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THE INFLUENCE OF METHOHEXITAL SODIUM AND HALOTHANE
ON MITOCHONDRIAL MONOAMINE OXIDASE ACTIVITY UNDER
NORMOBARIC AND HYPERBARIC CONDITIONS

DISSERTATION

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

By

Joel Milton Weaver, II, B.S. Ph., D.D.S.

* * * * *

The Ohio State University
1976

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To my profession, and especially to those areas relating to pain and anxiety control, I dedicate my life's future work.
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INTRODUCTION

Literature Review

I. Theories of General Anesthesia -- Mechanisms and Sites

Although the state of general anesthesia has been recognized for well over a century (Erving, 1933), the fundamental mechanism involved in its production is still unknown. There have been many hypotheses and theories concerning that mechanism throughout the literature, but no single one has been supported with sufficient evidence to merit distinction as the fundamental cause and effect relationship of the general anesthetic agent on a biological system.

The more recent discovery of the chemically inert gas xenon as an anesthetic agent (Cullen and Gross, 1951) has all but eliminated the popularity of purely chemical theories of general anesthesia, and it has brought to the forefront those which involve physical or physico-chemical aspects.

The physico-chemical hypotheses and 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. The investigations of Miller (1961) and Pauling (1961, 1964), who worked independently, led to the theory that some form of hydrate
microcrystals appearing in the aqueous layers of nervous tissue results in general anesthesia. Other investigators found no correlation between the solubility of gases in water and their anesthetic potency (Miller, et al., 1965). Most authorities believe that the critical phase for anesthetic action is not aqueous but rather is non-aqueous (Miller, et al., 1965; Miller, et al., 1967; Eger, et al., 1969).

Considerably more research has been done in the area of the lipid layers as the site of action of general anesthetics. Very early, 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. Later, K. Meyer (1937) suggested that "... narcosis commences when any chemically indifferent substance has attained a certain molar concentration in the lipids of the cell ... depend(ing) on the nature of the animal or cell but ... independent of the narcotic".

Using principles of thermodynamics, Mullins (1954) developed a physical theory of narcosis which involved the formation of a stabilized lattice structure in the non-aqueous phase 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 size of the anesthetic. This tends to support the possibility that changes in the dimensions of cell membranes by anesthetics are the basis for the mechanism of action of general anesthesia.
The critical volume hypothesis, as it is presently named (Miller, et al., 1973), states that anesthesia occurs when the volume of the hydrophobic region is expanded beyond a definite critical volume by absorption of inert molecules. This expansion of a biological membrane causes an increase in its fluidity, which may subsequently cause some permeability change to result in interference with normal physiological processes. Thus, the state of general anesthesia is achieved (Miller, et al., 1973).

Although structural knowledge of cellular membranes is still incomplete, much has been learned about them within the past 50 years (Douglas and Zuckerman, 1976). Most membranes are thought to exist as a mosaic combination of fluid lipids and globular proteins. The lipids are generally arranged in a bimolecular layer, with the hydrophobic ends of the molecules facing each other at the center of the membrane and the hydrophilic ends facing the membrane surfaces. These inner and outer membrane surfaces are covered by a layer of globular protein (Davson and Danielli, 1935).

Danielli (1936) suggested that the globular proteins changed in conformation as they contacted the lipid portion of the membrane. This change caused the protein to "unfold" and resulted in a continuous protein sheath. This was thought to provide strength and stability for the membrane (Fig. 1).
FIGURE 1

Classical Membrane Structural Model by Danielli.

The inner lipid bilayer and the outer layer of globular proteins are separated by "unfolded" proteins.
The melting transitions of the lipids are within normal physiologic temperature ranges. This fact suggests 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 (1973).

II. Influences of Pressure on Anesthetic Action

In 1942, it was reported that the inhibition of bacterial luminescence by alcohol, urethane, and other anesthetic agents could be reversed by a high hydrostatic pressure applied to the system (Johnson, et al., 1942a; Johnson, et al., 1942b). Johnson and Eyring (1948) postulated that anesthetics cause a reversible protein denaturation. The increased volume of the protein was predicted to be reversed by pressure. Johnson and Flagler (1950) reported that tadpoles of Rana sylvatica, which were anesthetized in 3 to 6 percent alcohol at 22° 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 per square inch (p.s.i.). 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.
Similar experiments (Lever, et al., 1971) were conducted with the anesthetic agents $N_2$, SF$_6$, pentobarbital Na, ether and halothane in Italian crested newts (*Triturus cristatus ornifex*) and mice. 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. Calculations made by Brink and Posternak (1948) estimated that the critical anesthetic concentration in the lipids is approximately 0.05 M, and the partial molar volumes of the anesthetics are between 50 to 100 cc (Walkley and Jenkins, 1968). Lever, et al. (1971), believed that the change in the lipid portion of the membrane could be transmitted to a functionally vital protein and make it unable to function properly. The end result would be the state of general anesthesia. An expansion of the lipid in this instance would be approximately 0.4 percent, and the calculated pressure of approximately 100 atmospheres would reverse this expansion process. They further predicted that the expansion of the lipid phase by hydrogen gas would be exactly reversed by the pressure necessary to produce hydrogen solution in the lipid. Thus hydrogen would be an excellent gas for reversing anesthesia. However, Brauer and Way (1970) demonstrated that narcosis could be produced in mice exposed to 130 atmospheres of hydrogen.

It was suggested (Johnson and Bangham, 1969; Johnson and Miller, 1970) that disordering or distortion caused by anesthetics could reduce
the control or alter the permeability and function of cellular membranes. The application of pressure could restore membrane control and thus reverse the anesthetic state. This is supported by the fact that anesthetics increase the cation permeability of lysosomes by increasing the freedom of motion of the lipid molecules, especially at the aqueous-lipid interface (Johnson and Bangham, 1969). 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, other 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).

Miller (1974a) stated that although it seems clear that anesthetics act at some non-polar 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, it is a physical rather than a chemical interaction at some biological site which produces anesthesia. Since the strength of this interaction depends 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 erythrocyte 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 MAC of the anesthetic agents. This eight percent reduction in hemolysis was accounted for by a 0.4 percent expansion in surface area.

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 changing the lateral phase separation-controlled environment of the nervous membrane solvated proteins necessary for nervous function. A second finding 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, P., 1891; Cattell, 1936; Lever, et al., 1971).

Thermodynamic analysis showed that the inhibition mechanism of anesthetics on firefly extract is identical to the reversible thermal inactivation of the enzyme. Thus the original folded luciferase becomes unfolded by the expansion caused by the anesthetic agent at the enzymes' hydrophobic sites (Ueda and Kamaya, 1973).

A controversy has arisen concerning the anatomical location of the anesthetic-induced blockade of the neuron. One hypothesis states that anesthetic actions inactivate lipoproteins essential for synaptic transmission (Eyring, et al., 1973). In experiments using humans anesthetized with thiopental, nitrous oxide–oxygen and halothane, no significant change in ulnar nerve conduction velocity was noted before and during the anesthesia (Thornton, et al., 1968). Another hypothesis, however, suggests that the reversible anesthetic-induced blockade 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).
III. Mitochondria and Anesthetics

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., 1974b). 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 on mitochondrial state 3 glutamate oxidation by a combination of nitrous oxide and halothane is additive (Nahrwold and Cohen, 1973). However, some investigators believe that depression of respiration does not predict in vivo anesthetic activity (Nahrwold, et al., 1974a).

