyde was the product obtained in all preparations except the peritoneal exudate cell, which produced indoleacetic acid. The TLC plates were also run on the Baird Atomic Radiochromatographic scanner indicating high purity of the labeled tryptamine as well as reconfirming the absence of non-metabolized tryptamine in the toluene extract.

RESPIRATORY STUDIES

Respiratory studies of brain mitochondria, lysed brain mitochondria and synaptosomes were carried out using a polarographic technique. The protocol utilized a Yellow Spring Instrument Company Model 5331 oxygen probe in conjunction with a Sargent-Welch Model S R G recorder. Respiratory reaction medium (1.1ml), consisting of
Thin layer chromatography of substrate and extracted products of MAO reaction samples were spotted on silica gel plates and allowed to migrate 16 cm in a butanol-acetic acid-H$_2$O (25:4:10) solvent system.

A. Radioactivity of $^{14}$C tryptamine standard.

B. Relative positions of standards located with iodine vapors and ultraviolet light.

C. Extracted reaction product from brain mitochondria, synaptosomes and brain slice.

D. Extracted product from PEC.
Counts per min.

- Counts per min.

**FIGURE 6**

1. TRYPTAMINE
2. INDOLE ACETIC ACID
3. INDOLE ACETALDEHYDE
75mM KCl, 50mM tris-HCl, 12.5mM \( \text{K}_2\text{PO}_4 \), 5mM \( \text{MgCl}_2 \) and 1mM EDTA was added to the oxygen electrode chamber. Two hundred microliters of the monoamine oxidase enzyme preparation were added to the chamber and time given for the system to equilibrate. After a baseline was established either 50μl of glutamate (0.2M) or 50μl of sodium succinate (0.2M) was added. Mitochondrial respiration as studied by the phosphorylation reaction, is dependent on several factors; the presence of oxygen, oxidizable substrate, inorganic phosphate and ADP. The concentrations of inorganic phosphate, ADP and ATP can affect the rate of mitochondrial respiration. Lehninger (1965) has described various "states" or processes in mitochondrial respiration. State 1 consists of mitochondria in the presence of oxygen, but without ADP or substrate. In State 2 the substrate is present, but not ADP. State 3 is the active state of respiration in the presence of ADP. When all the ADP is phosphorylated, respiration returns to a slower rate. State 4 which is when all reactants are present, but the acceptor is used up.

The experiment is carried out by adding all the reactants to the oxygen electrode chamber except ADP and the mitochondria preparation. The baseline for maximum oxygen concentration can be established once 200μl of the mitochondrial preparation is added. Since all reactants except ADP are now present, the mitochondria are essentially in State 4. Subsequent addition of ADP, 3μl of 0.1M ADP, produces the active State 3 "burst" of respiration and when all the acceptor (ADP) is
phosphorylated, the mitochondrial respiration returns to the State 4 rate.

The respiratory control index (RCI), which is a measure of the ratio of respiratory rates, was calculated by determining the ratio of the slope of State 4 to that of State 3 on the recording graph. The usual RCI values for succinate are 3 to 5.

**DRUGS USED**

**Methohexital:**

Methohexital sodium (Brevital) solution was prepared fresh for each experiment. Through preliminary experiments in our laboratory, (Weaver, 1976) a 20 to 30 minute preincubation time prior to the monoamine oxidase assay was sufficient for maximal effect of the methohexital. The structure is shown in Figure 7. Dose response curves were first carried out to determine the inhibitory 50 concentration over a 20 minute reaction period. Later experiments using anesthetic concentrations of $10^{-7}$M employed reaction times of 1.5, 3.0 and 6.0 minutes.

**Lilly 51641:**

Lilly 51641 (N-(2,5(0-chorophenoxy)-ethyl) cyclopropylamine) — see Figure 7 — is an irreversible inhibitor of monoamine oxidase. Fuller (1968) has reported Lilly 51641 as being a type "A" monoamine oxidase inhibitor,
FIGURE 7

Chemical structure of methohexital and Lilly MAO inhibitors 51641 and 54761.
N-(2-O-chlorophenoxy)-ethyl cyclopropylamine (Lilly 51641)

N-phenacylcyclopropylamine (Lilly 54761)
Lilly 54761:

Lilly 54761 (N-phenacylacyclopropylamine) (Figure 7) is a proposed irreversible inhibitor of type "B" monoamine oxidase; equivalent in potency to pargyline (Fuller and Roush, 1972).

CHAMBER FOR INSTILLING HYPERBARIC CONDITIONS

The chamber was a stainless steel cylinder made by Parr Instrument Company, Model 526HC with an outside diameter of 11.5cm, a height of 28.0cm, wall thickness of 1.0cm and operating volume of 950.0ml. The open end was flanged so that the cap containing the pressure gauge could be sealed in place with a retaining ring that compresses the "o" ring rubber seal to maintain a pressure-tight fit.

The temperature regulator (Figure 8) was designed to maintain the water bath inside the chamber at \(37°C \pm 0.25°C\). Both the heat exchanger and the temperature sensor were located within the chamber.

The circuitry for the proportional temperature controller is illustrated in the schematic diagram (Figure 8). A silicon diode, D-9, was the temperature reference probe which was immersed in the water contained in the temperature reference test tube inside the chamber. The forward-biased silicon junction had a characteristic temperature coefficient of 2.2mV/°C which was applied to an operational amplifier, A-1. The amplifier A-1, along with circuitry labeled "Zero trim" allowed for setting the meter reading at 0°C when the sensor was in an
FIGURE 8

Schematic diagram of the temperature regulating system used in conjunction with pressure chamber.
ice bath. The amplifier A-2 conditioned the signal to such an extent that a 200 micro-ampere meter would indicate the temperature of the diode over a range of -15°C to 45°C. The amplifier A-3 compared the output of A-1 with the "Temperature Set Control" and its (A-3) output was amplified by A-4 to drive the transistor Q3. The Q3, which determines the gate bias on transistors SCR Q1 and SCR Q2, controlled the amount of current which flowed through the bath heater coil. The greater the current, the more heat that was generated.

The electrical current generates a quantity of heat according to the equation $P = VI$, where $P$ is the heat in watts, $V$ is the applied voltage (12.6 volts average), and $I$ is the current (7 amperes maximum). The heat was dissipated on the heat exchangers inside the chamber, and subsequently transferred to the water bath.

