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STUDIES OF BRAIN ACETYLCHOLINESTERASE AND Na^{+}-K^{+}-ATPase: INTERACTIONS WITH THE GENERAL ANESTHETIC CYCLOPROPANE

BY

Usha Maheswari

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ABSTRACT

Studies of Brain Acetylcholinesterase and Na^+-K^+ -ATPase: Interactions with the General Anesthetic Cyclopropane

The interactions of a general anesthetic, cyclopropane with brain macromolecules, i.e., both membrane-bound and "purified" forms of acetylcholinesterase (AChE) and Na^+-K^+-ATPase from beef brain were studied. In addition, the possibility of a functional relationship between the two enzyme systems was examined using a brain microsomal fraction containing AChE and "ion-transport" ATPase.

Cyclopropane inhibited all forms of AChE activity, the inhibition being greater (35%) at low substrate and less at high substrate concentrations. The inhibitory effect was observed at low gas pressures (0.1 atm) and was reversible. Cyclopropane inhibition in all AChE preparations occurred by a mechanism involving mixed kinetics. This suggested the interaction of the gas at more than one site on the enzyme molecule. By analogy with known interactions of xenon with heme proteins, the competitive component of inhibition was interpreted in terms of gas binding at some "non-polar" region on the active site. An additional such region of the molecule distinct from the active site, but capable of modifying reaction rate is suggested by the non-competitive component of cyclopropane inhibition.

Na^+-K^+-ATPase in all preparations studied was inhibited by cyclopropane. Inhibition was reversible and both pressure and temperature dependent. From control experiments with

other gases and hydrostatic pressure, the inhibition appeared to be specific for cyclopropane. The inhibition of Na^{+}-K^{+}-ATPase by cyclopropane was substrate promoted following "non-competitive" kinetics. Cyclopropane inhibited the enzyme reaction at or before the phosphorylation stage, with no significant effect on the dephosphorylation of protein. These results were interpreted in terms of the cyclopropane interaction with enzyme to modify the influence of substrate on the possible "regulatory" site or sites.

In a membrane preparation, ATP inhibition of cation "stimulated" AChE occurred under conditions optimal for "ion-transport" ATPase activity, suggesting a possible functional relationship between the two enzyme systems. However, other nucleotides (GTP, UTP) and ATP in the presence of single cation also partially blocked the "ion-activated" AChE activity. A relatively pure AChE preparation (no measurable Na^{+}-K^{+}-ATPase activity) was also activated by the cations and this activation was reversed by ATP. From these findings one could conclude that ion activation and nucleotide inhibition of AChE represent individual properties of the enzyme which may be of importance in the regulation of enzyme activity during excitation.

I. INTRODUCTION

Anesthesia has been defined as a reversible state of unconsciousness. In addition to depression of the nervous system, pharmacological agents that cause anesthesia may alter functions throughout the organism (28). A large number of chemical compounds with marked structural heterogenity are capable of exerting anesthetic action such as gaseous anesthetics (176), volatile anesthetics (178,179) and barbiturates (157). Also, hypothermia and electric stimulation of certain regions of the brain can cause conditions approaching general anesthesia. There have been many studies on the mechanism of narcosis, yet the basic question of what physical or chemical changes in the central nervous system cause the state of general anesthesia is unsolved. Anesthetic molecules have been postulated to interact with nerve cell components, i.e., lipid (46,114), water (113,127,128), protein (147,151) or membranes (16,120). Unitary theories ascribe anesthesia to a primary action in a simple phase such as hypotheses including the Meyer-Overton theory based on the correlation of anesthetic potency with oil/water partition ratios (112,126), the thermodynamic approach (55), the microcrystal hydrate theory (113,127), and cell permeability changes (66) interfering with ion movements. Simultaneous actions presumably occur in more than one phase.

There has been many studies on the biochemical actions of anesthetic drugs. Quastel (135) demonstrated _in vitro_ inhibition of oxygen uptake by brain after exposure to

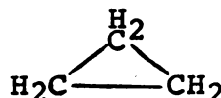
barbiturates, chloral hydrate and urethane. He suggested that these anesthetics act via depression of the respiratory enzymes at the level of NADH dehydrogenase. Xenon has been shown to increase the rate of oxygen consumption in mouse liver and brain slices while it depresses anaerobic glycosis (169). Chance and Hollunger in 1963 (33) demonstrated that amytal in low concentrations inhibits mitochondrial metabolism at a) electron transfer between NAD and flavoprotein and b) at energy transfer and the oxidation of succinate at the mitochondrial level.

McIlwain in 1962 suggested that anesthetics do not primarily act at the level of energy producing mechanisms, because *in vivo* creatine phosphate levels increase and inorganic phosphate concentration decreased while oxygen consumption is depressed during anesthesia (110). He further indicated that anesthetics could inhibit ionic movements across the membrane during excitation (110).

The gaseous anesthetics in general and the inert gases (xenon, cyclopropane, nitrous oxide, ethylene) in particular, have received little attention from the standpoint of possible mechanisms underlying their anesthetic properties. The so called "inert gaseous anesthetic agents" exert their biological effects without undergoing any change in their own chemical structure, yet these molecules must ineract with some of the constituents of the system in which they produce anesthesia. It has been suggested by Featherstone et al (53,54), that these agents cannot interact with membrane or protein as a whole,

but can only associate with small groups of atoms which constitute these large macromolecules. It is pertinent to mention how these small molecules can produce functional changes. The inert gas anesthesics cannot form covalent, ionic or hydrogen bonds under physiological conditions but only secondary weak bonds involving Van Der Waals' forces, and the duration of such bonds is very short (35,54). The most interesting of Van Der Waals' forces is the London force which explains attractive force between such gas molecules which have no permanent dipole moments. The existence of such interactions has been well documented in studies of the association of xenon and cyclopropane with proteins of established structures (53,54,147,149). In 1951 Cullen and Gross (39) conclusively demonstrated that the inert gas xenon was an anesthetic in man at atmospheric pressure. Discovery of the anesthetic properties of xenon stimulated interest in the metabolically inert gases which are known to be narcotic, although several are not anesthetics at less than one atmosphere pressure.

Xenon is a monoatomic gas with atomic number of 54 and atomic weight of 131.3. Cyclopropane is the simplest hydrocarbon with structural formula:



Waters and Schmidt (1934) published the first clinical report on the use of cyclopropane as a general anesthetic (181). It is a colorless gas, heavier than air and is stored under pressure as a liquid in metal cylinders at 37°C, its water

solubility is .204 ml gas/ml of water (53). It is explosive and anesthetic concentrations vary from 10-30 vol. percent. It is a safe anesthetic and does not affect the cardiovascular system. It is eliminated entirely by lungs (185) and recently ^{14}C-cyclopropane has been shown to be converted to ^{14}C-CO_2 in rats (178).

The anesthetic action of ethylene ($H_2C{=}CH_2$) was first noted in 1865 but the gas was first used for anesthesia by Lewis in 1923 (103). It is a colorless gas with an unpleassant taste and is stored in metal cylinders. It is lighter than air and not explosive.

Xenon is not only an anesthetic in man but also in mice, rabbits, rats and monkeys (131). It has been shown to block impulse transmission in isolated perepheral nerve fibers (27). Cyclopropane at a concentration of 40% has been shown to depress electrical activity at preganglionic regions in dogs (56). In addition, it has been shown that cyclopropane depresses cerebral oxygen consumption (8). Xenon and cyclopropane at atmospheric pressures reversibly inhibit the aerobic and anaerobic growth of E. coli (64). These gases also affected NADH oxidase and succinic dehydrogenase by 20% at 1 atmosphere in E. coli (64). Using x-ray diffraction techniques specific binding has been demonstrated of xenon to hemoglobin (149) methemoglobin, metmyoglobin (150) and of cyclopropane to myoglobin (104,148). Binding of these gases occurred at a position in the myoglobin equidistant from the proximal histidine and heme and between a nonpolar and a partially polar area perhaps due mainly to London

interactions (147). This is not a generalized phenomenon as is suggested by the fact that xenon does not bind to hen egg lysozyme, chymotrypsin, and horse heart cytochrome (151). In addition, krypton, ethylene, acetylene and ethane do not bind to myoglobin as do xenon and cyclopropane (104). Helium group gases have been shown to inhibit active sodium transport in frog skin although at extremely high pressures (61). Anesthetics interfere with the movement of both Na^+ and K^+ during the action potential in nerves (119) and have been also shown to interfere with the Na^+ pump mechanism (119).

There have been only a few studies on the actions of these inert gases on brain enzymes. Trevor et al (176) reported inhibition of Na^+-K^+-ATPase by cyclopropane at pressures above one atmosphere. "Ion-Transport" ATPase has also been shown to be sensitive to ether (75), halothane (75, 101) and ethanol (74,75,172). Recently Roberts et al (138) have demonstrated inhibition of eel AChE by ether. A "membrane bound" AChE from brain tissue has been shown to be activated by helium group gases (153) although at extremely high pressures.

A general blockade of excitable neurons could result in the observed neurochemical changes associated with the actions of anesthetics. It is generally accepted that the transmission of excitatory activity across junctional regions in mammals occurs through the mediation of chemical substances such as acetylcholine, norepinephrine, gamma-aminobutyric acid, and 5-hydroxytryptamine. ACh is established to be a neuro-

transmitter agent at parasympathetic neuroeffector junctions, at skeletal myoneural junctions, in the ganglia of the autonomic nervous system and at certain synapses in the central nervous system. Norepinephrine is a chemical transmitter at postganglionic sympathetic nerve endings and with epinephrine is released from the adrenal medulla. Special mechanisms exist at each functional site for the disposition or removal of the transmitter, ensuring precise control of the transmitter concentration. Three potential pathways available are: a) diffusion from the cleft, 2) destruction by enzyme activity, and c) reuptake of the transmitter into the nerve ending (58). For example, the termination of norepinephrine action is via reuptake into the nerve, although two enzyme systems, i.e., monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT), are capable of destroying the transmitter, these probably are not very important in terminating sympathetic activity (58). Similarly ACh action is presumably terminated via inactivation by AChE. Thus, the enzyme AChE performs a key function in the control of excitability and of information transfer between cells. Another important enzyme system in the establishment, maintainence, and control of cerebral excitability is "Ion-Transport"-ATPase. This enzyme system is of importance for regulating ionic concentration gradients and thus ionic fluxes. An interaction with Na^+-K^+-ATPase would restrict the ability of the nerve cell to restore ionic gradients following excitation, with a resultant loss of neuronal excitability. A general description of these two enzyme systems, i.e., AChE and Na^+-K^+-ATPase will be

included here to permit descriptions of drug interactions subsequently.

Acetylcholinesterase (EC 3.1.1.7) appears to be an integral part of most excitable membranes and has been found in the innervated tissues of all vertebrates and invertebrates studied so far. The main sources are brain and nervous tissues (122,123,177), electric organ (140,141,191) and muscle end plates. Cholinesterases have been described to be of many types and have been differentiated by their location, substrate specificities, kinetic properties and by the actions of selective inhibitors. The hydrolytic action of AChE via inactivation of ACh presumably plays an important role in the control of excitability at post-synaptic sites (123). The enzyme may also regulate the levels of neurotransmitter in the pre-synaptic terminals and has been suggested to constitute an integral component of the cholinergic receptors (35,100). There is a considerable amount of histochemical evidence for central cholinergic transmission mechanisms and thus AChE can be anticipated to have importance in central synaptic transmission (110).

During the hydrolysis of ACh, an acetylated enzyme is formed as an intermediate and choline is liberated. The choline is probably taken up by nerve endings and used for transmitter synthesis but the acetylated enzyme is subsequently split by water to give acetate and free enzyme. From studies on AChE from electric tissue and erythrocytes it has been suggested that the active site of the enzyme consists

of an anionic component and an esteratic site. The anionic site, consisting of one or more negative groups which interact by ionic bondings with the cationic N^+ atoms of the choline residue, may be involved in the initial contact between enzyme and substrate. The esteratic site containing a serine-OH, an acid site and a basic site is involved in ester bond activation. The principal forces of attraction between the substrate and the enzyme are considered to be via coulombic and hydrophobic forces (188). A weak covalent bond between the basic group of the esteratic site and the electrophilic carbonyl C atom of the ester is subsequently formed.

Current concepts of the molecular and submolecular events involved in AChE activity and its modification by pharmacologic agents have developed from studies on the unpurified forms of the mammalian enzyme and on drug actions in higher animals in vivo. AChE activity is influenced by many agents such as neuromuscular blocking agents (34,95), indirectly acting parasympathomimetics, parasympatholytics (91,193) and certain gaseous anesthetics (153).

There are numerous compounds that inhibit AChE at the esteratic site in a manner similar to the reaction of acetylcholine with the enzyme, such as organophosphates, carbamates and sulfonates (145,187). Most of these agents react with the enzyme to transfer an acid function, i.e., a phosphoryl, a carbamyl or a sulfonyl group to the enzyme. Unlike the acetylated enzyme, these intermediate complexes involving phosphorylation, carbamylation and sulfonation are relatively stable and react with water slowly. Quaternary ammonium

compounds, at appropriate concentrations, inhibit AChE (189) in a manner distinct from the phosphates. This involves the formation of a readily dissociable complex with a negatively charged group on the enzyme, presumably the anionic subsite of the active surface because of the competitive nature of the interaction. Recently evidence has accumulated indicating that AChE possesses in addition to an anionic site in the catalytic center, perepheral anionic sites, where pharmacologic agents might bind and exert a regulatory role on the enzyme activity (34,85,91). Belleau and his co-workers, using enzyme from the electric organ of electrophorus electricus or from bovine erythrocytes (13,14) have shown at least two anionic sites on the protein molecule. From experiments with eserine and atropine, Kato et al (86,87) have also demonstrated the presence of two distinct binding sites on the surface of the enzyme extracted from the head ganglia of squid. Eserine was shown to bind to the active center and atropine to another anionic site distinct from the catalytic site. In addition, experiments with neuromuscular blocking agents such as gallamine and d-tubocurarine have indicated the possibility of binding sites which are non-catalytic (34). It has been shown that small quaternary ammonium ions and inorganic ions may bind to allosteric sites on the enzyme (34,37). Ca^{++}, for example, which has stimulatory action on the enzyme, is considered to bind at an allosteric site, i.e., the beta-anionic site as it does not compete with acetylcholine for the catalytic anionic site (alpha). On the other hand, Mg^{++} and Na^{++} (143,144) seem to

bind at both alpha and beta sites. According to Roufogalis et al (142), there is another site on the protein, the (gamma-anionic) site where gallamine can bind. This suggestion is based on the observation that tetraethyl ammonium (TEA), tetramethyl ammonium (TMA) or Ca^{++} do not antagonize the inhibition of AChE by gallamine and that the alpha and beta sites are most likely occupied by these cations. In summary, most studies on the properties of the purified forms of eel AChE suggest that the protein possesses a "regulatory" site distinct from the active site and thus AChE is an allosteric enzyme (34,91).

AChE, from mammalian sources, has been less extensively studied in this regard, but there is some evidence suggesting that mammalian enzyme has properties similar to eel AChE (7,14, 193). AChE from beef brain (32), bovine erythrocytes and membrane-bound and solubilized rat brain preparations (193), have been shown sensitive to inorganic salts, gallamine, d-tubocurarine, TMA and edrophonium. These studies also indicate the existence of multiple anionic sites on brain AChE which may be associated with regulatory effects.

The availability of purified forms of AChE from a mammalian source is important to facilitate our understanding of the hydrolytic mechanisms of the enzyme and its modification by various pharmacologic agents. The electric tissue of _Electrophorus electricus_ and _Torpedo marmorata_ provide a rich source of the enzyme and have been used extensively for purification attempts. AChE was first obtained in a semi-purified form by extraction from electric tissue of _Torpedo marmorata_ in

1938 (121). In the early 1940's, a 300-fold purification was obtained by ammonium sulfate fractionation and analytical centrifugation by Rothenberg and Nachmansohn (141). Various chromatographic procedures, such as ion exchange, and gel filtration were developed in the late 1950's and Kremzner and Wilson (94) further attempted to purify the enzyme using such procedures. The chromatographic method of Wilson (94) was slightly modified by Leuzinger and Baker (99) to achieve large-scale purification and finally, crystallization of the enzyme. Recently the application of affinity chromatography was introduced (38) and was applied by Berman and Young (18) to the purification of electric eel AChE. These methods reduced the purification procedure to several steps and tremendously increased the yield. Since then the technique has been modified and scaled up (82,140), so that relatively large amounts of the enzyme are available for more extensive studies.

Studies on the regional distribution of AChE activity in the brain show that the caudate nuclei contain high concentrations of the enzyme that are apparently located in the membrane components. Thus, caudate nuclei tissue of a larger mammalian brain appeared to be a good starting material for the purification of enzyme.

In brain, as contrasted to eel AChE, the major problem had been to solubilize the enzyme from membranes. A number of approaches have been used to achieve this and can be grouped as a) the use of surface active agents (67,97), b) organic

solvent extraction (97,98), c) treatment with proteolytic enzymes (42,47,67,97), and d) the use of chelating agents (29).

Among the surface active agents, most of the cationic and anionic detergents inactivated the enzyme (67,97) so only the non-ionic agents were of use. The use of non-ionic detergents did separate the protein from membranes, but influenced further separation (67,97,160,161). Organic solvents have also been used to remove lipids and subsequently release AChE into a soluble form. The most successful study using an organic solvent was that reported by Jackson and Aprison (78) which resulted in an overall purification of 32-fold.

Recently solubilization has been achieved by the use of a proteolytic enzyme, elastase (83) and of chelating agents such as EDTA (29,30). The yield with the latter is high and the membrane components are exposed to a much milder treatment with presumably minimum physicochemical changes in the macromolecule.

Chan et al have reported a modified method for solubilization of the enzyme with EDTA (30). Recently using affinity chromatography coupled to gel filtration with Sephadex G-200, the brain enzyme has been obtained as three peaks each with a specific activity of approximately 600,000 umoles ATC/mg protein/h. The three peaks have been characterized and their molecular weights have been found to be 130,000, 270,000 and 395,000 (31,32).

The Na^{+}-K^{+}-Mg^{++} dependent adenosine triphosphatase (ATP phosphohydrolase, EC 3.6.1.3) was first described by Skou (164) in the crab nerve and has been studied by many authors (3,6,59,70). Enzyme activity has been demonstrated in a wide variety of tissues such as kidney (96,133,182), red blood cells (132,144), perepheral nerve (21,164,166), brain (159, 164,165), liver (49,54), skeletal and cardiac muscles (19, 23,155) and secretory glands (158), etc. It has been found in most species ranging from man to microorganisms (24,57, 63,65,157) with some quantitative differences, but basically the enzymes possess the same characteristics. The highest activities of Na^{+}-K^{+}-ATPase have been demonstrated in the brain and tissues concerned with secretion (20,60,164,165). Studies on the regional distribution of brain show that the highest enzyme activity is found in cerebral cortex, in sub-cellular fractions containing nerve endings (24,25,72,133,146). Isolated synaptic vesicles, however, contain very little activity (4,62,133).