Monoamine oxidase has been shown to be a reliable marker for liver mitochondria (De Champlain, et al., 1969), especially in isolated outer membrane preparations (Schnaitman, et al., 1967; Schnaitman and Greenawalt, 1968; Greenawalt and Schnaitman, 1970). 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 permeability or
accessibility of substrate to the enzyme in the presence of anesthetic-
induced membrane volume expansion, disorientation or fluidity. Likewise,
Izumi, et al., (1967; 1969) and Youdim and Sandler (1968) showed 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
isometric tension response of isolated cutaneous canine vein preparations
to exogenous or endogenous 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 release of norepinephrine, possibly by reducing the influx of
calcium ions which are necessary for nerve-stimulated release of norepi-
nephrine 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 proposi-
tion was supported by their observation that halothane did not increase
the response to tyramine when monoamine oxidase was 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. 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). Guinea pig liver mitochondria are useful for several reasons. The size of the liver is appropriate for the yield of fresh, whole mitochondria needed for many types of experiments. Guinea pig liver mitochondrial MAO exhibits exceptional thermal stability necessary for prolonged incubations at 37°C (Costa and Greengard, 1972b). Tryptamine is often useful since it is a more universal substrate than serotonin for the multiple forms of monoamine oxidases (Shih and Eiduson, 1971).

IV. High Pressure Neurological Syndrome

Effects of Pressure Alone

In studies involving the high pressure reversal of the general anesthetic state, unanesthetized control animals exposed to pressure exhibited a high pressure neurological syndrome, characterized by hyperexcitability, tremor, convulsions and death. Furthermore, the addition of anesthetic agents, such as nitrogen or pentobarbital, blocked the onset of tremor and convulsions and elevated the lethal pressure (Lever, et al., 1971).
Hyperexcitability and tremor occurs in man at approximately 20 atmospheres of pressure (Barnard, 1972). Convulsions occur in experimental animals, including primates, at pressures of 60 to 100 atmospheres. Consequently, man has been unable to surpass the pressure barrier of approximately 60 atmospheres while breathing a helium and oxygen mixture (Miller, 1974b). (Nitrogen is eliminated from the breathing mixture, because it is a narcotic.)

The rate of compression influences the convulsive threshold in mice (Beaver, et al., 1975). Slower rates apparently allow the animal to adapt, and thresholds are elevated. This effect is first apparent in newborn mice at 12 days of age. Reserpinized mice lose this ability to adapt to slow increases in pressure at about this same age. The initial change is seen at day 8 and is well defined at day 12.

Monoaminergic Interrelationships

Reserpine, which tends to deplete the brain of serotonin and other monoamines, lowers the convulsive threshold of high pressure in squirrel monkeys and mice, although high doses of reserpine are anticonvulsant (Beaver, et al., 1975). This latter possibly relates to its tranquilizing and sedative properties similar to the more commonly used anticonvulsant drugs. Tranylcypromine, a monoamine oxidase inhibitor which tends to increase monoamines including serotonin, blocks this convolution threshold-lowering effect when given prior to low doses of reserpine. Amphetamine, which blocks the neuronal re-uptake of monoamines and
therefore increases amine concentration at receptor sites, reverses the reserpine effect.

Experiments with whole animals have revealed that anesthetics, analgesics, anticonvulsants, and monoamine oxidase inhibitors lead to increases in serotonin levels in the brain (Bonnycastle, et al., 1962). Para-chloro-phenylalanine (PCPA), which decreases serotonin by inhibiting tryptophan hydroxylase (Fig. 2), has been shown to decrease the anticonvulsant action of trimethadione in animals given the convulsant pentylenetetrazole (Diaz, 1976). PCPA has been known to prolong post-decapitation convulsions in mice (Myslinski, et al., 1976). Conversely, Lilly 110140 (3-\{p-trifluoromethylphenoxy\}-n methyl-3-phenylpropylamine), which prevents neuronal uptake of serotonin, has been shown to increase the anticonvulsant action of trimethadione (Diaz, 1976).

These data support the possibility that drugs or processes which bring about decreases in serotonin content in the brain may be responsible, at least in part, for the production of convulsions and/or for their antagonism of anticonvulsant agents. Other drugs or processes which lead to increases in serotonin content or availability at receptor sites in the brain may be anticonvulsants or add to anticonvulsant effects of other agents.

Serotonin levels in the brain have many other interesting correlations with drug effects. Rats that are fed a tryptophan-poor diet have decreased levels of serotonin, and are more sensitive to electroshock-induced painful stimulation (Lytle, 1976). When these rats are then
FIGURE 2

Typical Metabolic Pathway for the Monoamine, Serotonin.
allowed adequate amounts of tryptophan, serotonin levels and electroshock thresholds return to control values. Other animals on tryptophan-deficient diets show even greater pain sensitivity when given PCPA, which further lowers serotonin levels, or show analgesia when given Lilly 110140, which increases serotonin levels (Lytle, 1976). Furthermore, tryptophan-deficient rats do not demonstrate an analgesic response to morphine (Lytle, 1976).

The neutral amino acids such as valine, phenylalanine, tyrosine, leucine and isoleucine normally compete with tryptophan for uptake into the brain. Excess quantities of any one of these amino acids will essentially block the uptake of the others. Rats which were injected with valine, phenylalanine, or leucine have been shown to have reduced tryptophan levels in the brain, and consequently reduced levels of serotonin. These rats were predictably more sensitive to electroshock-induced painful stimulations than controls (Messing, et al., 1976).

Another study indicated that both control and morphine-injected rats become more sensitive to electroshock-induced painful stimulation when exposed to a high pressure environment (Tofano and DeBoer, 1975). These data suggest that the serotonin levels in the brain, or in some particular area of the brain, correlate with thresholds of analgesia, and that pressure can alter these analgesia thresholds.

In summary, general anesthetics are known to be associated with increases in brain levels of serotonin, and high pressure can reverse the anesthetic state. Increased analgesia is associated with increases
in serotonin, and decreased analgesia is associated with decreases in serotonin. Pressure reverses analgesia and promotes hyperalgesic states. Anticonvulsants are associated with increases in serotonin; high pressure not only reverses anticonvulsant action but also produces convulsions.

**STATEMENT OF PROBLEM**

Since many investigators believe that the anesthetic effect on biological membranes is not merely restricted to nervous tissue but is rather a generalized anesthetic-membrane phenomenon, it seems appropriate that almost any available biological membrane for which a marker of permeability exists could be useful for the elucidation of anesthetic and pressure-induced membrane interactions. The mitochondrion contains considerable stores of monoamine oxidase in its outer membrane, and this enzyme has been used as a marker for the presence of that membrane. Monoamine oxidase, as a membrane-bound enzyme, may be useful for the determination of anesthetic-induced changes in permeability to radioactive substrate. These alterations should be easily detected by measuring similar changes in radioactive product formation. Pressure-induced alterations in substrate permeability should also be detected by this procedure.

