1969

Central Nervous System Responses to Anticholinesterases in Rabbits: Evidence for a Non-Inhibitory Action and for an Adrenergic Link

William G. Van Meter

Loyola University Chicago

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CENTRAL NERVOUS SYSTEM RESPONSES TO ANTICHOLINESTERASES
IN RABBITS: EVIDENCE FOR A NON-INHIBITORY ACTION AND FOR AN
ADRENERGIC LINK

by
William G. Van Meter A.B., B.S.

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

1969
LIFE

William G. Van Meter was born in Springfield, Illinois on May 4, 1933.

He attended and was graduated from primary and secondary schools in Lincoln, Illinois.

He was graduated from Knox College, Galesburg, Illinois in 1955 and received his A.B. degree in Liberal Arts having majored in biology.

His career in scientific research began at the Thudichum Psychiatric Research Laboratory of the Galesburg State Research Hospital in 1955. He spent the next 5 years under the guidance and supervision of Dr. Harold E. Himwich, Director of Research.

He attended Drake University College of Pharmacy in Des Moines, Iowa from 1960 to 1963 and was graduated with a B.S. in Pharmacy.

After working in the Department of Pharmacology at Abbott Laboratories from 1963 to 1964, he was accepted for graduate study in the Department of Pharmacology and Therapeutics, Loyola University Graduate School in 1964.

His military service includes active duty in 1956 as a commissioned 2nd Lieutenant in the Medical Service Corps of the U.S. Army. After continuing in the Active Army Reserves for 8 years he retired at the rank of Captain.
In 1956 he married the former Beverley Ann Barr and they have a daughter, Susan Elizabeth, born in 1965.

Mr. Van Meter's publications include the following:


ACKNOWLEDGMENTS:

I wish to express a deep personal debt of gratitude to Dr. Harold E. Himwich whose encouragement, guidance and supervision during my early formative years as a scientist have been of an incalculable value to me.

Dr. Alexander G. Karczmar has given me the opportunity of graduate study. His support, encouragement and personal interest in my graduate study and research has been both generous and genuine. His contributions to the present dissertation are gratefully appreciated.

I wish also to thank Dr. Alexander H. Friedman for his interest and assistance. In particular I wish to thank him for acting as surrogate advisor in the absence of Dr. Karczmar who has been on sabbatical leave this past year.

Finally, I wish to acknowledge the able assistance of the Departmental Laboratory Technician, Mr. Lionell Barnes, for his help in the assay of norepinephrine.
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<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>AMN</td>
<td>atropine methyl nitrate</td>
</tr>
<tr>
<td>aMPT</td>
<td>alpha methyl para-tyrosine</td>
</tr>
<tr>
<td>AntiChE</td>
<td>anticholinesterase</td>
</tr>
<tr>
<td>AtSO₄</td>
<td>atropine sulfate</td>
</tr>
<tr>
<td>ChE</td>
<td>cholinesterase</td>
</tr>
<tr>
<td>ChEI</td>
<td>cholinesterase inhibitor</td>
</tr>
<tr>
<td>COMT</td>
<td>catechole-0-methyl transferase</td>
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<tr>
<td>CPZ</td>
<td>chlorpromazine</td>
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<tr>
<td>D(-)-INPEA</td>
<td>D(-)-1-(4’Nitrophenyl)-2-Isopropyl-aminoethanol HCl</td>
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<tr>
<td>DFP</td>
<td>diisopropyl phosphonofluoridate</td>
</tr>
<tr>
<td>DHMA</td>
<td>dihydroxymandelic acid</td>
</tr>
<tr>
<td>DHPG</td>
<td>dihydroxyphenylglycol</td>
</tr>
<tr>
<td>DOPA</td>
<td>dihydroxyphenylalanine</td>
</tr>
<tr>
<td>Eserine</td>
<td>physostigmine</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamineoxidase</td>
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<td>MAOI</td>
<td>monoamineoxidase inhibitor</td>
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<td>MHPG</td>
<td>methoxyhydroxy phenylglycol</td>
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<td>norepinephrine in the free form</td>
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<td>NM</td>
<td>norepinephrine in the bound form</td>
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<td>Pargyline,</td>
<td>N-benzyl-N-methyl-a-propynylamine HCl</td>
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<td>Sarin, (GB)</td>
<td>isopropyl methylphosphonofluoridate</td>
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<td>VMA</td>
<td>vanillyl-mandelic acid</td>
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<td>BOA</td>
<td>basal olfactory area</td>
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<td>BP</td>
<td>blood pressure</td>
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<td>CBL, Cblm,</td>
<td>cerebellum</td>
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<td>Cerebell</td>
<td>cerebellum</td>
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<td>CTX, Ctx</td>
<td>cerebral cortex</td>
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<td>EKG</td>
<td>electrocardiograph</td>
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<td>INTRG.</td>
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</tr>
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<td>L-RPC</td>
<td>left-right posterior motor cortex</td>
</tr>
<tr>
<td>LAC</td>
<td>left anterior cortex</td>
</tr>
<tr>
<td>LH</td>
<td>left hippocampus</td>
</tr>
<tr>
<td>LN</td>
<td>lenticular nucleus</td>
</tr>
<tr>
<td>MB</td>
<td>midbrain</td>
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</table>
MP, Med-Pons Medulla - Pons
O.C. Occipital cortex
RAC Right anterior cortex
RF Reticular formation
RH Right hippocampus

III. ABBREVIATIONS OF WEIGHTS AND MEASURES

cm centimeter
c/s cycles per second
Gm. gram
Kg kilogram or $10^3$ Gm
mcgm microgram or gamma or $10^{-6}$ Gm
mg milligram or $10^{-3}$ Gm
uv microvolt or $10^{-6}$ Volts
sec second
msec millisecond or $10^{-3}$ seconds
mm millimeter or $10^{-3}$ meters

IV. ABBREVIATIONS OF ROUTES OF ADMINISTRATION

i.c. intracarotid
i.p. intraperitoneal
i.v. intravenous
i.vt. intravertebral
### V. ABBREVIATIONS OF GENERAL REFERENCE TERMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CRO</td>
<td>cathode ray oscilloscope</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalograph</td>
</tr>
<tr>
<td>PE</td>
<td>polyethylene</td>
</tr>
<tr>
<td>RB</td>
<td>rebinding (of NE at the membrane)</td>
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PART I. EVIDENCE FOR AN ACTION OF ANTICHOLINESTERASES ON THE CENTRAL NERVOUS SYSTEM OF RABBITS NOT ATTRIBUTABLE TO ENZYME INHIBITION
I. INTRODUCTION

Electrocorticographic responses to the administration of DFP to cats and monkeys consisted of EEG arousal which was attenuated by atropine (Wescoe et al. 1948). Johns et al. (1951) found that the amount of theophylline required to induce cortical EEG seizure activity was decreased by as much as one-sixth when given after DFP. Atropine blocked the convulsions but its effectiveness was a function of the ratio of DFP to theophylline. Johns et al. concluded that atropinic antagonism of DFP-theophylline convulsions occurred when the concentration and hence the effect of theophylline was minimal. Bouzarth and Himwich (1952) evaluated the mechanisms of seizures induced by DFP. They noted that the order of appearance of seizures was thalamus, caudate, limbic cortex, and finally motor cortex. However, later findings have indicated the hippocampus as a primary site of convulsant action in the CNS due to its low epileptogenic threshold (Liberson and Akert 1955). Sarin-induced convulsions were attenuated by atropine and by PAM (2-pyridin-aldoxime methiodide), which antagonized the convulsive phase of sarin but not the EEG desynchronization. The latter finding with PAM is of interest since it is a quaternary
compound which probably does not gain access to the CNS. Wills (1963) discussed the pharmacological antagonism of anticholinesterases and suggested that the EEG seizure activity was the result of stimulation of diencephalic brain sites.

Eserine, in low doses has been shown to evoke a transient EEG arousal that is associated with the reversible nature of enzyme inhibition (Longo and Silvestrini 1957). Holmstedt (1963) pointed out that neostigmine had an excitatory effect on the neuromuscular junction after cholinesterase had been inactivated by DFP. Other experiments were discussed that utilized the neuromuscular junction responses as evidence of anticholinesterase drug effects that were independent of cholinesterase inhibition.

Beněšová et al. (1964) reported that amitryptiline and other thymoleptics decreased the duration of Eserine-induced arousal and that chlorpromazine and reserpine pretreatment prolonged it. The authors attributed the prolonged response to the antiadrenergic effects of Eserine. However, Benson (1948) had shown "adrenalin" of the swine caudate to inhibit butyrylcholinesterase and acetylcholinesterase as measured manometrically. He proposed the adrenalin-potentiating effect on ACh was attributable to inhibition of cholin-
esterase as well as promoting the synthesis of ACh.

Goldberg et al. (1963, 1965) found that cholinesterase had to be inhibited greater than 21 to 50 per cent in order to observe behavioral effects. Also they found that Eserine and Carboryl (naphthyl N-methyl carbamate) had central agonist properties in addition to inhibition of cholinesterase. These responses were antagonized by atropine.

Bito et al. (1967) noted that chronic administration of ecatiophate failed to produce enduring effects, but blocked the response to the presence of carbachol in the eye of the dog. Other responses of the iris were normal and blocked by atropine. They concluded that ecatiophate had anti-parasympathomimetic effects.

The present study evaluates EEG changes in the presence of protected and of markedly inhibited cholinesterase. These findings are correlated with biochemical determinations of cholinesterase activities as well as with histochemical evidence for irreversible enzyme inhibition.
I. LITERATURE REVIEW

Survey of the literature of CNS effects of anticholinesterases and its antagonism by anticholinergics was facilitated by existent reviews, which were extensively utilized due to the excessive number of publications on cholinesterases and anticholinesterases that have appeared in recent years (250-300 per year, Augustinsson 1963).

Diamant (1954) tabulated the results of investigation of CNS effects from: (1) the reversible inhibitors, Eserine and neostigmine, and (2) the irreversible organophosphorus inhibitors, DFP, TEPP, parathion, and tabun. Miller et al. (1938 op.cit.) observed that Eserine induced an increase in electrical activity of the cortex when applied topically to the cortex of cats. Anticholinesterase action on spinal cord reflex activity of dogs was shown by Bülbbring and Burn (1941 op.cit.). These investigators administered Eserine arterially into the spinal cord and noted that the effects on the lower leg reflexes were antagonized by atropine but were enhanced by "adrenalin". Williams (1941 op.cit.) noted that Eserine enhanced petit mal EEG paroxysms in humans (cf. Williams and Russel (1941 op.cit.), who also observed atropinic antagonism of Eserine). Darrow et al. (1944 op.cit.) observed an
increase in the fast frequencies of the EEG of cats and a decrease in high amplitude slow waves. Atropine antagonized the responses. Hyde et al. (1947 op.cit.) found that topically applied Eserine potentiated the effects of strychnine and pentylenetetrazole on the EEG. Bradley and Elkes (1953 op.cit.) noted only a moderate increase in electrocortical activity subsequent to the intraperitoneal administration of 0.5-1.0 mg/Kg of Eserine. EEG responses to the administration of DFP consisted of cortical desynchronization followed by convulsive activity (Wescoe et al. 1948 op.cit., Freedman et al. 1949 op.cit., Essig et al. 1949 op.cit., Bouzarth and Himwich 1952 op.cit. and Johns et al. 1951). Furthermore, mechanisms of action in addition to cholinesterase inhibition were proposed by several authors (Verbeke 1949 op.cit., Koppanyi and Karczmar 1951 op.cit., Michaelis et al. 1949 op.cit. and Brooks et al. 1949 op.cit.). In general, the mechanisms of action were concluded to be: (1) of a direct pharmacodynamic nature on the effector cells, (2) inhibition of cholinesterase along with the pharmacodynamic action, (3) histological cellular damage, or (4) metabolic effects on cellular respiration.

Holmstedt (1959) reviewed the effects of organophosphorus cholinesterase inhibitors on EEG activity. The
literature revealed a prolonged EEG arousal with subconvulsant doses of DFP and sarin while higher doses provoked diffuse convulsive EEG patterns. Holmstedt pointed out that the CNS effects of these anticholinesterases could be obtained without damage of the blood brain barrier (BBB).

In addition to inhibition of cholinesterase, sarin, DFP, and Eserine had been reported to evoke pressor responses.

Heath (1961) discussed the protective effect of reversible inhibitors (Eserine) on the subsequent administration of irreversible inhibitors (TEPP, DFP). He also proposed that reversible anticholinesterases, given in the presence of irreversible inhibitors, would enhance the degree of enzyme inhibition. However, he pointed out that these results varied with species, drugs, and doses.

Loshadkin and Smirnov (1962), in a review of chemical and toxicological aspects of organophosphorus anticholinesterases, discussed drug action on choline-reactive systems. They defined a choline-reactive system as that sequence of biochemical events through which ACh is released from nerve terminals and acts on "choline receptors". They then equated the choline receptor with choline-reactive system.

Two features common to AChE and choline receptors were suggested; first, both were highly specific for the same
substrate (ACh); second, excess concentrations of substrates prevented inhibition of ChE by organophosphorus inhibitors. Karasik et al. (1946, 1947, 1948 op.cit.) as well as Župančič (1953 op.cit.), proposed that cholinesterases and choline receptors were one and the same. The reviewers pointed out that while the function of choline receptors was specific and associated with the presence of sulfhydryl groups, the action of sulfhydryl containing inhibitors of ChE was non-specific. Loshadkin and Smirnov proposed that organophosphorus agents which competed with ACh for ChE, could react with the choline receptors. Similar to the review of Diamant (1954), the hypothesis for a combined enzyme inhibition with pharmacodynamic action on choline-reactive systems was suggested. Examples from the literature were cited by Loshadkin and Smirnov (1962) to include:

(1) McNamara et al. (1954 op.cit.), who compared the response to DFP and TEPP with the histochemical demonstration of ChE in various organs. They concluded that inhibition of enzyme alone was insufficient to account for mechanisms of toxic action.

(2) Holaday et al. (1954 op.cit.), who investigated pre- and postganglionic action potentials in the presence of DFP and compared their findings with the degree of inhibition
of total and specific cholinesterases. It was concluded that changes in transmission of the superior cervical ganglion under the influence of DFP could not be explained by inhibition of enzyme alone and a direct action of DFP on choline receptors was proposed.

(3) Roeder and Kennedy (1955 op.cit.), who showed that sarin, tabun, and soman competed with ACh for choline receptors at synaptic junctions.

(4) Liljestrand and Zotterman (1954 op.cit.), who found that DFP sensitized choline receptors of the carotid body to the action of choline esters.

(5) Turpaev and Putintseva (1957 op.cit.), who showed Fosfakol (diethyl p-nitrophenyl phosphate) to act on isolated intestine in the presence of totally inactivated cholinesterase.

(6) Komissarov (1957 op.cit.), who concluded that the action of Fosfakol on reflex conduction in rabbit spinal cord, would not be explained on the basis of enzyme inhibition alone.

(7) Seminov (1957 op.cit.), who showed that the bronchospasm induced by Armin (ethyl p-nitrophenyl ester of ethylphosphonic acid), included a cholinomimetic action along with the inhibition of enzyme.
(8) Murtha et al. (1955 op. cit.), Feldberg (1954 op. cit.), and Hobbiger (1954 op. cit.) were also listed as concluding that the action of anticholinesterases was in some way associated with a direct action on choline receptors.

It should be emphasized that direct actions of the anticholinesterases were proposed in addition to the evidence for inhibition of enzyme.