"Ion-transport"-ATPase is intimately associated with cellular membranes and perhaps is a component of a lipoprotein complex. The enzyme differs from other ATP hydrolyzing enzymes, in that a) it requires both Na^{+} and K^{+} in addition to Mg^{++} for maximum activity, b) ATP appears to be the major substrate, since with other nucleotides such as inosine (ITP) guanosine (GTP) and uridine (UTP) triphosphates as substrate, phosphatase activity is quite low, and c) effects of inhibitors (139). The stimulated enzyme is distinguished from the "Basic" Mg^{++}-ATPase

in that a) it is specific for ATP, b) it is inhibited by low concentrations of cardiotonic steroids and c) it is characterized by high activation energy (62,139).

It is necessary to discuss the possible mechanisms of ATP hydrolysis in order to understand the mechanism of active cation transport and the action of various pharmacologic agents. There is strong evidence that ATP is hydrolyzed through a multiple reaction sequence (3,6,163) and various steps are summarized in Figure 1. It has been well established that there is an Mg^{++}-Na^{+} -dependent phosphorylation of the protein and this phosphorylated intermediate is an acyl phosphate (3,5,71) characterized as a glutamyl-gamma-phosphate residue (3,71). Nucleotides other than ATP, which are poor substrates for $Na^{+}K^{+}$-ATPase activity, such as CTP, UTP, and ITP, can also phosphorylate the enzyme (175). There is strong evidence from studies with electroplex enzyme that the phosphoenzyme undergoes a Mg^{++} dependent transition from a high energy form (E_1 P) to a low energy form (E_2-P). Subsequently, K^{+} promotes dephosphorylation of the E_2-P form (51,52,162). The dephospho enzyme (E_2) has a high affinity for K^{+} and is interconvertible with E_1, a species of high Na^{+} affinity (162,163) which thus completes the enzyme catalytic cycle. Other reactions such as ADP-ATP exchange and enzyme-substrate binding will be discussed later in the Discussion.

The ubiquitous occurrence of Na^{+}-K^{+}-ATPase in cells that are capable of pumping sodium and potassium against a concentration gradient, its chemical characteristics, and anatomical

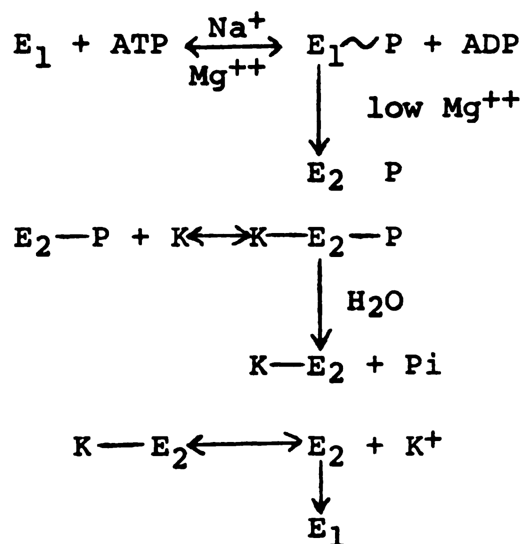


Figure 1. Scheme for ATP hydrolysis

localization suggest that this enzyme is involved in some way with the active transport of these cations (22,132,133, 165,183). Changes in enzyme activity appear to be correlated with the changes in electrolyte movements in kidney (165, 182,184), red blood cells (69,182), brain slices (165,183) and perhaps other organs. A relationship between cation transport, amino acids (12,164) and sugars (110,164) transport has also been suggested.

In red blood cells, the link between enzyme activity and cation transport is more easily demonstrated using the technique of reversible hemolysis (69). The parallelism between Na^+-K^+-ATPase activity as measured by the ATP hydrolysis and the active transport of Na^+ and K^+ as determined by the flux of radioisotopes across cell membranes, give strong support to the close association of the two. In tissue slices of brain and kidney, ouabain inhibits coupled transport of Na^+ and K^+ and depresses metabolism as well as inhibiting Na^+-K^+-ATPase activity in the broken cell homogenates of these organs (182,184). The activity of ouabain inhibited ATPase is relatively high in neural tissues such as brain, electric organs of eel, kidney and certain glands, where rapid active extrusion of Na^+ ion occurs following depolarization. By contrast, it is relatively low in red blood cells (23,24) and toad bladders (20) which transport cations slowly.

It is now well established that Na^+-K^+-ATPase activity is specifically inhibited by cardiac glycosides (6,59,157, 159). The sensitivity of the enzyme activity to cardiac

glycosides ranges widely, i.e., from 10^{-4}M to 10^{-9}M, depending upon experimental conditions, time of exposure to the drug, temperature and species involved (59). Recent studies have suggested that there may be a causal relationship between the inhibition of enzyme activity and inotropic effect of cardiac glycosides (107,155,156). Schwartz and others (107,155,157) have shown a parallel relationship between the degree of binding and the extent of inhibition of Na^{+}-K^{+}-ATPase activity by cardiac glycosides in electric organ and dog heart. However, the above relationship has been questioned by various workers (59,70), since no quantitative correlation have been found between the inhibition of Na^{+}-K^{+}-ATPase activity _in vitro_ and the inotropic effect _in vivo_. In addition, there are many compounds which inhibit the enzyme activity that do not have a positive inotropic effect on heart, such as oligomycin (11,170,179), sulfhydryl blocking agents (51), sodium azide (137), and chlorpromazine (2).

An understanding of the function of Na^{+}-K^{+}-ATPase and indeed the possible elucidation of the mechanism of Na^{+}-K^{+} transport depends in part on the purification of the enzyme. Conventional procedures such as ammonium sulphate precipitation and ion exchange column chromatography used in the purification of many soluble enzymes have not been used successfully for the purification of Na^{+}-K^{+}-ATPase due to its particulate nature. Detergent treatments have been applied in the attempted isolation of the enzyme from various tissues. Problems with the anionic agent deoxycholate include

inhibition or stimulation of enzyme activity (80,115), a rather low yield in terms of its solubilization (173) and the possible influence of the detergent on the physical properties of the enzyme (90). Similar problems were observed by the use of nonionic detergents Triton X-200 or Lubrol. For example, Lubrol W effected 40% solubilization of brain Na^+-K^+-ATPase, but caused a marked stimulatory effect at low concentrations and inhibition at higher concentrations above 4 mg/ml (173). Hokin and his colleagues extracted this enzyme from brain microsomes using 10-20 mg/ml Lubrol and further purified by centrifugation and column chromatography (111,159).

The high concentrations of detergents used in these studies are known to cause enzyme inhibition (159,160). Lower concentrations of Lubrol WX (1-2 mg/ml) were shown (159) to be quite effective in extracting the enzyme from both brain and cardiac tissue, but the sensitivity of the preparations to cardiac glycoside inhibition was somewhat different from the enzyme in particulate material. Fractionation with discontinuous density gradient resulted in a partial purification (115) but did not give homogenity. Column chromatography and acrylamide gel electrophoresis have resulted in the loss of enzyme activity (115). Hokin (71) has recently reported a purification scheme from beef brain microsomes which involves solubilization with Lubrol and chromatography on 6% agarose. Na^+-K^+-ATPase was eluted as a single peak with molecular weight of approximately 670,000

(71). The same apparent molecular weight was obtained with a Lubrol solubilized NaI treated beef brain enzyme by Uesugi et al (177). They did not suggest, however, what percentage of this molecular weight is due to bound Lubrol. So far, the protein has been solubilized, yet not completely purified.

AChE and Na^+-K^+-ATPase may both be associated with nerve conductions and synaptic transmission. The enzyme activities are widespread and associated with excitable cell membranes, i.e., both are membrane-bound. In brain tissues, both enzymes display maximal activity in microsomal fractions (109). It has been suggested by Kalandarishvili (81) that AChE and "ion-transport"-ATPase are located in close association with each other in the microsomal fractions of rat brain. This was based on the fact that uniform parallel changes occurred in the activities of these enzymes following purification procedures. An examination of the sedimentation properties of the two "solubilized" enzymes using zonal ultracentrifugation by Shirachi et al (20) also suggested the possibility of a physical association between the two enzyme systems. A possible functional interrelationship between AChE and "ion-transport"-ATPase has been suggested by Fahn (50,51,52) and Kometiani et al (93). This suggestion is based on a) the inhibition of ion-transport-ATPase by ACh and the inhibition of AChE by ATP, b) ouabain inhibition of AChE activity, and c) the demonstration that inhibition of both enzymes occurred only in the presence of Na^+, K^+ and Mg^{++} ions.

There has been a lack of information on the effects of inert gas anesthetics on brain enzyme-systems, i.e., AChE

and "ion-transport"-ATPase, the importance of which in the CNS has already been discussed. Cyclopropane was selected in the present study because it is less expensive than xenon and chemically unreactive. Since purified forms of a mammalian brain AChE were available, it appeared possible to study gas interactions analogous to myo/hemoglobin studies with inert gases.

In the present study, an attempt was made to study the following:

A. The interaction of cyclopropane with both membrane bound and the purified forms of AChE from ox brain caudate tissue. This involved characterization of the enzymes, determining conditions required for optimum gas inhibition, the reversal of the effect and the kinetics at two different gas pressures.

B. The interactions of various gases including cyclopropane, nitrogen (N_2), oxygen (O_2), and compressed air were examined with semipurified preparations of Na^+-K^+-ATPase from ox brain cortical tissue. This phase of the study included characterizing the enzyme systems, optimum conditions for gas effects, controls using gases such as N_2, O_2, and hydrostatic pressure and the effect of cyclopropane as a function of substrate concentration. In addition, the site of cyclopropane action in the ATP hydrolytic reaction sequence and the influence of substrate concentration on that site was examined.

C. The final question was to examine the possibility of a functional relationship between the two enzyme systems using a brain microsomal preparation containing both AChE and Na^+-K^+-ATPase. This section included the study of the effects of the cations Na^+, K^+ and Mg^{++} either alone or in various combinations, the influence of nucleotides (ATP, GTP, and UTP) and ouabain effects on AChE activity. In addition, a comparative investigation of the effects of ATP and cations on different preparations of brain AChE of varying degrees of purity was carried out.

II. MATERIALS AND METHODS

A. Materials

Acetylthiocholine (ATC), butyrylthiocholine iodide, ouabain, tris-ATP and trizma base were obtained from Sigma Chemical Co. (St. Louis, Mo.); 5,5-dithiobis-(2-nitrobenzoic acid) was from Aldrich Chemical Co., Inc. (Milwaukee, Wisc.); affinose-202, was from Bio-Rad. Labs (Richmond, Calif.); Sephadex G-200, was from Pharmacia (Uppsala, Sweden); edrophonium chloride was a gift from ICI America, Inc.; gases were obtained from Ohio Medical Products (Madison, Wisc.); aquasol and ATP (p32) was obtained from New England Nuclear. $AT^{32}P$ had a specific activity of 14.6 curies/mmole. Other reagents or chemicals used were of reagent grade.

B. Methods

1. Preparation of Acetylcholinesterase

a) Solubilization and Fractionation

Bovine brains were obtained from a local slaughter house and were kept on ice during transportation. Dissection was done by quickly removing the membranes and blood vessels located over the caudate with tweezers. Using a spatula, the caudate was then carefully stripped away from the internal capsule minimizing the presence of all white matter. A 15% (w/v) homogenate was prepared in 0.32 M sucrose containing 1 mM EDTA, at pH 6.9. The homogenate was centrifuged according to the scheme shown in Figure 2 in a Lourdes Model A_2 Beta-fuge using the 9RA rotor and a Spinco Model L centrifuge using the 30 rotor at 0-4°C. The supernatant fraction 1 was

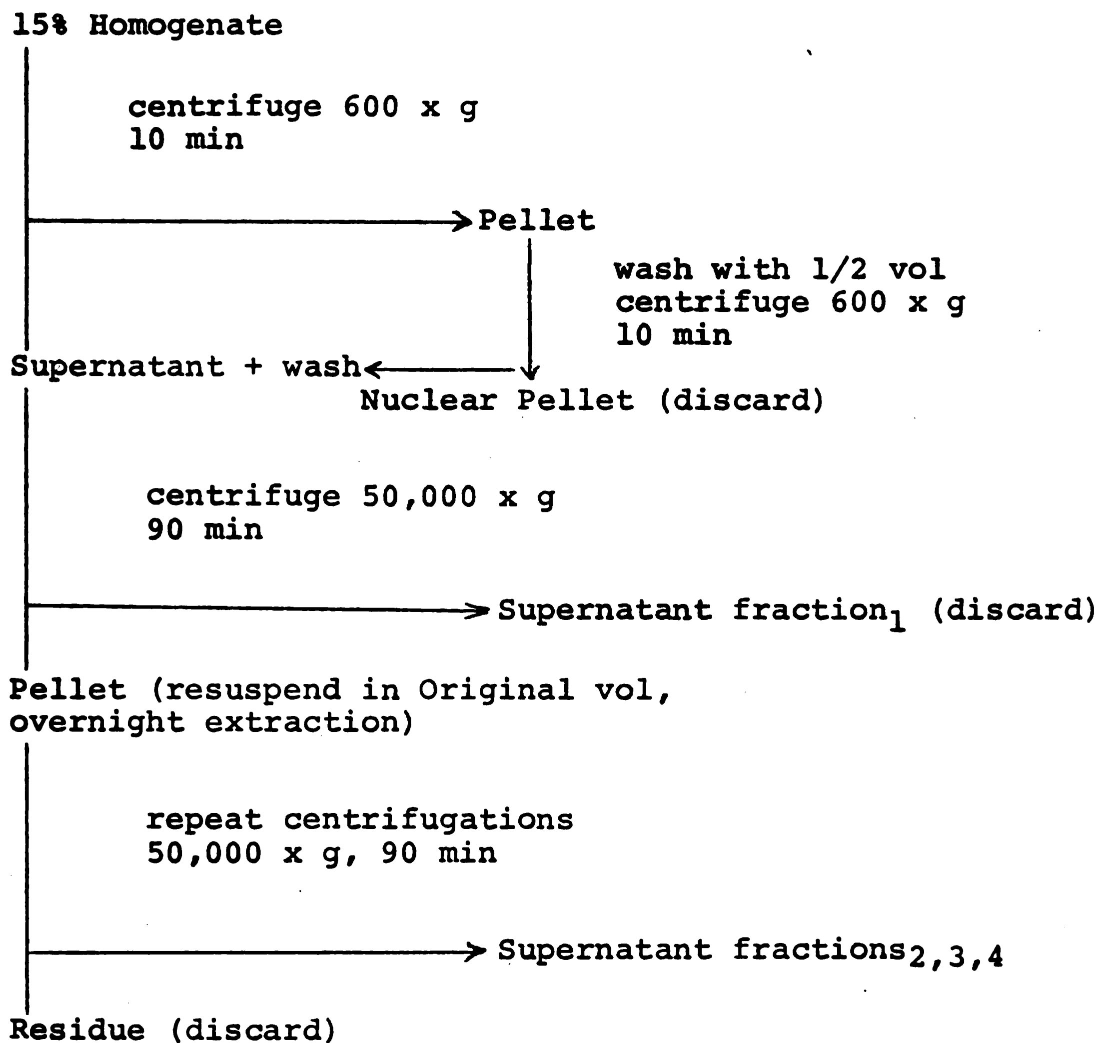


Figure 2. Fractionation scheme for AChE solubilization.

discarded because of low AChE content. The pellets were then suspended in the original volume of 0.32 M sucrose in 1 mM EDTA, pH 6.9. The suspension was stirred slowly overnight in the cold room at 4°C and then centrifuged to give supernatant 2. Supernatant fractions 3 and 4 were obtained similarly after overnight suspension of the pellets and centrifugation. Fractions 2, 3, and 4 were dialyzed overnight at 4°C against 1 mM phosphate buffer, pH 7.0. The supernatants were then centrifuged at 80,000 x g for 75 minutes to remove any residue and assayed for AChE activity and protein.

b) Ammonium Sulphate Precipitation

12.5 g of $(NH_4)_2SO_4$ was added slowly with constant stirring to every 100 ml of the combined supernatant fractions. The mixture was stirred for 30 minutes and centrifuged at 14,000 x g for 40 minutes. The supernatant fluid was removed for further precipitation by adding 26.5 g of $(NH_4)_2SO_4$ to every 100 ml and the above procedure was repeated. The second protein precipitate was redissolved in the smallest possible volume of 10 mM phosphate buffer pH 7.0 (100 ml; 5-6 mg of protein/ml), dialyzed overnight against 1 mM phosphate buffer (pH 7.0) and centrifuged at 14,000 x g for 40 minutes to remove any insoluble residue.

c) Affinity Chromatography

The affinity gel was synthesized from modified Affinose-202 and m-trimethylammoniumaniline as described by Chan et al (30). AChE preparations obtained after $(NH_4)_2SO_4$ precipitation were applied to a 5-10 ml bed volume of the affinity gel. The

bound material was washed with (750-1000 ml) 100 mM NaCl at pH 8.0. The elution of AChE was carried out with 10 bed volumes of 10 mM edrophonium chloride in 100 mM NaCl at pH 8.0. The eluted fractions (each equal to a bed volume) were dialyzed overnight to remove the edrophonium chloride. AChE activity and protein concentration of each fraction were determined and active fractions were pooled, concentrated and then applied to a Sephadex G-200 column.

d) Sephadex G-200 Filtration

One ml of the concentrated AChE preparation was applied to a previously equilibrated Sephadex G-200 column (1.3 x 100 cm). Elution was carried out with 200 ml of 30 mM phosphate buffer pH 7.0, at a flow rate of 7-8 ml/h. Fractions of 1.8 ml were collected and assayed for AChE activity. Active fractions were pooled and concentrated. The single peak of AChE obtained by affinity chromatography was eluted as three active peaks (A, B, and C). The average specific activity of each peak was 580 (A), 535 (B), and 610 (C) mmoles of ATC hydrolyzed/mg of protein/h.