The presence of an intact outer mitochondrial membrane may be necessary to detect membrane permeability alterations. Lysed or broken
outer membranes may contain monoamine oxidase which has free accessibility to substrate via ruptured areas or broken, open ends.

Therefore, if general anesthetics increase the fluidity and decrease the substrate permeability in the intact outer mitochondrial membrane, product formation would be decreased. Likewise, if high pressure decreases membrane fluidity and increases its permeability, in spite of the presence of anesthetic agents, the product formation by the enzyme should increase.
METHODS AND MATERIALS

I. Mitochondrial Isolation

Our procedure for isolation of mitochondria was patterned after Schneider and Hogeboom (1950) but was sufficiently modified to warrant a full description (Fig. 3). All solutions, glassware, and tissue are maintained at 0° to 4° C. Each unanesthetized male guinea pig weighing between 400 and 600 grams was sacrificed at 8:30 a.m. by decapitation or cervical dislocation. The liver was quickly removed, weighed, and chilled on ice. The tissue was minced with sharp scissors and then mixed with a solution containing 0.25 M sucrose, 0.01 M tris HCl, and 0.001 M EGTA (pH -- 7.8). Approximately 9.0 cc were used per gram of liver, and homogenization was then accomplished by using five strokes with each of two pestles in a Dounce tissue homogenizer.

The homogenate was then placed in a refrigerated centrifuge and spun at 800 x g for ten minutes. The resulting pellet was discarded, and the supernatant centrifuged at 10,000 x g for ten minutes. This pellet was resuspended in 40 cc of sucrose-tris-EGTA (pH -- 7.5) with the use of a teflon-glass hand homogenizer and centrifuged at 35,000 x g for ten minutes. The light fluffy layer above the tan mitochondrial pellet was removed by rinsing several times with sucrose-tris (0.25 M sucrose- 0.01 tris HCl, pH -- 7.5). The mitochondria were resuspended
FIGURE 3

Flow Diagram of Intact Mitochondrial Isolation Procedure.
FLOW DIAGRAM OF MITOCHONDRIAL ISOLATION

LIVER HOMOGENATE (9.0cc sucrose-tris* - EGTA, per gram of tissue)

\[ 800 \times G \times 10' \]

- **Pellet**: RBC's, cell walls, nuclei
- **Supernatant**: Mitochondria, Microsomes

\[ 10,000 \times G \times 10' \]

- **Pellet**: Mitochondria
- **Supernatant**: Microsomes

\[ 35,000 \times G \times 10 \]

- **Pellet**: Mitochondria
- **Supernatant**: Contaminants

\[ 35,000 \times G \times 10' \]

- **Pellet**: Resuspended in 5.0cc of sucrose-tris
- **Supernatant**: Contaminants

*Tris (Hydroxymethyl) Aminomethane
in the sucrose-tris and centrifuged at 35,000 x g for ten minutes. The final pellet was then resuspended in 5.0 cc of sucrose-tris.

The mitochondrial suspension was assayed for protein content by the biuret method similar to that of Jacobs (Jacobs, et al., 1956). An aliquot of the resuspended pellet was diluted, one part in five, with sucrose-tris, and 0.1 cc of this was added to each of three tubes. A like volume of sucrose-tris was also added to each of two control blanks. To each tube was added 0.2 cc of 20 percent Triton-X 100 solution, followed by 1.2 cc of water and 1.5 cc of biuret reagent. 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 mp, and the protein content was calculated as follows:

\[
\frac{\text{optical density}}{0.095} \times \frac{10}{1} \times \frac{5}{1} = \text{mg protein/cc}
\]

The fresh whole mitochondrial suspension used for monoamine oxidase assays was then adjusted to 0.10 mg protein/cc by dilution with sucrose-tris solution.

II. Mitochondrial Outer Membrane Isolation

The isolation of the outer membrane of the mitochondria (Fig. 4) was a slight modification suggested by Doctor C.D. Stoner of his own method (Stoner and Sirak, 1969). Mitochondria were prepared in the
FIGURE 4

Flow Diagram of Isolated Outer Mitochondrial Membrane Procedure.
FLOW DIAGRAM OF MITOCHONDRIAL OUTER MEMBRANE ISOLATION

**MITOCHONDRIAL SUSPENSION**

Layered over 1.2 M sucrose - 5mM Na-pipes*  
PH 7. -  
100,000 X G X 1 hour

---

Pellet  | Supernatant  
Mitochondria | Microsomal Contaminants

Resuspend  
0.25 M sucrose - 5mM Na-pipes (pH 7.0)

To each 0.1cc of Mitochondria Suspension, add 8.9 cc of  
25 milliosmolar sucrose - 5mM imidazole acetate.  
PH 7.0

INCUBATE 15' on ice

Add 1cc of 218 milliosmolar sucrose  
100,000 X G X 1 hour

---

Pellet  | Supernatant

Resuspended in  
0.25 M sucrose - Na-pipes

Place over continuous gradient ranging from 0.5 M to  
2M sucrose - 0.5mM Na-pipes - 0.034% heparin

Centrifuge 70,000 X G X 1 hour

---

Colored band containing MAO activity removed from top of gradient column and suspended in 0.25 M sucrose - 5mM Na-pipes

*Piperazine -N-N' -bis [2-ethane Sulfonic Acid]
usual manner, and the concentration of protein standardized by the biuret assay. The resulting suspension was layered over a 1.2 M sucrose solution buffered with sodium-pipes (Fig. 4) \{5 \text{ mM, pH} = 7.0\} and centrifuged at 100,000 \times g for one hour to remove any possible contaminants. Resuspension was accomplished in 0.25 M sucrose buffered with sodium-pipes (5 mM, pH = 7.0), and 0.1 cc of this was added to 8.9 cc of 25 mM sucrose solution buffered with imidazole acetate (5 mM, pH = 7.0). This was incubated in an ice bath for 15 minutes, followed by the addition of 1 cc of 218 mM sucrose. The preparation was then centrifuged at 100,000 \times g for one hour. The resulting pellet was resuspended in 0.25 M sucrose buffered with sodium-pipes (5 mM, pH = 7.0) and placed over a continuous sucrose gradient, ranging from 0.5 M to 2.0 M of sucrose buffered with 5 mM sodium-pipes, pH = 7.0 and 0.034 percent heparin. This was then centrifuged at 70,000 \times g for one hour. The outer membranes were in the thin layer at the suspended-pellet-sucrose gradient interface.

The monoamine oxidase assay presented below was used to standardize the activity of this preparation to the same range as that of the whole mitochondria suspension. Standardization was necessary to ensure that excess substrate was always present during the reaction.

The quality of the outer mitochondrial membrane preparation was confirmed not only by the presence of MAO activity but also by electron microscopy and respiratory studies.
III. Mitochondrial Respiratory Studies

An aliquot of the mitochondrial suspension was adjusted to 2.0 mg protein/cc with sucrose-tris buffer. A Yellow Springs Instrument Company Model 5331 Oxygen Probe was utilized for mitochondrial respiratory studies in conjunction with a Sargent-Welch Model S R G Recorder. To the oxygen electrode chamber was added 1.1 cc of respiratory medium consisting of:

- 75 mM KCl
- 50 mM tris-HCl
- 12.5 mM K2HPO4
- 5 mM MgCl2
- 1 mM EDTA

Then 50 µl sodium succinate (0.2 M) was added, followed by 100 µl of mitochondrial suspension. State four respiration, which represents the idling state of mitochondrial respiration, was observed in the recording. Upon the addition of 3 µl of 0.1 M ADP, active State 3 respiration occurs. When the acceptor (ADP) is completely phosphorylated, the mitochondrial respiration returns to State 4.