Handbuch der Experimentellen Pharmakologie 15: "Cholinesterases and Anticholinesterase Agents" provided considerable information pertinent to the present investigations. Evaluation of brain AChE activities by biochemical and histochemical methods was adequately reviewed (Koelle 1963 op. cit. and Augustinsson 1963 op. cit.).

Machne and Unna (1963) reviewed the CNS effects of anticholinesterases. Their discussion revealed that desynchronization of the EEG, attenuation of recruited potentials and convulsive EEG patterns could be evoked by the i.v. administration of irreversible or reversible inhibitors. They pointed out that anticholinesterases enhanced the level of spontaneous electrical activity while depressing the response to electrical stimulation in isolated cortex. Topical application of inhibitors yielded conflicting results also. However, Sigg et al. (1965) observed an enhance-
ment of the cortical dendritic response following the intrapial administration of Eserine in cats. They postulated a muscarinic effect on dendrites since the response was sensitive to the blocking action of atropine.

The review of Machne and Unna (1963) was controversial in that continued reference was made to the CNS effects of quaternary compounds, atropine methyl nitrate and neostigmine. It has been established that these drugs penetrate the blood brain barrier (BBB) to little if any extent (Longo 1962).

Longo (1962) presented the most significant information of pharmacological EEG research in rabbits. He reviewed the effects of reversible and irreversible inhibitors of cholinesterases and his discussion was in agreement with Machne and Unna with regard to the responses to Eserine, DFP, atropine, and scopolamine. However, Longo and others (Hance et al. 1963) have failed to support the contention of Machne and Unna with regard to quaternary compounds.

Karczmar (1966) reviewed the pharmacology of anticholinesterases in extenso. Actions of anticholinesterases on the reticular formation and cortex were included and EEG desynchronization was seen as a characteristic feature of i.v. or topically applied Eserine. Cholinergic mechanisms in the
reticular formation responsible for EEG desynchronization were discussed (Rinaldi and Himwich 1955 op. cit.). The effect of anticholinesterases on cholinergic thalamocortical pathways was also presented. Recent studies of Shute and Lewis (1963, 1965, 1966) have shown that cholinesterase-containing fibers exist throughout the brain. In particular, these fibers run through the ascending reticular activating system from the medulla-pons (Holmes and Wolstencroft 1964) through the forebrain. Acetylcholine release at the cortex has been evoked by electrical stimulation of the midbrain reticular formation as well as "spontaneously" (Mitchell 1963 and Krnjević 1967). A cholinergic nature of CNS transmission has been implied by the nicotinic pharmacological responses of Renshaw cells, (Curtis and Eccles 1958) which were associated with the early responses of motor axon stimulation, while late muscarinic responses have also been observed (Curtis and Ryall 1964). However, motoneuron firing has been shown to be depressed by NE in the lumbar spinal cord of cats (Weight and Salmoiraghi 1967). Therefore, sites of action for anticholinesterases exist throughout the CNS and their activity can be evaluated at all levels from cord to telencephalon. In addition,
functional interaction with adrenergic systems is probably a factor in CNS responses to anticholinesterase drugs (Marczynski 1967).
II. METHODS AND MATERIALS

A. GENERAL

Experimental animals\(^1\) were adult, male, New Zealand albino rabbits of approximately 2.5-3.5 Kg body weight. Larger animals were used for the experimental preparations in which the basilar artery was ligated at the midpontine level in order to facilitate the required surgical approaches. A standard laboratory animal diet was provided with food and water available ad libitum. The animals were retained in the animal quarters for a minimum of one week prior to use and were brought to the laboratory on the day of the experiment.

B. HISTOLOGICAL METHODS

1. Thionin and Eosin Staining Procedure

A combination thionin and eosin staining procedure was used that revealed cell bodies and fibers in the central nervous system. This staining procedure was compatible for use with frozen sections (Conn et al. 1962). Sagittal sections were prepared from brain tissue of untreated rabbits sacrificed by air embolism via the marginal ear vein. The brains were kept intact and rapidly removed beginning at the

\(^1\)Scientific Small Animal Laboratory and Farm, Inc., Arlington Hts., Ill.
olfactory bulbs and extending to the calamus scriptorius, placed in a beaker of saline for removal of excess blood and bone fragments, and then transferred to a saline dampened gauze square for removal of meninges. The removal of these membranes prevented the knife from snagging during sectioning which would result in artifacts across the surface of the section. The brains were then divided in halves by passing a large spatula through the midsagittal plane. A flattened piece of freezer wrap was placed on the floor of the cryostat chamber and the half brain specimen was placed on it with the medial surface directed down. This last procedure froze the brain with the medial surface flat and permitted orientation of the plane of section parallel to the knife edge. The result was a marked reduction in the loss of important midline structures during the sectioning procedure. Brains were frozen at -20°C to -30°C at least 4 hours and in most cases 24 hours. Sections were cut at a thickness of 32 microns and were mounted in permount medium.

Verification of electrode implantation site was visualized after the staining procedure had been completed. However, it was possible to trace the electrode tracts while sectioning and the subsequent staining procedures were not
required. Details of the staining schedule are listed in appendix A.

2. Thiocholine Histochemical Procedure

The in vitro histochemical procedure as outlined by Koelle (1951, 1955) for the localization of cholinesterases was modified to include in vivo and in vitro procedures (Koelle 1957). Control sections were obtained from rabbits sacrificed by air metabolism. Brains were prepared in the same manner as described above except that the frozen sections were 16 microns in thickness. Details of the staining schedule are listed in appendix A.

Irreversible inhibition of cholinesterase was induced by the intravenous administration of DFP (1.0-3.0 mg/Kg) or of sarin (20.0-50.0 mcgm/Kg). Brains were quickly removed after the animals had convulsed and died. Frozen brain sections from these animals were processed through the same staining procedure as controls (vide supra). Untreated control sections were run concurrently with the sections that had been exposed to anticholinesterases in vivo and served as internal controls for the histochemical demonstration of the enzyme.

Physostigmine (100.0-150.0 mcgm/Kg i.v.) was used to inhibit cholinesterase reversibly. It was given 5 minutes
prior to DFP or sarin which were given in the doses indicated above. After the animals convulsed and died, the brains were removed for histochemical analyses. The same staining procedure for cholinesterases was used throughout.

C. BIOCHEMICAL METHODS

1. Warburg Procedure

A standard manometric procedure for the determination of cholinesterase activity was used (Koppanyi and Karczmar 1964). Brains of animals sacrificed by air embolism were divided at the midsagittal plane and one-half was added to bicarbonate buffer (1:50 w/v). Ultrasonic homogenization of the tissue required less than 2 minutes and a chilled solution was used to reduce the effects of heat. Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE, ChE) activities were determined using methacholine and butyrylcholine as substrates. Control untreated brain samples were included with the experimental samples and readings were taken at ten-minute intervals for up to an hour. Microliters CO₂ formed per unit time are expressed as percentages.

Experimental samples from animals included those treated (1) with sarin (20.0 mcgm/Kg i.v.), (2) with DFP (1.0 mg/Kg i.v.), (3) with sarin in the presence of atropine
methyl nitrate (1.0 mg/Kg i.v.), and (4) with sarin followed by atropine sulfate (5.0 mg/Kg i.v.). The rabbits were prepared for EEG recording (vide infra) and were maintained with artificial respiration after exposure to toxic amounts of the anticholinesterases. Histochemical analysis (vide supra) was carried out on the other half of these brains. The biochemical determination of AChE and BuChE activities served as a control measure to correlate with histochemical findings and electrophysiological responses during conditions in which the enzyme activity was irreversibly inhibited.

D. PHYSIOLOGICAL METHODS

1. Electroencephalography

EEG recordings from unanesthetized restrained animals in acute experiments necessitated development and modification of equipment. An animal restraining board fitted with a headholder was used for immobilization of the rabbits during the recording sessions. Openings cut into the bottom of the board permitted access to the ventral anatomy of the rabbit. Thus, tracheotomy as well as arterial and venous cannulation could be carried out under local anesthesia. The rabbit could then be returned to the supine position for implantation of brain electrodes without being released from
Electrode Hub Diagram: Diagram of design of electrode hub. Scale is 0.25 inch to 1.0 mm. Construction is of brass.
ELECTRODE CONSTRUCTION

Figure 2

Electrode Hub

Nichrome Wire

#37 Gauge Insulated Stranded Wire

Heat Shrinkable Tubing

PE #60 Tubing

Completed Electrode

Scale: ___ cm
Legend for Figure 2

Steps in the Construction of EEG Electrodes: The components of an electrode are laid out in figure 2. The electrode hub (cf. Electrode Hub Diagram) two twisted 0.010 inch diameter formvar insulated nichrome wires, and three insulated stranded wires are assembled as indicated to form the completed electrode as seen on the right side of the figure according to the scale.
the restraining board.

The steps in the construction of the concentric tripolar cortical and subcortical EEG electrodes are illustrated in figure 2. A detailed description is provided in appendix A.

The procedure for implantation of electrodes was carried out under local anesthesia. An incision was made in the scalp that extended from the nasion, caudally to the cervical vertebra. After the calvarian surface was exposed and local tissue and bone bleeding controlled, the coronal and midsagittal bone sutures were located. The interstice of the sutures was used as an anterior-posterior zero (AP zero) reference point. Sawyers' atlas for the rabbit diencephalon (1954) was used as a guide for the coordinates. The skull was trephined at selected sites by a No. 51 drill held in an X-Acto model makers pin vise. This size of opening in the bone permitted a tight fit of the electrode shank so that further fixation of the electrode to the skull was generally unnecessary. However, dental acrylic could be used to affix the electrode to the bone. After cutting the nichrome wire depth electrodes to the appropriate length, they were manually implanted according to selected stereotaxic coordinates (see appendix A). The electrode hub recorded EEG activity from the cortex while the depth electrodes in that same assembly
recorded activity from selected subcortical structures. Bipolar electrical stimulation of brain sites was carried out through the appropriate depth electrodes. The coordinates of brain sites for recording and for stimulating are designated in appendix A. They are: basal olfactory area (BOA), lenticular nucleus (LN), left or right anterior motor cortex (L,RAC), left or right posterior cortex (L,RPC), reticular formation of the brain stem (RF), occipital cortex (O.C.), and anterior dorsal thalamic nuclei (ADN). Electrode placement was verified histologically at the termination of the experiment. During the experiment, the characteristics of the EEG patterns as well as the responses to electrical stimulation of the brain were used as an index of the accuracy of electrode placement. Bipolar recording technique was routinely used and the electrode combinations are as indicated in the relevant figures.

A minimum of 30 minutes was allowed for the recovery of the experimental subjects after the implantation procedure was completed. A dimly lighted room was used for recording sessions. A seven channel, ink writing Grass Model IIID electroencephalograph was used. Recording speed and amplification are as indicated by the calibra-
tions in the figures. Six of the seven channels were used for obtaining EEG information and the remaining channel recorded either blood pressure or heart rate. Heart rate was obtained from leads placed across two limbs. The blood pressure was recorded directly from the femoral artery via a Statham pressure transducer and was amplified by a low level D.C. Grass preamplifier. Pressure was usually recorded as a mean value by using the 1/2 amplitude high frequency control on the recorder. Periodic control recordings were obtained at a full frequency amplification in order to observe the wave form. Blood clotting was controlled by the use of heparinized saline (20 units/ml) in the cannula.

After recovery, EEG activity was evaluated to determine if the implantation of electrodes had produced extensive brain damage. EEG alerting responses to stimuli of sound (clapping of the hands), touch (lightly stroking the animal's back), and pain (pinching the hind leg of the rabbit) were observed. These also gave an indication of the general condition of the animal. Saline injections were given as a control measure with the volume equivalent to the volume of the drug to be given.
2. Ligation of the Basilar Artery

Drug injections into the arterial circulation of the brain minimizes effects due to peripheral actions of drugs. Procedures used include injections into either the vertebral or carotid arterial blood supplies in animals with their basilar artery ligated at the midpontine level (Jolly 1956 and Magni 1962). This method was applied to the present study.

The animals were anesthetized with diethyl ether and 2% procaine local anesthesia prior to a tracheotomy. Following surgery, ether was expelled in the respired air and the local anesthesia was maintained at wound sites. The rabbits were immobilized with curare and supported by artificial respiration. The trachea above the point of insertion of the cannula and the larynx were removed. The musculature at the base of the brain was retracted to expose the basi-occipital bone. Ligation of veins encountered during this procedure was critical. An 8mm x 5mm rectangular area of outer bone was removed by drilling two holes through the outer layer, 2 mm anterior to the foramen magnum and then working cephalad with rongeurs. Bone bleeding was controlled by the use of bone wax. The basilar was exposed by removing the rectangular inner layer of bone
beginning at the caudal border of the opening and exposing the artery through the dura along the ventral midline of the pons. After rupturing the dura, the basilar was freed by a small blunted dental pick. Caution was required at this point, to prevent rupture of the artery or of its penetrating branches. The artery was ligated by passing a 6-0 suture under it with a miniature blunted "U" shaped needle and then tying the suture with forceps. The opening in the basioccipital bone was packed with gelfoam. Implantation of electrodes (vide supra) required that the rabbit be placed on its abdomen after which it was placed on its back for the remainder of the experiment.

The jugular and subclavian veins were exposed and separated from the underlying subclavian and innominate arteries. The origin of the vertebral artery was exposed and the omocervical and internal mammary arteries ligated. The subclavian artery was cannulated (PE#60); so that the tip of the cannula rested approximately 5.0 mm from the origin of the vertebral artery. Intravertebral arterial injections were carried out by occluding the subclavian artery between the carotid and the vertebral arteries and then injecting retrogradly through the cannula. After the injection, the subclavian was released. The bifurcation of
the common carotid was exposed and the external branch cannulated (PE#10) with the tip of the cannula at the bifurcation. Intracarotid arterial injections were then made without interruption of the blood flow (fig. 17). Electrical activity of the brain stem posterior to the position of the tie could be obtained by inserting an electrode through the opening used to approach the basilar. Mineral oil was applied in place of the gelfoam. Blood pressure could also be recorded by cannulating the contralateral external carotid.

The functional state of the brain was revealed by the spontaneous EEG and its response to the control EEG arousal stimuli of sound, touch, and pain. Physiological saline at room temperature injected intracarotidly or intravertebrally served as a control for EEG responses to anticholinesterases (AntiChE's).

The distribution of materials injected into these two arterial paths was shown in two different preparations (fig. 18) by the administration of a monastral blue dye diluted 1:1 with saline. The aorta was clamped about 5 seconds after the dye had been given and prevented a generalized distribution by recirculation.
E. PHARMACOLOGICAL METHODS

1. Drugs and Dosages

Atropine methyl nitrate was used as a peripherally acting anticholinergic that does not readily penetrate the blood brain barrier (BBB). Untoward cardiovascular responses to centrally acting anticholinesterases were adequately attenuated by intravenous doses of 0.5-2.0 mg/Kg.

Physostigmine (Eserine) was used as a reversible inhibitor of cholinesterases that readily penetrated the BBB. EEG desynchronization without seizure activity was found, during preliminary studies, to be evoked by intravenous doses of 100-150 mcgm/Kg. EEG and classical grand mal convulsive episodes resulted from doses of 200.0 mcgm/Kg or greater.