2. Microsomal Fractions

Caudate nucleus tissue cleared of white matter and blood capillaries was homogenized in 9 volume of 0.32 M sucrose containing 1 mM EDTA adjusted to pH 6.9 with tris (hydroxymethyl) aminoethane. Subcellular fractionation was carried out in a Lourdes Model A_2 Betafuge using a 9RA rotor according to the fractionation scheme shown in Figure 3. The final microsomal pellets were resuspended in 0.32 M sucrose containing 1 mM

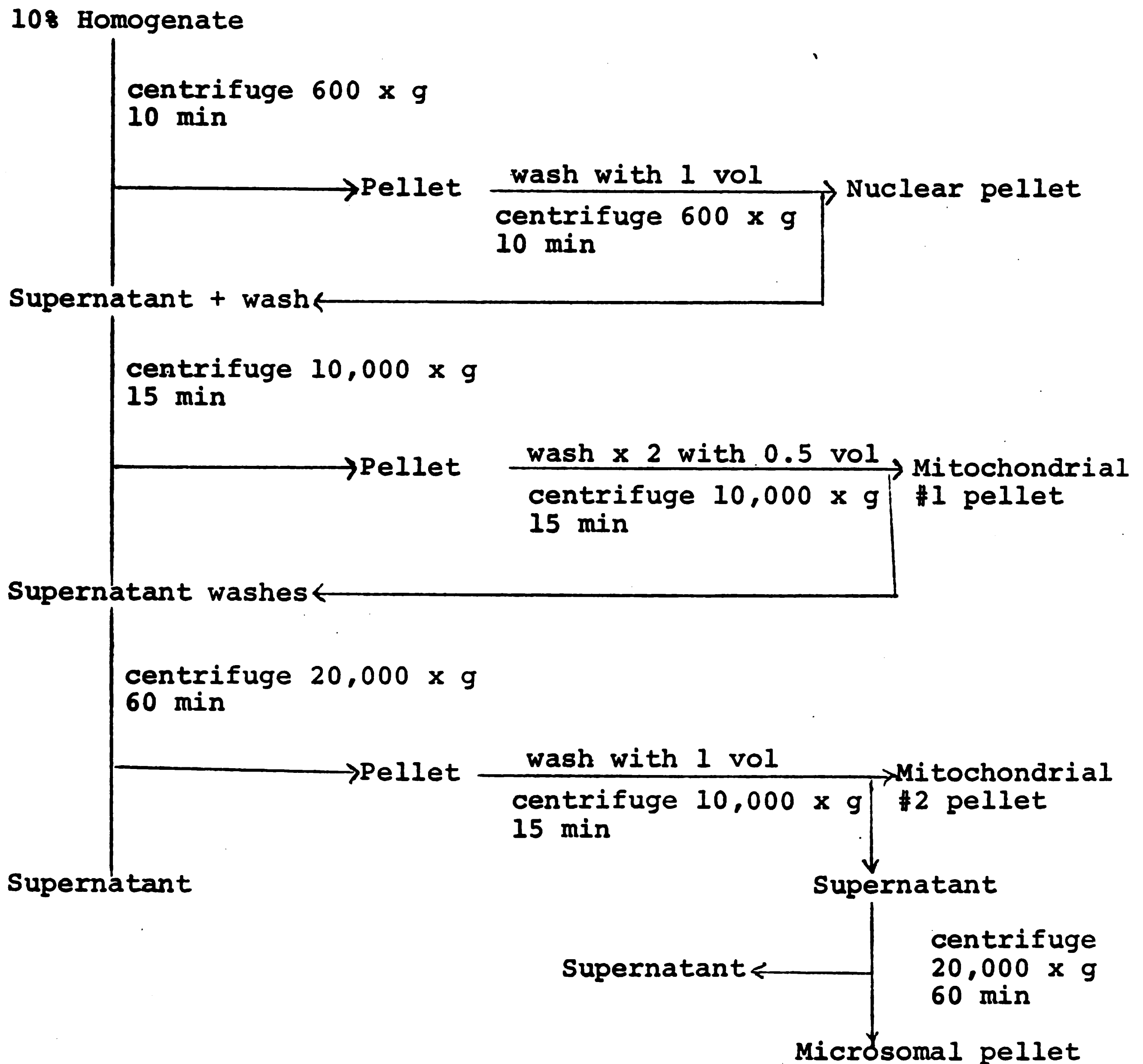


Figure 3. Fractionation scheme for microsomal fraction.

EDTA with a Dounce homogenizer. The final protein concentration was 16-20 mg/ml. Small aliquots of the suspension were stored at -10°C until use.

3. Preparation of Synaptosomal Fractions

A 10% homogenate of the caudate tissue was prepared in 0.32 M sucrose. Subcellular fractionation of the homogenate was carried out by the procedure of De Robertis et al (41) which is presented in Figure 4. The crude mitochondrial fraction was submitted to osmotic shock in distilled water and centrifuged at 20,000 x g for 30 minutes. The pellets were resuspended in 0.32 M sucrose and carefully layered on a sucrose density gradient and centrifuged at 50,000 x g for 2 hours. Finally, 5 layers and a pellet were obtained. Fractions 2 and 3 were separated carefully and combined for measuring AChE activity and protein concentration. Fraction 1 consisted principally of myelin, fraction 4 contained synaptic membrane with some mitochondria and fraction 5 was predominantly mitochondrial in nature.

4. Preparation of Na^+-K^+ Adenosinetriphosphatase

a) Homogenization and Subcellular Fractionation

The cerebral cortices (gray matter) of beef brains were cleared of membrane and blood capillaries. Gray matter was scraped off with a spatula and homogenized in 9 volumes of 0.32 M sucrose containing 1 mM EDTA, pH 6.9. Subcellular fractionation of the homogenate was carried out according to the fractionation scheme previously discussed and shown in

10% Homogenate

centrifuge 900 x g
10 min

Pellet | Supernatant

centrifuge in 1/2
original vol
centrifuge 900 x g
10 min

Nuclear Pellet (discard) ——> Supernatant

centrifuge 11,500 x g
20 min

Mitochondrial Pellet | Microsomal Supernatant$_1$

resuspend in 1/2
original vol
centrifuge 11,500 x g
20 min

Mitochondrial Pellet | Microsomal Supernatant$_2$

osmotic shock
resuspend in distilled water
1 g tissue/10 ml distilled water
let stand for 30 min at 0°-4°C
then centrifuge 20,000 x g
30 min

Pellet M_1 | Supernatant

resuspend in 0.32 M sucrose
apply to 4 layer sucrose
density gradient
centrifuge 50,000 x g
2 hrs

Clear Layer | Five Particulate Layers

Figure 4. Subcellular fractionation scheme for isolation of synaptosomal acetylcholinesterase

Figure 3 in a Lourdes Model A_2 Betafuge at 0-4°C using a 9 RA rotor. The final pellet (microsomal) some of which probably also contained mitochondria was resuspended with a Dounce homogenizer in 0.32 M sucrose EDTA solution and protein concentration was adjusted to 18-22 mg/ml. The microsomal fraction was stored at -10°C until used or treated either with lubrol or NaI as desired.

b) Lubrol Extraction of the Microsomal Fraction

A 0.4% lubrol solution (w/v) in 0.32 M sucrose containing 1 mM EDTA, pH adjusted to 6.9 with tris buffer was added to an equal volume of the microsomal fraction, resulting in a final lubrol concentration of 0.2%, which was found to be optimal for Na^+-K^+-ATPase extraction (160). This suspension was homogenized in a Dounce homogenizer (B pestle) using 10 strokes. It was kept on ice for 15 minutes. The suspension was then centrifuged in a FA 40 rotor of a Spinco Model L centrifuge at 100,000 x g for 1 hour. The supernatant was removed and stored in a refrigerator for immediate use of frozen at -10°C for longer storage (3 weeks) and the pellet was discarded. The supernatant had a protein concentration of 2.5-3 mg/ml.

c) Sodium Iodide Treatment of the Microsomal Fraction

The microsomal fraction was diluted with 0.32 M sucrose 1 mM EDTA solution to give a protein concentration between 2-5 mg/ml. The NaI treatment was carried out by the procedure of Schwartz et al (157) with the following minor modifications.

The precipitate was washed with 5 mM EDTA and was centrifuged at 50,000 x g for 15 minutes. The above washing and centrifugation was repeated twice. The final pellet was resuspended in 0.32 M sucrose to give a protein concentration of 1-1.2 mg/ml and kept frozen until use.

5. Assays and Procedures

a) Acetylcholinesterase

AChE activity was determined using the thiocholine colorimetric procedure of Ellman et al (47) at 25°C using a Perkin-Elmer Model 356 two wavelength double-beam Spectrophotometer connected to a Perkin-Elmer Model 165 Recorder. The reaction mixture contained phosphate buffer, 0.1 M; 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 10 mM; acetylthiocholine, 1 mM; protein and deionized water in a final volume of 3.0 ml. Tris buffer, 75 mM, was used instead of phosphate in some of the experiments. To study the effect of cyclopropane all the contents except the substrate were placed in the optical cuvette, a modified Thunberg tube (Figure 5). The desired gas pressure was measured to the nearest milimeter by means of a meter scale attached to a closed end manometer. For gas pressures above one atmosphere a specially designed stainless steel chamber with a quartz cell was used (Figure 6). The gas was introduced in to the system at the desired pressure and 5 minutes were allowed for gas equilibration before starting the reaction with substrate ATC (0.1 mM to 10 mM). The control AChE activity was measured in the presence of air at the same pressure. The

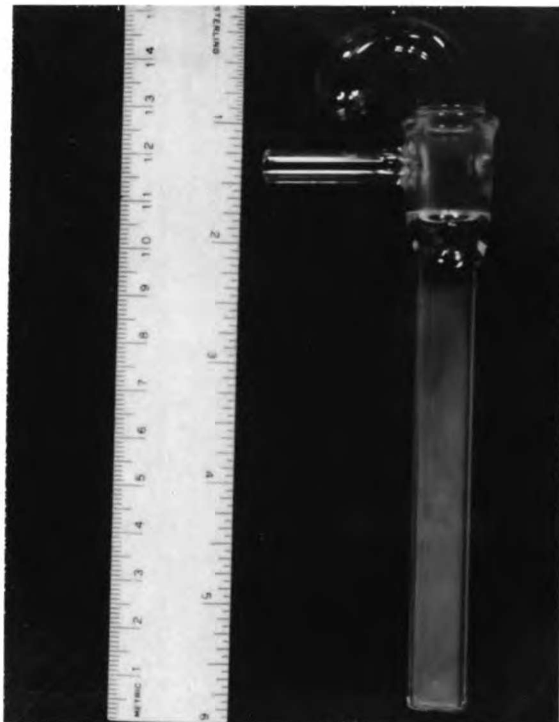


Figure 5. Thunberg tube with an optical cuvette.

Figure 6. Airtight steel chamber with a quartz cell.

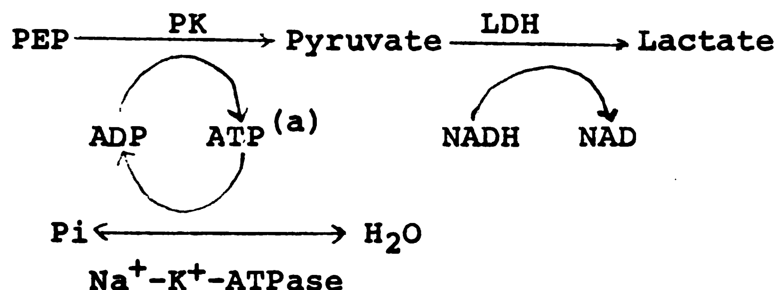
effects of cyclopropane were also examined on DTNB and non-enzymatic hydrolysis of ATC.

To study the relationship between AChE and Na^+-K^+-ATPase, AChE activity was measured at 37°C. The solutions were mixed with a stirrer and incubated at 37°C for 10 minutes. In all the experiments (apart from those where the experimental conditions are specifically mentioned), the final concentrations of NaCl were 110 mM; KCl, 10 mM; $MgCl_2$, 5 mM; and ATP, 5 mM. The final protein concentrations ranged from 200 to 300 ug/ml. ATP was added to the reaction mixture just before the substrate (acetylthiocholine), however, preincubation with ATP (up to 15 minutes) did not change the degree of inhibitory effect on AChE activity. The pH was measured before and after the reaction and did not change more than 0.05 pH units. The term "basic" AChE activity is used to describe AChE activity without the addition of any salts and "stimulated" AChE activity is that activity in the presence of salts. Basic AChE activity (acetylthiocholine as substrate) was 27.2 umoles/mg protein/h at pH 8.0 and 37°C. Activity with butyrylthiocholine as substrate was approximately 1% of this value.

b) Na^+-K^+-Adenosinetriphosphatase

The solution used for the assay of ATPase activity contained ATP (tris salt), 5 mM; KCl, 10 mM; NaCl, 110 mM; $MgSO_4$, 5 mM; tris buffer, 75 mM; and the enzyme in a total volume of 0.5 ml. Protein concentration was 200-300 ug/ml. A 0.25 ml aliquot was taken after the reaction was stopped

with .05 ml of 10% perchloric acid (PCA) and phosphate was determined by the method of Martin and Doty (106). Na^+-K^+-ATPase activity was also determined by recording the rate of oxidation of NADH spectrophotometrically, at a wavelength of 340 mu, employing a linked enzyme system by the procedure of Albers and Koval (6). The reaction scheme is as shown below:



Each cuvette contained (in final concentration) $MgCl_2$, 5 mM; NaCl, 110 mM; KCl, 10 mM; tris HCl, 25 mM; (pH 7.5), tris ATP, 2.5 mM; NADH, 0.5 mM; phosphoenolpyruvic acid and 0.02 ml of a combined pyruvate kinase-lactic dehydrogenase suspension in a final volume of 1.9 ml. The reaction was started after proper temperature equilibration, by the addition of Na^+-K^+-ATPase preparation (containing 50-100 ug of lubrol solubilized protein). Reaction (a) is rate limiting, and the oxidation of NADH is directly proportional to the amount of ATP hydrolyzed by Na^+-K^+-ATPase. This linked procedure is superior to the standard phosphate method in that a) it constantly removes ADP which is inhibitory to the ATPase reaction, b) a constant steady state level of substrate ATP is maintained, and c) it monitors a continuous reaction for a longer period of time. It also enables one to study the initial reaction rates. Some problems of this system should also be mentioned

since pyruvate kinase requires K^+-ion, Mg^{++}-dependent ATPase cannot be directly measured. However, this problem is insignificant, since the preparation used contained very low amounts of Mg^{++}-ATPase and Mg^{++}-K^+-ATPase activities. It was important, therefore, to study the effects of gases on pyruvate kinase and lactate dehydrogenase to make any reasonable statement of gas effects on Na^+-K^+-ATPase.

c) Lactate Dehydrogenase

Lactate dehydrogenase (LD) activity was estimated spectrophotometrically by following NADH oxidation at 340 mu by the procedure of Kubowitz and Ott (95). The reaction medium contained potassium phosphate, 50 mM (pH 7.4); NADH, 0.17 mM; and sodium pyruvate, 0.7 mM; in a final volume of 3 ml. The reaction was started with the enzyme preparation (.005 units activity) after temperature equilibration and followed in a Perkin Elmer Model 356 two wavelength double-beam Spectrophotometer.

d) Pyruvate Kinase Lactate Dehydrogenase

The combined PK-LD activity was recorded spectrophotometrically by following NADH oxidation at 340 mu by the procedure of Neglein (124). Each cuvette contained: NADH, 0.15 mM; PEP, 1.78 mM; $MgSo_4$, 10 mM; KCl, 45 mM; tris buffer, 50 mM; and .005 units of PK-LD suspension in a final volume of 3.0 ml. The reaction was started by adding ADP, 1.5 mM.

6. Protein

Protein was determined by the procedure of Lowry et al (102). Bovine serum was used as standard. Since some of the preparations contained sucrose and sucrose lubrol, their effects were observed on the Lowry's procedure. There was no effect of sucrose or lubrol on this method.

7. Gasing Procedures

a) Gas Manifold Technique

To study the reversibility of cyclopropane inhibited Na^{+}-K^{+}-ATPase, a gas manifold technique was used and the apparatus is shown in Figure 7. All the solutions mentioned before in the phosphate assay procedure were placed (the total volume in this case was 2.5 ml, because it was difficult to draw aliquots using a syringe) in the flasks with side arms sealed with rubber caps. The flasks were attached to a vacuum line and the contents were degassed using a vacuum pump (Hyvac 7, Central Scientific Co.). The reaction was started by the addition of substrate ATP through the side arm. Control readings were taken every minute for 4 minutes. Gas was introduced into the system and equilibration was facilitated by magnetic stirring. The gas pressure was read on the pressure gauge and remained constant at the given temperature. Aliquots were removed through the side arms of the vessels and reaction was stopped by adding 0.05 ml of 10% perchloric acid. The same apparatus was used to study the effects of cyclopropane on the phosphorylation and the dephosphorylation of the enzyme.

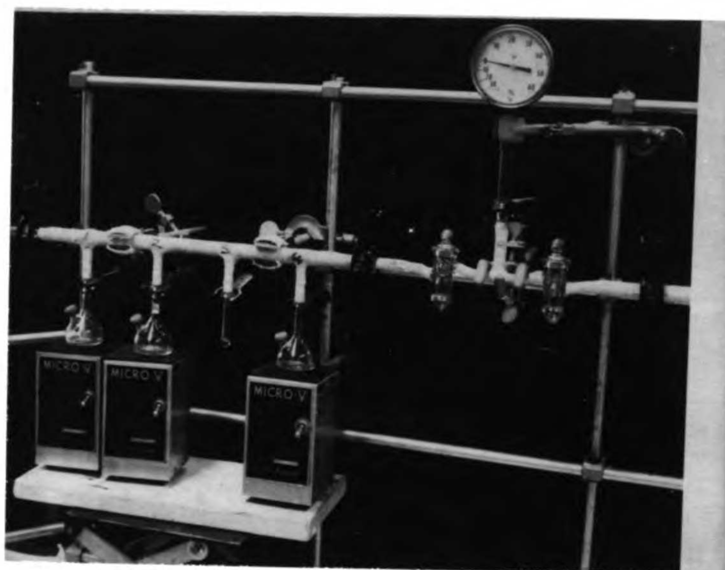


Figure 7. Airtight reaction chamber apparatus.

b) Pressure Bomb

The lubrol solubilized microsomal ATPase was subjected to various pressures of cyclopropane and other gases in a pressure bomb which is shown in Figure 8. It was constructed from stainless steel and holds twelve specially designed reactions tubes. These tubes have a central well and a side arm. Into the side arm substrate ATP was added and other reactants (ions, buffer, protein and water) were placed in the central well to give a final volume of 0.5 ml at pH 6.9. Since the enzyme activity and the gas effects were measured at different temperatures (15°-37°), the pressure bomb had to be left overnight in a temperature controlled room to achieve the desired temperature. The tubes containing the reaction mixture were placed in the air tight bomb and degassed on the vacuum for 1 minute. Gas was then added to the desired pressure and the entire system was allowed to equilibrate for 15 minutes before the reaction was started with ATP. The bomb was tipped 90° in the opposite direction from the original positions, 10 times. The reaction was allowed to proceed for a specified time, 10-20 minutes.