The regulator switch was placed on "set", and the "temperature control" is turned to the desired temperature, i.e. 37°C. The regulator switch was then placed on "sense", and the system generated heat as necessary to maintain a constant temperature inside the chamber. The temperature was maintained at 37°C ± 0.2°C for the reaction time. Pressure increased the temperature, therefore it was necessary for the bath water to be approximately 20°C initially in order to allow for the temperature change with pressurization to 100 atmospheres. Equilibration to 37°C time was maintained within a 45 second interval.
STATISTICAL METHODS

Dose response curves were obtained in order to determine the inhibitory dose\(_{50}\) (ID\(_{50}\)) for each of the drugs used. Plots of percent inhibition monoamine oxidase versus negative log concentration were used to demonstrate the relative potencies of these drugs. Data obtained from the 1.5, 3.0 and 6.0 minute reactions were plotted in two ways. These reactions were plotted as product formation versus time and the 6.0 minute points were analyzed by paired Student \(t\) test to determine significance. In order to further analyze these reactions using a computer assisted program for slope analysis the data was plotted as product formation versus log time. The best fit slope was supplied to the data generated from 1.5, 3.0 and 6.0 minute reactions. To test the difference between two different slopes representing control versus drug treated or two different drug treated experiments, a calculated slope having half the value between the two was used for statistical comparison. If each slope was significant from the estimated half-way slope then the original slopes were significant from each other. The statistical analysis system was designed and implemented by Mr. Indrayan, Department of Statistics, The Ohio State University, Columbus.
RESULTS

CELLULAR AND SUBCELLULAR INTEGRITY AND VIABILITY STUDIES

The integrity of all biological preparations used was established before any experiments were done.

Peritoneal Exudate Cells (PEC):

Each mineral oil injected guinea pig yielded 3 to 6 X 10^9 peritoneal exudate cells. The trypan blue dye exclusion test indicated that more than 90 percent of the cells in each preparation were viable. Electron photomicrographs of a PEC pellet (Plate 1) and migrating macrophage (Plate 2) indicated that the cells had intact membranes and were otherwise normal. In addition, the macrophages used in the enzymatic studies were also found to migrate in a normal mushroom shape pattern.

Synaptosomes:

An electron photomicrograph (Plate 3) of a typical synaptosomal preparation shows synaptic junctions with intact neuronal membranes, mitochondria and neuronal vesicles. Respiratory studies were conducted on portions of the pellet not used in MAO studies as further confirmation of subcellular integrity. A high respiratory control index (RCI) is
Macrofages from peritoneal exudate cell pellet isolated from the guinea pig following centrifugation at 6500 X g for ten minutes. Macrophages were fixed in buffered glutaraldehyde and postfixied with osmium tetroxide. Integrity of the matrix as well as cellular and subcellular membranes is shown.

Magnified X 5900.
PLATE II

Migrating macrophage taken from migration chamber. Cells were fixed in situ on glass coverslips with paraformaldehyde and glutaraldehyde then postfixed with osmium tetroxide and potassium ferrocyanide.

Magnified X 5900.
Synaptosomal preparation from guinea pig brain following centrifugation at 11000 X g for 20 minutes. The pellet was fixed in buffered glutaraldehyde and postfixed with osmium tetroxide.

Magnified X 51000.

S — Intact Synaptic Junctions
NM — Neuronal Membranes
M — Mitochondria
V — Neuronal Vesicles
considered by Lehninger (1965) to be the primary measure of the presence of intact mitochondria and their associated functioning enzymatic processes such as Kreb's cycle activity. Four separate synaptosomal preparations produced an average RCI value of 3.36 (Table 1) which is within the normal range of 3.0 to 5.0 for succinate substrates (Ozawa, et al., 1966). Synaptosomes were found to have approximately one-fourth the MAO specific activity of isolated brain mitochondria.

**Intact Brain Mitochondria:**

Electron microscopy revealed that the intact brain mitochondrial preparation (Ozawa, et al., 1966) had 20 percent contamination with synaptosomes. Since, approximately 6.0 percent of the amount of labeled product measured in the enzymatic reactions was that of synaptosomal mitochondrial MAO activity. An electron photomicrograph (Plate 4) indicates the integrity of the mitochondrial membranes and inner cristae. The mitochondria appear to be normal and intact. The respiratory control index for intact brain mitochondria (Ozawa, et al., 1966) was greater than 3 (Table 1) indicating coupled respiration present.

**Lysed Brain Mitochondria:**

The product obtained by sonicating whole mitochondria was examined under the electron microscope. This preparation contained
# TABLE 1

**RESPIRATORY CONTROL INDEX (RCI)† WITH SUCCINATE AS SUBSTRATE**

<table>
<thead>
<tr>
<th></th>
<th>WHOLE BRAIN MITOCHONDRIA*</th>
<th>WHOLE BRAIN MITOCHONDRIA**</th>
<th>SONICATED BRAIN MITOCHONDRIA</th>
<th>SYNAPTOSOMES</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>n=5</td>
<td>n=3</td>
<td>n=4</td>
<td>n=4</td>
</tr>
<tr>
<td></td>
<td>6.64</td>
<td>6.20</td>
<td>1.00</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td>4.10</td>
<td>2.10</td>
<td>1.50</td>
<td>4.05</td>
</tr>
<tr>
<td></td>
<td>2.55</td>
<td>1.56</td>
<td>2.0</td>
<td>3.20</td>
</tr>
<tr>
<td></td>
<td>4.16</td>
<td></td>
<td>1.0</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>4.87</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td>4.46</td>
<td>3.28</td>
<td>1.30</td>
<td>3.36</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>±0.66</td>
<td>±1.46</td>
<td>±0.20</td>
<td>±0.26</td>
</tr>
</tbody>
</table>

*Whole mitochondria prepared by technique of Ozawa, et al. (1966).*

**Whole mitochondria prepared by technique of Barberis and McIlwain (1972).*

†See METHODS for RCI calculation.
PLATE IV

Mitochondrial pellet from guinea pig brain following centrifugation at 11000 x g for 20 minutes. The pellet was fixed in buffered glutaraldehyde and postfixfixed with osmium tetroxide.

Magnified X 51000.
fractured, disrupted membranes with no evidence of whole mitochondria being present (Plate 5). Sonicated mitochondria showed no evidence of respiration (Table 2), in contrast with the whole mitochondrial preparation.

REVERSIBILITY OF MAO INHIBITION

Figure 9 presents data accumulated from a series of experiments conducted to probe the reversibility of the enzymatic inhibition. The inhibitory effect of methohexital on intact brain mitochondria was overcome by washing, which was not the case with Lilly 51641 or Lilly 54761. The Lilly inhibitors are therefore more strongly bound to the mitochondrial MAO than is methohexital.

MAO ACTIVITY IN NEURONAL TISSUE UNDER NORMOBARIC CONDITIONS

The plots of percent MAO inhibition versus the logarithm of inhibitor concentrations were derived from 20 minute reactions. The brain slice (Figure 10), synaptosomes (Figure 11), and brain mitochondria (Figure 12) all demonstrate that methohexital was a considerably weaker inhibitor of monoamine oxidase than either of the two Lilly compounds, 51641 and 54761. All CNS preparations gave an ID₅₀ of 10⁻²M with methohexital except the brain slice which required a slightly higher dose to achieve the same effect (Table 2). Lilly compound 54761, like methohexital required the same concentration to
PLATE V

Sonicated mitochondrial pellet from guinea pig brain following centrifugation at 40000 X g for 30 minutes. The pellet was fixed in buffered glutaraldehyde and postfixied with osmium tetroxide.