DFP and sarin, which are irreversible inhibitors of cholinesterase that readily penetrate the BBB, have marked effects on the electrical activity of the CNS (Diamant 1954). DFP was diluted with saline prior to injection and used within 15 minutes. EEG desynchronization was evoked by 0.5 mg/Kg i.v. and convulsions and death by 2.0-3.0 mg/Kg i.v. as determined in preliminary studies. If additional doses were to be given, artificial respiration was usually needed. Sarin was diluted with saline and used within 15 minutes. EEG arousal was elicited by 10.0 mcgm/Kg i.v.
while 20 mcgm/Kg i.v. evoked an alert pattern followed by convulsive activity. Doses of 50.0 mcgm/Kg i.v. or greater, evoked a prompt EEG alert pattern followed by convulsions. Death occurred within minutes.

In the preparation with the basilar artery ligated at the midpontine level, doses were given via the internal carotid (i.ct.) or the vertebral artery (i.vt., cf. figs. 21, 23). Drugs were not administered on the basis of unit body weight because preliminary studies showed that the responses to the drugs were determined by the specific dose given and not by drug wt. per unit body wt. This dose is referred to in the figures as "total dose".

The dosages of anticholinesterases included (1) Eserine given alone as well as in the presence of the irreversible inhibitors and (2) DFP or sarin given alone as well as in the presence of Eserine. The administration of Eserine was given to protect cholinesterase from irreversible inhibition by subsequent injections of DFP or sarin even in amounts that evoked convulsions and death. On the other hand, initial administration of DFP or sarin permitted the observation of the effects of Eserine in the presence of irreversibly inhibited enzyme.
III. RESULTS

A. CONTROL OBSERVATIONS

1. Histological

The results of the thionin and eosin staining procedure on the rabbit brain sections are revealed in figures 3a through 3d. Line drawings accompany these figures and provide an identification of relevant anatomical sites. Verification of sites of electrode implantation in experimental animals was readily obtained after this master set of control sections had been prepared. Anatomical sites of electrode implantation for recording and/or stimulation are as indicated in the appropriate sections. In addition, this sagittal section atlas of the rabbit brain served as an orientation for histochemical studies of cholinesterase localization. The results of the thiocholine procedure are illustrated in figures 5a through 5c. These sections are also accompanied by line drawings to indicate significant anatomical areas. The areas with the most intense stain reveal the highest concentration of cholinesterase. These sites are the caudate nucleus, lenticular nucleus, anterior dorsal nuclei of the thalamus, pulvinar and reticular thalamic nuclei, superior colliculus, amygdaloid nuclei,
and hippocampus. Other areas that revealed an intense stain are the nuclei cuneatus and gracilis of the pontomedullary area as well as midbrain masses such as the nucleus rubra and substantia nigra.
### SAGITTAL BRAIN SECTIONS

#### LIST OF ABBREVIATIONS:

<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>a.c.</td>
<td>anterior commissure</td>
</tr>
<tr>
<td>a.c.p.</td>
<td>anterior cerebellar peduncle</td>
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<tr>
<td>a.d.n.</td>
<td>anterior dorsal thalamic nuclei</td>
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<td>a.m.n.</td>
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</tr>
<tr>
<td>cen. ov.</td>
<td>centrum ovale</td>
</tr>
<tr>
<td>cer. ped.</td>
<td>cerebral peduncle</td>
</tr>
<tr>
<td>claus.</td>
<td>claustrum</td>
</tr>
<tr>
<td>i.coll.</td>
<td>inferior colliculus</td>
</tr>
<tr>
<td>s.coll.</td>
<td>superior colliculus</td>
</tr>
<tr>
<td>cor. rad.</td>
<td>corona radiata</td>
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<tr>
<td>fim.</td>
<td>fimbria of the hippocampus</td>
</tr>
<tr>
<td>forn.</td>
<td>fornix</td>
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<tr>
<td>Abbreviation</td>
<td>Full Term</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>fr.ctx.</td>
<td>frontal cortex</td>
</tr>
<tr>
<td>d.hpc.</td>
<td>dorsal hippocampus</td>
</tr>
<tr>
<td>v.hpc.</td>
<td>ventral hippocampus</td>
</tr>
<tr>
<td>i.m.l.tr.</td>
<td>intermediolateral thalamic tract</td>
</tr>
<tr>
<td>l.gen.</td>
<td>lateral geniculate body</td>
</tr>
<tr>
<td>l.t.n.</td>
<td>lateral thalamic nuclei</td>
</tr>
<tr>
<td>lemn.</td>
<td>lemnisci</td>
</tr>
<tr>
<td>ma.b.</td>
<td>mamillary bodies</td>
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<tr>
<td>mam.th.tr.</td>
<td>mamillothalamic tract</td>
</tr>
<tr>
<td>m.lat.n.</td>
<td>mediolateral thalamic nuclei</td>
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<tr>
<td>med.n.</td>
<td>medial thalamic nuclei</td>
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<td>Meynert's tract</td>
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<tr>
<td>m.c.p.</td>
<td>mid-cerebellar peduncle</td>
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<tr>
<td>n.amb.</td>
<td>nucleus ambiguous</td>
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<tr>
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<td>caudate nucleus</td>
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<tr>
<td>n.gr., cun.</td>
<td>cuneate and/or gracilis nuclei</td>
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<tr>
<td>n.i.p.</td>
<td>interpenduncular nucleus</td>
</tr>
<tr>
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<td>lenticular nucleus</td>
</tr>
<tr>
<td>n.ol.</td>
<td>olivary nuclei</td>
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<td>occipital cortex</td>
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<tr>
<td>op.rad.</td>
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</tr>
<tr>
<td>p.c.</td>
<td>posterior commissure</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>p.c.p.</td>
<td>posterior cerebellar peduncle</td>
</tr>
<tr>
<td>pons</td>
<td>pons</td>
</tr>
<tr>
<td>pul. n.</td>
<td>pulvinar nucleus</td>
</tr>
<tr>
<td>pyrm. tr.</td>
<td>pyramidal tract</td>
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<tr>
<td>r.n.</td>
<td>red nucleus</td>
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<tr>
<td>ret. sub.</td>
<td>reticular substance</td>
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<tr>
<td>ret.th.n.</td>
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</tr>
<tr>
<td>thalamus</td>
<td>thalamus</td>
</tr>
<tr>
<td>sep. pel.</td>
<td>septum pellucidum</td>
</tr>
<tr>
<td>III.N.tr.</td>
<td>oculomotor nerve tract</td>
</tr>
<tr>
<td>III.n.</td>
<td>oculomotor nucleus</td>
</tr>
<tr>
<td>VI.n.</td>
<td>abducent nucleus</td>
</tr>
</tbody>
</table>
Figure 3a

medulla

pyrm.tr.

pons

ret.sub

lema

ret.sub.

i.m.i.tr.

cer. ped.

sub nig.

v.hpc

amyg.

op.tr.

pre op. ctx

caps.e

ac.

cer. ped.

ref. th.n.

a.m.n.

fim.

n.cau.

p.cen. ctx.

c.c.

cen. gr.

i.coll.

c. coll.

occ. ctx.

cen. ctx.

d.c.

med.n.

a.d.n.

r.n.

lat.n.
MODE OF ACTION OF THIOCHOLINE PROCEDURE:

Reaction "A"

\[
2 \text{CH}_3\text{COSCH}_2\text{CH}_2\text{N(CH}_3\text{)}_2\text{OH} + (\text{NH}_2\text{CH}_2\text{COO})_2\text{Cu} + 2\text{H}_2\text{O} + \text{ChE}
\]

\[
\text{(Acetyltiocholine)} \quad \text{(Copper Glycinate)} \quad \text{(Water)} \quad \text{(Cholinesterase)}
\]

\[
\text{Cu(SCH}_2\text{CH}_2\text{N(CH}_3\text{)}_2\text{OH)}_2 + 2\text{NH}_2\text{CH}_2\text{COOH} + 2\text{CH}_3\text{COOH}
\]

\[
\text{(Copperthiocholine)} \quad \text{(Glycine)} \quad \text{(Acetic Acid)}
\]

Reaction "B"

\[
\text{Cu(SCH}_2\text{CH}_2\text{N(CH}_3\text{)}_2\text{OH)}_2 + (\text{NH}_4)_2\text{S}
\]

\[
\text{(Copperthiocholine)} \quad \text{(Ammonium Sulfide)}
\]

\[
\text{CuS} + 2\text{NH}_4\text{SCH}_2\text{CH}_2\text{N(CH}_3\text{)}_2\text{OH}
\]

\[
\text{(Copper Sulfide)} \quad \text{(Ammoniumthiocholine)}
\]
Postulated Sequence of Events for Histochemical Thiocholine Procedure that Reveals the Distribution of Cholinesterase:
The mode of action of the thiocholine procedure is postulated to occur as two reactions. Reaction "A" involves the interaction of acetylthiocholine with cholinesterase in the incubation medium that contains copper glycinate. The intermediate copper thiocholine thus formed takes part in reaction "B" with the ammonium sulfide (developer solution). Copper sulfide is the resultant product that is present at those sites in which cholinesterase is to be found.
Irreversible inhibition of cholinesterase is shown in the results of the thiocholine procedure applied to animals injected with DFP 2.0-5.0 mg/Kg i.v. or with sarin 20.0-100.0 mcgm/Kg i.v. Brain sections of these experimental subjects are shown in figures 6a through 6c. The blank stained sections appeared as a translucent film on the microscopic slides after the thiocholine staining procedure and necessitated marked overdevelopment and overexposure during photographic processing. Counterstaining procedures were avoided so that only the localization of cholinesterases was revealed. Areas of shading that appear on the blank sections reflect differences in tissue density and outline anatomical structures but do not indicate a deposition of copper sulfide which would suggest the presence of cholinesterase. On the other hand, reversible inhibition of cholinesterase by Eserine in amounts of 50.0-150.0 mcgm/Kg i.v. did not prevent the selective staining of brain cholinesterase. These sections stained similar to the untreated controls (cf. fig. 29 and fig. 5a).

The brain sites supplied by the vertebral and carotid blood supplies are shown by the distribution of monastral blue dye as seen in figure 18. The position of the arterial pressure deadpoint that results from the meeting of the
two pressure heads of cerebral arterial blood flow, the vertebral supply and the internal carotid supply, was firmly established by ligation of the basilar artery at the midpontine level. Dye injection through the vertebral artery produced the results shown in figure 18 upper section. The vertebral arterial contribution to the blood supply of the brain and drugs injected into this artery initially infuse upper spinal cord and the bulbar pontine reticular substance in the caudal brain stem. The section of brain in the lower half of figure 18 reveals that brain sites cephalad to the position of the ligature receive blood from the internal branch of the carotid via the circle of Willis. Thus, the more rostral brain stem and the cerebral hemispheres are exposed initially to drugs given by the intracarotid route of administration. Diagrammatic illustration of the ventral surface of the rabbit brain is shown in figure 17 and is described in the legend for that figure.

2. Biochemical

The results of the manometric determination of the activity of AChE and of BuChE are expressed in Table I and appendix A. Raw data was expressed as microliters of CO₂ evolved from brain tissue read at the times and under the
### Table I. Correlation of Histochemical and Biochemical Cholinesterase Data

<table>
<thead>
<tr>
<th>Drug</th>
<th>Histochemical</th>
<th>Biochemical</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ChE Stain</td>
<td>AChE Activity(^1)</td>
</tr>
<tr>
<td>Control</td>
<td>Present</td>
<td>100.0</td>
</tr>
<tr>
<td>Sarin</td>
<td>Absent</td>
<td>1.6</td>
</tr>
<tr>
<td>DFP</td>
<td>Absent</td>
<td>8.2</td>
</tr>
<tr>
<td>Sarin and Atropine Methyl Nitrate</td>
<td>Absent</td>
<td>7.7</td>
</tr>
<tr>
<td>Sarin and Atropine Sulfate</td>
<td>Absent</td>
<td>10.6</td>
</tr>
</tbody>
</table>

\(^1\)Expressed as per cent of control (100.0%).

\(^2\)BuChE activity at 100.0% was only 10–15% of AChE control (cf. text and appendix A).
conditions referred to in the figures and Methods. Brain tissue controls as well as reagent controls were run with each experimental determination. The enzyme activity of the control samples was arbitrarily designated as 100%. Experimental values were expressed as a percentage of their respective control. While the enzyme activity of BuChE and of AChE is set equal at 100% (Table I), actual BuChE activity was far less than that of AChE (appendix A). BuChE activity was only 10% to 15% of the activity of AChE.

3. Electrophysiological

The electrodes as constructed provided few difficulties and artifact free recordings were constantly obtained. Verification of the site of implantation was determined histologically as well as from the characteristic type of EEG pattern or of response to electrical stimulation. Thus, cortical slow waves and spindle activity, hippocampal theta rhythm, occasional olfactory bursts, and classical recruitment were all used as indices of accurate placement. Typical EEG patterns in the control animal are represented by the tracings found in figures 7 through 10. In general, a spectrum of EEG activity was shown in the rabbit. The predominant pattern was one of slow wave sleep
Control EEG Pattern Response to Stimulation: The resting EEG pattern (left side of tracings) is interrupted by the sound of hand clapping (arrows) to evoke a desynchronized EEG alert pattern. This is brief in duration (right side of figure) as the pattern returns to one of slow wave sleep.
Control EEG Pattern Response to Stimulation: The resting EEG pattern (left side of tracings) is interrupted by a desynchronized EEG alert pattern on the presence of tactile stimulation (arrows) elicited by lightly touching the back of the rabbit. Note that the response to the stimulation outlasts the duration of stimulus presence (right side of figure) as compared with the response to sound in the previous figure.
Control EEG Pattern Response to Stimulation: Pain stimulation induced by pinching the hind leg of the rabbit evokes a desynchronized EEG pattern (arrows). Note that the response persists after the stimulation has terminated.
Control EEG Pattern Response to Stimulation: Electrical stimulation of the mesodiencephalic reticular formation (arrows, 200 c/s 2.0 volts, 0.7 msec duration) elicits an EEG arousal response that outlasts the period of stimulation.
with random 8-14 c/s activity appearing in the cortices. This synchronized pattern was periodically interrupted by an abrupt desynchronization evoked by either an uncontrolled stimulation or by the presence of sound, tactile, or painful stimuli (figs. 7, 8, 9), as well as by the direct electrical stimulation of the reticular formation of the midbrain. As is seen in figure 7, the response to sound was generally not an enduring one and the tracings reverted back to the synchronized state within a few seconds after the stimulus was withdrawn. Hippocampal leads showed a marked hypersynchronization during the presentation of the stimulus as contrasted to the cortical responses during the EEG arousal state. The other control figures showed a similar pattern of response. Tactile stimuli provided an EEG alert pattern but this desynchronization generally was more persistent as shown in figure 8 in which a painful stimulus was used to elicit an abrupt arousal as indicated by the arrows. Direct electrical stimulation of the midbrain reticular formation at the parameters indicated in figure 10 elicited a response similar to that from painful stimuli in that the desynchronization was long lasting.

All animals were tested for these responses as indications of a general physiological state during a control
period. The characteristics of the tracings as well as histological identification verified site of implantation. And the responsivity of the rabbit to the several stimuli as indicated above showed whether or not extensive damage had occurred during the implantation procedure, such as a subdural hematoma or massive trauma to the brain tissue resulting from displacement of the electrode. Animals not responsive to the stimuli in a manner similar to these just described were rejected as experimental subjects.