The gas was then released from the chamber and 0.05 ml of 10% perchloric acid was rapidly added to each reaction tube to stop the reaction. Each tube was immediately shaken 3-4 times so that any reaction occurring in the side arm was also stopped. An aliquot of 0.25 ml of the reaction mixture was taken for phosphate determination. Since cyclopropane was used at gas pressures above one atmosphere, it was impor-

Figure 8. Stainless-steel pressure bomb.

tant to rule out the effects of pressure per se. So the enzyme activity was also measured in the presence of air, nitrogen, and hydrostatic pressure (1-4 ATM). The apparatus used to study the effect of hydrostatic pressure on lubrol Na^{+}-K^{+}-ATPase is shown in Figure 9.

8. Labelling Procedure

The NaI treated enzyme was labelled by the addition of ATP (γ-^{32}p) 10^{6}cpm by the procedure of Nagano et al (123). The final concentration of $AT^{32}p$ in the reaction medium was 0.5-5 mM. The reaction was carried out at 0°C using the gas manifold apparatus. All the contents of the assay medium except the substrate $AT^{32}p$ were placed in the flasks which were continuously stirred. The reaction was started by injecting $AT^{32}p$ from the side arm using a Hamilton Syringe. The reaction was stopped after 15 seconds by injecting 3 ml of ice cold 3% perchloric acid containing 10 mM NaH_2PO_4 and 0.1 mM ATP as diluting carriers. To study the effects of cyclopropane the contents were equilibrated with the gas at the desired pressure for 10 minutes before starting the reaction with the $AT^{32}p$. Gas effects were observed a) on the phosphorylation of protein, i.e., in the presence of sodium and magnesium ions and no potassium, and b) on the dephosphorylation of protein where all the above ions were present. The centrifugation, washings and homogenizations of the labelled protein were carried out as described by Nagano et al (123). The final precipitate was suspended uniformly in 2 ml of water and 6 ml of aquasol. A portion of the supernatant was saved

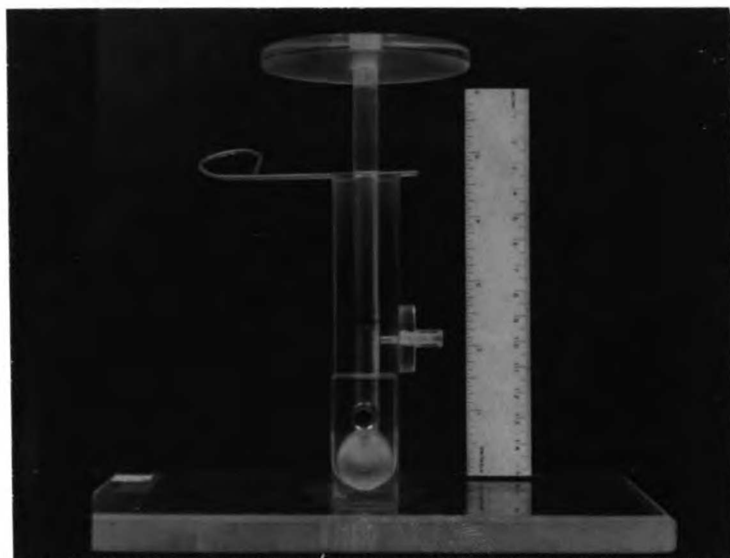


Figure 9. Hydrostatic pressure apparatus.

after each washing. Radioactivity was measured in the final pellet and in each wash using a Packard Tricarb Liquid Scintillation Spectrometer, Model 3375. The efficiency of the system was determined using internal standard method (180) and it varied from 79-85%.

III. RESULTS

A. Interactions of Cyclopropane with Brain Acetylcholinesterase

1. Properties of a Membrane Bound and Purified Forms of Brain Acetylcholinesterase

Since most of the studies of gas interactions with AChE were carried out on a membrane-bound (synaptosomal) and three purified forms of the enzyme from beef caudate tissue, the general properties of these preparations were examined (Table I). The average specific activity of synaptosomal AChE was 45 umoles of ATC hydrolyzed/mg protein/h. The substrate optimum, pH and Km values were found to be similar to those values found for other preparations of brain enzyme. The average specific activity of the three purified forms was 580 (A), 535 (B), and 610 (C) mmoles of ATC hydrolyzed/mg protein/h. These three forms exhibited some differences in kinetic properties. Forms A and B showed similar pH optima (8.0), substrate optimum (1 mM ATC) and apparent Km values (.14-.15 mM). Form C with the smallest molecular weight had a broad pH optimum (7.5-8.0), lower substrate optimum (0.5 mM) and was characterized by a higher Km value (.23 mM).

2. Effect of Varying Cyclopropane Pressure on Enzyme Activity

Since it is possible to obtain increased gas solubilities with increasing gas pressures AChE activity was measured over a pressure range of .025 to 3 ATM of cyclopropane and air in all the preparations. Results are shown for Form C in Figure 10 but quantitatively similar results were obtained for the membrane-bound and the purified forms. The degree of

TABLE I. PROPERTIES OF MEMBRANE BOUND AND

MULTIPLE FORMS OF BRAIN AChE

AChE Form	Mol. Wt.	Specific Activity (mmol of ATC/mg/h)	pH Optimum	Substrate Optimum (mM)	Km (mM)
A	390,000	580	8.1	1.0	0.14
B	270,000	535	8.0	1.0	0.15
C	130,000	610	7.5-8.0	0.5	0.23
Membrane Bound		.045	8.0	1.0	0.10

AChE forms A, B, and C from bovine caudate nucleus tissue were separated by Sephadex G-200 filtration affinity chromatography. Membrane-bound AChE was isolated from nerve endings. Their molecular weights were determined as described in Methods. Enzyme activity was determined by the acetylthiocholine (ATC) method at 25°C and pH 8.0. Data given represent the average of 3 experiments.

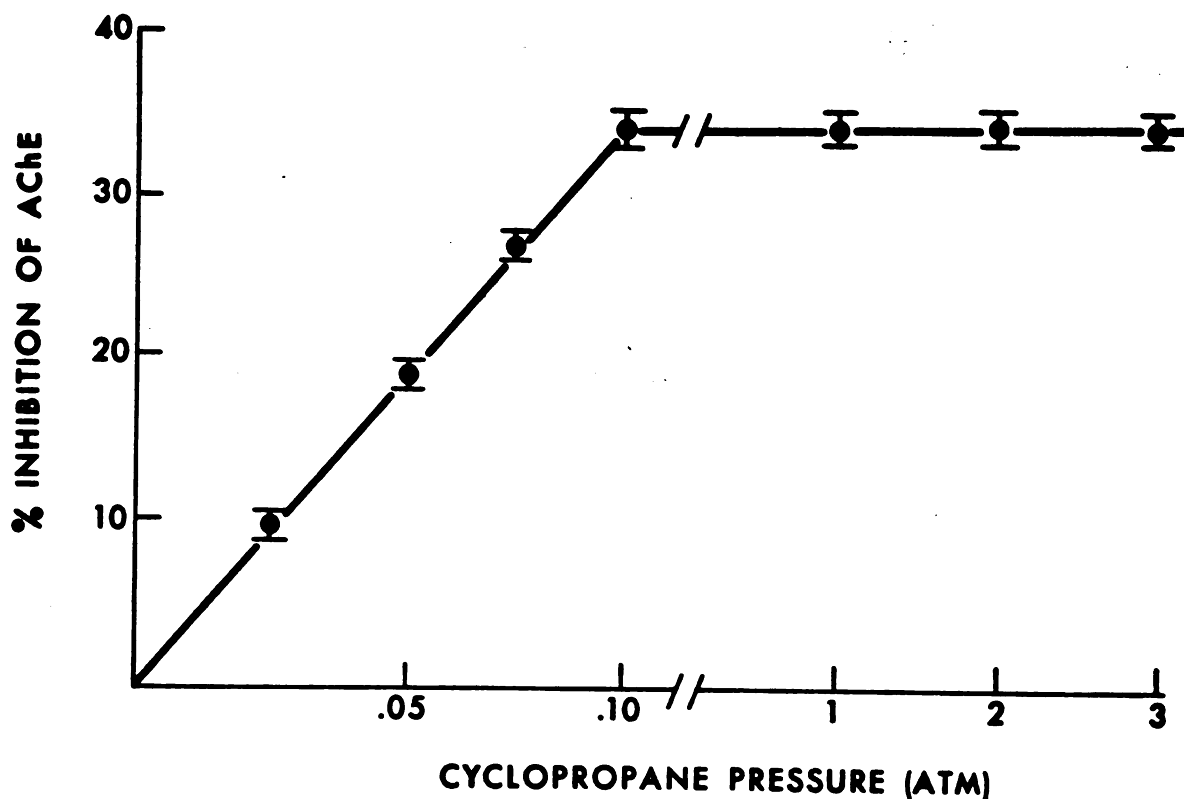


Figure 10. Pressure effect of cyclopropane on purified acetylcholinesterase, Form C. AChE activity was determined at room temperature at optimum pH (8.0) and at a substrate concentration (0.05 mM). Control AChE activity was 610,000 umoles ATC hydrolyzed/mg protein/hour. Each point is the mean of 4 separate experiments. Vertical bars represent S.E.M.

inhibition increased from 8% to 35% with increasing gas pressures up to 0.1 ATM and then remained constant (35%) over a range of 0.1-3.0 ATM of cyclopropane.

3. Sensitivity of Various AChE Preparations to Cyclopropane

The effect of cyclopropane was examined on AChE activity present in tissue homogenate, a mitochondrial fraction, a membrane-bound (synaptosomal) preparation and on the three purified forms at two substrate concentrations. The influence of cyclopropane at 0.1 atmosphere on these different preparations is shown in Table II. All these preparations were inhibited by cyclopropane and the degree of inhibition was less (15%) at the higher concentrations of the substrate and greater (35%) at the low concentrations of ATC used. There was no significant difference regarding sensitivity of the membrane-bound and the purified forms to cyclopropane at either substrate concentration.

4. Effect of Pre-Incubations with Cyclopropane

The influence of pre-incubation of all enzyme forms with the gas was studied since it was possible that the effect of cyclopropane on enzyme activity could vary with time of exposure to the gas. The incubation medium, i.e., all the reaction constituents except the substrate, was exposed to cyclopropane at 0.1 atmosphere for various periods of time (0-30 minutes) at room temperature. In the control experiments, the entire system was pre-incubated with air at 0.1 atmospheres for the specified time. At each time point, the reaction was started by the addition of the substrate ATC from the side

TABLE II. EFFECT OF CYCLOPROPANE ON

DIFFERENT AChE PREPARATIONS

Preparation	Specific Activity (mmol ATC/ protein/hr)					
	Low [S]			High [S]		
	Control	Gas	% Inhibition	Control	Gas	% Inhibition
Homogenate	.011	.007	37	.027	.0227	15
Mitochondrial Fraction	.007	.004	42	.018	.015	16
Synaptosomes	.015	.010	33	.040	.0346	13
Pure Form A	216 ± 5	155 ± 3	28	580 ± 6	511 ± 8	12
Form B	185 ± 8	135 ± 4	27	535 ± 5	467 ± 4	12
Form C	220 ± 6	144 ± 4	34	610 ± 6	500 ± 6	18

Control AChE values were taken in air (0.1 ATM). Cyclopropane was used at a pressure of 0.1 ATM. Low [S] refers to 0.1 mM ATC except in case of Form C where it was 0.05 mM ATC. High [S] was 1.0 mM ATC except for Form C where it was 0.5 mM ATC. Data represents mean of 4-5 experiments ± standard errors.

arm of the Thunburg tube. There was no significant difference in percent inhibition in all preparations at 0 and 30 minutes and the results of pre-incubation for Form C are shown in Table III. The results indicated that the onset of gas effect was immediate and eliminated the need for pre-incubation for subsequent experiments.

5. Reversal of AChE Activity after Cyclopropane Inhibition

Reversibility of the cyclopropane effect was examined in the AChE preparations mentioned to check the possibility of irreversible enzyme denaturation. Similar results were obtained for all of them. Figure 11 shows the reversal of gas effect on the purified form C at a gas pressure of 0.1 atmosphere. Control rate was determined for two minutes in the presence of air at 0.1 atmosphere. Then the reaction contents were degassed using a vacuum pump and cyclopropane was introduced at 0.1 atmosphere. The rate of ATC hydrolysis was measured again for 2-3 minutes. Cyclopropane inhibited the enzyme activity by 35% at 0.5 mM ATC. After removal of the gas, air was introduced again at the same pressure and the enzyme activity returned to approximately 95% of the initial control value, demonstrating reversibility of the cyclopropane effect. These experiments for measuring enzyme activity took about 12 minutes and the reaction rate was linear for about 15 minutes.

TABLE III. EFFECT OF PREINCUBATION WITH CYCLOPROPANE

ON THE AChE ACTIVITY

Time (minutes)	Enzyme Activity (mmol ATC/mg protein/hr)		
	Control	Cyclopropane (0.1 ATM)	% Inhibition
0	220 $\pm$ 6	144 $\pm$ 4	34
5	218 $\pm$ 5	140 $\pm$ 2	35
15	220 $\pm$ 6	140 $\pm$ 4	36
30	215 $\pm$ 3	141 $\pm$ 3	34

Assays were performed on purified form C at room temperature using 0.5 mM ATC. Control values were taken in air at 0.1 atmospheres. Figures represent mean values for 4 different experiments $\pm$ standard errors.

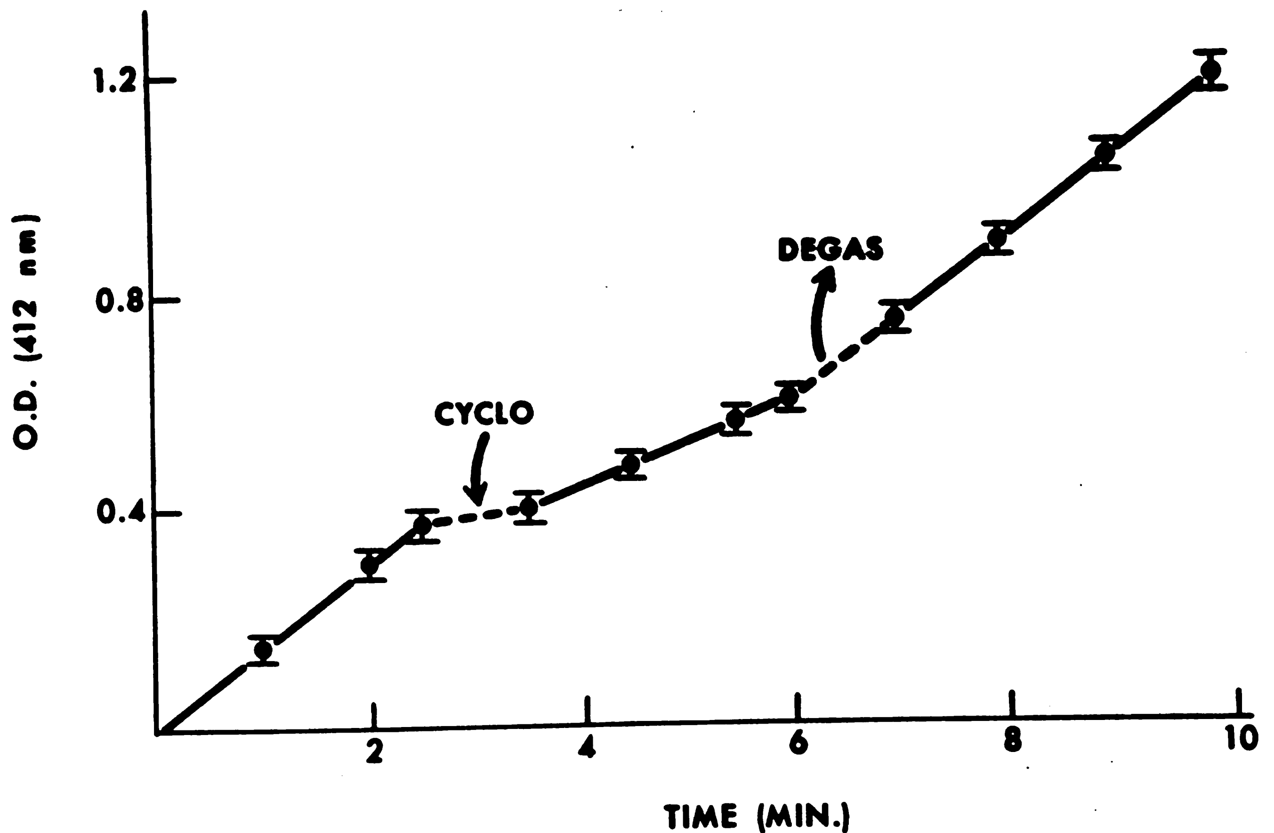


Figure 11. Return of acetylcholinesterase activity (Form C) following degasing. Control AChE activity at pH 8.0 and 0.05 mM ATC was taken for 3 minutes in the presence of air (0.1 atm). Cyclopropane at a gas pressure of 0.1 atm inhibited AChE activity. Each point represents mean of 4 different experiments. Vertical bars represent S.E.M.

6. Effect of Varying ATC Concentrations on the Cyclopropane

The effect of various substrate concentrations (.05-.5 mM ATC) was examined on the enzyme activity in the presence of air (control) and cyclopropane at a gas pressure of 0.1 ATM on the membrane-bound and the purified forms of AChE. A Lineweaver-Burk plot of cyclopropane effect on the purified form C at a gas pressure of 0.1 ATM is shown in Figure 12, but similar results were obtained for all the AChE preparations. Mixed inhibition was demonstrated by kinetic analysis and the most simple interpretation of this type of inhibition being that it involves both the competitive and non-competitive interactions of cyclopropane with the enzyme.