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 in the range of 3 to 5 (Sordahl, et al., 1971).
IV. **Electron Microscopy**

Electron microscopy was performed on both the mitochondrial pellet and the lysed mitochondrial outer membrane pellet. Fixation was accomplished in phosphate buffered glutaraldehyde (pH -- 7.3) for 30 minutes. The pellet was then rinsed in phosphate buffer and post-fixed for one hour in osmium tetroxide. Dehydration occurred in one-half hour steps in consecutive ethanol concentrations of 50 percent, 70 percent, 95 percent and 100 percent (absolute). The pellet then was treated for one-half hour in propylene oxide, followed by one hour in 50 percent propylene oxide and 50 percent Spurr low viscosity imbedding medium, and finally for one hour in 100 percent Spurr medium. Polymerization was allowed to take place for a minimum of eight hours in an oven at 70°C. This was then sectioned with a Dupont diamond knife in a Sorvall Porter-Blum MT-1 Microtome to a 600 to 900 Å thickness. These sections were picked up on 300 mesh copper grid, stained with uranyl acetate and lead citrate, and viewed through a Philips 300 electron microscope.

V. **Monoamine Oxidase Assay**

The monoamine oxidase activity assay was a slight variation of that developed by Nagatsu (1973) {Fig. 5}. Reagents include the following:

- Tryptamine-C¹⁴ bisuccinate (0.05 mCi/0.5 cc)
- Tryptamine HCl solution with above C¹⁴ isotope (0.1 μCi/50 λ and 51 mM/50 λ)
FIGURE 5

Monoamine Oxidase Assay Procedure.
MONOAMINE OXIDASE ASSAY

TRYPTAMINE

\[ ^{14}C \text{CH}_2\text{-CH}_2\text{-NH}_2 \xrightarrow{\text{MAO}} \text{INDOLE} \]

\[ \text{ACETALDEHYDE} \xrightarrow{\text{Aldehyde dehydrogenase}} \text{INDOLE} \]

\[ \text{ACETIC ACID} \]

EXTRACTED AND COUNTED

EXTRACTED AND COUNTED
HCl (2N)

Toluene scintillation fluid PPO \{2,5-di-(phenyloxazolyl)\} 4 gm and POPOP \{1,4-di-2-(5-phenyloxazolyl) benzene\} 0.05 gm dissolved in 1 liter toluene

Toluene

Sucrose-tris

Phosphate buffer

To each test tube was added 150 µl of phosphate buffer, 50 µl of stock radioactive tryptamine solution and 100 µl of a fresh, whole mitochondrial suspension which was adjusted to a protein content of 0.1 mg/cc. The isolated outer mitochondrial membrane assays also utilized 100 µl of the membrane suspension which was previously assayed and diluted to activity similar to whole mitochondrial monoamine oxidase activity. Blank control tubes were used in the assay by substitution of 100 µl of a mitochondrial suspension which had been previously inactivated by heating for 40 minutes at 98° C. Water at 37° C was also added to another tube which was utilized for the temperature sensing device of the incubation bath inside the pressure chamber.

All tubes were incubated at 37° ± 0.25° C for 20 minutes, then placed in an ice bath to stop the reaction immediately. Two hundred microliters of 2N HCl were added to acidify the product which was then extracted by adding 6.0 cc of toluene and vortexing for 15 seconds. Each tube was centrifuged for ten minutes at low speed which produced an adequate organic solvent-water separation. A 3.0 cc aliquot of the
toluene layer was pipetted into a vial containing 10 cc of scintillation fluid and was then placed in a Packard Tri-Carb scintillation spectrophotometer.

The counting efficiency of the Packard Tri-Carb was accomplished as follows: 0.1 cc of a standard toluene-\textsuperscript{14}C solution and 2.9 cc of toluene were placed in a vial containing 10 cc of scintillation fluid. The vial was placed in the counter, and after one hour temperature equilibration, the counts per minute were determined and compared to the known disintegrations per minute of this standard. The ratio of these two numbers provided an efficiency of 87.5 percent. The calculation of the amount of product formed was as follows:

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

\[
\{\text{Tryptamine (51 nM)}\} \ \text{nM} \times \frac{1 \text{ minute}}{20 \text{ minutes}}
\]

The product formed and extracted is indole acetaldehyde and/or indole acetic acid, depending on the presence or absence of the aldehyde dehydrogenase enzyme necessary to form the acid. The nature of the product(s) is not important since they are both radioactive and extracted by toluene (Nagatsu, 1973). To discount the possibility of substrate contamination of the product, the results of the blanks are subtracted from those of the reaction tubes.
Some kinetic factors of our MAO assay were studied in preliminary experiments which revealed that the concentration of substrate was at least ten times more than the standardized enzyme could biotransform in 20 minutes. Thus, the amount of product formation compared to the excess of substrate was minimal, and any change in product formation should be a result of some action on the enzyme or its associated membrane and not that of product feedback inhibition or substrate limitation. Furthermore, the amount of product formed was directly proportional to time, as was determined at five minute intervals during these initial procedures.

The purity of the tryptamine substrate and MAO product was determined by thin layer chromatography on silica gel G plates with n-butanol, acetic acid, and water (25:4:10 v/v/v) as the developing system and iodine vapors for localization under ultraviolet light. Radiochromatograms were then made from the plates with a Baird scanner. The silica gel was subsequently removed in 0.5 cm segments and counted by the liquid scintillation procedure. The results verify the high purity of the substrate and indicate that the only detectable contamination of the product of the MAO reaction is a very minute amount of tryptamine which is compensated for by subtraction of the boiled blank from the reaction.

VI. The Pressure Chamber

The chamber is a custom-made device which was machined from a solid brass cylinder (Fig. 6). Its dimensions are as follows:
FIGURE 6

Pressure Chamber.

Arrows indicate circulation of heated water for internal heat exchanger. Above the exchanger is the internal temperature sensor.
<table>
<thead>
<tr>
<th>Outside diameter</th>
<th>5.56 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>15.0 cm</td>
</tr>
<tr>
<td>Wall thickness</td>
<td>1.0 cm</td>
</tr>
<tr>
<td>Volume</td>
<td>125.0 cc</td>
</tr>
</tbody>
</table>

The open end is threaded so that the cap can be screwed tightly to the base, compressing the round "O" ring rubber seal to maintain a pressure-tight fit.

The temperature regulator (Fig. 7) is designed to maintain the water bath inside the chamber at $37^\circ \pm 0.25^\circ$ C. The heat sources are external to the chamber, but the temperature sensor is located within the chamber.

A recirculating water system, utilizing a B. Braun, Melsungen (West Germany) constant temperature pump, is used as the heat transfer device. The water enters the transistor and the load resistor heat exchangers where additional heat may be added if needed. It then enters the pressure chamber where heat is transferred via the internal heat exchanger to the internal water bath which surrounds and warms the reaction test tubes. The water then returns to the pump for recycling.