Magnified X 100000.
<table>
<thead>
<tr>
<th>DRUG</th>
<th>(n) BRAIN SLICE</th>
<th>(n) BRAIN SYNAPTOSOMES</th>
<th>(n) CNS MITOCHONDRIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>54761 (Lilly)</td>
<td>(6) $2.5 \times 10^{-6}$</td>
<td>(6) $2.1 \times 10^{-6}$</td>
<td>(6) $2.1 \times 10^{-6}$</td>
</tr>
<tr>
<td>51641 (Lilly)</td>
<td>(6) $3.5 \times 10^{-5}$</td>
<td>(6) $2.1 \times 10^{-6}$</td>
<td>(6) $1.65 \times 10^{-7}$</td>
</tr>
<tr>
<td>METHOHEXITAL</td>
<td>(6) $9.5 \times 10^{-2}$</td>
<td>(6) $2.35 \times 10^{-2}$</td>
<td>(6) $2.1 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

Pressure (100 atms. He)

<table>
<thead>
<tr>
<th>DRUG</th>
<th>(n) BRAIN SLICE</th>
<th>(n) BRAIN SYNAPTOSOMES</th>
<th>(n) CNS MITOCHONDRIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>54761 (Lilly)</td>
<td>(6) $3.95 \times 10^{-6}$</td>
<td>(6) $1.75 \times 10^{-6}$</td>
<td>(6) $3.95 \times 10^{-6}$</td>
</tr>
<tr>
<td>51641 (Lilly)</td>
<td>(6) $1.7 \times 10^{-5}$</td>
<td>(6) $1.4 \times 10^{-6}$</td>
<td>(6) $8.0 \times 10^{-7}$</td>
</tr>
<tr>
<td>METHOHEXITAL</td>
<td>(6) $10.1 \times 10^{-2}$</td>
<td>(6) $1.56 \times 10^{-2}$</td>
<td>(6) $2.25 \times 10^{-2}$</td>
</tr>
</tbody>
</table>
FIGURE 9

MAO activity in brain mitochondria after a 30 minute incubation with the various drugs and washed twice before reacting with substrate. The control preparations were handled in the same manner as the drug treated. Columns B, C, D, E are individually significant (p < 0.05) from control. A, B and C are significantly different from each other according to paired Student's t analysis.

n = 6 for each group.
FIGURE 9

- **Control**
- **5 x 10^{-2} M**
- **5 x 10^{-2} M**
- **1 x 10^{-5} M**
- **1 x 10^{-5} M**

**A**

**B**

**C**

**D**

**E**

**Moles Activity Per mg Protein**
FIGURE 10

Inhibition of monoamine oxidase activity in guinea pig cortical slice by Lilly 51641, Lilly 54761 and methohexital. In this Figure and subsequent Figures, values are presented as means and standard errors (S.E.M.) for duplicate determinations from three or more guinea pigs.
FIGURE 10

Inhibition of Monoamine Oxidase

- Lilly 54761
- Lilly 51641
- Methohexital

Log Dose (M)

% Inhibition of Monoamine Oxidase

0  20  40  60  80  100

9  8  7  6  5  4  3  2  1

FIGURE 10
FIGURE 11

Inhibition of guinea pig synaptosome monoamine oxidase by indicated drugs.
FIGURE 11
Inhibition of guinea pig brain mitochondria monoamine oxidase by indicated drugs.
FIGURE 12

% INHIBITION OF MONOAMINE OXIDASE

\[ \text{Log Dose (M)} \]

-三角形- LILLY 54761
-圆圈- LILLY 51641
-实心圆- METHOHEXITAL
inhibit MAO by 50 percent regardless of the enzyme preparation employed (Table 2). Incremental logarithmic doses of Lilly 51641 were necessary to demonstrate the same level of inhibition when the complexity of the neuronal biological system was increased from isolated mitochondria to synaptosomes to tissue slice (Table 2).

MAO ACTIVITY IN VISCERAL TISSUE UNDER NORMOBARIC CONDITIONS

MAO Activity in Peritoneal Exudate Cells:

The studies of monoamine oxidase activity carried out in peritoneal exudate cells gave four types of information: 1) basal levels; 2) functional activity; 3) MAO types and 4) effects of inhibitors. Normally migrating macrophages were found to metabolize 580 picomoles of tryptamine per $1 \times 10^7$ cells (76.0 picomoles/mg protein) in a 20 minute reaction period. The relationship of cell migration to drug dose is indicated in Figure 13. The comparison of the three drugs, i.e. methohexital, Lilly 51641 and Lilly 54761 indicates that migration inhibition is produced by all three drugs at about the same dose level, $5 \times 10^{-4}$M. Plate 6 displays a typical effect of methohexital on macrophage migration, control on the lower figure, 50 percent inhibition upper left, occurred at a dose of $1 \times 10^{-3}$M, and complete inhibition, upper right, occurred at a dose of $5 \times 10^{-3}$M. This dose of methohexital necessary to produce complete inhibition of migration inhibited MAO by only 50 percent. Conversely, migration was not significantly inhibited
FIGURE 13

Inhibition of guinea pig peritoneal exudate cell (PEC) migration. Control migrations were carried out by incubating PEC at 37°C for 20 to 24 hours in MEM-PS medium. Drug treatment involved the reaction of PEC with various concentrations of either methohexital or the Lilly compounds. Asterisk indicates the first point of significance as compared to control (p <0.05).
FIGURE 13
Migration of guinea pig peritoneal exudate cells and inhibition with methohexital. PEC were centrifuged 280 X g for ten minutes, the pellet placed in migration chambers and allowed to migrate on glass coverslips 20 to 24 hours.

Light Microscope, Magnified X 10.

Lower Figure: Control migration.
Upper Left: 50 percent inhibition with methohexital.
Upper Right: Complete inhibition with methohexital.
by concentrations of the MAO inhibitors that inhibited MAO activity by approximately 85 percent. The percent MAO inhibition produced by the irreversible MAO inhibitors at various concentrations is shown in Figure 14. Monoamine oxidase present in PEC was approximately 1000-fold more sensitive to Lilly 51641, the "A" type inhibitor than Lilly 54761, the "B" type inhibitor. The ID$_{50}$'s were $4.6 \times 10^{-8}$M and $3.25 \times 10^{-5}$M respectively (Table 3). From Figure 14 and Table 3, it is apparent that methohexital is a considerably weaker inhibitor of MAO than either of the irreversible MAO inhibitors in PEC.