B. Interactions of Various Gases with Brain Adenosinetriphosphatase

1. Properties of Lubrol Solubilized Na^+-K^+-ATPase

Most of the studies of gas interactions with Na^+-K^+-ATPase were carried out on the lubrol solubilized form of the enzyme from beef cerebral cortex. The general properties of this preparation are given in Table IV. Ion activation conditions, substrate and pH optima and apparent Km values were similar to those values found for other preparations of the brain enzyme (156,159,176). Since it was anticipated that some experiments would be carried out at different temperatures, the enzyme reaction rate was measured at 15, 25, and 35°C (Figure 13). At the lowest temperature, reaction rate was linear up to 30 minutes, while at the higher temperature 15

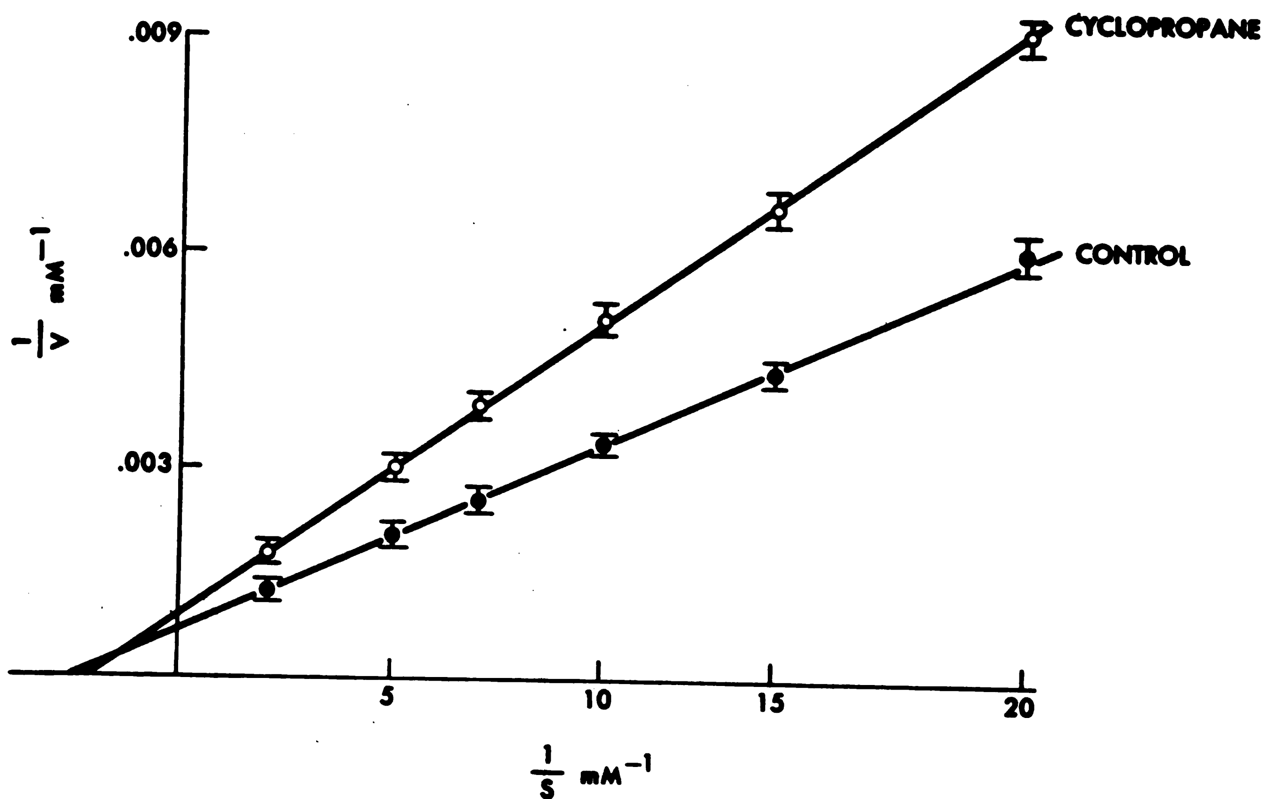


Figure 12. Lineweaver-Burk plot of inhibition of purified acetylcholinesterase, Form C by cyclopropane (0.1 atm). Enzyme activity was determined at optimum pH (8.0) and substrate concentrations ranged from 0.05 to 0.5 mM ATC. Control enzyme activity was 610 mmoles ATC hydrolyzed/mg protein/hour at optimum substrate concentration (0.5 mM).

TABLE IV. PROPERTIES OF LUBROL EXTRACTED Na^+-K^+-ACTIVATED ATPase FROM BEEF BRAIN

I. Effect of Na^+ and K^+

  A. Activation by Na^+ with 10 mM K^+

    1. Maximal - 100 mM Na^+

  B. Activation by K^+ with 100 mM Na^+

    1. Maximal - 10 mM K^+

  C. No activation by Na^+ or K^+ alone

II. Enzyme concentration

  A. Linear - 20-200 ugm Prot/ml incubation media

  B. Apparent Km - 5.0 x 10^{-4} M

  C. Substrate inhibition - 7.5 mM ATP and above

IV. pH curve

  A. Optimal - pH 6.8 in 100 mM imidazole

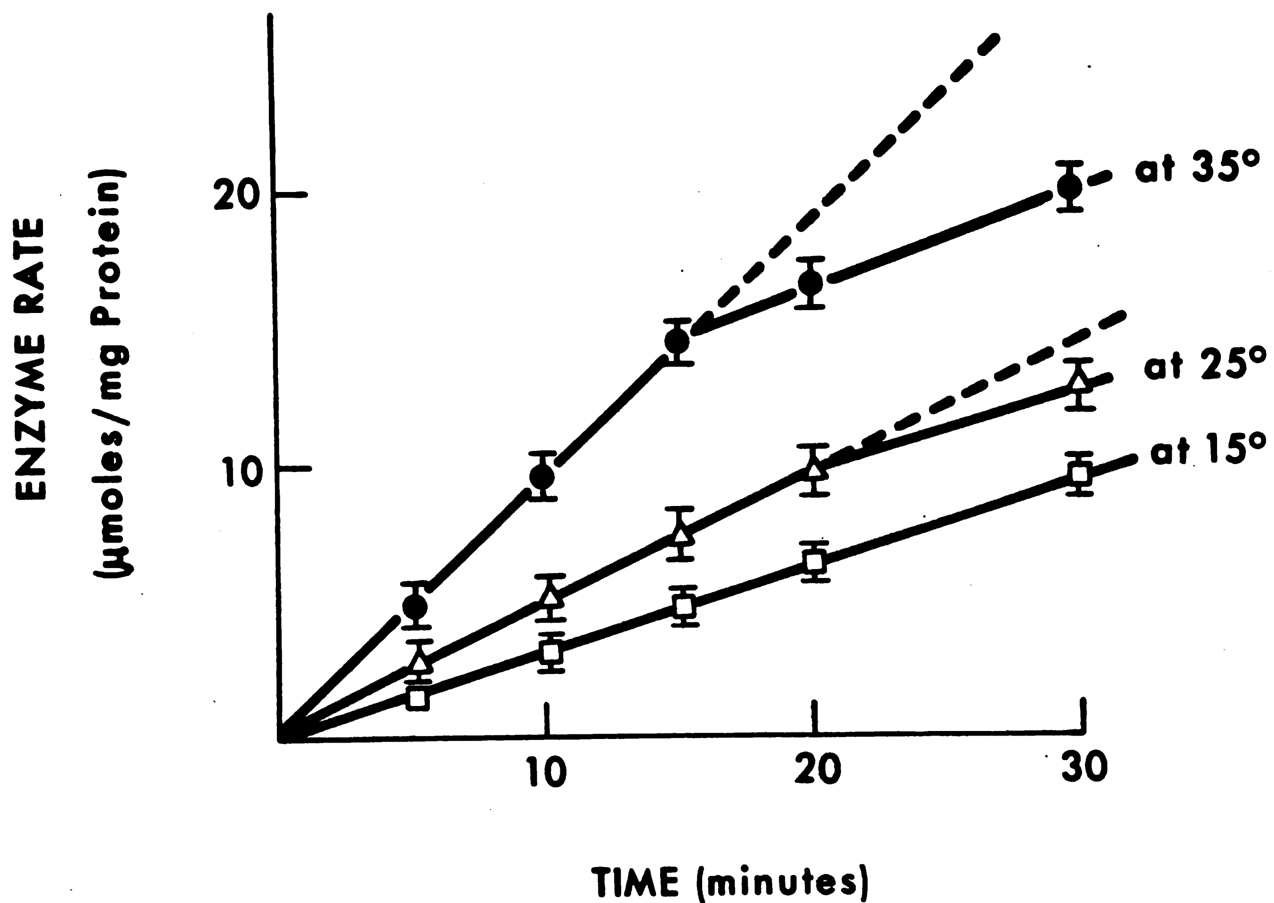


Figure 13. Changes in lubrol solubilized Na^{+}-K^{+}-ATPase activity with time at 35°, 25°, and 15°C at pH optimum (6.9) and substrate optimum (5 mM ATP). Each point represents the mean of 3-4 separate experiments. Vertical bars represent S.E.M.

minutes was judged to be the maximum period over which a reaction could be examined while still maintaining linearity. Na^{+}-K^{+}-ATPase activity present in microsomal fractions or obtained by NaI treatment were not characterized so thoroughly since their properties have been well documented (155,156).

2. Effect of Various Cyclopropane Pressures on Lubrol Na^{+}-K^{+}-ATPase

Lubrol Na^{+}-K^{+}-ATPase activity was measured over a pressure range of 1-4 atmospheres of cyclopropane at optimum substrate (5 mM ATP) concentration at 37°C. Results of this experiment are shown in Table V. The degree of inhibition increased from 12% to 55% approximately with increasing gas pressures. This phenomenon may reflect the increased solubility of cyclopropane with increasing gas pressures. Since only a slight effect (10%) was observed at one atmosphere of gas, higher pressures will be used in subsequent experiments.

3. Sensitivity of Various Preparations of Na^{+}-K^{+}-ATPase to Cyclopropane

The effect of cyclopropane was also examined on Na^{+}-K^{+}-ATPase activity present in cerebral tissue homogenate, microsomal fraction prepared by differential centrifugation and on the enzyme preparation derived by NaI treatment of the microsomal pellet. The influence of cyclopropane at 3.5 atmospheres on these different preparations is shown in Table VI. All preparations were inhibited by the gas with no significant difference in the degree of inhibition. Cyclopropane at the pressure used in these experiments had no significant effect on Mg^{++}-ATPase activity (data not shown).

TABLE V. INFLUENCE OF CYCLOPROPANE PRESSURE

ON Na^{+}-K^{+}-ATPase ACTIVITY

Cyclopropane (P-ATM)	Enzyme Activity (umoles Pi/mg protein/hr)			
	Control	Cyclopropane		% Inhibition
1	58 ± 2	51	1	12
2	55 ± 2	41	2	26
3	60 ± 1	38	1	36
4	58 ± 1	25	.5	60

Assays were performed at 37°C at pH optimum (6.9). Control values were taken at the optimum concentration of ATP (5 mM) at atmospheric pressure. Data represents mean value of 3-4 separate experiments ± standard erros of mean.

TABLE VI. EFFECT OF CYCLOPROPANE ON VARIOUS

Na^{+}-K^{+}-ATPase PREPARATIONS

Preparation	Specific Activity (umoles Pi/mg/hr)		% Inhibition
	Control	Cyclopropane 3.5 ATM	
Homogenate	16	10	38
Microsomes	26	15	42
Na I	43	22	49
Lubrol	58	28	51

Experiments were performed at 37° at optimum pH (6.9) and at 5 mM ATP concentration. Control values were taken at one atmospheric pressure. Figures represent mean values of 4 different experiments.

4. Effect of Pre-Incubation with Cyclopropane on the Lubrol Na^{+}-K^{+}-ATPase Activity

Since it was possible that the degree of cyclopropane inhibition of enzyme activity could vary with time of exposure to the gas, the influence of pre-incubation of enzyme with the gas was studied. Lubrol Na^{+}-K^{+}-ATPase preparation together with all reaction constituents except substrate were exposed to cyclopropane at 3.0 atmospheres for 5, 15, and 30 minutes at 37°C. At each time, the reaction was initiated by the addition of substrate from the side arm of the reaction vessel. In the control experiments the entire system was degassed for one minute and left for the specified time before starting the reaction with ATP. There was no significant difference in the degree of inhibition from 5 to 30 minutes as is shown in Table VII. For all subsequent experiments 10 minute pre-incubation time was selected.

5. Reversal of Cyclopropane Inhibitory Effect

Although cyclopropane affected the enzyme activity at pressures below 1 atmosphere, significant inhibition required higher gas pressures. Under such conditions it was possible that the enzyme preparation could undergo an irreversible denaturation. To examine this possibility, experiments were performed using the 'Gas-Manifold' apparatus described in 'Methods.' Control rates of lubrol enzyme activity were determined at 2 and 4 minutes in the presence of air at atmospheric pressure. The entire system was then degassed and cyclopropane was introduced at 2.3 atmospheres. Samples were taken for determination of enzyme reaction rate at 8 and

TABLE VII. EFFECT OF PREINCUBATION WITH CYCLOPROPANE ON ENZYME ACTIVITY

Time (minutes)	Enzyme Activity (umoles/mg/hr)		% Inhibition
	Control	Cyclopropane 3 ATM	
5	38 ± 1	19 ± 1	47
15	39 ± 1	20 ± 1	48
30	43 ± 1	22 ± 0	48

Assays were performed at 37°. Media was degassed and preincubated for the specified times with or without gas. Figures represent mean values for 3 distinct experiments ± standard error of mean.

10 minutes. The system was then degassed to remove cyclopropane and air permitted to enter at atmospheric pressure. The enzyme reaction rate was then determined at 14 and 16 minutes. The results of these experiments are shown in Table VIII. Cyclopropane at 2.3 atmospheres caused a 35% inhibition of Na^{+}-K^{+}-ATPase activity. After removal of the gas, the enzyme activity returned to approximately 95% of the initial control value, demonstrating almost complete reversibility of the cyclopropane effect. These experiments involved estimation of enzyme activity over a 16 minute time period and at 25°C, Na^{+}-K^{+}-ATPase reaction rate was linear for 20 minutes (Figure 13).

6. Sensitivity of Lubrol Na^{+}-K^{+}-ATPase to N_2, or O_2 and Hydrostatic Pressure

Since gas pressure above 1 atmosphere was required to observe a marked effect of cyclopropane on Na^{+}-K^{+}-ATPase activity, it was necessary to determine if cyclopropane inhibition was specifically due to cyclopropane or due to pressure per se. In these experiments, the influence of nitrogen, oxygen and hydrostatic pressure all at 4.3 atmospheres was examined on lubrol Na^{+}-K^{+}-ATPase preparation (Figure 14). The control value represents the enzyme activity in the presence of air at atmospheric pressure. Hydrostatic pressure, nitrogen and oxygen all stimulated the enzyme activity by 10-12% unlike cyclopropane, which at the same pressure, inhibited the enzyme activity by approximately 50%. Subsequent attempts were made (see later) to examine the nature of the small but

TABLE VIII. REVERSAL OF CYCLOPROPANE INHIBITED Na^+-K^+-ATPase

Time (minutes)		Enzyme Rate umoles/mg protein	% Change
2-4	Air atmospheric pressure	$0.73 \pm .04$	-
8-10	Cyclopropane 2.3 ATM	$0.47 \pm .06$	-35
14-16	Air	$0.70 \pm .04$	-4

Control enzyme rate was determined at room temperature, at pH 6.9 and at optimum substrate concentration (5 mM ATP) in the presence of air at atmospheric pressure. Figures represent mean values of 4 distinct experiments $\pm$ standard errors of mean.

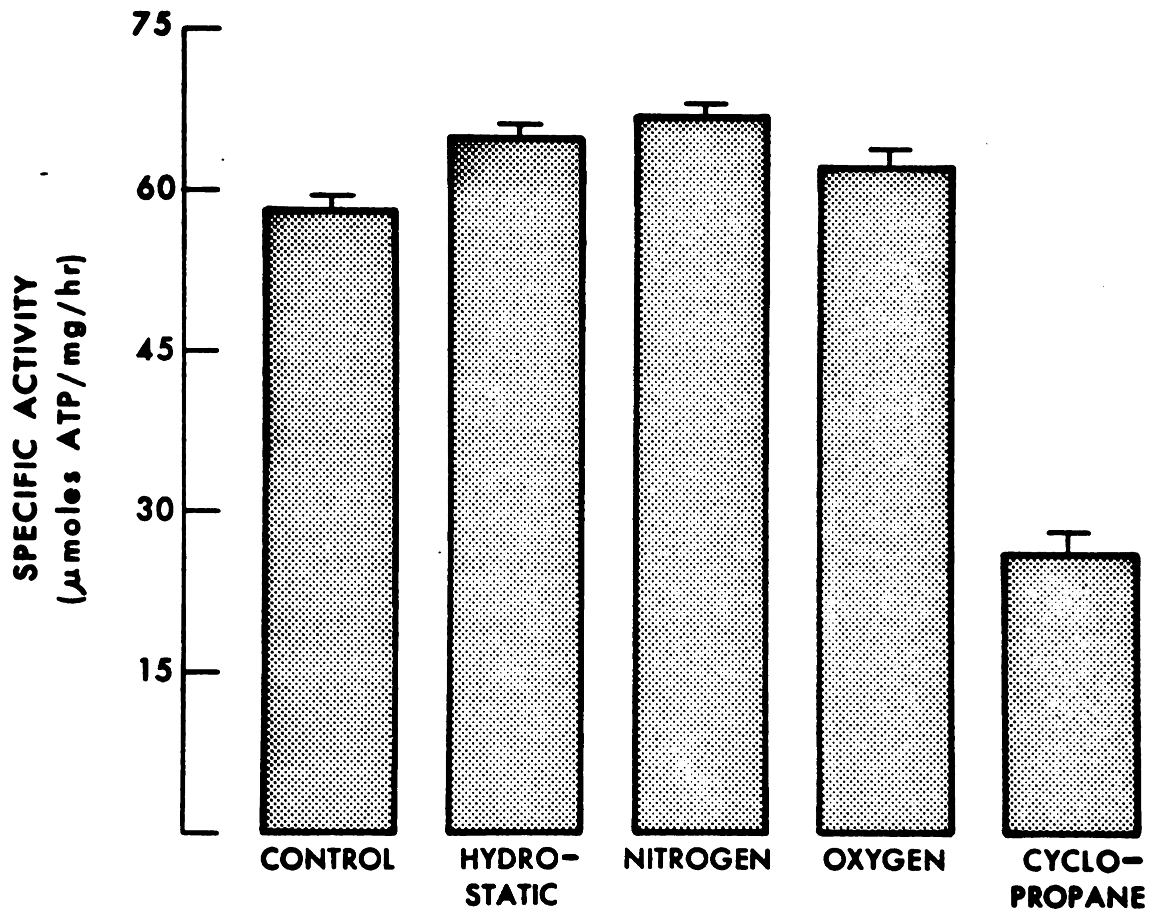


Figure 14. Effect of hydrostatic pressure, nitrogen, oxygen and cyclopropane (4.3 atm) on lubrol Na^+-K^+-ATPase activity. Control enzyme activity was 58.5 umoles Pi/mg protein/hour, at 37°C, pH 6.9 and substrate optimum (5 mM ATP) in the presence of air at atmospheric pressure. Data represents mean of 4 individual experiments. Vertical bars represent S.E.M.

significant stimulating action of the other gases. The data clearly suggests that while pressure per se may influence Na^{+}-K^{+}-ATPase activity, the inhibitory action of cyclopropane is specific for this gas.