The circuitry for the proportional temperature controller is illustrated in the schematic diagram (Fig. 7). A silicon diode, D-1, is the temperature probe which is immersed in the water contained in the temperature reference test tube inside the chamber. The forward-biased silicon junction has a characteristic temperature coefficient of $2.2 \text{ mV/}^\circ\text{C}$ which is applied to an operational amplifier, A-1. This amplifier conditions the signal to such an extent that a 200 micro-amp. meter will indicate the temperature of the diode over a range of $-15^\circ$ C to $45^\circ$ C. This
FIGURE 7

Schematic Diagram of Chamber Temperature Control Regulator.
D1
IN4148

Temp. Sensor Diode (Encapsulated)

ΔV/ΔT = approx. 2.2mV/°C

+15

390Ω

DZ 9.5

7.5K

5K

39K

5K

Temp. Set
10K

0.1µF

470K

A1

100p

200K

1.5K

15M

A3

+15

100mA PWR SPL

120v ac

Q1 2N6387

4x (6.3v @ 3.0A)

120v ac

Power gnd

From Pump

H₂O

Water-cooled heat sinks

To chamber heat exchanger

H₂O

Water-cooled heat sinks

To chamber heat exchanger
signal is applied to an operation amplifier, A-3, as is a reference voltage from the "temperature set" control. The operational amplifier, A-3, acts as a comparator, and its output, amplified at A-4, controls the amount of current passing through transistors Q-1, Q-2 and the load resistors (2 ohm).

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 is dissipated on the water-cooled heat sinks, transferred to the water, and subsequently to the chamber heat exchanger and bath. A light-emitting diode, D-3, is used to indicate when heat is being generated.

The temperature controller is calibrated by immersing the sensor in ice water and adjusting the "zero control" to 0° C on the meter scale. Water at 45° C, checked by a Yellow Spring Instrument Company temperature gauge, is then used to adjust the "calibrate control" for the proper meter reading. This is repeated several times since the two adjustments are interactive.

The regulator switch is placed on "set", and the "temperature control" is turned to the desired temperature, usually 37° C. The regulator switch is then placed on "sense", and the system generates heat as necessary to maintain a constant temperature inside the chamber.
The pre-incubated mitochondria are added to two test tubes for duplicate sampling technique. Pre-incubated, heat-inactivated mitochondria are added to a third tube to serve as a control blank. To the fourth tube is added water from the pre-incubation bath in an amount equivalent to the other three tubes. This last tube serves as the temperature control by immersion of the heat sensor.

When pressure is applied to the chamber, the rate of compression is approximately 50 p.s.i. per second. At this rate the increase in temperature due to pressure does not allow the temperature to pass the 37°C point.

VII. Drug Experiments

*Methohexital*

Methohexital sodium (Brevital®, Lilly) was prepared fresh daily in a stock concentration of 5 percent w/v and then diluted to the various working concentrations. Equal volumes of the whole mitochondrial suspension or the outer membrane suspension were pre-incubated with the barbiturate for 20 minutes in a water bath. It was found in preliminary experiments that this pre-incubation time prior to the monoamine oxidase assay was sufficient for maximal effect of the methohexital.
Halothane

Halothane (Fluothane®, Ayerst) was administered to equal volumes of mitochondria or their outer membranes as a saturated solution of the anesthetic in sucrose-tris buffer. The concentration of stock halothane-sucrose-tris solution was equal to that of halothane in water (0.345 gm/100 cc). It was previously determined that approximately 30 µl of halothane (Sp. G., 1.863) would dissolve in a capped test tube filled with 16 cc of either sucrose-tris buffer or water. This amount corresponds with the above stated water solubility of halothane. The pre-incubation step with halothane takes place in nearly filled, capped tubes in a water bath at 37°C for one hour to allow for adequate equilibration with the anesthetic.

VIII. Statistical Methods*

Analysis of variance is used to determine the least significant difference (LSD) of the adjusted means. This provides a very powerful generalized statement concerning the significance of differences between sets of data. The statistical formula at a 95 percent level of confidence for all data is:

\[ \text{LSD}(0.05) = t \sqrt{\frac{2s^2}{N}} \]

NOTE: \( s^2 \) = error mean square

* Statistical analysis system designed and implemented by Anthony James Barr, James Howard Goodnight, Department of Statistics, North Carolina State University, Raleigh, North Carolina, August, 1972.
The difference between the means of two sets of data must be greater than the LSD value to demonstrate that the means are significantly different.
RESULTS

I. Preliminary Studies on Intact Mitochondria

Before proceeding to the anesthetic and high pressure effects on mitochondrial membranes, it was apparent that the quality of the mitochondrial product first had to be established. Electron photomicrographs of a portion of the mitochondrial pellet revealed a normal appearing group of intact, apparently non-traumatized mitochondria (Plate 1, 2) with intact outer membranes and inner cristae. No gross foreign contaminants were observed.

Respiratory studies were conducted on another portion of the initial pellet and were also done at regular intervals throughout the research period to ensure quality control of the product. A high respiratory control index (R.C.I.) is 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. Five separate whole mitochondrial preparations produced an average R.C.I. value of 3.16, which is within the normal range of 3.0 to 5.0 for succinate substrates (Table 1).

Experiments with the intact mitochondria revealed that monoamine oxidase activity was also present. The kinetic factors of the monoamine oxidase reaction were examined and thoroughly tested.
PLATE 1

Intact Mitochondrial Pellet, Guinea Pig Liver.

Fixed in Buffered Glutaraldehyde; Post-fixed with Osmium Tetroxide.

Magnified x 37,125.
PLATE 2

Mitochondrial Pellet, Guinea Pig Liver.

Fixed in Buffered Glutaraldehyde; Post-fixed with Osmium Tetroxide.

Magnified x 74,250.

Thin Dark Band on Circumference is the Outer Membrane.
## TABLE 1

**RESPIRATORY CONTROL INDEX (RCI) WITH SUCCINATE**

| RCI* | 3.3 | 3.7 | 2.8 | 3.0 | 3.0 |

Mean — 3.16 ± 0.15 S.E.M.

*Whole Mitochondria, N = 5*
The rate of MAO product formation by the standard concentration of mitochondria employed was time dependent. The amount of product formed when the reaction was permitted to approach equilibrium, i.e., completion, over an eight-hour period in the closed chamber was approximately equal to the excess of substrate added. This indicates that the calculated excess of oxygen in the chamber was sufficient to oxidize all of the substrate, and that the amount of substrate added to reaction was approximately equal to ten times the amount needed for the reaction time of 20 minutes. Therefore, the rate limiting factor of the reaction during the constant incubation time period was the amount of monoamine oxidase present. Since the concentration of mitochondria added to the reaction was standardized, the amount of product formation in any single assay series depends upon the influences of drugs or pressures on the system.

Small changes in the pH of buffer solutions at high pressures have been shown to occur. These changes are in the range of 0.1 to 0.2 pH units (Newman, et al., 1973). No changes in product formation were observed when the buffer is adjusted to various pH readings within a range of 7.2 to 7.8 under normal atmospheric pressure conditions. This suggests that small changes in pH have little or no effect on the rate of product formation in the MAO assay procedure.
The size of our chamber is very limited and permitted only four test tubes to be used during each 20 minute assay. The length of time of each series of experiments was so long (ten hours) that the monoamine oxidase activity slowly decreased with time in a linear manner. Therefore controls were run on both ends of the time period to correct for aging. This correction is denoted on data graphs as "time adjusted".