B. EXPERIMENTAL OBSERVATIONS

1. Histological

Figure 30 reveals a blank thiocholine result following the administration of 50.0 mcgm/Kg i.v. of sarin and its subsequent antagonism by atropine sulfate (5.0 mg/Kg i.v.). This result could not be differentiated from those brain sections found in animals receiving only an irreversible inhibitor (cf. figs. 6a, 6b, 6c). Similar results were noted with rabbits pretreated with sarin or DFP and subsequently exposed to convulsant doses of Eserine. On the other hand, irreversible inhibition of cholinesterase by sarin (50.0-100.0 mcgm/Kg i.v.) was blocked by the prior administration of Eserine (50.0-150.0 mcgm/Kg i.v.). Convulsions and death occurred but histochemically, the
brain sections showed areas of intense staining comparable to controls (fig. 29, cf. figs. 5a, 5b, 5c).

2. Biochemical

The effects of AntiChE's on the activity of cholinesterase are indicated in Table I and appendix A. Sarin (20.0 mcgm/Kg i.v.) markedly reduced AChE activity as did DFP. Neither atropine sulfate nor atropine methyl nitrate altered this effect as inhibition of enzyme activity continued at a high level. BuChE activity also was greatly reduced under these same conditions and while the per cent of reduction is less, again there was approximately 90% less activity compared to that of AChE (cf. appendix A).

3. Electrophysiological

Control tracings prior to the administration of sarin are shown in figure 11. The response to sound stimulation was similar to that obtained previously (cf. fig. 7) and indicated a satisfactory preparation. Sarin (20.0 mcgm/Kg i.v.) evoked a classical grand mal EEG convulsive pattern as shown in figure 12. A slight rise in mean arterial blood pressure and bradycardia was noted. Atropine sulfate (5.0 mg/Kg i.v.) given at the height of the convulsive activity antagonized it and restored the tracings to a non-convulsive pattern typical of that seen after atropine
CONTROL EEG—PRIOR TO ADMINISTRATION OF SARIN

Control EEG Pattern: Resting EEG pattern with slow wave EEG sleep interrupted by the clapping of hands to induce a sound stimulation (arrows) with resulting EEG alert pattern.
Initial Sarin Seizure: Grand mal type convulsive pattern induced by the i.v. administration of 20 mcgm/Kg of sarin. This figure reveals the onset of the seizure which quickly develops into a diffuse pattern of convulsive EEG activity. Note the onset of the pattern at the right side of the tracings and the slight rise in mean blood pressure. Sarin was injected approximately 2 minutes earlier.
Antagonism of the Sarin Seizure: Atropine sulfate 5.0 mg/Kg i.v. given at the height of the anticholinesterase induced convulsion, antagonizes the seizure and reveals a control pattern similar to that seen after only atropine. Uninterrupted brain wave tracings show slow waves and occasional 8-14 c/s spindles. These tracings were made approximately 15 minutes after those seen in figure 12.
Additional Dose of Sarin Provokes a Second Convulsion: The administration of a second injection of sarin (40 mcgm/Kg i.v.) in the presence of atropine as a continuation of the previous record, again evokes a diffuse EEG seizure pattern with a more marked pressor response perhaps due to the higher dose. Moreover, atropine sulfate (5.0 mg/Kg i.v.) given at the arrow rapidly antagonizes the convulsive pattern (cf. Figure 13).
only (Longo 1962). Blood pressure and heart rate changes were also antagonized as shown in figure 13. EEG convulsions were readily initiated by the administration of another dose of sarin (40.0 mcgm/Kg i.v.) as seen in figure 14. Blood pressure was elevated and a slight bradycardia was detected. The seizure pattern which began in the hippocampus (left side of figure) spread to the rest of the brain and a second dose of atropine sulfate (5.0 mg/Kg i.v.) was given at the height of the convulsion (arrow). The EEG tracings showed a cessation of the grand mal waves indicating the onset of antagonism (cf. right side of fig. 14). However, shortly after atropine had been given, the brain waves began to show an increased delta activity as indicated in figure 15. The rabbit then failed to respond to control stimuli which indicated an atropinic blockade. The mean blood pressure was also at pre-sarin levels. An additional injection of sarin (40 mcgm/Kg i.v.) again evoked a convulsive EEG pattern but did not induce a pressor response (fig. 16).

Ligation of the basilar artery had no effect on the electrical activity of the brain which showed the same EEG and reaction to stimuli as that seen in the control animals.
Injections of 2.0 ml of saline at the rate of 1.0 ml/20 seconds into either the carotid or the vertebral arterial circulation did not alter the spontaneous EEG activity or blood pressure. Control tracings of synchronized EEG activity are shown in figure 19 and of desynchronized activity in figure 20.

The response to the intracarotid administration of sub-convulsant doses of Eserine occurred in two stages shown in figures 21 and 22. Synchronized EEG activity appeared in the cortices with a slight transient decrease in blood pressure that appeared within seconds after the drug administration (arrow), and persisted for about 1 minute. Desynchronized EEG activity then appeared which was the second phase of the response. This EEG alert persisted for up to 15 minutes and was accompanied by a pressor response that began after the onset of the EEG arousal (fig. 22 (arrow)). EEG tracings resumed pre-drug characteristics 30 to 40 minutes after the injection.

Sarin administered intravertebrally (10 mcgm/Kg , fig. 23) evoked an initial convulsion in the hippocampus that was somewhat localized. Figure 24 reveals the diffuse convulsive activity that followed approximately 20 seconds after the appearance of the limited seizure.
Figure 15

ATROPINE ANTAGONISES 2nd SARIN INJECTION

EEG Pattern of 2nd Atropine Antagonism of 2nd Sarin Seizure: Brain wave tracings taken shortly after the preceding Figure show the antagonism of the sarin grand mal seizure activity by atropine sulfate. Note also the lowering of the mean blood pressure to pre-convulsion levels.
Sarin (40 mcgm/Kg i.v.) Again Evokes a Convulsion in the Presence of Atropine:

The administration of an additional dose of 40 mcgm/Kg i.v. of sarin provokes a seizure in the presence of atropine. This tracing is a continuation of the previous recordings. Note that the seizure is evoked but that the level of atropinization is sufficient to block the pressor response (See mean blood pressure). The seizure developed approximately 2 minutes after sarin was given.
Basilar Artery Ligation

carotid ext.
carotid int.
carotid comm.
cerebral ant. art.
cerebral mid. art.
cerebral post. art.
communicating post. art.
mesencephalic art.
cerebellar sup. art.
basilar art.
spinal sup. art.
vertebral art.
omocervical art.
subclavian art.
innominate art.
mammary int. art.
Anatomical Considerations in Basilar Artery Ligation in the Rabbit: The position of the ligation of the basilar artery is at the point of reference as indicated in the figure for 'basilar art.' The external carotid cannulation extends to the bifurcation of the internal and external carotids. The subclavian artery is also cannulated. The omocervical and internal mammary arteries are tied while the innominate is raised by means of a loop of suture thread at the time of injection. This latter procedure prevents injected materials from passing into the common carotid and then into the forebrain via the internal carotid and Circle of Willis. Injections into the external carotid follow the flow of blood into the internal carotid and the Circle of Willis thus supplying the anterior brainstem and telencephalon. The injection of substances into the subclavian artery with the innominate temporarily occluded courses up the vertebral and basilar to the position of the tie. This type of administration will supply the bulbar pontine reticular formation and those structures in the medulla and parts of the pons (cf. Figure 18 Dye Distribution).
Figure 18
Legend for Figure 18

Distribution of Monastral Blue Dye in Two Rabbit Brains with Ligation of the Basilar Artery at the Mid-Pontine Level: In the brain represented by the upper section, the injection was made into the vertebral artery. Note that the distribution of dye is restricted to the caudal pontine-medullary area which includes the bulbar-pontine reticular formation.

The lower half of the figure is from a brain in which the injection of the dye was made into the internal carotid from the external carotid. Note the limitation of the dye distribution to those structures more anterior to include the mesodiencephalic reticular substance.
Control EEG Pattern: Synchronized or slow wave EEG sleep pattern in the resting animal with the basilar artery ligated at the mid-pontine level. Note the high amplitude, slow frequency patterns primarily in the cortices (LAC-BOA, RAC-LN, L-RPC) while the LN-BOA is primarily a low amplitude tracing and the leads from the hippocampus reveal marked theta rhythm, (LH-LN and RH-BOA). The blood pressure (BP) is recorded as a mean pressure.
Control EEG Pattern: Desynchronized EEG activity during the control phase reveals a decreased amplitude and faster frequency of the tracings. Note the marked theta rhythm, especially in the leads from the hippocampus. The basilar artery was ligated at the mid-pontine level.
Initial Response to an Intracarotid Injection of Eserine with the Basilar Artery Tied: Eserine (100 mcgm total dose) administered at the arrow elicits an increased EEG sleep pattern shortly after the injection (right side of figure). This effect is accompanied by little if any change in mean blood pressure. The synchronized pattern is most evident in channels 2, 3, and 6.
Pressor Response and EEG Alert after Intracarotid Eserine in the Basilar Tied Preparation: Subsequent to the previous record (-Response "A") an EEG desynchronization occurs followed by a rise in mean blood pressure (beginning at the arrow). The time lapse between the two figures, Response "A" and Response "B", is approximately two minutes.
Figure 23

INTRAVERTEBRAL SARIN—LOCAL CONVULSION

Initial Response to the Intravertebral Arterial Administration of Sarin in the Rabbit with the Basilar Tied: Sarin (10 mcgm total dose) given intravertebrally to the rabbit with the basilar artery ligated at the mid pontine level induces a biphasic response. The initial phase consists of a localized seizure limited somewhat to the hippocampus (LH-LN and RH-BOA). Note the reflection of the seizure in the posterior cortices overlying the dorsal hippocampus. The 2nd phase is in the following figure.
Second Phase of Intravertebral Sarin: Subsequent to the previous response in the preceding figure, grand mal type EEG seizure activity spreads to the entire brain as is evident from these tracings. Note the typical seizure activity as intermittent spike activity is associated with high frequency burst activity. The time between the tracings in figures 23 and 24 is approximately 20 seconds.
Sarin Convulsion in the Presence of Eserine: The intracarotid administration of sarin (50 mcgm total dose) elicits a grand mal convulsion (right side of tracings) in the presence of a subconvulsant dose of eserine. Note the fall in mean blood pressure and the initial phase of its recovery on the far right side of the tracing. Eserine (100 mcgm/Kg i.v.) had been given approximately 10 minutes prior to these tracings. Sarin was given about 30 seconds before the seizure activity.
Recruitment During the Control Phase Prior to the Administration of Eserine Followed by Sarin: Stimulation of the anterior dorsal thalamic nuclei elicits typical recruitment responses from the cortices. The stimulus of 8.0 c/s, 0.8 msec and at a voltage greater than threshold was presented as indicated by the arrows.
Intravenous Administration of Eserine Antagonizes Thalamo-Cortical Recruiting Responses: The injection of 100.0 mcgm/Kg i.v. of Eserine has antagonized the evoked thalamo-cortical recruited potentials from stimulation of the thalamic nuclei at the same values of stimulation as during the control period. Stimulus presented as indicated by the arrows. The recording was made 7.0 minutes after the administration of the drug.
Intravenous Administration of Sarin Elicits Seizure in the Presence of Eserine:

Sarin was injected (50 mcgm/Kg i.v.) in the presence of 100 mcgm/Kg i.v. of Eserine. Eserine had been injected 8.0 minutes prior to the administration of the Sarin and the sarin induced convulsion appeared within one to two minutes. Note the diffuse nature of the seizure and the artifact in the EKG which resulted from the fasiculations elicited by the anticholinesterases. Histochemical analysis of this brain revealed absence of irreversible inhibition of acetylcholinesterase (cf. Figure 29).

Figure 28
Sarin Convulsion Does Not Irreversibly Inhibit Cholinesterase in the Presence of Eserine: Histochemical analysis by the thiocholine procedure reveals cholinesterase in the sagittal section of the rabbit brain. Note the dense areas representing sites of cholinesterase in the caudate nucleus, superior colliculus, thalamic nuclei, amygdala, and medullary nuclei. Pretreatment with Eserine has prevented the irreversible inhibition as normally seen with this dose of sarin (figure 6a). This figure represents the brain from the animal used in the experiment shown in the figure 28.
Legend for Figure 30

Sagittal Section of Rabbit Brain Exposed to Sarin and Atropine Sulfate and Processed by the Thiocholine Procedure for Demonstration of Cholinesterase: The brain used in this figure represents the effect of treatment with an irreversible inhibitor of cholinesterase, sarin. A sarin induced convulsion (50 mcgm/Kg i.v.) had been antagonized by atropine sulfate (5.0 mg/Kg i.v.). Note the absence of intense areas of staining as compared with the control sections (cf. Figure 5a). The photographic processing of these "blank" sections required intense over-development while the control cholinesterase sections did not.
On the other hand sarin, given by the carotid route of administration, did not reveal a localized seizure followed by a diffuse one but rather showed only a generalized convulsive pattern (fig. 25). Pretreatment with Eserine (100 mcgm/Kg i.v.) had evoked a desynchronized EEG with an elevated blood pressure (see left side of tracings fig. 25). Sarin injected intracarotidly on this background of reversibly inhibited AChE not only evoked seizure activity but also induced a marked transient depressor response and bradycardia.

Figure 26 shows cortical recruitment evoked by thalamic stimulation as indicated in the legend (see also Part II). A control response to the dose of Eserine that attenuated recruitment and induced a desynchronized EEG pattern was obtained prior to the intravenous administration of 50.0 mcgm/Kg of sarin (fig. 27). Diffuse EEG convulsive episodes occurred after the administration of sarin (fig. 28) and the animal died shortly after these tracings were made. Subsequent histochemical analysis of the brain showed an absence of irreversible enzyme inhibition (fig. 29).
IV. DISCUSSION

Anatomical findings in the rabbit brain stained with thionin and eosin agreed with the stereotaxic atlas of Monnier and Gangloff (1961), but contained greater detail (cf. Fikková and Maršala 1960, Sawyer et al. 1954, Meesen and Olszewski 1949, Young 1936, Kappers et al. 1936, and Winkler and Potter 1911). Tissue processing with frozen sections permitted the determination of the sites of implantation of electrodes in less than an hour as compared to a formalin-fixed, frozen section method (McBride and Klemm 1968).

The thiocholine procedure (Koelle 1955) was applied to rabbit brain tissue and subsequently modified (see Methods). Histochemical evidence of irreversible inhibition of cholinesterase or of protection from irreversible enzyme inhibition was of interest in the current studies. Therefore, only the thiocholine procedure that revealed all cholinesterases was used. Furthermore, the rabbit brain has been reported to contain little BuChE in contrast to other species (Gerebtzoff 1959). But the brains of different mammalian species appear to have greater AChE than BuChE activity (Augustinsson 1948, Ord and Thompson 1950).
Localization of cholinesterases in the non-drugged rabbit brain (figs. 5a, 5b, 5c), agreed with previous observations in rats (Koelle 1963) and in rabbits (Gerebtzoff 1959), though the procedures differed. No attempt was made to evaluate cellular localization of the enzyme in the current studies. The thiocholine procedure of Koelle (1949, 1950, 1951, 1955) is basically an in vitro method that uses combinations of inhibitors and substrates that are specific for AChE and BuChE. Counterstaining as well as post-fixation with formalin are included. A disadvantage of this method is the diffusion artifact that appears following long term incubation. Prefixation in 10% formalin was introduced as a modification of Koelle's method (Cöers 1953, Couteaux 1958, and Gerebtzoff 1959) and the diffusion artifact was minimized as a result of inactivation of cholinesterase by the fixative (Taxi 1952). Bergner and Bayliss (1952) have proposed a mode of action for the thiocholine histochemical process and this appears in figure 4.