**MAO Activity in Liver Slice Under Normobaric Conditions:**

The plot of percent MAO inhibition versus the logarithm of inhibitor concentration for liver slice (Figure 15) depicts that methohexital is a considerably weaker inhibitor of MAO than either of the Lilly compounds. The monoamine oxidase present in the liver slice and liver mitochondria appear to be equally sensitive to either of the Lilly inhibitors as indicated in Table 3.

**HYPERBARIC CONDITIONS**

In all the preparations indicated above, the reduced MAO activity induced by the drugs, i.e. methohexital, Lilly 51641 and Lilly 54761 was
FIGURE 14

Inhibition of guinea pig peritoneal exudate cell monoamine oxidase by Lilly 51641, 54761 and methohexital. In this Figure and subsequent Figures, values are presented as means and standard errors (S.E.M.) for duplicate determinations from three or more guinea pig preparations.
FIGURE 14

% INHIBITION OF MONOAMINE OXIDASE

- LOG DOSE M

△ LILLY 54761
○ LILLY 51641
● METHOHEXITAL
TABLE 3

MAO INHIBITORY DOSE 50's IN PERIPHERAL PREPARATIONS

Molar concentrations of drug necessary to produce 50% inhibition

No Pressure

<table>
<thead>
<tr>
<th>DRUG</th>
<th>(N) LIVER SLICE</th>
<th>(N) MITOCHONDRIA</th>
<th>(N) MACROPHAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>54761 (Lilly)</td>
<td>(6) 2.14 X 10^-6</td>
<td>(3) 2.5 X 10^-6</td>
<td>(6) 3.25 X 10^-5</td>
</tr>
<tr>
<td>51641 (Lilly)</td>
<td>(6) 1.00 X 10^-6</td>
<td>(3) 1.4 X 10^-6</td>
<td>(10) 4.6 X 10^-8</td>
</tr>
<tr>
<td>METHOHEXITAL</td>
<td>(6) 2.9 X 10^-2</td>
<td>(10)* 4.5 X 10^-2</td>
<td>(10) 4.75 X 10^-2</td>
</tr>
</tbody>
</table>

Pressure (100 atms. He)

<table>
<thead>
<tr>
<th>DRUG</th>
<th>(N) LIVER SLICE</th>
<th>(N) MITOCHONDRIA</th>
<th>(N) MACROPHAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>54761 (Lilly)</td>
<td>(6) 5.6 X 10^-6</td>
<td>(3) 2.25 X 10^-6</td>
<td>(10) 2.1 X 10^-5</td>
</tr>
<tr>
<td>51641 (Lilly)</td>
<td>(6) 2.9 X 10^-6</td>
<td>(3) 2.0 X 10^-6</td>
<td>(10) 4.85 X 10^-8</td>
</tr>
<tr>
<td>METHOHEXITAL</td>
<td>(6) 7.1 X 10^-2</td>
<td>(10)* 5.5 X 10^-2</td>
<td>(10) 1.4 X 10^-1</td>
</tr>
</tbody>
</table>

*Data from Weaver, 1976
FIGURE 15

Inhibition of monoamine oxidase activity in guinea pig liver slice by the indicated drugs.
FIGURE 15

- % INHIBITION OF MONOAMINE OXIDASE
- \( \log \text{Dose M} \)

- \( \triangle \) Lilly 54761
- \( \circ \) Lilly 51641
- \( \bullet \) Methohexital
not overcome by 100 atmospheres of helium pressure. Tables 2 and 3 reveal no significant difference between the ID\textsubscript{50}'s under normobaric and hyperbaric conditions.

INFLUENCE OF METHOHEXITAL ON MAO INHIBITION BY LILLY 51641 AND 54761

Normobaric Conditions:

As previously noted, pressure did not antagonize the barbiturate effect as measured by ID\textsubscript{50}. It was therefore postulated that two conditions were present that precluded such action. Firstly, the concentrations of methohexital showing inhibition were of a toxic nature, and secondly, 20 minutes probably was too long a reaction time to capture the expected changes. Therefore, a shorter reaction period proposed along with a concentration (10\textsuperscript{-7}M) of methohexital calculated to be equivalent to an anesthetic blood level (Gardier, et al., 1963) which did not inhibit the MAO enzyme. Under these conditions, methohexital had an effect on the pattern of inhibition produced by both the Lilly inhibitors 51641 and 54761. Methohexital enhanced the inhibitory activity of Lilly 54761 in synaptosomes (Figure 16) and the lysed brain mitochondrial preparations (Figure 17). The barbiturate reversed the inhibitory activity of Lilly 54761 on peritoneal exudate cells but potentiated the inhibition produced by Lilly 51641 (Figure 18). When methohexital was combined with the Lilly MAO inhibitors, no quantitative alteration of inhibition by the latter drugs was apparent in brain
MAO activity in guinea pig synaptosomes and its inhibition by Lilly inhibitors alone and in combination with methohexital. The experimental procedure is described in "METHODS". For this Figure and subsequent Figures, the values are presented as means with S.E.M.; three determinations were completed in duplicate for each point on the curves.

Statistical analysis using Student's $t$ test ($p < 0.05$) at 6.0 minutes and slope analysis ($p < 0.05$) indicates:

1. The curve C and curves below are significant from control (A).
2. Curves C and D are significant from each other.
3. Neither curves A and B or E and F are significant from each other.
FIGURE 16
FIGURE 17

MAO activity in guinea pig lysed brain mitochondria and its inhibition by Lilly inhibitors alone and in combination with methohexital. Data was obtained as described in Legend of Figure 16.

Statistical analysis using Student's t test (p <0.05) at 6.0 minutes and slope analysis (p <0.05) indicates:

1. The curve plotted at F and curves below are significant from control (A).

2. Curves C and D are significantly different from each other.

3. Neither curves A and B or E and F are significant from each other.
FIGURE 17

- Control
- Methohexital Control 10^-7 Molar
- Lilly 54761
- Lilly 54761 and Methohexital
- Lilly 51641
- Lilly 51641 and Methohexital

n Moles Product per mg. Protein

Time (min.)

1.0 2.0 3.0 4.0 5.0 6.0
FIGURE 18

MAO activity in guinea pig peritoneal exudate cells and its inhibition by Lilly inhibitors alone and in combination with methohexital. Data was obtained as described in Legend of Figure 16.

Statistical analysis using Student's $t$ test ($p < 0.05$) at 6.0 minutes and slope analysis ($p < 0.05$) indicates:

1. The curve plotted at D and curves below are significant from control (A).
2. Curves C and D and E and F are significant from each other.
3. Curves A and B are not significant from each other.
Figure 18
slice (Figure 19) or intact brain mitochondria (Figure 20). In each of the preparations, methohexital alone had no significant effect on MAO activity. The irreversible inhibitors always were able to effect a change from control during the 6.0 minute reaction period regardless of the presence of methohexital.