II. Preliminary Studies on Isolated Mitochondrial Outer Membranes

Our initial attempt to obtain the isolated outer membrane preparation by pressure-lysis failed. However, some very interesting results were obtained. Whole mitochondria were placed in a chamber and pressurized with 2200 p.s.i. of nitrogen for 20 minutes. The pressure was then released as quickly as possible in an attempt to rupture the outer membrane and pull it away from the remainder of the mitochondrion.

Electron microscopic examination of this "exploded" pellet revealed nearly normal appearing, apparently intact mitochondria (Plate 3) compared to control (Plate 4). Only a slight vacuolization within the inner membrane and an occasional enlarged intermembrane space differentiated these mitochondria from control. These small changes could perhaps be a miniature model of the rapid decompression sickness known to deep sea divers as the "bends".

Respiratory studies were also performed on a portion of these mitochondria. The R.C.I. values obtained for this group were equal to those
PLATE 3

Mitochondrial Pellet, Guinea Pig Liver.

Fixed in Buffered Glutaraldehyde; Post-fixed with Osmium Tetroxide.

Magnified x 10,300.

Pressurized with Nitrogen at 2,200 p.s.i., then rapidly decompressed.
PLATE 4

Mitochondria Pellet, Guinea Pig Liver.

Fixed in Buffered Glutaraldehyde; Post-fixed with Osmium Tetroxide.

Magnified x 10,300.

Non-pressurized Control.
of non-pressurized control, and this indicated that the mitochondria were still intact.

Monoamine oxidase assays of the decompressed and control mitochondria revealed that the amounts of radioactive product formed were equal.

Osmotic lysis of fresh, whole mitochondria was then performed to attempt to separate the outer and inner membranes. The product obtained was examined under the electron microscope. This preparation contained fractured, thread-like membranes and no evidence of whole mitochondria (Plate 5).

Another preparation of osmotically-lysed outer mitochondrial membranes was tested for respiratory activity. After repeatedly confirming respiration with whole mitochondria used for control, the isolated outer membranes showed no evidence of respiratory control. The concentration of the membranes used in the previous procedure was examined by monoamine oxidase assay and resulted in MAO activity 1000 times higher than normally used. This indicated that the outer membranes were in fact used in the respiratory studies; the lack of respiratory control indicated that the outer membranes were no longer a component of intact mitochondria.

The bulk of the protein of a mitochondrion is contained within the confines of the inner membrane. The protein content of the thin outer membrane is, therefore, extremely small in comparison. To assay this
PLATE 5

Isolated Mitochondrial Outer Membranes,
Guinea Pig Liver.

Fixed in Buffered Glutaraldehyde; Post-fixed with Osmium Tetroxide.

Magnified x 100,000.
isolated outer mitochondrial membrane preparation for protein content, in an attempt to standardize it, would leave insufficient material for monoamine oxidase assays. Therefore protein determinations were not done. Instead, the preparation was diluted until MAO product formation was within the range of the standardized concentration of whole mitochondria. Excess substrate was maintained in subsequent MAO reactions with the concentration of the isolated outer membranes standardized by this method.

III. Intact Mitochondria -- Effects of Anesthetics

*Methohexital*

The monoamine oxidase activity of intact mitochondria was significantly decreased from control in both the non-pressure and pressure groups when methohexital is added at various concentrations (Fig. 8). Pressure significantly increased MAO activity when compared to the non-pressure group (Fig. 8).

*Halothane*

When halothane was administered in increasing doses to intact mitochondria, monoamine oxidase activity was decreased significantly in both the non-pressure and pressure groups (Fig. 9). Pressure significantly increased MAO activity when compared to the non-pressure group (Fig. 9).
Guinea Pig Liver Intact Mitochondria.

Drug — Methohexital

No pressure — 1 atmosphere

Pressure — 100 atmospheres helium (1500 p.s.i.)

Product — nM product/mg protein/20 min reaction

Analysis of Variance Indicates a SIGNIFICANT (p < 0.05):

1. Decrease in MAO Activity with Methohexital (No Pressure).

2. Decrease in MAO Activity with Methohexital (Pressure).

3. Increase in MAO Activity with Pressure compared to No Pressure.
WHOLE MITOCHONDRIA

N = 11
METHOHEXITAL
\( \bar{x} \pm S.E. \)

\[ \begin{array}{c|c|c|c|c|c}
\text{LOG DOSE (mg\%)} & 0.15 & 1.5 & 15 & 150 \\
\hline
\text{n moles Product} & \text{NO PRESSURE} & \text{1500 psi PRESSURE} & \text{(TIME ADJUSTED)} \\
\end{array} \]
FIGURE 9

Guinea Pig Liver Intact Mitochondria.

Drug — Halothane

No pressure — 1 atmosphere

Pressure — 100 atmospheres helium (1500 p.s.i.)

Product — nM product/mg protein/20 min reaction

Analysis of Variance Indicates a SIGNIFICANT (p <0.05):

1. Decrease in MAO Activity with Halothane (No Pressure).

2. Decrease in MAO Activity with Halothane (Pressure).

3. Increase in MAO Activity with Pressure compared to No Pressure.
WHOLE MITOCHONDRIA
N=10
HALOTHANE
(TIME ADJUSTED) X ± S.E.

- NO PRESSURE
- 1500 psi PRESSURE

n moles Product

DOSE (mg %)

C 17. 51. 85. 170. 255.
**Pressure Alone**

Intact mitochondrial monoamine oxidase activity significantly increases as the pressure increases from one atmosphere to 100 atmospheres, i.e., 1500 p.s.i. (Fig. 10). This increase in activity with pressure appears to be reversible. Intact mitochondria were pre-incubated at 37° C in either a non-pressure or a pressure (1500 p.s.i.) environment. An aliquot of the previously pressurized mitochondria was placed in one reaction tube, and an aliquot of the non-pressurized was placed in another. These tubes were then placed in the chamber under no pressure conditions for the usual MAO assay. The amount of product formed was the same for each tube (Table 2). This procedure was repeated with another set of tubes, except that the chamber was pressurized to 1500 p.s.i. The amount of product formed was the same for each tube. However, the increased MAO activity due to pressure was again apparent. If pressure had produced an irreversible change in the mitochondrion during the pre-incubation period, its product formation would have been different from the control.

IV. **Isolated Outer Mitochondrial Membranes — Effects of Anesthetics and Pressure**

Both methohexital and halothane significantly decrease the MAO activity of the outer membrane preparations (Fig. 11, 12). However, pressure fails to increase MAO activity significantly in either case.
Guinea Pig Liver Intact Mitochondria.