7. Influence of Temperature on Cyclopropane Inhibition of Na^{+}-K^{+}-ATPase

The results of studies on the influence of temperature (15°-35%) on the enzyme inhibition by cyclopropane at 3.5 atmospheres are shown in Table IX. Cyclopropane inhibited the enzyme activity by 50% at the higher temperature and the degree of inhibition increased to 70% at 15°C as might be predicted by the increased gas solubility at the lower temperatures. An arrhenius plot of log of specific activity versus reciprocal of absolute temperature is shown in Figure 15. Inflections occur in the plots between 25-30° points, both in the absence and presence of cyclopropane.

8. Effect of N_2, Compressed Air and O_2 on Enzyme Activity as a Function of Substrate Concentration

The effects of N_2, compressed air and oxygen were examined on Na^{+}-K^{+}-ATPase activity at 4.3 atmospheres, since no significant effects were observed in the presence of various substrate (ATP) concentrations and the results of this experiment is shown in Table X. At lower substrate concentrations, both N_2 and compressed air inhibited the lubrol Na^{+}-K^{+}-ATPase activity by 13-17% but stimulation was observed at the higher concentrations of ATP. Similar results were obtained with oxygen (data not shown). Since hydrostatic pressure also stimulated the enzyme activity by approximately

TABLE IX. TEMPERATURE EFFECT ON Na^{+}-K^{+}-ATPase ACTIVITY AND CYCLOPROPANE INHIBITION OF ENZYME SYSTEM

Temperature (°C)	Enzyme Activity (umoles/mg protein/hr)		% Inhibition
	Control	Cyclopropane 3.5 ATM	
35°	53 ± 2	25 ± 1	52
25°	21 ± 1	9 ± 0	56
20°	11 ± 1	4 ± 0	60
15°	5 ± 0	1 ± 0	71

Enzymatic assays were performed in the reaction media buffered with 20 mM imidazole at pH 6.9 at 5 mM ATP concentration. Data represents mean values from 3 separate experiments ± standard error.

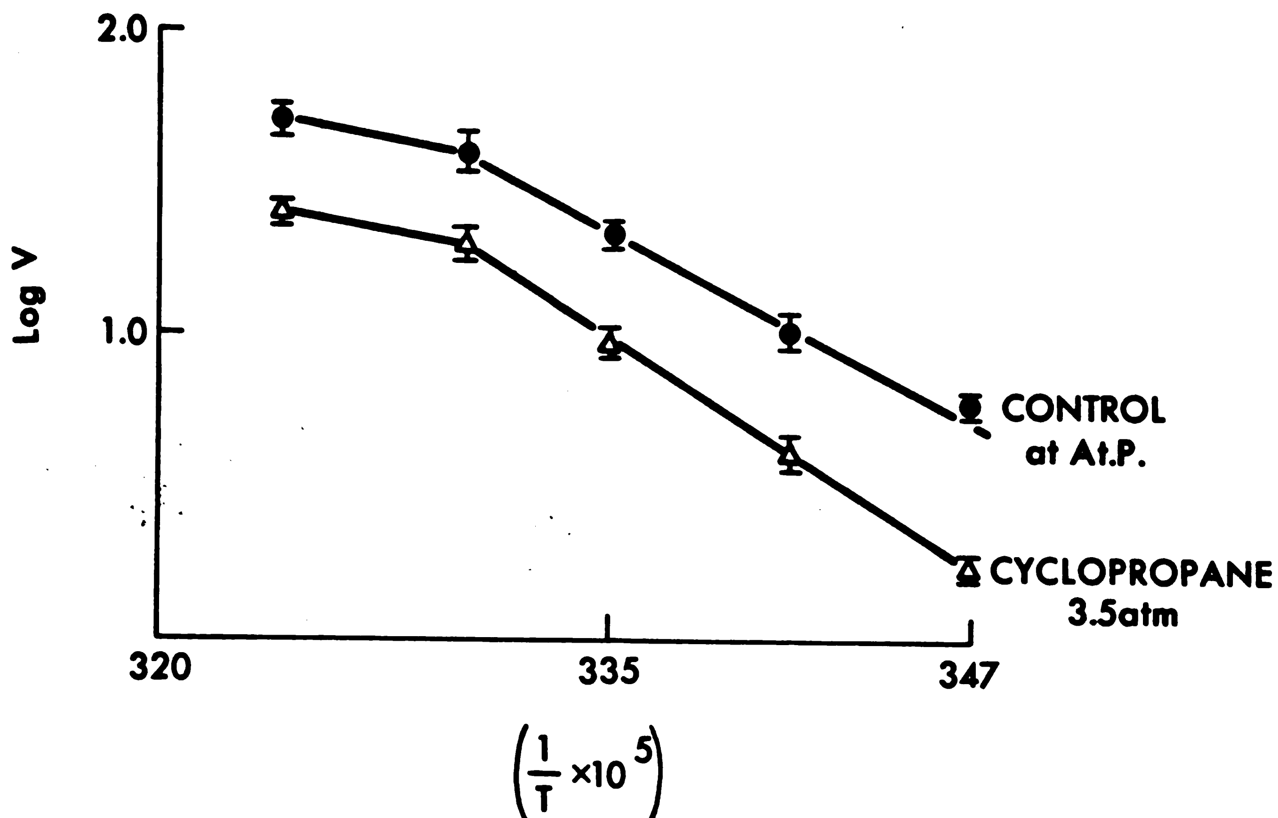


Figure 15. Arrhenius plot of inhibition of Na^{+}-K^{+}-ATPase activity by cyclopropane. Control enzyme activity at 35°C was 58.5 umoles/mg protein/hour at optimum pH (6.9) and at 5 mM ATP. Temperature varied from 15° to 35°. Each point represents mean of 4-5 distinct experiments. Vertical bars represent S.E.M.

TABLE X. EFFECT OF 4.3 ATM of N_2 AND COMPRESSED AIR ON Na^+-K^+-ATPase ACTIVITY

[ATP Concentration] mM	Control Specific Activity (umoles/mg protein/hr)	N_2	% Change	Compressed Air	% Change
.5	27 ± 0	22 ± 0	-17	23 ± 1	-13
1.0	40 ± 0	33 ± 1	-17	35 ± 0	-12
2.0	48 ± 1	49 ± 0	+1	49 ± 1	+1
3.0	51 ± 2	61 ± 1	+19	59 ± 2	+13
5.0	58 ± 1	72 ± 2	+24	69 ± 0	+19

Effect of 4.3 atmospheres of N_2 and compressed air was determined on the Na^+-K^+-ATPase activity at 37°C. Substrate concentration ranged from .5 to 5 mM ATP. Control enzyme activity was measured in the presence of air at 1 atmosphere. Figures represent mean values of 3 distinct experiments ± standard error.

12% (Figure 14) one could interpret these gas effects to be non-specific. However, this does not appear to be the case, as at the lower substrate concentrations, hydrostatic pressure also stimulated Na^{+}-K^{+}-ATPase activity unlike with N_2, O_2, and compressed air, inhibition of the enzyme activity was observed.

9. Kinetics of Cyclopropane Inhibition of Na^{+}-K^{+}-ATPase

The effect of various substrate concentrations (0.5-5 mM ATP) on the activity of lubrol enzyme at atmospheric control and at gas pressures of 3.5 and 2.0 atmospheres was examined. The results indicated that a concentration of 5 mM ATP gave optimal enzyme activity in the presence of air at atmospheric pressure and also maximum inhibition of the enzyme activity at both gas pressures used. Reciprocal Lineweaver-Burk plots of the enzyme activity versus substrate concentrations are shown in Figure 16. The degree of inhibition increased with increasing substrate concentration, in other words inhibition by cyclopropane appeared to be substrate-promoted. This type of inhibition is not frequently observed and has been referred to as 'Exclusive C Type' or uncompetitive inhibition (137).

10. Cyclopropane Action on Phosphorylation and Dephosphorylation of Na^{+}-K^{+}-ATPase

A number of steps have been postulated in the ATP hydrolytic reaction sequence (3,6,163,170). Perhaps the two with which there is most agreement are those involving Na^{+} and Mg^{++}-dependent phosphorylation and the subsequent K^{+}-dependent dephosphorylation of the enzyme. Cyclopropane effect at 2.5

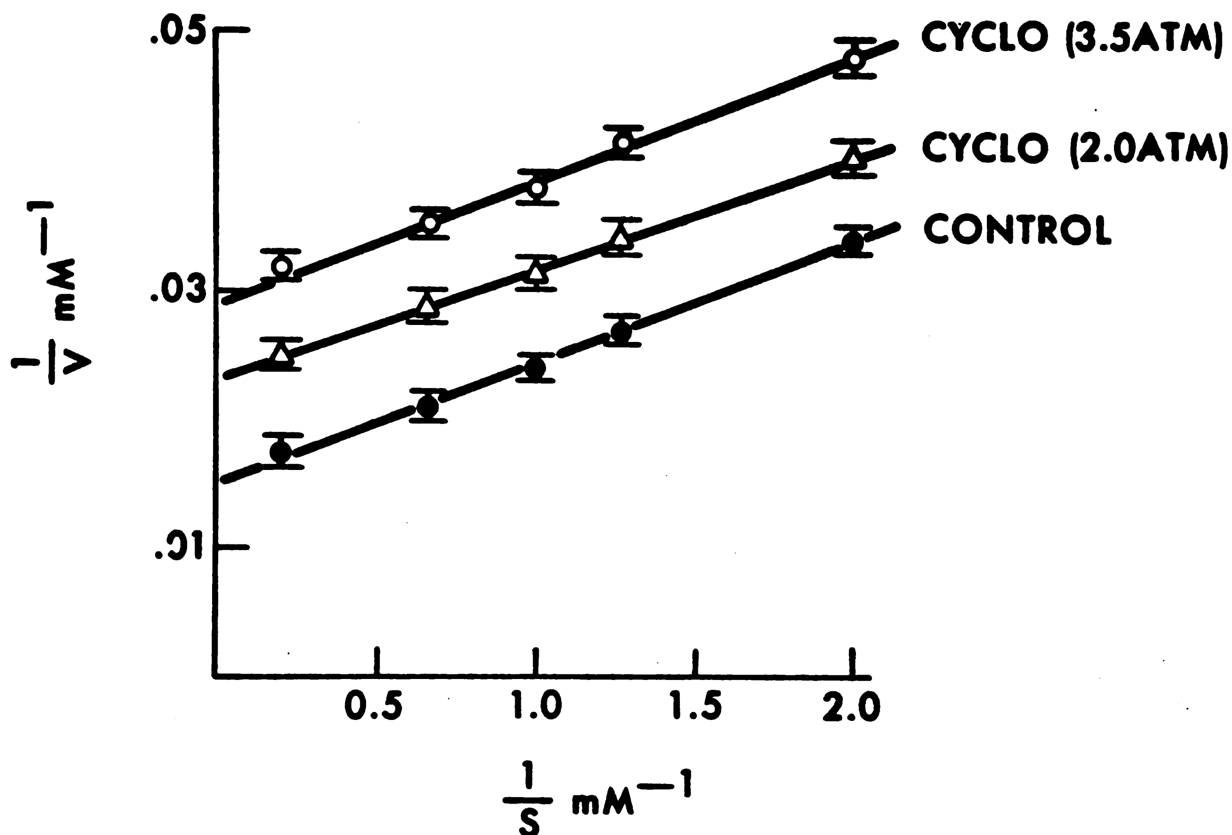


Figure 16. Lineweaver-Burk plot of inhibition of Na^{+}-K^{+}-ATPase activity by two concentrations of cyclopropane. Substrate concentration ranged from 0.5 to 5 mM ATP. Control enzyme activity at 37° was 58.5 umoles/mg protein/hour at optimum pH (6.9) in the presence of air at atmospheric pressure. Each point represents mean value of 4 distinct experiments. Vertical bars represent S.E.M.

atmosphere was examined on the phosphorylation and dephosphorylation of the NaI treated Na^{+}-K^{+}-ATPase and the results are shown in Table XI. The phosphorylation of the protein occurred quickly (15 seconds) in the presence of Na^{+} and Mg^{++} ions in the presence of air at atmospheric pressure. Cyclopropane at 2.5 atmospheres inhibited this step by approximately 39%. Quantitatively, the same degree of inhibition (35%) was observed when Na^{+}, K^{+}, and Mg^{++} ions were present in the incubation medium, demonstrating that cyclopropane did not inhibit the dephosphorylation of the enzyme. This is a very indirect method of studying gas effect on the dephosphorylation of the enzyme. The effect of different concentrations of K^{+} was studied on Na^{+}-K^{+}-ATPase activity in the presence of cyclopropane at 4 atmospheres. The results of this experiment are shown in Table XII. Increasing concentrations of K^{+} up to 50 mM did not change the degree of cyclopropane inhibition of Na^{+}-K^{+}-ATPase.

11. Influence of Substrate Concentration on Cyclopropane Inhibition of Phosphorylation

The influence of $AT^{32}p$ concentrations (.5-5 mM) on the phosphorylation of NaI treated Na^{+}-K^{+}-ATPase in the presence of air at atmospheric pressure and cyclopropane (2.5 atm), was examined and the data is shown in Table XIII. The degree of inhibition was 8% at the low substrate concentration and increased to 36% at the optimum substrate concentration, demonstrating again that the inhibition of phosphorylation of the protein is substrate promoted. A Lineweaver-Burk plot of

TABLE XI. EFFECT OF CYCLOPROPANE ON p32 INCORPORATION INTO A BRAIN ATPase PREPARATION

Additions	Phosphorylated Protein (umoles p32/mg protein)	% Change
$Na^+ + Mg^{++}$	220 $\pm$ 6	-
$Na^+ + Mg^{++}$ + Cyclopropane	135 $\pm$ 3	-38
$Na^+ + Mg^{++} + K^+$	140 $\pm$ 4	-
$Na^+ + Mg^{++} + K^+$ Cyclopropane	88 $\pm$ 3	-34

The microsomes 1-1.2 mg/ml were incubated with Na^+, 110 mM; K^+, 10 mM; Mg^{++}, 5 mM; EDTA, 5 mM; in tris buffer 40 mM at pH 7.7. Reaction mixture was equilibrated with or without cyclopropane (2.5 ATM) for 10 minutes at 0° stirring continuously. The figures represent mean of 4 to 5 distinct experiments $\pm$ standard error.

TABLE XII. EFFECT OF CYCLOPROPANE ON Na^{+}-K^{+}-ATPase ACTIVITY AT DIFFERENT POTASSIUM CONCENTRATIONS

K^{+} Concentration (mM)	Enzyme Activity (umoles Pi/mg Protein/hr)		% Inhibition
	Control	Cyclopropane 4 ATM	
2.5	19 ± 1	12 ± 1	38
5.0	20 ± 1	11 ± 0	43
10.0	20 ± 1	12 ± 1	38
25.0	20 ± 0	13 ± 1	39
50.0	21 ± 2	12 ± 0	42

Assays were performed at room temperature in 20 mM imidazole buffer (pH 6.9) at optimum substrate concentration (5 mM). Figures represent mean values of 4 experiments ± standard errors.

TABLE XIII. EFFECT OF CYCLOPROPANE ON THE PHOSPHORYLATION OF PROTEIN AT VARIOUS [S] CONCENTRATIONS

ATP Concentration (mM)	Phosphorylated Protein (umoles p32/mg Protein)		% Inhibition
	Control	Cyclopropane (2.5 ATM)	
0.5	125 ± 5	114 ± 3	8
1.25	166 ± 4	126 ± 3	24
2.5	210 ± 7	150 ± 5	28
5.0	280 ± 8	180 ± 6	35

Proteins 1-1.2 mg/ml were incubated with Na^{+}, 110 mM, Mg^{++}, 5 mM, EDTA, 5 mM in tris buffer, 40 mM at pH 7.7. Reaction mixture was continuously stirring at 0°. Time was allowed for gas to equilibrate. Values represent mean of 4 separate experiments ± standard error.

the data is shown in Figure 17 and this type of inhibition is referred to as "uncompetitive" (137).

C. A Study of the Possible Functional Relationship Between Na^+-K^+-ATPase and AChE

1. Properties of Microsomal AChE and Na^+-K^+-ATPase

In most of these studies, a microsomal preparation from ox caudate nucleus tissue was used, so the general properties of AChE and Na^+-K^+-ATPase were examined (Table XIV). The substrate optimum (.75-1 mM ATC), pH optimum (7.8) and apparent Km values for AChE were found to be similar to those values found for other brain preparations. For Na^+-K^+-ATPase, the substrate optimum was 5.0 mM ATP, and pH optimum 6.8. Assays were performed in 75 mM tris buffer and there was no effect of tris buffer on AChE and Na^+-K^+-ATPase activity up to a concentration of 200 mM. Both monovalent and divalent cations stimulated AChE and Na^+-K^+-ATPase activities. Optimal activation of AChE activity occurred with 110-150 mM Na^+, 5 mM Mg^{++} and 30 mM K^+. Maximal activation of "Ion-Transport"-ATPase activity also occurred with the same ionic concentrations except for the concentration of K^+ which gave optimal stimulation at 10 mM.