The biochemical study of Aprison et al. (1954) showed variations in AChE activity in several parts of the rabbit brain with the highest values appearing in the caudate nucleus. Our histochemical data (figs. 5a, 5b, 5c), in general correlated with their observations and the most
intense stain for AChE appeared in the caudate nucleus. The inhibition of brain cholinesterase by the organophosphates (figs. 6a, 6b, 6c), corroborate previous biochemical findings (Holmstedt 1963). Also, the irreversibility of the inhibition was reflected by the negative (blank) histochemical stain for cholinesterases (figs. 6a, 6b, 6c, 30). Atropine sulfate and atropine methyl nitrate failed to antagonize this inhibition of cholinesterases as seen with histochemical observations (fig. 30) and biochemical findings (Table I). Atropine sulfate has been shown to produce a considerable increase in the release of acetylcholine from the cortices of several species (Beani et al. 1964, Polak 1967, Beani et al. 1968). However, the mechanism for producing this effect is unclear and Beani et al. (1968) summarized some of the tentative explanations. Mechanisms proposed, include the following: (1) blockade of postsynaptic sites to prevent uptake of released ACh (Celesia and Jasper 1966), (2) blockade of a negative feedback mechanism that results in an increased rate of firing of corticopetal cholinergic paths (McIntosh 1963), and (3) blockade of a negative feedback mechanism that is located intracortically and acts on cholinergic terminals (Polak 1967). Furthermore, the anticholinesterase properties of
atropine might be a factor (Vincent and Parant 1956, Todrick 1954, and Gyermek 1955). It has been shown that the acute toxicity of anticholinesterases can be additive and perhaps reveal potentiation (Frawley et al. 1952, 1957). This would result in an increased accumulation of ACh. Cholinergic desynchronization of the EEG would be blocked by the competitive antimuscarinic actions of atropine at post-synaptic sites. While our data did not show an increased inhibition of cholinesterases in the presence of atropine, the enzyme was already maximally inhibited (fig. 29, cf. Koppanyi and Karczmar 1951). Based on the observations cited above (Table I), the presence of the anticholinergics did not reduce cholinesterase inhibition.

The EEG of the rabbit at rest and during activation was similar to that described by Longo (1962) (figs. 7, 8, 9, 10). Intravenous sarin (20.0 mcgm/Kg, fig. 11), elicited a convulsive pattern (cf. Longo 1962) and the dose approximated the LD 50 for rabbits (Holmstedt 1963). Atropine antagonism of the EEG response to sarin has been known (Machne and Unna 1963) and present findings are similar. The reciprocal nature of this response (figs. 13, 14, 15) as pointed out by Longo (1962) depends on the amounts given. This reciprocal antagonism of anticholinesterases and anti-
cholinergics at the central nervous system in terms of the basic pharmacology of atropine (Goodman and Gilman 1965) suggests a competitive antagonism. The onset of anti-cholinesterase-induced convulsions is accompanied by hypertensive blood pressure responses which persist for the duration of that paroxysmal EEG activity (Longo 1962). This was found to be the case in the presence of reciprocal antagonism concomitant with EEG changes. However, at a sufficiently high dose of atropine (fig. 16, 10.0 mg/Kg i.v. total dose), blood pressure failed to rise though the EEG and behavioral response to sarin was a convulsion.

Activity of brain cholinesterases after the first injection of sarin and after the AntiChE had been antagonized by atropine showed marked attenuation (Table I). Furthermore, the histochemical evidence supported this finding (figs. 6a, 6b, 6c). Therefore, after the first injection of sarin, cholinesterase was irreversibly inhibited and after repeated reciprocal antagonism with atropine the irreversible nature of the inhibition was unchanged (cf. Koppanyi and Karczmar 1951). Effects of the irreversible anticholinesterase, when produced in the presence of maximally inhibited enzyme, cannot be attributed to irreversible
inhibition. Reciprocal antagonism of these effects with atropine also occurred in the same manner as seen after the initial dose of sarin and on a background of irreversibly inhibited brain cholinesterase.

Experiments were then designed to protect brain cholinesterases from irreversible inhibition (cf. Koelle 1957), and this data is shown in figure 29. Eserine readily penetrated the BBB and produced a transient sequence of effects on the EEG (Longo 1962 and Longo and Silvestrini 1957) which were also noted in the current studies (figs. 21, 22, and Part II). Marked attenuation of thalamocortical recruitment (see Part II) is a characteristic feature of the action of Eserine in rabbits and the studies of Hance et al. (1963) revealed that EEG desynchronization and marked attenuation of the surface negative component of the recruited potentials were the most significant effects produced in cats. Figure 27 shows the degree of attenuation of recruitment and the EEG arousal evoked by Eserine as compared to a pre-drug state during a moderately desynchronized EEG background (fig. 26). These responses served as controls for the effectiveness of that dose of Eserine. In the presence of Eserine, the administration of 50.0 mcgm/Kg i.v. of sarin, a dose that approximates 2 times the LD 50 for rabbits
(Holmstedt 1963), evoked a convulsion (fig. 28). At this time, brain cholinesterase was reversibly inhibited by Eserine (Long 1963). Histochemical data (fig. 29), showed that irreversible inhibition of the enzyme had not occurred and areas of intense staining appear comparable to the untreated rabbit brain (figs. 5a, 5b, 5c). Protection of cholinesterase from irreversible inhibition by the prior administration of a reversible inhibitor was used by Koelle (1946, 1957). He noted that the concentrations of AChE varied according to the activity and dose of the reversible inhibitors but not the irreversible ones. In earlier studies, Koelle (1946) suggested that Eserine could be displaced from cholinesterase by DFP. Our data indicates that brain AChE reversibly inhibited by Eserine is not susceptible to marked irreversible inhibition by sarin or DFP. The lack of agreement may be in the amounts of drugs given. However, the time of sacrifice after the administration of the irreversible inhibitor may have greater importance. Attenuation of recruitment by DFP in the presence of Eserine is reversible following the first exposure but repetition of this procedure results finally in an EEG response and attenuation of recruitment that is consistent with irreversible inhibition (unpublished preliminary studies). Histochemical evaluation of
these rabbit brains revealed irreversible inhibition of cholinesterase. The seizure induced by sarin in the presence of Eserine does not appear to be due to irreversible inhibition of that enzyme which normally occurs with sarin given alone (cf. fig. 30).

The biphasic response to the intracarotid administration of Eserine was somewhat unexpected. The transient fall in blood pressure and concomitant synchronization of the EEG could be attributed to a muscarinic action of Eserine (fig. 21). The subsequent EEG arousal (fig. 22) could be due to nicotinic actions of Eserine and/or the increased concentration of ACh that attends inhibition of AChE. Silvette et al. (1962), Domino (1967), and Longo et al. (1967) have shown EEG desynchronization to be the initial response to the intravenous administration of nicotine. An increased synchronization of activity may follow. In addition, Schaeppi (1968) injected nicotine intracarotidly or intravertebrally into cats and found that either route of administration elicited results similar to the intravenous injections. However, the intravertebral injection appeared to be more effective and produced direct effects on cardiovascular centers in the brain that were independent of the effects of nicotine on the EEG. Schaeppi attributed the EEG arousal to an action
on an unspecified ascending desynchronizing system and the subsequent EEG synchronization on another unspecified ascending synchronizing system. Longo (1966) showed that atropine blocked the EEG and motor convulsions evoked by cortical application of a combination of Eserine and acetylcholine. Furthermore, Domino (1968) found mecamylamine to be an ineffective antagonist of either Eserine or pilocarpine-induced EEG and behavioral arousal in cats. Atropine readily blocked these responses. Responses to Eserine seen in the current experiment (figs. 21, 22) may be the result of muscarinic-cholinergic stimulation since atropine antagonizes them (Longo 1962). In addition, the pressor response to the intracarotid administration of Eserine supports the findings of Varagić and Krstić (1966). These authors noted that the Eserine evoked pressor response in rats was blocked by lesions in the lateral hypothalamus.

Intravertebral administration of sarin produced a biphasic response (figs. 23, 24) of convulsive EEG patterns that were initially localized and then developed into a grand mal pattern. Intracarotid administration of sarin in the presence of Eserine (fig. 25) also elicited a seizure pattern which had a diffuse and abrupt onset. The convulsive activity of sarin did not appear to be restricted to specific
sites of the forebrain and hindbrain, and occurred in the presence of Eserine-inhibited enzyme (fig. 29). Microinjections of sarin given bilaterally into the lateral reticular nucleus of the medulla of rabbits resulted in apnea, which could be antagonized by atropine given intravenously (Stewart and Anderson 1968). Hypotensive responses also were evoked and were antagonized by atropine. Microinjections of sarin into more medial areas of the reticular formation produced tachypnea, bradycardia, and hypotension. Tachycardia was not seen but hypertensive responses could be evoked. All of these responses were antagonized by atropine. The bradycardia was of interest because it could be blocked by a bilateral vagisection and could not be elicited by microinjections of sarin into the area of the nucleus solitarius or the dorsal nucleus of the vagus. Stewart suggested that rhythmicity of the medullary respiratory center is under a muscarinic inhibitory control in a way analogous to the relationships found between vagus nerve and cardiac pacemaker.

Cholinoceptive neurones of the ponto-medullary brainstem of cats revealed excitatory nicotinic and inhibitory muscarinic responses to iontophoretically applied drugs (Bradley et al. 1966). Dihydro beta-erythroidine blocked the excitatory
responses while atropine antagonized both excitatory and inhibitory effects. In addition, Bradley and co-workers noted that the iontophoretic application of Eserine and neostigmine potentiated the effects of ACh and also had actions *per se* different from ACh. Eserine had an excitatory effect on neurons that were inhibited by ACh.

Cholinergicity of the ascending pathways of the CNS has been implied by several lines of evidence. Cholinoceptivity of the brainstem neurons has been established (*vide supra*). Cortical cholinergic mechanisms have been discussed by Krnjević (1967) who pointed out the presence of ACh sensitive cells in the cortex as well as choline acetylase and AChE. Evidence for cholinergic innervation arriving from subcortical origins was based on electrophysiological, histochemical, and biochemical data. ACh-sensitive cortical cells responded to thalamic stimulation and these responses were potentiated by ACh or Eserine. Atropine antagonized these responses. AChE-staining fibers that projected to the forebrain could be traced to several subcortical sites to include the septum pellucidum, lenticular nucleus, and the midbrain reticular formation. Cortical release of ACh, measured biochemically, showed that as cerebral activity decreased during anesthesia, so did the amount of ACh being
Shute and Lewis (1966, 1966a) have compared their findings for cholinergic pathways in the rat brain with the histochemical fluorescence data for monoaminergic pathways (Dahlström and Fuxe 1964). Shute and Lewis concluded that the 2 ascending systems paralleled one another but that electrocortical arousal was more likely a cholinergic event rather than monoaminergic. They cited as evidence their observations that monoaminergic cells did not extend to the forebrain telencephalon as did the cholinergic ones. However, Andén et al. (1966) proposed a forebrain extension of monoaminergic pathways. Cuculic et al. (1968) postulated that the final link in the electrocortical arousal was a cholinergic one. They arrived at their conclusions from data obtained by the direct application of cholinolytic substances on the cortex. Atropine antagonized both cholinomimetic and adrenomimetic agonists (cf. White and Daigneault 1959).

Therefore, the evidence of cholinergic pathways from the reticular formation to the cortex presents multiple sites of action for anticholinesterases. Anticholinesterases can act not only indirectly, by enhancing the action of ACh, but also directly as was shown by Bradley
et al. (1966) at the cellular level and also in the current study where it was demonstrated that anticholinesterases could evoke responses in the presence of inhibited enzyme. The presence of parallel monoaminergic and cholinergic pathways suggests cholinergic-adrenergic interrelationships. Part II of the dissertation presents evidence for an adrenergic-cholinergic interaction at the thalamocortical level.
V. SUMMARY

Figure 31 reviews histochemical, electrophysiological, and biochemical evidence for the direct action of anticholinesterases. The "Histochemical Data" shown in the column on the left hand side of the figure (read down) indicates that Eserine evoked an EEG alert at which time the administration of sarin or DFP evoked a convulsion and death. The thiocholine procedure for cholinesterase revealed a stain which indicated non-irreversible inhibition of the enzyme. On the other hand, histochemical data also showed that if sarin or DFP were given first (2nd column Histochemical Data) then an EEG alert would also be evoked during which time the administration of Eserine evoked a convulsion and death as before. However, the thiocholine procedure now revealed a blank result which indicated irreversible inhibition of enzyme.

The "Electrophysiological Data" shown in the middle column reflects the reciprocal antagonism between anticholinesterase and anticholinergic drugs. Sarin evoked an EEG alert which led to a convulsion and was antagonized by atropine. Subsequent injections of sarin (2nd Sarin Injection; 3rd Sarin Injection) again evoked EEG alert patterns
and convulsive episodes which were similarly antagonized by atropine. Biochemical Data (right hand column) revealed that the activity of ChE was irreversibly inhibited approximately 95% after the first injection of sarin and that atropine antagonism did not change this effect of sarin. Thus, after the initial, irreversible inhibition of ChE, the interaction between sarin and atropine occurred in the presence of maximally inhibited enzyme.

Therefore, in summary:

(1) EEG responses to the administration of anticholinesterases were evaluated in the presence of (a) irreversibly inhibited enzyme and (b) in the presence of cholinesterase that was protected from an irreversible inhibitor by the presence of a reversible inhibitor.

(2) Reciprocal antagonism between sarin and atropine was repeatedly demonstrated in the same animal. Biochemical and histochemical findings indicated maximal irreversible inhibition of brain cholinesterase after the first injection of sarin.

(3) Sarin evoked grand mal seizures and death when given after Eserine. Histochemical data showed that sarin failed to irreversibly inhibit brain cholinesterase.
Figure 31

DIRECT ACTION OF ANTICHE'S

Histochemical Data

<table>
<thead>
<tr>
<th>Eserine</th>
<th>Sarin-DFP</th>
<th>Sarin</th>
</tr>
</thead>
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Electrophysiological Data

<table>
<thead>
<tr>
<th>EEG Alert</th>
<th>EEG Alert</th>
<th>EEG Alert - Convulsion</th>
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<table>
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<tr>
<th>Atropine - Antagonism</th>
<th>Atropine - Antagonism</th>
</tr>
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</table>

Biochemical Data

<table>
<thead>
<tr>
<th>ChE Inhibition After Sarin:</th>
<th>ChE Inhibition After Sarin and Atropine:</th>
</tr>
</thead>
</table>

| > 95% | > 95% |

<table>
<thead>
<tr>
<th>EEG Alert - Convulsion</th>
<th>EEG Alert - Convulsion</th>
</tr>
</thead>
</table>

ChE Inhibition After Sarin and Atropine:

| > 95% |

<table>
<thead>
<tr>
<th>Thiocholine Stain</th>
<th>Thiocholine Stain</th>
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<table>
<thead>
<tr>
<th>Blank</th>
<th>3rd Sarin Injection</th>
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<tr>
<th>EEG Alert - Convulsion</th>
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(4) A direct action of anticholinesterases is proposed in addition to inhibition of enzyme.
PART II. EVIDENCE FOR AN ADRENERGICALLY MEDIATED ACTION OF ANTICHOLINESTERASES IN THE CENTRAL NERVOUS SYSTEM OF RABBITS
I. INTRODUCTION

Electrocortical desynchronization by cholinomimetics and anticholinesterases with the demonstration of antagonism by antimuscarinic drugs has suggested cholinergically-mediated events (Wescoe et al. 1948, Funderburk and Case 1951, Bradley and Elkes 1953). Mediation by adrenergic mechanisms has also been proposed (Bonvallet et al. 1954, Dell 1961). However, difficulty in interpretation of results has occurred since the administration of norepinephrine (NE) or epinephrine (EPI) resulted in EEG desynchronization, or synchronization or no effect (see Longo 1962 and Baust and Niemczyk 1964 for reviews). The monoamines, NE, EPI, and dopamine (DA) penetrate the blood brain barrier (BBB) to little if any extent (Weil-Malherbe 1961) and therefore CNS responses attributable to a direct action following intravenous administration is questionable. Adrenomimetic drugs such as amphetamines and other phenylethanolamines that readily gain access to the CNS showed EEG desynchronization consistently which was associated with behavioral arousal in unanesthetized preparations (Longo and Silvestrini 1957, Bradley 1968). Inhibition of monoamineoxidase followed by the administration of L-DOPA, the precursor of NE and EPI,
resulted in an EEG and behavioral arousal associated with marked increases in brain biogenic amine levels (Everett 1961). Furthermore, reserpine-depletion of biogenic amines and behavioral sedation in monkeys has been reversed by the administration of a monoaminoxidase inhibitor (MAOI) plus L-DOPA (Everett et al. 1963).