Drug -- None

No pressure -- 1 atmosphere

Pressure -- Various pressures used

Product -- nM product/mg protein/20 min reaction

Analysis of Variance Indicates a SIGNIFICANT (p <0.05):

1. Increase in MAO Activity with Pressure compared to No Pressure.
WHOLE MITOCHONDRIA
N=6
INCREASING PRESSURE
NO DRUG
(TIME ADJUSTED)

Pressure (PSI)

n moles Product

C 100 300 500 1000 1500

PRESURE (PSI.)
**TABLE 2**

REVERSIBLE PRESSURE EFFECT*

<table>
<thead>
<tr>
<th></th>
<th>MAO Assay (No Pressure)</th>
<th>MAO Assay (Pressure)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM product/mg protein/20 minutes</td>
<td>nM product/mg protein/20 minutes</td>
</tr>
<tr>
<td>No Pressure</td>
<td>Pressure</td>
<td>No Pressure</td>
</tr>
<tr>
<td>Pre-incubated</td>
<td>Pre-incubated</td>
<td>Pre-incubated</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.19</td>
<td>4.15</td>
<td>4.96</td>
</tr>
<tr>
<td>4.10</td>
<td>4.16</td>
<td>5.02</td>
</tr>
</tbody>
</table>

\[ \bar{X} = 4.15 \pm 0.05 \]
\[ \bar{X} = 4.15 \pm 0.01 \]
\[ \bar{X} = 4.99 \pm 0.03 \]
\[ \bar{X} = 4.99 \pm 0.01 \]

\[ *N = 2 \]
FIGURE 11

Guinea Pig Liver Isolated Lysed Outer Mitochondrial Membranes:

Drug -- Methohexital

No pressure -- 1 atmosphere

Pressure -- 100 atmospheres (1500 p.s.i.)

Product -- nM product/MAO adjusted activity/20 min reaction

Analysis of Variance Indicates a **SIGNIFICANT** (p <0.05):

1. **Decrease** in MAO activity with **Methohexital** (No Pressure).

2. **Decrease** in MAO activity with **Methohexital** (Pressure).

(No Significant Pressure Effect Compared to No Pressure)
ISOLATED MITOCHONDRIAL OUTER MEMBRANES

N = 5

METHOHEXITAL

$\bar{x} \pm S.E.$

- NO PRESSURE
- 1500 psi. PRESSURE
  (TIME ADJUSTED)

n moles Product

LOG DOSE (mg%)
FIGURE 12

Guinea Pig Liver Isolated Lysed Outer Mitochondrial Membranes.

Drug — Halothane

No pressure — 1 atmosphere

Pressure — 100 atmospheres (1500 p.s.i.)

Product — nM product/MAO adjusted activity/20 min reaction

Analysis of Variance Indicates a SIGNIFICANT (p <0.05):

1. Decrease in MAO activity with Halothane (No Pressure).

2. Decrease in MAO activity with Halothane (Pressure).

(No Significant Pressure Effect Compared to No Pressure)
ISOLATED MITOCHONDRIAL OUTER MEMBRANES

N = 5
HALOTHANE
(TIME ADJUSTED) X ± S.E.

- NO PRESSURE
- 1500psi PRESSURE

n moles Product

DOSE (mg %)

C 17 51 85 170 255

17.5 1.8 5.1 7.0 25.5
DISCUSSION

I. Effects of Anesthetics and Pressure on Membranes

A compilation of the results obtained by many researchers in the field of anesthetic and pressure effects on membrane structure produces fairly sound evidence to support the concept that general anesthetics expand membranes and cause an increase in their fluidity. High pressures are believed to compress these expanded membranes and reduce their fluidity. The fact that pressure alone produces a neurological syndrome with convulsions in the intact animal suggests that control membranes, in the absence of anesthetics, may be compressed to such an extent that normal membrane fluidity is reduced beyond the critical point for its maintenance of proper function.

The critical volume hypothesis of Miller, et al. (1973) proposes that general anesthetic agents concentrate in the hydrophobic regions of membrane tissue and produce a net volume expansion and an increase in fluidity of the membrane. When this expansion reaches a critical volume, the membrane function is depressed, and the state of general anesthesia appears.

Pressure within the ranges which produce reversal of the anesthesia process has been calculated to compress the membrane by an amount equal to the anesthetic-induced expansion. Further compression of this system or of non-anesthetized controls causes a net compression and a reduction
in fluidity of the membranes until another critical volume is reached. The high pressure neurological syndrome then appears with its coarse tremors and convulsive episodes, conceivably as a result of the reduction of control or increased excitability of membrane function.

The mechanism which produces the loss or reduction of normal function of these altered membranes is unknown. Some researchers have proposed that changes in membrane fluidity are accompanied by changes in permeability and that this somehow interferes with membrane action. Most investigators believe that nearly all membranes are subject to the effects of anesthetics and that neurological membranes are either highly susceptible to these agents or else the decrement in function is most easily observed in them.

II. The Outer Mitochondrial Membrane MAO Model

Assuming that membrane permeability changes might be a result of anesthetic-induced fluidity alterations and that these could be observed in various types of simple membrane systems, the intact mitochondrial outer membrane, which contains membrane-bound monoamine oxidase as a marker, was chosen as our model. Guinea pig liver is especially suitable for these experiments because it is a rather easily obtainable, abundant source of highly heat stable mitochondria.
The results of the respiratory control experiments, electron microscopic analyses, and monoamine oxidase activity assays indicated that the isolation procedures employed produced excellent quality, intact guinea pig liver mitochondria and osmotically-lysed outer membrane preparations devoid of intact mitochondria.

The monoamine oxidase assay procedural experiments demonstrated that an adequate ambient oxygen supply is available inside the chamber to allow the reaction to approach completion or equilibrium. These studies also show that the concentration of tryptamine substrate present in the reaction is approximately ten times more than can be converted to product within the 20 minute incubation period. Since excess oxygen and tryptamine are present in the assay, the rate limiting factor is the amount of monoamine oxidase added to the reaction. The volume of the mitochondrial suspension or outer membrane preparation is constant for each assay, and their concentrations are likewise standardized. Thus, for any particular preparation used for any one series of experiments at a constant temperature, changes in product formation compared to controls are due to the experimental variables such as type and concentration of the anesthetic used and/or the state of pressurization inside the chamber.
III. Effects of Anesthetics on Membrane MAO Activity

The decrease in monoamine oxidase activity of intact mitochondria and isolated mitochondrial outer membranes by methohexital and halothane supports the belief expressed by Schneider and Gardier (1969) that the primary effect of general anesthetic agents on MAO is enzyme inhibition. This decrease in activity is dependent on the drug and its concentration but is apparently independent of the integrity of the membrane to which the enzyme is bound, since inhibition is seen in both intact and ruptured outer membrane preparations.

It is also possible that the anesthetics could produce alterations in the membranes which could then induce an enzyme conformational change less favorable to monoamine oxidase activity. This would result in decreased product formation. However, if MAO activity decreases as the anesthetic concentration increases, multiple inhibitory conformational changes would be needed to show product decreases at the various concentrations.

Monoamine oxidase inhibition may also be due to the property that general anesthetic agents increase the fluidity of the membrane and decrease membrane permeability to substrate. However, since the MAO in membrane fragments was just as susceptible to inhibition as it was in the intact mitochondria, this possibility cannot be strongly supported. Whereas substrate permeability decreases could be a determinant in the amount of product formation in the intact membrane, it is less likely to
be a factor in the lysed preparation which may provide free substrate access to the monoamine oxidase via fracture points or broken ends.