2. Effect of Cations on AChE Activity

The effect of Na^+, K^+, and Mg^{++} ions alone and in combination was examined on the microsomal AChE activity at pH 6.9 which is optimum for "Ion-Transport"-ATPase and at pH 8.0, optimal for AChE activity (Table XV). Each cation stimulated the AChE activity and the activation was greater at pH 6.9, than at pH 8.0. Ion activation appeared to be a complex

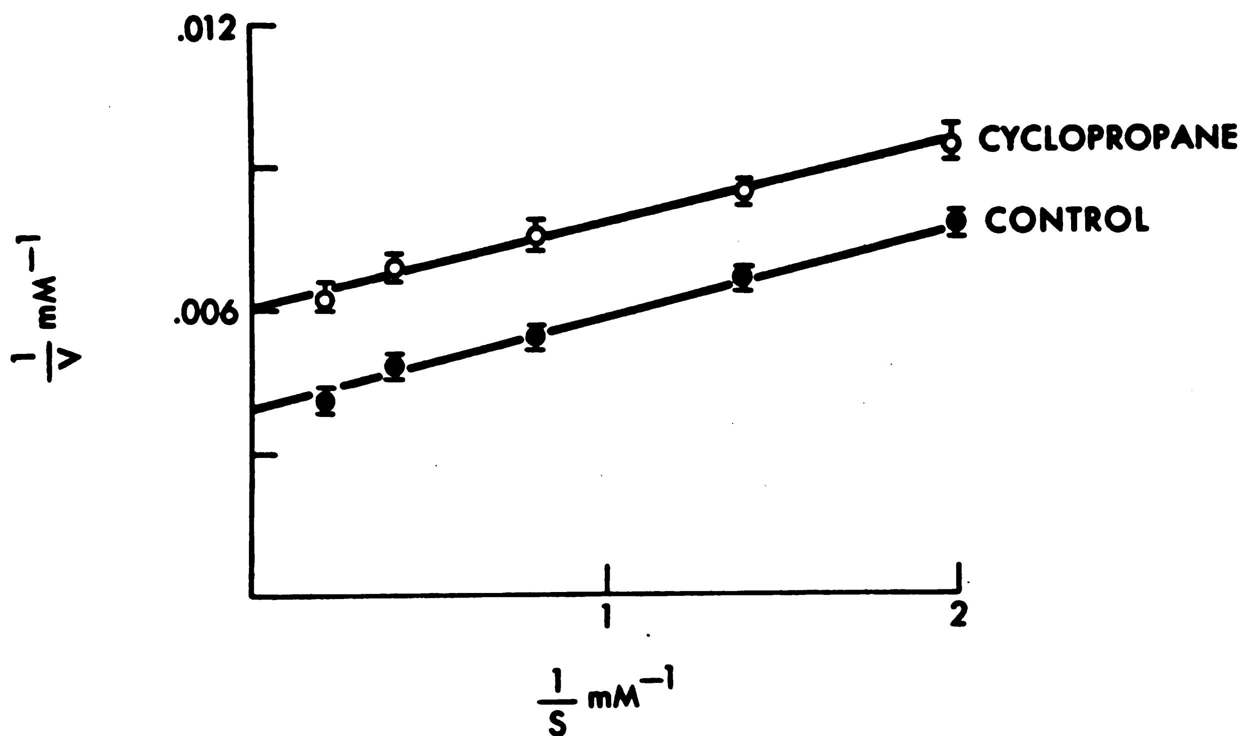


Figure 17. Lineweaver-Burk plot of cyclopropane inhibition (2.5 atm) of p32 incorporation (1/V) into a NaI treated microsomal fraction. Substrate concentration ranged from 0.5 to 5 mM $AT^{32}P$. Each point is mean of 4 different experiments. Vertical bars represent S.E.M.

TABLE XIV. SUMMARY OF PROPERTIES OF MICROSOMAL AChE AND Na^+-K^+-ATPase FROM BEEF BRAIN

AChE	Na^+-K^+-ATPase
1. Substrate Concentration	
a. Optimal at .75 - 1 mM ATC	a. 5.0 mM ATP
b. Apparent Km .1 mM	b. 4.8 x 10^{-4} M
c. [S] Inhibition 1 mM ATC	c. > 7.5 mM ATP
2. pH Optimum	
a. 7.8 in 75 mM tris	a. 6.8 in 100 mM imidazole
3. Effect of Tris Buffer	
a. No effect up to 200 mM	a. No effect up to 200 mM
4. Optimum Activation with Ions	
a. Na^+ 110-150 mM	a. Na^+ 110 mM
b. K^+ 30 mM	b. K^+ 10 mM
c. Mg^{++} 5 mM	c. Mg^{++} 5 mM

TABLE XV. ION ACTIVATION OF ACETYLCHOLINESTERASE ACTIVITY

Ions Added	% Activation	
	At pH 6.9	At pH 8.0
Mg^{2+}	24.5 ± 0.8	13.4 ± 0.9
K^{+}	8.5 ± 1.0	7.3 ± 0.6
Na^{+}	25.5 ± 0.4	3.5 ± 0.5
Na^{+} - K^{+}	21.0 ± 0.6	12.8 ± 0.8
Na^{+} - Mg^{2+}	26.5 ± 0.4	5.4 ± 0.5
K^{+} - Mg^{2+}	17.0 ± 1.1	9.8 ± 0.6
Na^{+} - K^{+} - Mg^{2+}	25.0 ± 0.8	8.7 ± 0.8

Control AChE activity at pH 8.0 and pH 6.9 was 27.2 and 20.3 (umoles acetylthiocholine hydrolyzed/mg protein/h), respectively at 37°C. The concentrations of ions used were Na^{+}, 110 mM; K^{+}, 10 mM; Mg^{2+}, 5 mM with 75 mM tris buffer. Standard deviations were derived from 4 enzyme preparations.

phenomenon, depending in part on pH. For example, at pH 6.9, Mg^{++} or Na^{+} alone or in combination activated AChE activity to the same extent (25%). At pH 8.0, Mg^{++} activated the enzyme more than K^{+} and K^{+} more than Na^{+}. Na^{+} and K^{+} activation seem to add, but when combined with Mg^{++}, they partially inhibit the Mg^{++} stimulation of AChE activity.

3. Effect of ATP on AChE Activity

Since optimal activation of the "basic" (without the addition of any cations) AChE activity was observed at the pH optimum for "Ion-Transport"-ATPase, the effect of ATP on the "basic" and "ion-stimulated" enzyme activity was examined (Table XVI). ATP at concentrations up to 10 mM had no effect on the "basic" AChE activity but 5 mM ATP in the presence of Na^{+}, K^{+} and Mg^{++} ions decreased the ion activation of AChE.

4. Effect of Varying ATP Concentration on Ion Activated AChE

ATP decreased the ion activated AChE activity in a concentration dependent manner as shown in Figure 18. Maximal decrease (80%) occurred at 5 mM ATP, the optimal substrate concentration for Na^{+}-K^{+}-ATPase activity. Higher concentrations of ATP (up to 10 mM) also exhibited the same degree of inhibition. The effect of ATP (5 mM) was also examined in the presence of Na^{+}, K^{+}, and Mg^{++} ions either alone or in various combinations at pH 6.9. ATP inhibition of ion activated AChE activity was also a complex phenomenon as shown by the results in Table XVII. There was a variable

TABLE XVI. EFFECT OF NUCLEOTIDES ON ACETYLCHOLINESTERASE ACTIVITY

Additions	Concentration (mM)	% Changes in Activity
ATP	5	
ATP	10	
Na^{+}-K^{+}-Mg^{2+}	110,10,5	+25.0 $\pm$ 0.8
Na^{+}-K^{+}-Mg^{2+} + ATP	110,10,5,5	+7.5 $\pm$ 0.4
Na^{+}-K^{+}-Mg^{2+} + UTP	110,10,5,5	+8.0
Na^{+}-K^{+}-Mg^{2+} + GTP	110,10,5,5	+10.1

Assays were performed at pH 6.9 at 37°C, in media containing 75 mM tris buffer. Percent changes are calculated from basic AChE activity (20.3) umoles/mg protein/h). When shown, $\pm$ refers to standard deviations derived from 4 distinct experiments.

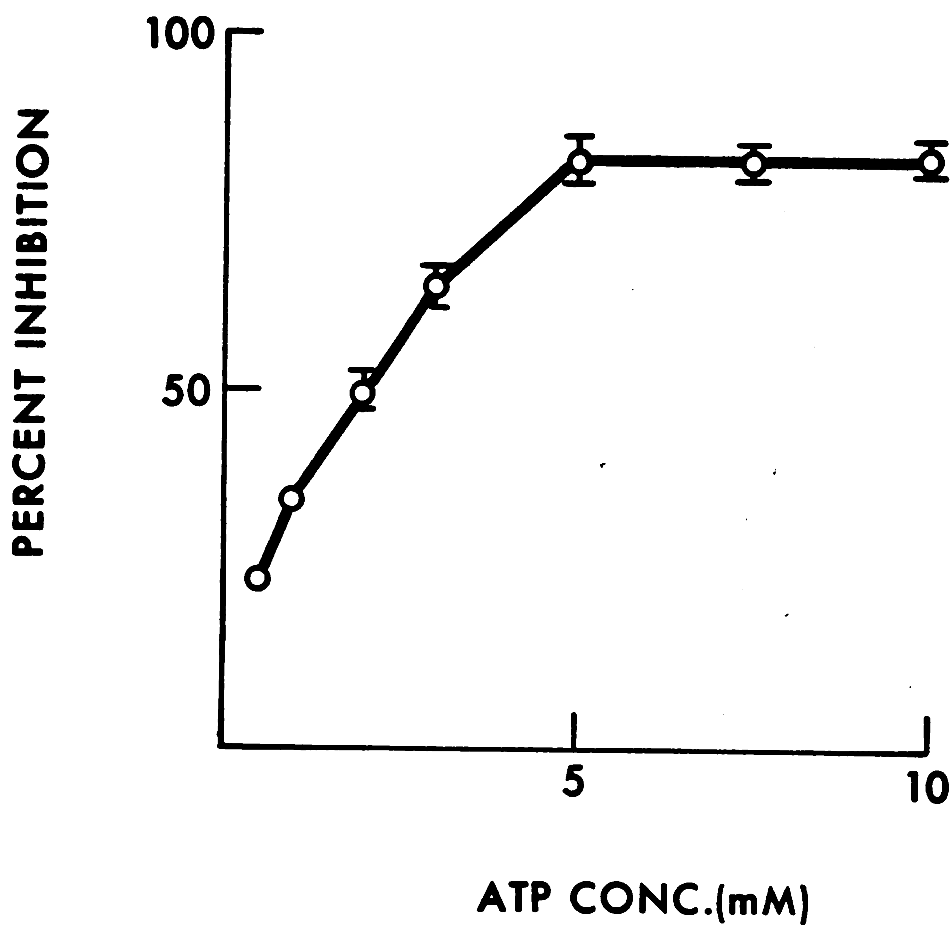


Figure 18. Effect of varying ATP concentration on Na^+-K^+ and Mg^{++} stimulated AChE activity at pH 6.9 with 50 mM tris buffer. Each point represents mean value derived from 4 separate experiments. Vertical bars represent S.E.M.

TABLE XVII. EFFECT OF ATP ON ACTIVATED

AChE ACTIVITY

Ions	% Activation	% Activation with 5 mM ATP
Mg^{2+}	24 ± 0.8	+0
Na^{+}	25 ± 1.0	+15.0 ± 0.5
K^{+}	8 ± 0.4	+7.5 ± 0.4
Na^{+}-K^{+}	21 ± 0.6	+16.5 ± 0.5
Na^{+}+Mg^{2+}	26 ± 0.4	+12.0 ± 0.8
K^{+}+Mg^{2+}	17 ± 1.0	-10.6 ± 0.4
Na^{+}+K^{+}+Mg^{2+}	25 ± 0.8	+7.5 ± 0.4

Experiments were performed at pH 6.9. Percent activation values were calculated from the basic AChE activity (20.3 umoles/mg protein/h). Concentration of Na^{+}, 110 mM; K^{+}, 10 mM; and Mg^{2+}, 5 mM were used. Each value represents the mean of 4 individual experiments. ± refers to standard deviations.

degree of inhibition depending on the combination of ions added. For example, in the presence of K^+ and/or Na^+ (but no Mg^{++}) ATP inhibited only the ion activation of AChE. When K^+ and/or Mg^{++} (but no Na^+) was added, ATP inhibited both "ion activated" and "basic" AChE activity. The activation of AChE by 5 mM Mg^{++} was completely inhibited by 5 mM ATP.

5. Effect of Varying Mg^{++} Concentration on ATP Inhibition of Ion Activated AChE

The complete inhibition of Mg^{++} activation of AChE by ATP suggested the possibility of complex formation between the cation and the nucleotide. However, this does not appear to be the case since increased concentrations of Mg^{++} up to 20 mM did not overcome the ATP inhibition of the Mg^{++} activated AChE activity (Table XVIII).

6. Effect of Other Nucleotides on "Stimulated" AChE

ATP inhibited the cation "stimulated" AChE activity under conditions optimal for Na^+-K^+-ATPase activity, i.e. [S] optimum, pH optimum and ionic concentrations. Other nucleotides such as GTP and UTP are known to be poor substrates for "Ion-Transport"-ATPase activity (70). Their effects were examined on the microsomal AChE activity in the presence of added ions and the results are shown in Table XVI. GTP and UTP were also effective inhibitors of cation "stimulated" AChE activity.

TABLE XVIII. ATP INHIBITION OF Mg^{2+}

ACTIVATED AChE

Mg^{++}(mM)	ATP (mM)	% Activation with Mg^{++}	% Activation with Mg^{++}+ATP
5	5	25.1 ± 0.7	1
10	5	24.8 ± 0.7	1
20	5	25.0 ± 0.7	1
10	10	25.6 ± 0.7	1

Assays were performed at pH 6.9 in 75 mM tris buffer at 37°C. Control AChE activity was 20.5 (umoles acetylthiocholine hydrolyzed/mg protein/h). Standard deviations were derived from 4 different experiments.

7. Effect of Ouabain

The effect of ouabain, an established inhibitor (23,70, 157,159) of Na^+-K^+-ATPase, was examined on the microsomal fraction which contained both the enzymes, i.e., AChE and "Ion-Transport"-ATPase. Ouabain at 10^{-5} M inhibited the Na^+-K^+ and Mg^{++} activated AChE activity by 60% as shown in Table XIX and Na^+-K^+-ATPase activity by 85-90% (results not shown). The inhibitory effect of ATP and ouabain on AChE was additive. Ouabain also inhibited a "pure" AChE preparation by approximately 50% in the presence of Na^+, K^+, and Mg^{++} ions. This "pure" preparation contained no measurable Na^+-K^+-ATPase activity.

8. Effect of Cations and ATP on Lubrol "Solubilized" and a "Pure" AChE Preparation

Effects of cations and ATP on the "microsomal" AChE activity were compared with a "Lubrol" extracted preparation and a "pure" AChE preparation. Na^+, K^+, and Mg^{++} activated each of these preparations both at pH 6.9 and 7.5 (Table XX). ATP (5 mM) partially inhibited this ion activation of AChE activity in each preparation with no significant difference. The most "purified" AChE preparation had no measurable Na^+-K^+-ATPase activity.

TABLE XIX. OUABAIN INHIBITION OF ION ACTIVATED AChE

Inhibitor Added	Concentration M	% Inhibition
Ouabain	10^{-5}	60
ATP	5×10^{-3}	60
Ouabain + ATP	10^{-5}, 5×10^{-3}	100

Experiments were done at room temperature, pH 8.0. Control AChE activity was 25.8 (umoles/mg protein/h). Reaction media contained 110 mM Na^+, 10 mM K^+, 5 mM Mg^{2+} and 75 mM tris buffer. Inhibitors were added before the substrate. Percent inhibition represents the mean of 4 individual experiments.

TABLE XX. EFFECTS OF IONS AND ATP ON

3 AChE PREPARATIONS

Additions	pH	Microsomal Enzyme % Change	Lubrol "Solubilized Enzyme % Change	"Pure" Enzyme % Change
$Na^{+}+K^{+}+Mg^{2+}$	6.9	+25.0	+21.0	+21.0
$Na^{+}+K^{+}+Mg^{2+}$+ATP	6.9	+7.8	+14.1	+6.1
$Na^{+}+K^{+}+Mg^{2+}$	7.5	+21.3	+20.2	+25.0
$Na^{+}+K^{+}+Mg^{2+}$+ATP	7.5	+6.1	+12.0	+12.2

Control AChE activity (umoles/mg/h) at pH 6.9 and 7.5 for the microsomal enzyme was 20.5 and 25.2, the Lubrol enzyme was 35.5 and 42.3 and the "pure" enzyme was 850 and 1000, respectively at 37°C. 110 mM K^{+}, 5 mM ATP were added with 75 mM tris buffer. Results given are mean values derived from 3 distinct experiments.

IV. DISCUSSION

While the phenomenon of anesthesia has been studied extensively, most propositions regarding the mechanism underlying anesthetic action are based on correlations with the physicochemical properties of anesthetic agents. Such correlations of anesthetic potency with physical or chemical properties of anesthetic agents may be suggestive but fail to prove how or where anesthetics act. In view of the marked chemical heterogenity of agents capable of exerting anesthetic action, any single theory proposed regarding mechanism of anesthesia is unlikely to explain completely the actions of all such compounds. Some proposals of the anesthetic mechanism are merely disciplines of certain effects of these agents which may have little to do with the actual state of anesthesia. However, the goal of the present study was not to deal with the problems of mechanism of anesthesia and no such direct connection is implied. The main objective was to describe actions of inert gas cyclopropane on certain biochemical properties of cerebral membrane components including the enzyme systems - acetylcholinesterase, involved in the termination of neurotransmitter actions, and "ion-transport"-ATPase, important in the restoration and maintainence of ionic gradients. These enzyme systems represent "model systems" and anesthetic actions on them may have nothing to do with the mechanism of anesthesia. However, the study of such actions may have some relevance in increasing our knowledge by which a small molecule like cyclopropane could associate with neural components and exert certain actions leading to physiological effects.

In addition, it was hoped that this study would provide an increased understanding of the "control mechanisms" involved in the regulation of neuronal excitability.

A. In the present study, interactions of cyclopropane with highly purified forms of brain AChE were investigated. In addition, a membrane-bound synaptosomal preparation was used for comparative purposes. Cyclopropane inhibited all forms of the enzymes studied in a pressure dependent manner (Figure 10). There was no marked difference regarding sensitivity of the three soluble forms or the synaptosomal AChE. The inhibition was observed to occur immediately on exposure of the reaction media to cyclopropane and preincubation with the gas up to 30 minutes did not change the degree of inhibition (Table III). Cyclopropane inhibition occurred at low gas pressures (0.1 ATM) and was completely reversible on degassing, both at high (3 ATM) and low gas pressures in all the AChE preparations (Figure 11). In the past, most of the studies with ethanol (172) and local anesthetics (168) have been done at relatively high concentrations of these agents. In none of these previous investigations has there been any documentation of reversal of inhibitory effects. Under these circumstances, it is difficult to rule out the possibility of denaturation or inhibition of the enzyme system by an irreversible process. Cyclopropane, like other "inerg gases" is very lipid soluble and therefore one might predict, that in the case of membrane-bound AChE, less gas pressure would be required than for the "soluble" purified forms to obtain the same degree of inhibition. However, this did not appear so, as the gas pressure required to obtain

optimal inhibition of both synaptosomal and purified forms was identical (Table II). The mechanism of cyclopropane inhibition is preliminarily at this stage, but kinetic analyses suggest a mixed type of inhibition (competitive and non-competitive) in all the AChE preparations studied (Figure 12). The simplest interpretation of such kinetic studies is that there must be at least two sites on the enzyme molecule with which cyclopropane could interact. It is interesting to note that the level of purity of enzyme did not alter the sensitivity or the type of inhibitory effect of cyclopropane. This is in agreement with the work of other investigators (193) on neuromuscular blocking agents which give similar effects on a membrane-bound and a soluble preparation of AChE. A number of compounds including neuromuscular blocking agents (13,15,34,91), succinylcholine (91,193) and decemethonium (91,142,193) give mixed inhibition of erythrocyte membrane-bound and semipurified eel AChE. The non-competitive component of inhibition is interpreted by these investigators in terms of the existence of a site distinct from the active site on the enzyme where these compounds bind and produce a conformational change. This site is referred to as a "regulatory site" which does not show any catalytic activity and contains at least two negative charges. The competitive component of inhibition may be interpreted as due to allosteric inhibition, which is induced by binding of these compounds at the regulatory site as well as due to a direct competition between the substrate and these agents at the active site.