EEG responses to cholinomimetics and adrenomimetics were readily blocked by atropine (White and Daigneault 1959) as well as by the alpha blockers phenoxybenzamine and chlorpromazine (Goldstein and Muñoz 1961, Muñoz and Goldstein 1961). Though anticholinergic and adrenergic blockers may share similar effects there are notable differences including the failure of amphetamine to reverse the EEG synchronization induced by scopolamine (Longo 1962). Interrelationships between cholinergic and adrenergic systems associated with EEG desynchronization and behavioral arousal have been suggested by White and Daigneault (1959), Bradley (1961), Dell (1961), and White et al. (1965). Desynchronization of the EEG by anticholinesterases is presumably evoked through cholinomimetic mechanisms that may be indirect via increases in acetylcholine (ACh) concentrations as well as direct actions (Bradley et al. 1966, cf. Part I). Amphetamine also evokes an EEG arousal through adrenomimetic
mechanisms that may be indirect as well as direct (Bradley 1961). Both classes of drugs have been demonstrated to act at a level of the mesodiencephalic activating system (Rinaldi and Himwich 1955a, 1955b, Van Meter and Ayala 1961). The threshold of thalamocortical recruitment evoked by stimulation of the anterior nuclei of the thalamus was increased by EEG arousal (Longo 1962). EEG desynchronization evoked by stimulation of the midbrain reticular formation or by anticholinesterases was a more effective antagonist of recruitment than amphetamine-induced EEG arousal, which did not attenuate recruitment (Longo and Silvestrini 1957).

The present studies were undertaken to evaluate the interrelationship between cholinergic and adrenergic mechanisms in the attenuation of thalamocortical recruitment by an anticholinesterase.
I. LITERATURE REVIEW

Early studies on evoked thalamocortical potentials in cats revealed augmenting potentials which followed high frequency stimulation (up to 120 c/s) of the lateral thalamic nuclei. These potentials were localized to the sensorimotor cortices and showed a biphasic positive-negative waveform. Stimulation frequencies of 5-15 c/s applied to midline thalamic nuclei evoked recruited potentials of 20-34 msec latency with a surface negativity and wide distribution over the cerebral cortex (Morison and Dempsey 1941-42, Dempsey and Morison 1941-42). Cortical and subcortical sites of action for generating recruited potentials were proposed (Verzeano et al. 1953). Ajmone-Marsan (1958) used cats anesthetized with pentobarbital and observed that the characteristic latency of recruitment was a function of the waxing and waning of the evoked responses. He concluded that thalamocortical recruitment resulted from activation of either longer or shorter chains of neurons and that the latency was not a function of conduction time between thalamus and cortex but occurred at the thalamic level. He postulated that during recruitment two separate but interrelated electrical fields arose in the brain and that these could account for the recruiting phenomena.
Jasper (1949) noted a diffuse projection system that ascended from the lower brain stem to cerebral cortex which included thalamocortical pathways necessary for recruitment. Longo (1962) gave a description of recruitment in rabbits and pointed out that Stoupel and Terzuolo (1954) also found recruited potentials in response to caudate nucleus stimulation. Bishop et al. (1961) observed that thalamic augmenting potentials and recruiting potentials were mutually occlusive, had similar excitation characteristics and distribution in the various layers of the cortex. They concluded that the difference between thalamocortical recruitment and augmenting potentials was an operational one in that stimulation of thalamic relay nuclei evoked augmenting potentials along with primary sensory projection responses. Velasco and Lindsley (1965) suggested that orbital cortex played a crucial role in regulation of thalamocortical recruitment in cats. They based their conclusions on ablation studies in which removal of orbital cortex alone abolished recruited potentials. Involvement of the limbic system was revealed by Sparks and Powell (1966) who observed that conditioning stimuli to the hippocampus, mamillary bodies, or septal nuclei attenuated late components of recruited potentials that had been elicited by stimulation of the anterior dorsal
nuclei of the thalamus. Sparks and Powell concluded that limbic projection to the anterior thalamus modified its excitability and this in turn influenced the level of cortical excitability. Spindle bursts during EEG slow wave sleep were also found to be abolished by orbito-frontal cortical ablations in gallamine immobilized cats (Velasco et al. 1968). It was concluded that the orbito-frontal cortex was an essential anatomical link in a thalamocortical synchronizing system for spindle bursts and recruitment.

With the introduction of fast wave or paradoxical sleep hypotheses (Dement 1958, Jouvet 1962), Yamaguchi et al. (1964) noted that elicitation of recruitment during paradoxical sleep was questionable. They also observed that the degree of attenuation of recruitment during paradoxical sleep was greater than that seen during relaxed wakefulness. In addition, these authors noted that high frequency stimulation of the thalamus evoked EEG and behavioral arousal. Giaquinto (1968) divided sleep and wakefulness into 4 cycles: (1) strong arousal, (2) relaxed wakefulness, (3) slow wave sleep, and (4) rapid sleep (paradoxical). He found a high threshold for recruitment, a high level of electromyographic activity and a desynchronized EEG during strong arousal; no paradoxical sleep, but low voltage fast EEG activity with
moderate electromyographic activity during relaxed wakefulness; a low threshold for recruitment during slow wave sleep; and paradoxical sleep revealed no difference in threshold for recruitment from that seen during relaxed wakefulness, a threshold which had been relatively high.

Abeles (1967, 1967a, 1967b) evaluated the excitability of brainstem (nucleus solitarius) and thalamic neurons involved in elicitation of EEG synchronization and desynchronization. He found that the chronaxie of "desynchronizing" neurons was less than that of "synchronizing" ones and concluded that desynchronization was more readily obtainable than synchronization. He postulated that there were not two different sets of neurons but probably a common set that responded to synchronizing or desynchronizing stimuli.

Serotonergic sleep (Koella and Czicman 1966, Jouvet 1967, Koella et al. 1968) has been induced by the intracarotid administration of serotonin which penetrated the blood brain barrier at the area postrema. Koella and Czicman postulated that the injected serotonin then stimulated the nucleus solitarius and serotonergic sleep was mediated via the tractus solitarius to the midline nuclei and diffuse projection system of the thalamus.
Anderson and Curtis (1964) found ACh-sensitive neurons of the ventro-lateral thalamus to respond with inhibition followed by excitation and the excitation was a mixed muscarinic and nicotinic type. However, McCance et al. (1968) evaluated the responses of the ventral thalamic nuclei to iontophoretically applied carbachol, ACh, acetyl beta-methacholine, butyrylcholine, propriocholine, nicotine, Eserine, neostigmine, tensilon, dihydro beta-erythroidine, hexamethonium, atropine, and hyoscine. These authors found that muscarinic agonists were more effective than the nicotinic ones. Atropine blocked most of the responses but dihydro beta-erythroidine was much less effective. Anticholinesterases enhanced the responses and McCance et al. concluded that cholinceptive responses of the ventral thalamic nuclei of the cat were pharmacologically muscarinic.

Correlation of activity from reticular and thalamic units with states of sleep, wakefulness, and anesthesia showed a general decrease in activity with variable patterns of bursts but no unique differences in activity that could be associated with any given state (Goodman and Mann 1967). It was concluded that the characteristics of spontaneous firing of thalamic and reticular units could not be used to differentiate between wakefulness, sleep, or anesthesia.
However, Phillis and Tebècis (1967) found that pentobarbital blocked the ACh excitatory and the NE inhibitory effects on thalamic neurons without altering the levels of excitation of the neurons which remained responsive. In addition, Findlay and Hayward (1966) noted bimodal firing of massa intermedia neurons of rabbits during synchronized sleep but electrical silence during desynchronization.

Recent studies of Andersen et al. (1967, 1967a) and Andersen and Andersson (1968) have revealed that the thalamus served as a pacemaker site during barbiturate anesthesia. Independent rhythmic discharges were generated through their respective projections to the cortex, to control the rhythms of that cortical "column". The thalamic origin of recruitment and spindle burst activity was postulated to occur as the result of post inhibitory excitation which induced an increasing synchronized discharge of thalamic neurons and this accounted for the waxing of potentials seen during recruitment. Eventually, these inhibitory neurons get "out of phase" and the waning of potentials results. Increased unit firing was demonstrated to correlate with increased amplitude of cortical response. In addition, a functional relationship between thalamus and caudate was implied by the
presence of caudate responses to thalamic stimulation.

Kaji et al. (1968) established the existence of connections from anterior thalamic nuclei to the caudate with their studies on recruiting responses in caudate nucleus. They observed that caudate recruitment depended on the level of cortical activity much in the same manner as thalamo-cortical recruitment. Unit analysis of caudate-cortical excitatory fiber tract responses in cats disclosed caudo-cortical connections of a direct nature rather than collateral or corticopetal tracts from lower levels (Rocha-Miranda 1965). This data contrasts with that of Demetrescu and Demetrescu (1962), who had found the activating or facilitatory effects of caudate activity seen during light barbiturate anesthesia, suppressed by reticular formation lesions. At the same time, depressant effects of the caudate were not altered by the lesions of the reticular formation.

Single shock stimulation of the caudate evoked spindle bursts, the threshold of which was raised by pentylene-tetrazole and decreased by chlorpromazine, pentobarbital and alpha blockers (Tokizane et al. 1957). It was concluded that reticular formation activity influenced the threshold of caudate spindles and that the spindles were a reflection of the state of cortical excitability. In a sequence of
studies on the caudate spindle (Buchwald et al. 1961, Heuser et al. 1961, Buchwald et al. 1961a, Buchwald et al. 1961b), it was found that (1) caudate spindles evoked by single shock stimuli to the head of the caudate nucleus were attenuated by high frequency stimulation of the midbrain reticular formation, hypothalamus, globus pallidus, or centrum medianum, as well as by catnip. High frequency stimulation of the mid-amygdaloid area, rostral, midline, and relay nuclei of the thalamus failed to block caudate spindles; (2) cortical sites of caudate spindling and recruitment were the same; (3) functional integrity of the anterior ventral nuclei of the thalamus was necessary for caudate spindle activity as well as for electrocortical arousal induced by stimulation of the caudate; and (4) ether and locally applied procaine blocked spindles while pentobarbital and sleep facilitated then. A "Caudate Loop" was proposed in the path from the reticular formation to the cerebral cortex. Afferent connections from anterior ventral thalamic nuclei to the caudate and back formed the loop. Ventral thalamic afferents also projected to the cortex. Low frequency stimulation of this loop antagonized the ascending arousal system and evoked spindle bursts and behavioral inhibition. It was suggested that this caudate inhibitory
loop functionally balanced the alerting system proposed by Moruzzi and Magoun (1949). Frequency, intensity, and anatomical site of stimulation were considered as critical factors.

Horvath et al. (1964) compared caudate-spindle thresholds with those evoked by internal capsule stimulation in flaxedilized cats, and found a lower threshold in the caudate. Goldring et al. (1963) had not been able to evoke caudate spindles in monkeys and had noted a neuroanatomical difference between the cat and the macaque monkey. Afferent sensorimotor cortical fibers of the cat run adjacent to the caudate while the sensorimotor cortex of the monkey was caudal to the caudate nucleus. These authors noted that internal capsular stimulation evoked recruitment and concluded that caudato-cortical responses should not be used as evidence for anatomical pathways.

An ascending inhibitory system that included the caudate nucleus was proposed by Demetrescu and Demetrescu (1962). Evoked potentials from optic tract and optic radiations of the visual system; from medial geniculate body stimulation of the auditory system; and from stimulation of the ventral posterior thalamic nuclei of the somesthetic system; were all depressed by 40-100 c/s stimulation of the caudate. On
the other hand, stimulation of the midline thalamic nuclei facilitated evoked potentials from all of the above areas where caudate stimulation had shown depression of responses. Caudate depression of responses was not antagonized by lesions of the reticular formation. Demtrescu and Demetrescu (1962a) proposed an ascending inhibitory path that extended from the pontine-reticular formation to the caudate nucleus and the cerebral cortex. This ascending inhibitory path was ventral to, and paralleled the ascending reticular activating system. The inhibitory system was under control of the activating one and operated via a feedback servo mechanism.

Eserine injected into the caudate nucleus, was found to block spindling and subsequently could evoke seizures (Rakic et al. 1962). These seizures could be antagonized by unilateral lesions in the cortex and rostral extralaminar thalamus, as well as bilateral lesions in the posterior hypothalamus and the posterior intralaminar thalamus. These findings were interpreted to mean that Eserine interrupted the inhibitory influence of the caudate and permitted the excitatory one to obtain (cf. Demetrescu and Demetrescu 1962). The result was a lowered seizure threshold.
Traczyk and Sadowski (1962) noted that slow intravenous infusion of Eserine increased spontaneous spindling followed by a desynchronization of the EEG. In subsequent studies Traczyk and Sadowski (1964) compared ACh concentrations in caudate with EEG activity in cerveau isole' cats. They found that EEG synchronization in midbrain transected animals was associated with high ACh concentrations and that desynchronization of the EEG was attended by low ACh content of the caudate. On the other hand, caudate-ACh concentrations were low in desynchronized controls; in cerveau isole' preparations receiving caudate stimulation at 5 c/s and 50 c/s; and after atropine. Eserine or caudate stimulation at high frequencies (300 c/s) in cerveau isole' preparations resulted in high concentrations of ACh in caudate nucleus. They concluded that the lack of agreement between stimulus response and drug actions on caudate nucleus was explained by the existence of and the operation of different (undefined) transmitter systems.

Collins and Simonton (1967) observed that caudate conditioning stimuli depressed responses in the limbic system of chloralosed cats. Extracellular microelectrode studies showed that caudate stimulation inhibited centrum medianum unit activity and this effect could be antagonized by strych-
nine and picrotoxin more than by pentylenetetrazole. Amphetamine, caffeine, imipramine, chlorpromazine, and mephenesin were without effect on the caudate inhibition of the centrum medianum. Caudate-induced inhibition of evoked responses in the limbic system was not antagonized by strychnine. These authors concluded that caudate conditioning of limbic responses resulted in strychnine-like action.