**Hyperbaric Conditions:**

Helium pressure of 100 atmospheres was applied to each of the preparations in which methohexital induced an alteration in the inhibitory activity of the MAO inhibitors. Hyperbaric conditions overcame the barbiturate enhancement of MAO inhibition produced by Lilly 54761 in synaptosomes (Figure 21), lysed brain mitochondria (Figure 22) and that of Lilly 51641 in peritoneal exudate cells (Figure 23). The inhibitory activity of Lilly 54761 in peritoneal exudate cells antagonized by methohexital under normobaric conditions was also overcome by pressure (Figure 23), but continued to have no apparent change in intact brain mitochondria (Figure 24). Identical hyperbaric conditions significantly increased monoamine oxidase activity in all control reactions except whole intact brain mitochondria. The rates of MAO activity depicted by the slope of the curves remain in relatively the same position with each other even under hyperbaric conditions (Table 4).
FIGURE 19

MAO activity in guinea pig cortical slice and its inhibition by Lilly inhibitors alone and in combination with methohexital. The experiments were carried out as described in "METHODS" with the exception of employing 3.0, 6.0 and 12.0 minute reaction periods rather than those stated. The values are presented as means with S.E.M.; three determinations were completed in duplicate for each point on the curves.

Statistical analysis using Student's t test (p < 0.05) at 6.0 minutes and slope analysis (p < 0.05) indicates:

1. The curve plotted at C and curves below are significant from control (A).

2. Neither curves A and B, C and D, or E. and F are significant from each other.
FIGURE 19

- Control
- Methohexital Control 10^-7 Molar
- Lilly 54761
- Lilly 54761 and Methohexital
- Lilly 51641
- Lilly 51641 and Methohexital

Time (min.)

Moles Product per mg. Tissue
FIGURE 20

MAO activity in guinea pig isolated brain mitochondria and its inhibition by Lilly inhibitors alone and in combination with methohexital. Data was obtained as described in Legend of Figure 16.

Statistical analysis using Student's t test (p <0.05) at 6.0 minutes and slope analysis (p <0.05) indicates:

1. The curve plotted at C and curves below are significant from control (A).
2. Neither curves A and B, C and D, or E and F are significant from each other.
FIGURE 20
Inhibition of MAO activity in guinea pig synaptosomes under hyperbaric conditions. Data was obtained as described in Legend of Figure 16.

Statistical analysis using Student's $t$ test ($p < 0.05$) and slope analysis ($p < 0.05$) indicates:

1. The curve plotted at C and curves below are significant from control (A).
2. Curves C and D are no longer significant from each other.
3. Neither curves A and B or E and F are significant from each other.
**FIGURE 21**

- **Control**
- **Methohexital Control 10^-7 Molar**
- **Lilly 54761**
- **Lilly 54761 and Methohexital**
- **Lilly 51641**
- **Lilly 51641 and Methohexital**

The graph shows the change in n moles product per mg protein over time (minutes) for different experimental conditions.
Inhibition of MAO activity in guinea pig lysed brain mitochondria under hyperbaric conditions. Data was obtained as described in Legend of Figure 16.

Statistical analysis using Student's $t$ test ($p < 0.05$) at 6.0 minutes and slope analysis ($p < 0.05$) indicates:

1. The curve plotted at F and curves below are significant from control (A).

2. Curves C and D are no longer significant from each other.

3. Neither curves A and B or E and F are significant from each other.
FIGURE 22

- Control
- Methohexital Control 10^-7 Molar
- Lilly 54761
- Lilly 54761 and Methohexital
- Lilly 51641 and Methohexital
- Lilly 51641

N Moles Product per Mg. Protein

Control M in a
Methohexital Control 10^-7 Molar
Lilly 54761
Lilly 54761 and Methohexital
Lilly 51641 and Methohexital
Lilly 51641

Time (Min.)

1.0 2.0 3.0 4.0 5.0 6.0

FIGURE 22

117
Inhibition of MAO activity in guinea pig peritoneal exudate cells under hyperbaric conditions. Data was obtained as described in Legend of Figure 16.

Statistical analysis using Student's $t$ test ($p < 0.05$) at 6.0 minutes and slope analysis ($p < 0.05$) indicates:

1. The curve plotted at F and curves below are significant from control (A).
2. Curves C and D, and E and F are no longer significant from each other.
3. Curves A and B are not significant from each other.
FIGURE 23

- CONTROL
- METHOHEXITAL CONTROL 10^-6 Molar
- LILLY 54761
- LILLY 54761 AND METHOHEXITAL
- LILLY 51641
- LILLY 51641 AND METHOHEXITAL

N Moles Product per 10^8 Cells

Time (min.)

1.0  2.0  3.0  4.0  5.0  6.0
FIGURE 24

Inhibition of MAO activity in guinea pig isolated brain mitochondria under hyperbaric conditions. Data was obtained as described in Legend of Figure 16.

Statistical analysis using Student's $t$ test ($p < 0.05$) at 6.0 minutes and slope analysis ($p < 0.05$) indicates:

1. The curve plotted at F and below are significant from control (A).

2. Curves A and B, C and D, and E and F remained not significant from each other.
FIGURE 24

- CONTROL
- METHOHEXITAL CONTROL 10^{-7} MOLAR
- LILLY 54761
- LILLY 54761 AND METHOHEXITAL
- LILLY 51641
- LILLY 51641 AND METHOHEXITAL

n Moles Product per mg. Protein

Time (min.)

1.0 2.0 3.0 4.0 5.0 6.0

2.30 2.10 1.90 1.70 1.50 1.30 1.10 0.90 0.70
TABLE 4
PRESSURE EFFECTS ON MAO ACTIVITY

<table>
<thead>
<tr>
<th>REACTION</th>
<th>SYNAPTOSONE FRAGMENTED MITOCHONDRIA</th>
<th>MACROPHAGE</th>
<th>INTACT MITOCHONDRIA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP</td>
<td>P</td>
<td>R</td>
</tr>
<tr>
<td>CONTROL</td>
<td>0.71</td>
<td>1.63</td>
<td>*</td>
</tr>
<tr>
<td>54761 REACTION</td>
<td>0.35</td>
<td>0.54</td>
<td>*</td>
</tr>
<tr>
<td>51641 REACTION</td>
<td>0.18</td>
<td>0.21</td>
<td>NS</td>
</tr>
<tr>
<td>Methohexital Control</td>
<td>0.59</td>
<td>1.57</td>
<td>*</td>
</tr>
<tr>
<td>54761 and Methohexital</td>
<td>0.23</td>
<td>0.43</td>
<td>*</td>
</tr>
<tr>
<td>51641 and Methohexital</td>
<td>0.16</td>
<td>0.20</td>
<td>NS</td>
</tr>
</tbody>
</table>