IV. Effects of Pressure on Mitochondrial MAO Activity

Pressure is believed by most investigators to cause a decrease in membrane fluidity and a generalized ordering or structuring of membrane components. This may cause an increase in the permeability of the membrane to tryptamine and result in larger amounts of product formation in intact membranes. In the intact membranes, pressure produced an increase in product formation compared to non-pressure groups in spite of the fact that product formation was decreased by the anesthetics in both instances. Increasing the pressure in the absence of anesthetic agents also increased product formation. The lysed mitochondrial outer membranes did not show increases in product formation with pressure. A pressure induced reordering of the fractured system may be impossible, since the tryptamine could have free access to the enzyme via fracture points and/or open ends of the membrane.

The possibility also exists that the pressure may cause a more favorable enzyme conformational change which could also increase product formation. However, this possibility does not tend to be supported by the isolated outer membrane experiments which did not significantly show product increases with pressure.
The results of the experiment in which whole mitochondria are pre-incubated at either one atmosphere or 1500 p.s.i. (100 atmospheres) and subsequently placed in reaction tubes with substrate, under conditions of no pressure and pressure, indicate that the pressure effect of increased product formation is reversible. The mitochondria pre-incubated with pressure produced the same amount of product, with and without pressure, as those which were pre-incubated in an open water bath.

In summary, halothane and methohexital decrease MAO activity. The outer membrane experiments indicate that anesthetic-induced membrane permeability changes may not be the mechanism of this MAO inhibition, i.e., indirect inhibition. Multiple enzyme conformational changes (as a result of anesthetic distortion of membranes) corresponding to the various anesthetic concentrations seem unlikely to be the mechanism, i.e., indirect-direct inhibition. The mechanism of MAO inhibition by the anesthetic agents appears to be direct enzyme inhibition, although a combination of the above mechanisms is possible.

Pressure increases MAO activity, irrespective of the presence or absence of anesthetic agents. However, since an intact membrane is necessary for this effect, it cannot be explained by a pressure-induced, more favorable enzyme conformational change, i.e., direct effect, nor by a membrane-mediated change in enzyme conformation, i.e., indirect-direct
effect. The increase in MAO activity with pressure appears to be a result of increased substrate permeability through a membrane which is made less fluid by pressure.

V. **Possible Clinical Applications of These Experimental Implications**

The relationship of the results of these data to other closely allied areas such as anesthesia and analgesia mechanisms, pressure reversal of anesthesia, and high pressure nervous system syndrome are, at present, highly speculative. However, changes in monoaminergic neuronal transmitters have been associated with anesthetics, sedatives, hypnotics, analgesics, anticonvulsants and susceptibility to high pressure effects. The possibility that anesthetics or high pressure may produce changes in monoamine storage, release, and/or metabolism by reversibly altering intact membrane permeability is intriguing.

Neurotransmitters and putative neurotransmitters in the central nervous system are the targets of much research activity; serotonin is an example. Increases in brain content of serotonin have been associated with analgesic states, prolonged sleep, general anesthesia, anticonvulsive effects, and increased thresholds for the onset of the high pressure neurological syndrome. Decreases in brain content of serotonin have been associated with hyperalgesia even in the presence of morphine, insomnia, elevated thresholds for general anesthetic induction, increased sensitivity to convulsants, and a lowered threshold for the onset of the high pressure neurological syndrome.
Serotonin levels are also involved with thermal regulatory mechanisms, although the response is quite variable among different animals.

The effect of an anesthetic agent on monoamine oxidase in or about a monoaminergic neuron in the brain may possibly increase levels of amines such as serotonin. This could result in the production of at least part of the anesthetic state. Pressure could possibly reduce amine levels by increasing monoamine oxidase activity and produce, at least in part, the phenomenon of pressure reversal of anesthesia. The pressure effect could also be responsible for decreasing normal serotonin brain content to such an extent that convulsions, i.e., high pressure neurological syndrome, occur. Anesthetic interference with serotonin-mediated thermoregulatory mechanisms may be responsible for the production of malignant hyperpyrexia in certain highly-susceptible, genetically predisposed individuals.

The results obtained from the experiments in which whole mitochondria were subjected to various helium pressures indicate that as the pressure increases to 1500 p.s.i., the permeability of substrate also increases. This occurrence may be the result of a pressure-dependent decrease in membrane fluidity and supports the concept that non-anesthetized biological membranes possess a degree of inherent fluidity. Further, the intact outer membrane of the mitochondrion appears to be a good model for determining membrane effects of anesthetics and pressure.
Many extensions of this problem should be investigated. Guinea pig liver slices would be the next highest organizational step in this present investigation. Because their environment is within the central nervous system, brain mitochondria should also be tested. Intraneuronal and extra-neuronal sources of monoamine oxidase may react differently under conditions of anesthesia and pressure.

The possibility that other membrane-bound enzymes may show similarities to mitochondrial monoamine oxidase poses another potential area of research. These suggestions, as well as other possibilities, justify the continuation of this problem to the potential ultimate end -- the definition of the mechanism of general anesthesia.
SUMMARY AND CONCLUSIONS

The results of these experiments demonstrate that methohexital sodium and halothane inhibit monoamine oxidase activity in guinea pig liver intact mitochondrial preparations which are subjected to either one atmosphere or 100 atmospheres of pressure during the reaction period. The pressurized experiments, however, showed a significantly increased monoamine oxidase product formation compared to the non-pressurized group. The concept that a decrease in membrane fluidity causes an increase in membrane ordering and substrate permeability is supported by these experiments.

The pressure-induced increased monoamine oxidase activity was shown to be a reversible phenomenon by the results of the experiments in which intact mitochondria were pre-incubated at one atmosphere or 100 atmospheres of pressure and then tested for MAO activity under equal conditions. The previously pressurized mitochondria produced the same amount of product as those which had been previously incubated at one atmosphere. This equality of activity occurred during non-pressurized and pressurized MAO reactions.

Monoamine oxidase activity was inhibited by methohexital sodium and halothane in the lysed mitochondrial outer membrane preparations subjected to either pressure or no pressure conditions. However,
pressure did not significantly increase monoamine oxidase activity in this preparation as it did in the intact membrane.

These data demonstrate that methohexital sodium and halothane inhibit monoamine oxidase and support the propositions of Schneider, et al. (1974) and Muldoon, et al. (1975) that this may be a direct enzymatic inhibition. Decreased MAO activity in the lysed membranes is probably not a result of decreased substrate permeability since the enzyme may have free access to substrate. The alternative of direct MAO inhibition by methohexital sodium and halothane appears to be better supported by the data.

The concept that membranes possess an inherent fluidity is supported by the fact that increasing pressures elevated MAO activity in "unanesthetized" intact mitochondrial membranes. This is thought to be due to a decrease in the normal fluidity and an increase in membrane structuring which allows for greater substrate permeability.

In conclusion, it has been demonstrated that the outer membrane of guinea pig liver intact mitochondria is a suitable model to study the membrane changes produced by methohexital sodium and halothane and that confirmation of these results can be obtained from observing similar or opposite effects in the fractured outer membrane preparation. The significance of this model is that it may be a useful tool in the determination of the mechanism(s) of action of general anesthetics and/or high pressure neurological syndrome.
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