Neurons of the caudate as well as the hypothalamus have been shown to respond to iontophoretically-applied ACh with either inhibition or excitation (Bloom et al. 1963, Bloom et al. 1964). High concentrations of ACh (Aprison and Nathan 1957) along with high synthesis rates (Feldberg and Vogt 1948) have been reported for caudate nucleus. Marked concentrations of AChE are also evident (see Part I). Stimulation of the ventral anterior thalamic nuclei evoked the release of ACh from the caudate. Also, the response to cholinergic stimulation of the caudate and the response to stimulation of the ventralis anterialis was the same (McLennan and York 1966). Therefore, a "cholinergic link" may exist between the thalamus and the caudate.

The Burn-Rand Concept (Burn and Rand 1959, Burn and Rand 1962, Burn and Rand 1965), suggested that stimulation
of postganglionic sympathetic nerve fibers evoked the release of ACh which then promoted the entry of calcium into the nerve ending and enhanced the release of bound NE. Norepinephrine then acted as a neurotransmitter having been released by a "cholinergic link". The cholinergic link hypothesis has been included in recent reviews (Burn 1966, Ferry 1966, Karczmar 1967, Marczynski 1967).

Karczmar (1967) pointed out evidence obtained by the use of anticholinesterases for a cholinergic link but limited most of his comments to the peripheral nervous system. Burn (1968) suggested that if acetylcholine was released by the stimulation of postganglionic sympathetic nerve stimulation and in turn released NE, then anticholinesterases should enhance the observed responses. Furthermore, anticholinesterases should reveal a greater facilitation of response at low frequencies of stimulation than at high ones, since the low frequency of stimulation would permit more opportunity for AChE-hydrolysis of ACh. The following evidence was presented by Burn (1968): Eserine increased the response of the atropinized cat nictitating membrane by 51 per cent at stimulation frequencies of 1 c/s, by 8 per cent at 10 c/s, and by 2 per cent at 20 c/s; con-
tractions of the atropinized taenia of the guinea pig was similarly affected; stimulation of the sympathetic fibers to the isolated rabbit nerve-heart preparation showed an enhanced chronotropic response to the administration of Eserine in the presence of atropine; results with contraction of the dog retractor penis muscle preparation showed a greater enhancement of contraction at low frequency stimulation than at high, after the administration of Eserine in the presence of atropine; reduction of femoral arterial blood flow following sympathetic nerve stimulation was enhanced by the administration of Eserine in the atropinized dog and low frequency stimulation was affected more than high; rabbit ear vasoconstriction was similarly enhanced by Eserine in the absence of atropine; and finally, renal blood flow, following sympathetic nerve stimulation, reflected greater vasoconstriction in presence of Eserine at stimulation frequencies of 1/sec than at 10 per sec. Hemicholinium reversed these responses and choline antagonized the effect of hemicholinium. In addition, hemicholinium has been shown to block the response to stimulation of postganglionic fibers in cat atria, guinea pig colon, rabbit ear vessels, and cat spleen, all of which
could be reversed by the administration of choline. Finally, botulinum toxin has been shown to block the response of cat tail pilomotor muscle to sympathetic stimulation (cited in Burn 1968).

Ferry (1966) reviewed the cholinergic link concept and concluded that the evidence was not strong enough to support the hypothesis. He pointed out that while the presence of ACh had been shown to result in the release of NE from the spleen, antidromic impulses could have been initiated in the "C" fibers. These antidromic impulses would pass up the splenic nerve to then act as a postganglionic stimulant which resulted in the passage of impulses back down the splenic postganglionic nerve and the release of NE at the nerve ending. In other words, ACh first stimulated postganglionic fibers which in turn released NE. Electron-microscopic investigations of Thoenen et al. (1966) revealed that the spleen of the cat showed only adrenergic nerve endings on the basis of osmiophillic granules that were observed, while the vas deferens of the guinea pig showed both adrenergic and cholinergic elements lying membrane to membrane and encased in the same Schwann cell. They studied the effect of anticholinesterases on sympathetic nerve
stimulation and ACh injection in the perfused spleen of the cat and found no change to nerve stimulation but an enhanced response to injected ACh. It was concluded that while the Burn-Rand Hypothesis may apply in some cases it should not be assumed to apply in all cases. Karczmar (1967) also pointed out that the cholinergic link concept may not apply to all species particularly in view of the evidence of Koelle that some sympathetic tracts of the cat histochemically demonstrate very little ChE as compared to the rabbit. Boura and Green (1965) suggested that the effects of the xylocholine and bretyllium were poor evidence in support of the cholinergic link since their duration of action was so brief in comparison with other adrenergic neuron blocking drugs. They pointed out that the contraction of the cat nictitating membrane to stimulation of the sympathetic nerve was not effectively blocked by hemicholinium but was blocked by adrenergic neuron blocking drugs. The histochemical data of Koelle would support this observation (vide supra). Birmingham (1966) interpreted the results of his experimental data to mean that a cholinergic link was not required to evoke a response in the post-ganglionic adrenergic nerve following preganglionic stimula-
tion. He concluded that anticholinesterase enhancement of guinea pig vas deferens response could be explained by alternative mechanisms. One suggestion was a purely cholinergic sequence of events involving postganglionic nerves and muscarinic smooth muscle receptors, while the other interpretation was a cholinergic mechanism with the preganglionic cholinergic nerves increasing the release of postganglionic NE.

Histochemical evidence has been put forward by Jacobowitz (1965) and Jacobowitz and Koelle (1965). Their studies have shown the existence of AChE and catecholamine fluorescence in the same nerve trunk, the guinea pig vas deferens. Koelle's Percussive Theory was invoked to explain the existence of presynaptic AChE in the ganglion (Koelle 1961, 1962). Rand and Wilson (1967) noted that bretylium and guanethidine antagonized the effects of ACh on the rabbit colon, responses to sympathetic and parasympathetic stimulation; blocked the response to vagal stimulation of rabbit atria; and blocked the effects of ACh on guinea pig ileum, rat bladder, and chick biventer cervicis muscle. They interpreted their results as being in agreement with a cholinergic link hypothesis. Varagić et al. (1962) and Varagić and Krstić (1966) noted that the hypertensive
response to Eserine seen in rats was blocked by reserpine, and lateral hypothalamic lesions. They concluded that the hypertensive response to Eserine was mediated via the CNS. Kaul and Grewal (1968) observed that Eserine increased the catecholamine output of the adrenals of rats by a factor of 3 to 4 times and that this effect was not seen in pithed animals. They concluded that peripheral release of catecholamines played little role in the hypertensive response to Eserine seen in rats. This conclusion was strengthened by their observation that adrenalectomy failed to decrease Eserine-induced hypertension and if anything increased it. Guth and Amaro (1969) suggested a possible cholinergic link in olivocochlear inhibition since atropine and nicotinic blocking agents failed to antagonize the blockade while hemicholinium was effective. Choline reversed the hemicholinium antagonism of inhibition of VIIIth nerve action potentials.

Van Meter and Karczmar (1967) proposed that the Eserine attenuation of thalamocortical recruitment was dependent on endogenous catecholamines in the CNS. In the presence of amines depleted by reserpine or αMPT, Eserine failed to attenuate recruitment while MAOI plus DOPA restored brain biogenic amines and also restored the effectiveness of
Eserine as a blocker of thalamocortical recruitment. It was concluded that an "adrenergic link" was involved in the Eserine antagonism of recruitment.

At about the same time, Marczynski (1967) expressed the opinion that some of the CNS responses of Eserine might be explained by the Burn-Rand Hypothesis. He pointed out that the optimal frequency for thalamocortical recruitment (5-7 c/s) had been shown to produce maximal release of ACh from the caudate. Therefore, the inhibitory "Caudate Loop" as proposed by Heuser et al. (1961) and the studies of Buchwald (1961) could well be the site of the "adrenergic link" for the anticholinesterase induced antagonism of thalamocortical recruitment as seen in the present studies.

Tremorine, a muscarinic agonist has been shown to evoke a release of brain catecholamines (Friedman and Anton 1967), and to antagonize reserpine or chlorpromazine induced sedation in rabbits (Leslie 1965). George et al. (1966) have shown that the active metabolite of tremorine, oxotremorine, induced tremor following microinjection into pontomedullary sites of the rat brain. Other sites of action were proposed and included the basal forebrain area. All effects were antagonized by atropine. On the other hand,
Connor et al. (1967) have noted that intracaudate injection of carbachol induced tremor which was antagonized by the subsequent administration of catecholamines into the caudate nucleus. Alpha agonists and blockers were without significant effects while beta agonists antagonized carbachol tremor and beta blockers inhibited that antagonism. In addition intracaudate administration of ATP blocked the tremor and thus indicated a beta "metabolic" effect. The authors concluded that a neurohumoral balance was necessary in the caudate for the regulation of physiological tremor. In other words, a cholinergic-adrenergic balance.

Interactions between cholinergic and adrenergic mechanisms in the CNS have been implicated in a series of studies. Longo (1956) noted that while atropine and scopolamine failed to antagonize the neurovegetative responses to direct electrical stimulation of the hypothalamus, EEG arousal was antagonized. White and Daigneault (1959) observed that atropine could block the EEG desynchronization resulting from brain stem lesions, from cholinomimetic agonists and from adrenomimetic agonists. They concluded that the same area of the brain stem was involved in EEG desynchronization evoked by cholinergic or adrenergic stimulation. Later
studies have shown that Eserine antagonized alpha blockade (White et al. 1965). Malcolm et al. (1967) have shown an interrelationship between cholinergic and adrenergic mechanisms at the level of the cerebral cortex. They interpreted their data to represent a scheme similar to that proposed by Eccles and Libet for the superior cervical ganglion of the rabbit (cited in Eccles 1964). That is, that the adrenergic "P" wave acted as a modulator to decrease sensitivity. Cuculic et al. (1968) studied the action of drugs applied to the cortices of rabbits and observed that cholinolytic agents applied to the cortex blocked desynchronization evoked by peripheral stimulation, and by intravenous anticholinesterases, or amphetamine. These workers suggested that the final corticopetal link in EEG desynchronization was located near the cortical surface and was cholinergic in nature.

Jouvet (1967) proposed that paradoxical sleep was mediated by NE. Microinjection of oxotremorine or carbachol into the ponto-mesencephalic reticular formation also induced paradoxical sleep and this was antagonized by atropine sulfate (George et al. 1964). Ledebur and Tissot (1966) induced paradoxical sleep by the administration of DOPA into
the bulbo-pontine reticular substance, while 5 hydroxytryptophan induced either a synchronized or a desynchronized EEG when it was given locally into the sleep centers of "Moruzzi" or of "Jouvet". Post-pontine transection of the medulla of rabbits resulted in an EEG arousal and Bueno et al. (1968a) proposed the existence of a synchronizing center in the lower brainstem. Precollicular decerebration of cats induced the random appearance of paradoxical sleep (Matsuzaki et al. 1967). The administration of Eserine evoked episodes of paradoxical sleep and atropine sulfate, but not atropine methyl nitrate, was an effective antagonist.

Sterman et al. (1965) had observed that slow wave sleep and paradoxical sleep showed circadian patterns. Reis et al. (1968) found circadian rhythm of NE concentrations to exist regionally in cat brain. A comparison of these two studies showed that peaks of NE concentration occurred in the superior colliculi, pons and substantia nigra that coincided with the paradoxical sleep patterns.

The study of the effects of intravenously administered NE, EPI, or DA in the CNS is limited because of the BBB (Weil-Malherbe 1961). The administration of a MAOI plus L-DOPA promotes the synthesis of these catecholamines and their effects on the CNS can be observed (Everett 1961).
Depletion of brain biogenic amines can be accomplished by the administration of tetrabenazine (Quinn et al. 1959), reserpine (Costa et al. 1962), or inhibition of synthesis by αMPT (Spector et al. 1965).

Hemicholinium (HC-3) has been shown to alter the synthesis of ACh by decreasing the availability of choline with a resulting depletion of transmitter. Stitzel et al. (1965) evaluated the effect of HC-3 on catecholamine-depleting actions of reserpine and other drugs. They reported that HC-3 had no effect on the depletion of catecholamines from the CNS but did alter the release from adrenal medulla. Their conclusion was that HC-3 had no effect on the release of NE from the brain. However, no considerations of the BBB were made and there was absence of controls on ACh levels in the brain in the presence of HC-3. Domino et al. (1968) found that HC-3 had little effect and was unpredictable when given intravenously in amounts of 5-10 mg/Kg as compared to the 10 mcgm/Kg dose reported by Stitzel et al. (1965).

Frazier et al. (1969) applied HC-3 to the inside of squid axons, blocked the action potential and could not reverse the block by giving choline. They concluded that HC-3 antagonized the early transient and late steady state com-
ponents of the membrane ionic conductances. In other words, they proposed that the effect of HC-3 was on membrane conductances rather than a cholinergic mechanism.

Nicotinic stimulation of the CNS is dependent on the dose of alkaloid being used. Nicotine per se in repeated low doses of 2 mcg/mg Kg i.v. over a period of 20 minutes has been reported to evoke an electrocortical arousal with an increase release of ACh, while higher doses reversed this effect and induced a sleep pattern with a decreased ACh release (Armitage et al. 1969). Longo et al. (1967) had found a species variability in response to nicotine. EEG convulsions were evoked in rabbits, guinea pigs, and rats but not in cats. Cats revealed only motor convulsions and not EEG paroxysms. Mecamylamine was a more effective antagonist of the nicotinic actions of both nicotine and arecoline than was dihydro beta-erythroidine. Westfall et al. (1967) concluded that nicotine released NE in the CNS and noted that DA concentrations were also down 30 minutes after injection. However, Domino (1967) pointed out that nicotine also released ACh, serotonin, and vasopressin in addition to the release of catecholamines in the CNS. He noted that a low dose of nicotine evoked an EEG desynchronization that correlated with a behavioral arousal but
felt that actions of nicotine were too wide spread and that it was a poor drug of choice for determination of specific effects.

While the concept of steady state synthesis rate of NE (Brodie et al. 1966a) has proved useful in evaluation of catecholamine action in the CNS, the determination of synthesis rates must be viewed with some caution. Thoenen et al. (1969) have found that tyrosine hydroxylase activity after the administration of drugs could be increased. Phenoxycbenzamine administration showed an increased NE synthesis which they attributed to an increased membrane transport of tyrosine and not due to increased enzyme activity. Nerve stimulation also evoked the same response, and according to Thoenen et al. two mechanisms could be involved, (1) rapid regulation by end product inhibition, tyrosine transport and cofactor concentrations and (2) increased enzyme activity due to an increased amount of enzyme protein, which would require several hours before an increased enzyme activity appeared.

Finally, the adrenergic mechanisms in the CNS have been chiefly concerned with DA and NE but recently evidence has been presented that suggests the synthesis of EPI in the CNS (Ciaranello et al. 1969 and Pohorecky et al. 1969). If
these results are accurate, the response of the EEG to the administration of alpha blockers such as chlorpromazine and phenoxybenzamine could be explained on the basis of an unmasked beta effect and additional considerations for the role of EPI and the proposed beta receptor cyclic 3',5' adenosine monophosphate must now be considered for the central nervous system as well (Robison et al. 1967 cited in Burn 1968).
II. METHODS AND MATERIALS

A. GENERAL

The experimental subjects and their maintenance was the same as described in Part I. In cases where chronic drug administration was indicated the animals were dosed in their cages in the animal quarters and were brought to the laboratory on the day of the experimental recording.

B. PHYSIOLOGICAL

1. Electroencephalography

EEG recordings were obtained from the animals as described in Part I.