VALUES ARE SLOPES REPRESENTING THE RATE OF MAO PRODUCTION UNDER NORMOBARIC, NP, (1 ATMOSPHERE PRESSURE), AND HYPERBARIC, P, (100 ATMOSPHERES HELIUM PRESSURE) CONDITIONS. STATISTICAL DETERMINATIONS, S, BY SLOPE ANALYSIS (p<0.05) INDICATE THE EFFECT PRESSURE HAS ALONE ON THE RATE OF MAO PRODUCTION, R.
Statistical Evaluation:

The six minute points of the above series of plots were subjected to Student's t test. Moreover, slope analysis was employed in analyzing the curves of these plots. Table 5 shows each system of statistical analysis congruent with the others.
TABLE 5

DIFFERENCES BETWEEN DRUG TREATMENTS AS ANALYZED
BY TWO STATISTICAL PROCEDURES

<table>
<thead>
<tr>
<th>MACROPHAGE</th>
<th>LYSED BRAIN</th>
<th>INTACT BRAIN</th>
<th>BRAIN MITOCHONDRIA</th>
<th>BRAIN MITOCHONDRIA SLICE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP P</td>
<td>NP P</td>
<td>NP P</td>
<td>NP P</td>
<td>NP P</td>
</tr>
</tbody>
</table>

Methohexital Control

VERSUS NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS

Non Drug Control

54761 VERSUS * * NS NS * * NS NS NS NS NS NS NS NS NS NS NS NS
54761 + Methohexital * * NS NS * * NS NS NS NS NS NS NS NS NS NS NS NS

51641 VERSUS * * NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS
51641 + Methohexital * * NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS NS

NP, 1 atmosphere pressure. P, 10^ atmosphere of helium pressure. T, data analyzed by Student's t
Test (p<0.05) for the 6 minute reactions. S, data analyzed by Slope analysis (p<0.05)
DISCUSSION

BASIS FOR THESIS HYPOTHESIS

The antagonism observed between pressure and anesthesia in intact animals suggests that pressure antagonism may be a promising criterion for identifying the effects of anesthetics on the physical chemistry of the cell membrane (Kendig, et al., 1975). Singer and Nicolson (1972) define the membrane to exist in a fluid state as follows, "a membrane is an oriented, two-dimensional, viscous solution of amphipathic proteins (or lipoproteins) and lipids in instantaneous thermodynamic equilibrium". Evidence indicates that anesthetics act primarily by increasing the fluidity of membranes, a phenomenon associated with membrane expansion (Seeman, 1973). A high-pressure environment can counteract this effect of anesthetics just as it antagonizes general anesthesia in vivo (Trudell, et al., 1973a). Hyperbaric conditions have reversed narcotized states in microbes, teleosts, amphibia and even vertebrates. Pressure within the ranges which produce reversal of the anesthesia process has been calculated to compress the membrane by an amount of approximately equal to the anesthetic-induced expansion shown by Seeman, et al. (1969) (Miller, et al., 1973).

The mechanism which produces the loss or reduction of normal function of a "fluidized" membrane is unknown. Anesthetics have been
shown to block conduction in electrically excitable axonal membrane, depress postsynaptic electrogenesis at chemical synapses (Thesleff, 1956; Bloom, et al., 1965) and also alter release of transmitter from the presynaptic terminal (Matthews and Quillian, 1964). These researchers and other have proposed that changes in membrane fluidity are accompanied by changes in permeability and that this somehow interferes with membrane action. Although nearly all membranes are subject to the effects of anesthetics (Richards, 1976), neuronal membranes are either highly susceptible to these agents or else the decrement in function is most easily observed in them (Miller and Pang, 1976).

If pressure restores the alteration of membrane geometry brought about by the anesthetic agent, it may be demonstrable at the cellular and subcellular level. This project's approach was to study the effect of an "anesthetic" on a membrane-bound enzyme, monoamine oxidase. In order to test the hypothesis that anesthesia alters membrane geometry in vitro, I have studied the effect of anesthetics on the activity of monoamine oxidase, an enzyme which is bound to a variety of biological membranes. My expectation was that an alteration in the geometry of the membrane to which the enzyme was bound or the enclosing membranes would be reflected by an alteration in the activity of the enzyme. Monoamine oxidase activity of the guinea pig brain was investigated in different biological preparations with the intent of finding variations in activity dependent on alterations in either the mother membrane or enveloping membranes.
EFFECTS OF METHOHEXITAL ON MAO INHIBITION BY LILLY COMPOUNDS

Central Nervous System Preparations:

If methohexital acts by changing membrane geometry, membrane permeability to the MAO inhibitors or substrate may be altered in the presence of anesthetic concentrations of methohexital. Alternatively, the drug could change the conformation of the enzyme and in that manner modify activity. In the latter case a more universal effect would be expected than in the former. Since hyperbaric conditions did not alter the methohexital ID_{50}, the hope remained that an alteration in protocol would prove more positive and informative. This was borne out by shortening the reaction and combining noninhibitory concentrations of methohexital with effective concentration of the irreversible inhibitors.

"Anesthetic" concentrations of methohexital potentiated the effect of the "B" type monoamine oxidase inhibitor in brain synaptosomes, but had no effect on intact mitochondria. Since methohexital alone was noninhibitory, it could have had no effect on substrate diffusion. These results suggest that methohexital acts on the synaptosomal membrane either to allow enhanced diffusion of the "B" type MAO inhibitor 54761. It is possible that methohexital enhanced the diffusion of 51641 but that the change was of such a quantitative nature as to be masked by its (51641) possible tighter binding characteristics. It is also possible that methohexital had "uncovered" previously unavailable enzyme sites. This is considered unlikely since
such an effect would have been expressed generally among the preparations which was not the case. The fact that cortical slice and intact mitochondria showed no alteration of MAO inhibition in the presence of the anesthetic may indicate that there is a lower response of intact membranes, to the effect of methohexital than to disrupted or previously disrupted preparations, i.e. fragmented mitochondria and synaptosomes. Kendrick, et al. (1977) have indicated the possibility that disruption of the mitochondrial alters its transport mechanism. Their work with rat synaptosomes demonstrating that uptake and efflux of cations became dependent on ATP in lysed mitochondria but are independent of this energy source in intact mitochondria. This indicates that a change in diffusion characteristics occurs once the membranes have been exposed to sonication. Lipid bilayer studies by Miller and Pang (1976) have reported that pentobarbital, as well as halothane fluidize a simulated synaptosomal membrane excluding the proteins.