Cyclopropane, xenon and other inert gases have been shown to interact with a number of proteins of well established structure (53,54,147,148,149). Schoenborn and colleagues (54,147), using x-ray diffraction techniques demonstrated that xenon and cyclopropane molecules bind to myoglobin at a position equidistant from the proximal histidine and heme. They further suggested that the presence of xenon does not alter the shape of the myoglobin molecule. The gas molecules were suggested to fit into a cavity between an area composed of the side chains of "non-polar" amino acids and the partially polar areas of heme and histidine. In more recent studies, involving estimation of binding constants, xenon was also shown to bind to hemoglobin and metmyoglobin at specific sites (159). In addition, the presence of xenon was shown to alter the functioning of myoglobin as evidenced by the shift of carbon-monoxide binding equilibrium (159), in contrast to previous observations (147). In all these cases, the position of xenon molecule was considered to be between a "non-polar" and a partially polar area, suggesting that the binding is due mainly to London interactions (35). If one considers this data interms of cyclopropane interactions with cerebral AChE, one might suggest that there must be some regions on the "active site" of the enzyme that resemble lipid, i.e., "non-polar" groupings, where cyclopropane could interact. This suggestion originated from the kinetic data which showed a competitive component of the mixed inhibition. By the same token, the non-competitive component of cyclopropane inhibition

of AChE presumably reflects the presence of another region on the enzyme molecule with similar non-polar or hydrophobic characteristics.

The previous discussion concerned interactions of cyclopropane with a cerebral enzyme protein of documented purity (30,31,32). The "model system" examined in the second part of the present study, was the interaction of cyclopropane with cerebral Na^+-K^+-ATPase, which represented studies on heterogenous preparations presumed to contain both protein and lipid components. To date, this enzyme has not been completely purified and one is forced to work with partially purified preparations.

B. Cyclopropane inhibited activity of both particulate and partially purified forms of Na^+-K^+-ATPase, in a pressure dependent manner with little effect on the Mg^{++} ATPase activity at the gas pressures studied (Table V). Preincubation of the enzyme with cyclopropane up to 30 minutes did not change the degree of inhibition (Table VII). Since gas pressures above one atmosphere were required to produce a measurable effect one is forced to question whether cyclopropane effects might be just due to pressure per se. This does not appear to be the case as judged by control experiments with N_2, O_2 and compressed air. All of these caused a slight stimulation (10%) of the enzyme activity, in contrast to the inhibitory effect of cyclopropane (50%) at the same pressure (Figure 14). To examine the effect of pressure alone, hydrostatic pressure was applied at approximately 4 atmospheres and this again

resulted in a small stimulation (10%) of enzyme activity. It is clear that other gases (N_2, O_2 and compressed air) and pressure per se can have effects on the Na^+-K^+-ATPase activity. This is in accord with the observed actions of these gases at high pressures on other enzymes (153). However, in the present study one must conclude that the inhibitory effect of cyclopropane on Na^+-K^+-ATPase, has some degree of specificity. The action of cyclopropane is more pronounced than that of other gases, is inhibitory rather than stimulatory, and is more selective for the "ion-transport"-ATPase rather than Mg^{++} ATPase. Several investigators have studied the effects of general anesthetics including ether (75), ethanol (74,75, 116,172,179), halothane (75,101,179), barbiturates (157), chloroform (73,74) on Na^+-K^+-ATPase. For the most part, concentrations of these agents used in _in vitro_ studies were higher than those anticipated to occur in the central nervous system _in vivo_. Each study failed to demonstrate any reversal of drug effects. It is important to rule out any irreversible denaturations of the enzyme system to make any physiologically relevant statement of the observed effects. Cyclopropane inhibition of Na^+-K^+-ATPase was reversible (95% approx.) as shown in Table VIII. The inhibition of Na^+-K^+-ATPase by cyclopropane was temperature dependent. Greater inhibition was observed at lower temperatures which could be explained by the increasing gas solubility at lower temperatures. The temperature coefficient (Q_{10}) for most enzymes is between 1 and 2 (43), but Lubrol Na^+-K^+-ATPase had a Q_{10} of 4, indicating

greater sensitivity of the enzyme system to temperature changes. N_2, O_2 and compressed air stimulated the enzyme activity at optimal (5 mM ATP) substrate concentration, while inhibition was produced at low ATP concentrations (Table X). This is a very unusual effect and may be due to the fact that the enzyme system was impure and heterogenous. On the other hand, it may reflect the possibility of different sites of action of such gases as a function of changes in conformation of the enzyme produced by different substrate concentrations. Nitrogen and other inert gases are known to inhibit certain oxygenases, although at higher pressures, perhaps by competition with molecular oxygen (153).

The kinetics of inhibition of membrane-bound "ion-transport"-ATPase by barbiturates (157), halothane (75,101,179) and diethyl ether (75) has been shown to be of non-competitive type. Inhibition by cyclopropane, however, is promoted by an increase in substrate concentration (Figure 12). This type of inhibition is referred to as "competitive" by Ebersole et al (45) or "exclusive C type" by Reiner (136). One interpretation of such an effect is that the inhibitor combines with the enzyme-substrate (E-S) complex and not with the free enzyme. Although this type of inhibition is rare, there are certain precedents (36,40,89,98,129,130) for this type of effect. 5-6-dichloro-2-amino benzimidazole (DCB), inhibits phenylethanolamine-N-methyl-transferase (PNMT) in an uncompetitive manner, suggesting that inhibitor and substrate bind independently to the enzyme (129). Fluoride ions

have been shown to inhibit a number of enzymes including cholinesterases (36), urease (40), enolase (130), and 5 adenylic-acid-deaminase (98) in an uncompetitive manner. A similar type of inhibition has also been observed with detergents which inhibit bovine liver glutamate dehydrogenase (89). Cimmasoni et al (36) suggested that the mechanism of this type of inhibition is that the inhibitor binds to the enzyme, only after the protein has undergone a conformational change imposed by the substrate. This E.S. complex with the inhibitor (E-S-I) remains in an undissociated form, which results in inhibition of the reaction.

In attempting to identify the site of cyclopropane action on Na^+-K^+-ATPase with more precision, the effect of cyclopropane was studied on individual stages of ATP hydrolysis using an NaI treated Na^+-K^+-ATPase. Cyclopropane inhibited the Na^+-Mg^+-dependent phosphorylation with little effect on the K^+-dependent dephosphorylation (Table XI). Although the latter conclusion is based on indirect evidence, it is supported by the following facts. First, increased concentrations of potassium up to 50 mM, did not change the degree of inhibitory effect (Table XII) and second, the degree of inhibition of the Na^+-Mg^{++}-dependent phosphorylation step, correlates well with the data obtained on the overall ATP hydrolysis.

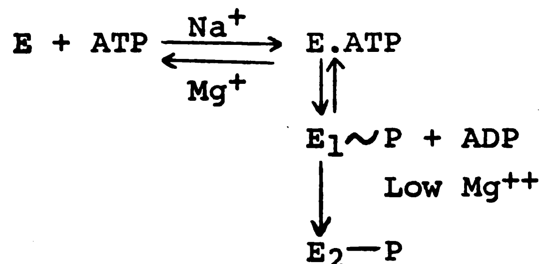
Cardiac glycosides are potent inhibitors of Na^+-K^+-ATPase, yet their mode of inhibition is unclear (23,70,157,160). In addition, Na^+-K^+-ATPase inhibition is partially reversed

by increasing concentrations of potassium (160). A competitive antagonism has been observed between ethanol inhibition of Na^{+}-K^{+}-ATPase and potassium (76), although its precise site of action in the ATP hydrolytic sequence has as yet not been determined. By analogy to these studies, if there is any actions of cyclopropane on the dephosphorylation step, it is probably not competitive with K^{+}. Therefore, essentially the effect of cyclopropane appears to be at a step prior to the release of phosphate from the E_2-P complex.

A (^{14}C) ADP-ATP exchange reaction has been observed in a variety of microsomal fractions including crab nerve, brain, kidney, and electric organ as part of the Na^{+}-K^{+}-ATPase system (5,137,170,174), constituting further evidence for the participation of the phosphorylated intermediate in the enzyme reaction. N-ethylimide (NEM), BAL-arsenite and oligomycin which all inhibit Na^{+}-K^{+}-ATPase are known to activate the exchange reaction (162,163,171). This ADP-ATP exchange reaction is observed only under specific conditions: a) very low Mg^{++} concentrations (0-0.15 mM), b) in soluble forms of the enzyme, and c) in the presence of certain agents which inhibit Na^{+}-dependent ATP hydrolysis (170). In the present study a particulate enzyme free from any detergent treatments was used in the presence of 5 mM Mg^{++}. This concentration is approximately 300 times more than required for the exchange reaction. In addition, no agent was used to inhibit Na^{+}-dependent ATP hydrolysis. Thus, one concludes that the unusual kinetics of inhibition are not the result of an action on the

back exchange reaction. Phosphorylation of the enzyme is generally assumed to be preceded by an enzyme substrate (E.S.) complex formation (11,125). This formation of E.S. complex is believed to be Mg^{++}-dependent by some (125), while others assume it is only Na^{+}-dependent (11). On the other hand, it may well be both Na^{+} and Mg^{++} dependent. Whatever cation(s) is involved, these investigators suggest that it is more plausible to assume that it is the initial interaction of enzyme with usbstrate rather than phosphorylation (E P) that creates a confirmational change in the enzyme. The altered enzyme molecule is then more favorable to the K^{+}-stimulated hydrolysis on the basis of oligomycin studies with Na^{+}-K^{+}-ATPase and the absence of formation of E P from nucleotides other than ATP (11).

In considering the mechanism of cyclopropane inhibition of Na^{+}-K^{+}-ATPase, one is forced to infer its nature by exclusion, rather than by a direct approach. As mentioned earlier, cyclopropane appears to inhibit the enzyme reaction at a step prior to dephosphorylation and the remaining scheme is shown below:



Cyclopropane does not affect: a) the exchange reaction for reasons mentioned before and b) the conversion of E_1 P to E_2-P is an unlikely site because of the unusual kinetics. However, cyclopropane does inhibit Na^+-Mg^{++}-dependent phosphorylation and does so in a substrate promoted manner. One possibility is that cyclopropane interacts with E.S. complex to form an E.S.I. (I being cyclopropane) complex. From kinetic studies, it would appear that cyclopropane binds at a site different from the substrate binding site. This site may be a "regulatory" site or a "modifying site" similar to that postulated before by other investigators. The precise nature of the site where cyclopropane might bind on the Na^+-K^+-ATPase, may be interpreted by analogy with cyclopropane interactions with AChE and its binding to other proteins of known structures as discussed previously. That is, that some region or regions of the enzyme molecule must contain hydrophobic regions with which cyclopropane could interact.

Cyclopropane effects are not limited only to membrane-bound enzymes, but also are known to occur with soluble enzymes (48,118,134). The administration of subanesthetic concentrations of cyclopropane and fluroxene increases adrenal tyrosine hydroxylase activity (118) a rate limiting enzyme involved in the synthesis of catecholamines from tyrosine. It has also been shown to stimulate PNMT activity and such an increase has been correlated with an increase in circulating catecholamines produced by cyclopropane which might be responsible for its cardiovascular effects (48,134). Both cyclopropane and xenon

have actions on flavoproteins. The succinic dehydrogenase activity (SDH) of mitochondrial fractions of rat liver and brain (180) and also of mutant-strains of E. coli (64) was stimulated by low pressures of cyclopropane.

C. Acetylcholinesterase from various sources has been reported to be activated by cations such as Na^{+}, Mg^{++}, Ca^{++}, K^{+} (9,34,105,190) while fluoride (36) and lithium chloride (26) inhibit the enzyme activity. Ca^{++} does not compete with ACh for the catalytic anionic site and presumably binds to an allosteric site (beta-anionic site) according to Roufgalis et al (142). Mg^{+} and Na^{+} inhibit substrate hydrolysis at low substrate concentrations and activate ACh hydrolysis at high substrate concentrations (143,144) and appear to bind to the catalytic anionic site (alpha) as well as at the beta-anionic site. The present investigation of microsomal AChE from beef caudate tissue also demonstrated stimulation of enzyme activity by cations such as Na^{+}, K^{+} and Mg^{++} (Table XV). Activation of the enzyme by cations was not influenced by the level of purity of the enzyme. Cation activation of AChE, seems to be a complex phenomenon depending on ionic species, concentrations of ions and pH of incubation media. An association of "ion-transport"-ATPase with AChE of the electrophorus electric organ has been suggested by Fahn (52) on the basis that there is a gradient of both enzyme activities along the length of the electric organ, being highest rostrally and lowest caudally. In addition,

calculations of enzyme activities in terms of protein concentration also yeilded similar qualitative results. Shirachi et al (20) also suggested the possibility of a physical association between the two enzyme systems based on examination of the sedimentation properties of the two "solubilized" enzymes using zonal ultra centrifugation techniques. In addition, to a morphological relationship, a functional relationship between the two enzyme systems has been proposed by Kometiani et al (93). The present investigation provided a certain amount of support for this suggestion in that "ion stimulated" AChE activity could be inhibited by ATP. This antagonism of ion activation of AChE occurred at the substrate (ATP) optimum (Figure 18), pH optimum and ion concentrations required for optimal "ion-transport"-ATPase activity. This would suggest the possibility of some form of control of AChE activity, at least that due to ion activation, by changes in the rate of reaction of Na^+-K^+-ATPase. However, such a proposal is not supported by other findings. For example, ATP in the presence of Na^+ and/or K^+ ions (no Mg^{++}) reversed ion activated AChE activity (Table XVII). Under these conditions since the enzyme has a mandatory requirement for Mg^{++} Na^+-K^+-activated ATPase is non-functional. Similarly ATP (5 mM) completely blocked Mg^{++} (5 mM) activation of AChE in the absence of Na^+-K^+ ions. Without Na^+ and K^+ ions, there is little ATPase activity. Increased concentrations of Mg^{++}, up to 20 mM did not reverse the ATP inhibitions of Mg^{++} stimulated AChE (Table XVIII), thus the possibility of a

complex formation between the Mg^{++} ion and the nucleotide ATP is unlikely. Other nucleotides such as GTP and UTP, although poor substrates for "ion-transport"-ATPase, were effective inhibitors of cation activated AChE (Table XVI).

Another test of the proposal of a functional relationship was to examine the influence of ouabain. The cardiac glycoside at concentrations which inhibited Na^{+}-K^{+}-ATPase activity by 85-90% also inhibited the ion stimulated AChE activity by 60% (Table XIX). However, any interpretation of this inhibitory effect in terms of a functional relationship between the two enzyme systems is limited by the observation that ouabain also inhibited a relatively pure preparation of AChE, containing no measurable Na^{+}-K^{+}-ATPase activity.

Perhaps the most convincing argument against a functional relationship comes from the studies on a highly purified form of AChE, which was also stimulated by Na^{+}, K^{+} and Mg^{++} ions. This activation was partially reversed by ATP, although the preparation contained no Na^{+}-K^{+}-ATPase activity (Table XX). Abdel Latif et al (1) have also examined AChE and Na^{+}-K^{+}-ATPase activities in the synaptosomal and microsomal fractions of developing rat brains. The effect of various inhibitors and activators on both enzymes showed no relationship. Similarly the pattern of development was different in both subcellular fractions. All these observations are more supportive of a functional independence of "ion-transport"-ATPase and AChE. However, one might conclude that ion activation and nucleotide inhibition of AChE may well represent characteristic properties of this enzyme which could be of

importance in the regulation of enzyme activity and hence, transmitter action during the excitatory process.

SUMMARY

Most of the work in the past on inert gases had been on the plasma proteins and not much on neural tissues. Interactions of cyclopropane with both membrane-bound and purified forms of brain AChE was of interest regarding the low concentrations of the gas required to observe the inhibitory effects. The gas pressure required was identical in all the preparations of AChE to obtain the same degree of inhibition. Cyclopropane inhibition of all AChE forms was reversible both at high (3.0 atm) and low (0.1 atm) gas pressures. Kinetic analysis showed mixed inhibition in both membrane-bound and pure forms of AChE. Since AChE from the mammalian brain source was available, it provided an excellent model analogous to xenon/myoglobin to investigate where and how cyclopropane interacted with the protein.

Cyclopropane also inhibited semi-purified forms of "ion-transport" ATPase, reversibly and in both pressure and temperature dependent manner. Cyclopropane inhibition of lubrol Na^{+}-K^{+}-ATPase was substrate promoted and from kinetic studies it appeared to interact at a site other than the active site on the enzyme molecule, perhaps a "regulatory site." Cyclopropane inhibited the enzyme reaction at or before the phosphorylation step, perhaps by altering the binding of substrate to the enzyme. Cyclopropane interactions with these enzyme systems does not explain the "*in vivo*" effects produced by this gas, but could provide an important tool to increase our understanding of the control mechanisms involved in cerebral excitability.

In the final section of the thesis, AChE of a membrane source and a relatively pure preparation was shown to be activated by cations including Na^{+}, K^{+} and Mg^{++} either alone or in combinations. ATP antagonism of the "ion activated" AChE occurred at conditions known to be optimal for Na^{+}-k^{+}-ATPase activity and thus favoured the proposed relationship. However, other nucleotides (GTP, UTP) and ouabain also inhibited the ion activated AChE activity. ATP also partially blocked activation of AChE by single cation. A pure preparation of AChE with no measurable Na^{+}-K^{+}-ATPase activity was also activated by cations and this cation activation was inhibited by ATP. These later findings did not support the suggested relationship between AChE and Na^{+}-K^{+}-ATPase. However, ion activation and nucleotide inhibition of AChE represent individual properties of the enzyme which may be of importance in the regulation of enzyme activity and, hence, transmitter action during the excitatory process.

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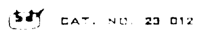
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