**Peritoneal Exudate Cells:**

Anesthetic concentrations of methohexital enhanced the inhibitory activity of 51641 but also significantly reversed the action of the "b" type monoamine oxidase inhibitor, 54761. Studies on phosphatidylcholine-cholesterol lipid bilayers reveal that some anesthetics (halothane and urethan) always fluidize membranes regardless of their composition. The anesthetic action of the barbiturate pentobarbital on biomembranes can either fluidize or "order" depending on the makeup of the membrane. Increasingly the negatively charged phosphatidylcholine
content tends to confer an ordering ability in some cases (Miller and Pang, 1976). With this evidence, it is possible to explain the opposite results found in peritoneal exudate cells. Neuronal membranes with known compositions of 30 to 50 percent cholesterol and 12 to 20 percent phospholipids are expected to have different permeability properties than the liver cell plasma membrane having much more protein (Adams, 1972; Gordesky and Mannetti, 1973). If the fluidized synaptosomal membrane enhances the diffusion of Lilly 54761, methohexital may have an "ordering" effect on the macrophage membrane having more protein in it. Therefore, the "ordering" of the macrophage may enhance the "A" type inhibitor, Lilly 51641, which is very effective in PEC while restricting the "B" type inhibitor, Lilly 54761. The amount of the protein present in a lipid bilayer exert additional influence on the fluidizing efficacy of anesthetics. Halothane which also fluidizes the bilayers has been reported to "order" rat brain synaptic membranes and consequently, it would seem likely to alter permeability (Rosenberg, et al., 1975).

It has been generally supposed that general anesthetics act in a nonspecific way to fluidize any biological membrane (Miller and Pang, 1976). Anesthetics have been shown to increase the permeability of some substances such as sucrose-triss and chloride (Miller and Hunter, 1970) while inhibiting the permeability of others such as phospholipid vesicles and valinomycin (Johnson and Gangham, 1969). Anesthetics have been described to both increase and decrease permeability of cations
through "fluidized" membranes (Lillie, 1923; Bangham, et al., 1965). The Na⁺/K⁺ ATPase of red blood cells and synaptosomes is unaltered at high, almost lytic doses of some anesthetics (Hale, et al., 1972; Levitt, 1975). Therefore, it is feasible that in the macrophage, methohexital can affect the selective permeability of each of the monoamine oxidase inhibitors, either to enhance the diffusion of one, Lilly 51641 or hinder the diffusion or block the enzymatic site for the other, Lilly 54761. Methohexital may in turn affect the synaptosomal membrane by enhancing the diffusion of only one inhibitor, Lilly 54761, and at the same time have no effect on the other, Lilly 51641.

Because of the characteristic composition of intact brain mitochondrial membranes, methohexital may have no effect on the diffusion of either MAO inhibitors.

An alternative explanation for methohexital's effects on the pattern of inhibition produced by the Lilly compounds, is that the barbiturate may act as a chaotropic agent by disrupting the delicate transport processes of the membrane or the structure of MAO. Chaotropic agents such as KSCN, KNO₃ and urea are known to significantly inhibit monoamine oxidase (Banerji, et al., 1977). It is possible that methohexital, being a lipophilic compound, is further enhancing the inhibition of the MAO inhibitors by either changing the sensitivity of the enzyme for the inhibitor or the substrate when it is in combination
with a Lilly compound. Presumably, methohexital would act indiscriminately as a chaotropic agent and therefore opposite effects observed in these preparations are difficult to explain.

EFFECT OF HYPERBARIC CONDITIONS ON BARBITURATE ALTERATION OF MAO INHIBITION

Lever, et al. (1971) have reported that hyperbaric conditions reverse the anesthetic action induced by pentobarbital in rats. The results obtained in each of the pressurized preparations confirm the findings of the before mentioned investigators. Helium pressure of 100 atmospheres reversed all anesthetic-induced alterations in monoamine oxidase inhibition by the Lilly compounds.

The pressure reversal of methohexital-induced enhancement of Lilly 54761 in synaptosomes and possibly lysed brain mitochondria under normobaric conditions can be explained by pressure restoring "order" to the fluidized membrane even in the presence of an anesthetic concentration of the barbiturate. However, this concept is not in accord with the explanation offered for the effects of methohexital on the PEC, since methohexital itself presumably "orders" the membrane with regard to Lilly 54761. If methohexital "ordered" the macrophage membrane such that it caused the inhibitory action of Lilly 51641 to be enhanced under normobaric conditions while restricting Lilly 54761, it seems contrary to the literature that pressure could "order" the membrane, causing increased permeability of Lilly 54761 to account for
the antagonism of inhibition under normobaric conditions. No direct explanation for this phenomenon is available, however, it is possible that in this cell type, methohexital has the ability to alter transport of the inhibitor. A precedent for this was established by Miller and Pang (1976) indicating that the barbiturate, pentobarbital, can either fluidize or "order" a biological membrane depending on its composition. Subsequent experimental evidence under hyperbaric conditions in our laboratory indicates that this is probably not the case, but rather that methohexital increased membrane fluidity and that this increase has an opposite effect on the transport mechanism of the two MAO inhibitors. It appears that a conclusion of pressure reversal can still be held despite the fact that identical hyperbaric conditions significantly increased monoamine oxidase activity in all control reaction. This is based on the fact that the control curves remain in the same previous relationship with each other as can be seen in the intact brain mitochondria (Figures 20 and 24). Therefore, pressure has overcome the methohexital-induced alterations in spite of the increase of activity per se brought about by pressure. Conversely, pressure may be "ordering" all the membrane preparations and therefore increasing the permeability of not only the substrate and methohexital, but also of the MAO inhibitors which would account for the large difference of effects of the inhibitors from controls in the synaptosomal preparation (Figure 21). The pressure effect may then be of a greater magnitude to mask the pressure reversal if it exists.
MAO ACTIVITY VARIATIONS WITH INCREASING BIOLOGICAL COMPLEXITY OF THE MEMBRANE PREPARATIONS

A correlation can be drawn when the results of the various brain preparations are compared in sequence of increasing number of membranes involved in substrate diffusion, i.e. intact mitochondria, synaptosomes, and tissue slice. Although the "B" type MAO inhibitor, like methohexital, was indifferent to the enzyme preparation employed based on ID\(_{50}\), incremental logarithmic doses of the "A" type inhibitor were necessary to demonstrate the same level of inhibition when the complexity of the membranes was increased from isolated mitochondria to synaptosomes to tissue slice. This suggests either that Lilly 51641 ("A" inhibitor) had more difficulty in diffusing through the membranes than either methohexital or Lilly 54761 ("B" inhibitor) or that the "A" isoenzyme is in some way less accessible to its inhibitor than is the "B" isoenzyme.

EVIDENCE FOR THE MECHANISM OF METHOHEXITAL'S ACTION

The series of experiments conducted to probe the reversibility of the enzymatic inhibition produced by either methohexital or the MAO inhibitors may provide an explanation for the weak inhibition of MAO by methohexital. The inhibitory effect of methohexital on intact brain mitochondria could be overcome by washing, which was not the case for either of the MAO inhibitors. From these data, it is presumable that
the inhibitors react in a different manner than methohexital. Metho­
hexital probably alters the mitochondrial membrane to affect monoamine
oxidase inhibition by the inhibitors whereas these latter bind cova­
ently to the enzyme. It is also possible that methohexital may be
weakly bound to MAO and easily modified by pressure.

Eluting the monoamine oxidase bound to its membrane from DEAE
cellulose with nonionic detergent, Triton X-100 abolished the
multiplicity of rat liver mitochondrial monoamine oxidase while having
minimal effects on total activity (Houslay and Tipton, 1975). The
decreased sensitivity to inhibitors such as chorgyllne or deprenyl, in
preparations rendered soluble by sonication and detergent treatment,
result from extracting different amounts or types of lipid material
being bound to the enzyme (Tipton, et al., 1976).

MONOAMINE OXIDASE ACTIVITIES SEEN IN TEST TISSUES

Fuller, et al. (1972) reported that type "A" monoamine oxidase
appears to be the predominant enzyme in mouse and rat brain, while "B"
type MAO prevails in the liver of these same species. The 20 minute
MAO assays on intact brain mitochondria indicated that its monoamine
oxidase was approximately ten-fold more sensitivie to "A" type inhibitor,
Lilly 51641 than the "B" type Lilly 54761, conforming to Fuller's findings
on whole brain homogenates. The results of the synaptosomal and liver
slice preparations, however, agree with more recent findings of Tipton,
et al. (1975) indicating both types are present in approximately equal quantities in guinea pig whole brain and liver homogenates.

In very few model systems is there a single monoamine oxidase isoenzyme. Currently, human blood platelets are a good model system for type "B" MAO whereas a line neuroblastoma cells are a model for type "A" monoamine oxidase. The MAO assay results from peritoneal exudate cells presented evidence for the macrophage as being a second and more easily obtainable model for type "A" MAO. The monoamine oxidase present in PEC was approximately 1000 to 10000 times more sensitive to type "A" inhibitor than that of the "B" type.

**CORRELATION BETWEEN MAO ACTIVITY AND MIGRATION**

Monoamine oxidase inhibitory doses did not relate to doses inhibiting migration of peritoneal exudate cells. Concentrations of methohexital producing complete inhibition of PEC migration inhibited MAO by only 50 percent therefore, no causal relationship between MAO activity and migration is possible. This conclusion was essentially confirmed with the Lilly compounds which demonstrated results opposite to methohexital in that MAO activity could be inhibited by 50 percent without the drugs significantly altering migration. The literature indicates that catecholamines, probably in association with changes in cyclic nucleotides, influence macrophage migration per se and oppose macrophage inhibitory factor (MIF) activity (Koopman and David, 1971;
Pick and Manheimer, 1972). This latter, i.e. antagonism of MIF is dose-dependent. The data presented suggests that migration and catecholamine biotransformation are not causally related.

PROPOSED EXTENSIONS OF THE PROBLEM

A number of unanswered questions and potential extension of the problem have been created by the data presented herein. To answer one of the initial proposals, whether different membranes are differently affected by anesthetics and pressure, experiments using halothane would provide a more suitable anesthetic than methohexital, because apparently it "fluidizes" membranes universally in contrast to methohexital. Evidence has been given that "anesthetics" affect the activity of a membrane bound enzyme, which are reversible by pressure. It would be of interest to study whether non-anesthetic agents have a similar effect, and whether these effects are reversible by pressure. Experiments carried out using Triton X-100 or another chaotropic agent which are known to inhibit MAO would be of interest because these agents would irreversibly alter the membrane geometry as well as the MAO binding sites. Experiments carried out using labeled selective substrates for either type "A" (serotonin) or type "B" (benzylamine) are needed to confirm type "A" MAO as being the prevailing type in PEC. Experiments using the herein model carried out with more selective tissue from the brain such as the reticular activating system may possibly show membranes highly susceptible to anesthetics and subsequent pressure
reversal as were cortical synaptosomes. The question whether a membrane bound enzyme used as a marker for studying anesthetic effects on membranes provides a valued model is still in question, however, the evidence presented herein gives supportive evidence confirming the membrane expansion theory as the mechanism of anesthesia.
SUMMARY AND CONCLUSIONS

The results of these experiments demonstrate that methohexital is a relatively weak inhibitor of monoamine oxidase in liver and brain slice, whole and lysed brain mitochondria, synaptosomes, peritoneal exudate cells compared with the irreversible monoamine oxidase inhibitors, Lilly compounds 54761 and 51641. These drugs inhibited monoamine oxidase under one atmosphere as well as 100 atmospheres; however, no reversal of the inhibitory action was observed with either inhibitor alone under hyperbaric conditions. These results along with the experiments that showed reversal of the methohexital effect by washing, suggest that methohexital may either act on the membrane itself rather than on the enzyme directly to bring about MAO inhibition or that methohexital is bound to MAO with less affinity.

When methohexital was used at anesthetic concentrations in combination with an inhibitory concentration of the monoamine oxidase inhibitors, the barbiturate quantitatively altered the pattern of inhibition caused by the irreversible inhibitors, Lilly 51641 and 54761. Noninhibitory concentrations of methohexital enhanced the inhibition of the "B" type inhibitor in lysed brain mitochondria and synaptosomes as well as that of the "A" type inhibitor in peritoneal exudate cells, while having no effect on either brain slice or intact brain mitochondria.
The inhibition by Lilly 54761 in peritoneal exudate cells was antagonized by methohexital. The various selective permeability alterations observed may be in agreement with Miller and Pang's (1976) work which indicated that barbiturates can either fluidize or "order" biological membranes depending on their composition.

Helium at 100 atmospheres reversed all the anesthetic-induced alterations present under normobaric conditions. Despite the fact that pressure alone increases monoamine oxidase activity, it affected the Lilly inhibitory action to such a degree that the reversal phenomenon is very likely to be real.

Monoamine oxidase activity in peritoneal exudate cells was considerably more sensitive to the inhibition by the type "A" inhibitor. These results may depict PEC as a suitable model of the isolated type "A" monoamine oxidase isoenzyme. This isoenzyme prefers serotonin as its substrate. Attempts to correlate MAO activity with macrophage migration offered no causal relationship between catecholamine metabolism and migration.

In conclusion, it has been demonstrated that membrane alterations and their pressure reversal brought about by methohexital can be studied at the cellular and subcellular levels using monoamine oxidase as a membrane marker. The significance of this model is that it is a useful tool in confirming the fluidized lipid hypothesis as an explanation for the possible mechanism of action of general anesthetics.
BIBLIOGRAPHY


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