2. Evoked Potentials

Thalamocortical recruited potentials were evoked by electrical stimulation of the anterior dorsal nuclei of the thalamus. Square wave pulses were delivered from Tektronix type 161 and 162 pulse and waveform generator combinations (see appendix B) and were monitored by CRO. Stimulus artifact was attenuated by means of a Bioelectric ISA stimulus isolation unit. Stimulus parameters consisted of a frequency of 8.0-8.5 c/s at durations of 1.0 msec or less. Threshold voltages varied from animal to animal, as well as, from that required during a synchronized background of EEG
activity compared with that required during a background of desynchronized EEG potentials. A stimulus voltage was determined that was sufficient to evoke recruitment during EEG alert tracings induced by peripheral alerting stimuli (see Part I), or even by direct electrical stimulation of the reticular substance. Stimulus parameters for reticular formation stimulation were 200 c/s, 0.2 msec, for 5-10 seconds. The voltage that was adequate to evoke recruitment during a period of EEG desynchronization was referred to as suprathreshold. The recruited potentials were recorded from the cortices and with the EEG were displayed on a Grass Model IIID electroencephalograph. In addition, recruitment was amplified and monitored by CRO. The experimental animals received no anesthesia except for 2% procaine applied to the wound sites. Restriction of movement was obtained by use of the animal restraining board fitted with a headholder as described in Part I. Electrode placement was verified as before (see Part I). Either arterial blood pressure (femoral artery) or heart rate was monitored with the EEG tracings.

Integrator analysis of recruited potentials was obtained by a relay controlled integration unit (see appendix B). The integrator was normally off except during the
stimulation and was triggered by the stimulator just prior to the appearance of the stimulus train at the electrode. The CRO sweep was also triggered in the same manner. In the case of data integration, the stimuli were presented for a period of two seconds which was adequate for showing the rising phase and the peak amplitude of the recruited potentials. Data from one channel was selected for analysis by this method and the selection of the channel was based on control observations from the leads exhibiting the most evident thalamocortical recruitment. Responses to the various drugs were evident and attempts to quantify them were not necessary. During any given observation period, the stimuli were presented once every 10 seconds for a period of 2 seconds.

Suprathreshold voltages for evoking recruitment were kept constant in any given experiment for the pre- and post-drug conditions.

C. HISTOLOGICAL

1. Thionin and Eosin Staining Procedure

The general staining procedure as outlined in Part I and appendix A was used to verify electrode placement. Also, electrode tracts were identified during the sectioning procedure that was carried out in the cryostat as
described in Part I.

D. BIOCHEMICAL

1. Fluorometric

Determination of norepinephrine (NE) levels was made by the method of Shore and Olin (1958) and is outlined in appendix B. The following brain parts of each of 54 animals were used: (1) cerebral cortex, (2) hippocampus, (3) cerebellum, (4) midbrain, and (5) medulla-pons. Animals were sacrificed either by air embolism or by decapitation. Control samples included untreated rabbits not subjected to other experimental procedures; as well as animals subjected to the experimental procedures excluding electrical stimulation and drugs. Determinations were also made in animals subjected to depletion of brain NE by reserpine or alpha methyl para-tyrosine, as well as animals in which NE levels had been raised by the administration of MAOI and L-DOPA (vide infra) (see Tables II and III).

E. PHARMACOLOGICAL

1. Drugs and Dosages

Brain catecholamines were depleted by the administration of reserpine (2.0 mg/Kg i.v.) daily for two days prior to the recording day (Table III). Inhibition of synthesis at
the tyrosine hydroxylase step was also used as a specific
method of lowering brain levels of NE. Alpha methyl para-
tyrosine (200 mg/Kg i.p.) was given as a liquid suspension
in tragacanth for two days preceding the recording day
(Table III, cf. Table II, and cf. Spector et al. (1965).
Following depletion of catecholamines, the tissue levels
in the brain were restored by the administration of
pargyline (12.5 mg/Kg i.v.) given one hour prior to the
injection of L-DOPA (100 mg/Kg i.p.). In addition, normal
tissue levels of brain biogenic amines could be raised by
the combination of the monoamine oxidase inhibitor and
L-DOPA (Table III, cf. Everett 1961).

Eserine salicylate was given intravenously in amounts
of 100.0-150.0 mcgm/Kg This dose range was found to be
adequate to evoke an EEG arousal and to markedly attenuate
or block recruitment in control animals (fig. 34).
Similarly, doses of (0.5-1.0 mg/Kg i.v.) DFP and 10.0-15.0
mcgm/Kg i.v. of sarin were found to be adequate in
eliciting the above effects. These anticholinesterases were
given to animals with modified tissue levels of NE. NE
concentrations in the brain were modified by: (1) reserpine
or alpha methyl para-tyrosine induced depletion, (2) deple-
tion followed by the restoration of NE concentrations
induced by the combination of pargyline and L-DOPA, and (3) by the combination of pargyline and L-DOPA without prior depletion of biogenic amines.

EEG tracings were made routinely and thalamocortical recruitment was evoked during control periods, in the presence of physostigmine, of DFP, and of sarin. These electrophysiological recordings were also obtained during conditions of NE depletion, as well as after the restoration of depleted NE. AntiChE's were administered in the presence of these altered states of brain NE concentrations. Tissue levels of brain NE were monitored in each case and these, along with the EEG and recruitment data, were correlated with the pharmacological procedures (Table III).

D(-) - INPEA, (D(-)-l-(4'Nitrophenyl)-2-isopropylamino-ethanol HCl) was administered to evaluate the effects of beta adrenergic blockade on EEG activity and on thalamocortical recruitment. Alpha adrenergic blockade was achieved by intravenous phenoxybenzamine (5.0 mg/Kg). Eserine (100 mcgm/Kg i.v.) was selected to evaluate antagonism of the EEG effects of the blockade of adrenergic receptors by an anticholinesterase. Thalamocortical recruitment was also evaluated in the presence of alpha blockade and in the presence of subsequent antagonism by an anticholinesterase.
Gross behavioral observations of the animals in their cages were made prior to the electrophysiological or biochemical observations in order that central and autonomic nervous system activity could be evaluated. Criteria used were (1) pupillary diameter, (2) GI tract activity (defecation), and (3) the general motor activity of the experimental animals compared to control subjects or to their own premedication activity.
III. RESULTS

A. CONTROL OBSERVATIONS

1. Histological

The results of the thionin and eosin staining procedure are presented in Part I together with the designation of the sites used for stimulation of the brain and for recording.

2. Biochemical

Norepinephrine concentrations from the brains of 5 control animals are presented in Table II. These values served as a reference for brain NE concentrations in untreated animals (see Table III).

Inhibition of synthesis with alpha methyl para-tyrosine showed a progressive decrease in NE concentration in rabbits sacrificed at 4, 16, and 24 hours after the drug (Table II). These values served as a reference for rabbit brain NE depletion by alpha methyl para-tyrosine in subsequent studies (see Table III).

Table II shows that reserpine induced a rapid fall in brain NE at 4 hours and that this degree of depletion was maintained in animals sacrificed at 16 and 24 hours after injection. These values served as a reference for brain NE
TABLE II. CONTROL BRAIN NE CONCENTRATIONSa

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Cortex</th>
<th>Hippocampus</th>
<th>Midbrain</th>
<th>Medulla Pons</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:00 (N=5)</td>
<td>238 (±105)</td>
<td>469 (±130)</td>
<td>360 (±81)</td>
<td>505 (±135)</td>
<td>235 (±56)</td>
</tr>
<tr>
<td>4:00 (N=3)</td>
<td>89 (±7)</td>
<td>122 (±54)</td>
<td>73 (±54)</td>
<td>133 (±31)</td>
<td>106 (±23)</td>
</tr>
<tr>
<td>16:00 (N=3)</td>
<td>87 (±24)</td>
<td>154 (±34)</td>
<td>93 (±30)</td>
<td>105 (±8)</td>
<td>199 (±6)</td>
</tr>
<tr>
<td>24:00 (N=3)</td>
<td>53 (±25)</td>
<td>66 (±28)</td>
<td>56 (±18)</td>
<td>62 (±24)</td>
<td>65 (±55)</td>
</tr>
</tbody>
</table>

Reserpine (2.0 mg/Kg. i.v.)

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Cortex</th>
<th>Hippocampus</th>
<th>Midbrain</th>
<th>Medulla Pons</th>
<th>Cerebellum</th>
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<tr>
<td>4:00 (N=3)</td>
<td>54 (±47)</td>
<td>267 (±154)</td>
<td>191 (±142)</td>
<td>209 (±164)</td>
<td>106 (±89)</td>
</tr>
<tr>
<td>16:00 (N=3)</td>
<td>48 (±33)</td>
<td>99 (±8)</td>
<td>70 (±16)</td>
<td>126 (±36)</td>
<td>76 (±33)</td>
</tr>
<tr>
<td>24:00 (N=3)</td>
<td>25 (±1)</td>
<td>61 (±17)</td>
<td>62 (±17)</td>
<td>33 (±19)</td>
<td>33 (±1)</td>
</tr>
</tbody>
</table>

αMPT (200 mg/Kg. i.p.)

a Expressed as ng/Gm tissue
depleted by reserpine in subsequent studied (see Table III).

3. Electrophysiological

Control EEG patterns and responses to stimuli were the same as described in Part I. In addition, thalamocortical recruitment was evoked during a background of synchronized and desynchronized EEG activity (figs. 32 and 33). Presentation of the stimuli is indicated in the figures and the parameters of stimulation are stated in Methods and Materials (vide supra).

B. EXPERIMENTAL OBSERVATIONS

1. Biochemical

Table III reveals that reserpine markedly depleted brain NE concentrations while reserpine treatment followed by pargyline and L-DOPA restored these concentrations towards control levels. Alpha methyl meta-tyrosine, also markedly depleted brain NE and this was likewise restored by the combination of monoamine oxidase inhibitor and L-DOPA. Finally, the MAOI and L-DOPA treatment resulted in an increase in brain NE.

2. Electrophysiology

The administration of physostigmine (150.0 mcgm/Kg i.v.) was followed by a period of approximately 2 minutes
of continuous slow wave EEG sleep. An uninterrupted
desynchronized EEG pattern followed and maximal intensity
appeared at about 5 minutes after the injection. During
the period of maximal intensity of EEG desynchronization,
the supra-threshold stimulus of the pre-drug state failed
to evoke a response and the recruited potentials were
attenuated (fig. 27, Part I). During the recovery from
Eserine (100 mcgm/Kg i.v.) recruitment reappeared while the
EEG was still desynchronized (fig. 35). EEG tracings re­
sumed pre-drug characteristics after 30-40 minutes. DFP,
on the other hand, elicited a desynchronized EEG pattern
that endured for more than 2 hours during which time re­
cruitment was attenuated (fig. 36). Scopolamine (100 mcgm/
Kg i.v.) readily antagonized both the EEG desynchroniza­
tion (fig. 37) and the attenuation of recruitment (fig. 38)
evoked by DFP. Chlorpromazine was also an effective
antagonist (fig. 39). Atropine sulfate (3.0 mg/Kg i.v.)
induced a slow wave EEG sleep pattern but did not alter
recruitment as is seen in figure 40. However, the adminis­
tration of sarin (15 mcgm/Kg i.v.) after that dose of
atropine resulted in an EEG alert pattern and attenuated
recruitment (fig. 41).
Control Thalamo-Cortical Recruitment: Thalamo-cortical recruitment evoked during a period of synchronized EEG activity. Note the waxing and waning of the potentials during the period of stimulation (arrows)
Control Thalamo-Cortical Recruitment: Thalamo-cortical recruitment evoked during a period of EEG activity that is desynchronous or alert. The waxing and waning of the recruited potentials appears during the period of stimulus application (between arrows) and is elicited by the same stimulation parameters as seen in the control thalamo-cortical recruitment during a period of synchronized EEG activity.
<table>
<thead>
<tr>
<th>Pharmacological Procedure</th>
<th>NE Brain Concentrations&lt;sup&gt;1&lt;/sup&gt;</th>
<th>EEG Pattern</th>
<th>Recruitment with Eserine</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CTX</td>
<td>HPC</td>
<td>MB</td>
</tr>
<tr>
<td>Control (N=6)</td>
<td>231(±94)</td>
<td>468(±118)</td>
<td>348(±73)</td>
</tr>
<tr>
<td>Reserpine (N=5)</td>
<td>55(±20)</td>
<td>64(±21)</td>
<td>38(±14)</td>
</tr>
<tr>
<td>Reserpine followed by MAOI &amp; L-DOPA (N=5)</td>
<td>160(±39)</td>
<td>335(±31)</td>
<td>233(±27)</td>
</tr>
<tr>
<td>αMPT (N=5)</td>
<td>36(±17)</td>
<td>48(±11)</td>
<td>63(±17)</td>
</tr>
<tr>
<td>αMPT followed by MAOI &amp; L-DOPA (N=5)</td>
<td>142(±36)</td>
<td>333(±31)</td>
<td>246(±36)</td>
</tr>
<tr>
<td>MAOI &amp; L-DOPA (N=5)</td>
<td>306(±61)</td>
<td>603(±174)</td>
<td>609(±40)</td>
</tr>
</tbody>
</table>

1 Expressed as ng/Gm tissue
**Legend for Table III**

**Summary Correlation of Pharmacological, Biochemical and Electrophysiological Data:** The table represents experiments in which pharmacological, biochemical, and electrophysiological data were correlated. The left side of the table represents the pharmacological procedure used to alter brain levels of catecholamines and in particular, norepinephrine. The values of brain tissue concentrations are represented as pg/Gm tissue for the selected brain parts as shown in the table. The effect of Eserine on thalamo-cortical recruitment in rows three and five is interpreted as meaning that the recruited potentials that were evoked in the presence of Eserine after depletion of norepinephrine by reserpine or alpha methyl para-tyrosine, were attenuated by Eserine after norepinephrine levels had been restored by the administration of MAOI and DOPA. See text for complete description.
Effect of Eserine on EEG and Recruitment: Eserine (150 mcgm/Kg i.v.) evokes an EEG desynchronization, markedly attenuates thalamo-cortical recruitment (arrows) and elicits a pressor response (cf. control figures). Stimulation was 8.5 c/s, 7.0 msec, at a voltage greater than threshold and it was presented as indicated by the arrows. Tracings were taken approximately 7.0 minutes after injection.
Appearance of Recruitment Prior to a Synchronized EEG after Eserine: During the course of recovery following the administration of 100 mcgm/Kg i.v. of Eserine, recruited potentials can be evoked (arrows) while the EEG tracings are still desynchronized. The pressor response is still evident (see BP). Stimulus parameters as in the previous tracing. Tracings were made 20 minutes after injection.
Figure 36

DFP ANTAGONISM OF RECRUITMENT

Persistent EEG Desynchronization and Attenuation of Thalamo-Cortical Recruitment

Induced by DFP: DFP (2.0 mg/Kg i.v.) administered approximately three hours prior to this tracing. Desynchronization of the EEG was evident at approximately three minutes after the injection and persisted. Recruitment was also attenuated for the same period of time. The stimulus applied between the arrows is 8.0 c/s, 0.9 msec, and a voltage greater than the threshold.
Scopolamine Reversal of DFP Induced EEG Desynchronization: Scopolamine (100 mcg/mL/Kg i.v.) antagonizes the EEG alert pattern elicited by the anticholinesterase and evokes a synchronized pattern. DFP (2.0 mg/Kg i.v.) two hours earlier and these tracings were made approximately twenty minutes after the injection of scopolamine.
Scopolamine Antagonism of DFP Fails to Alter Recruitment: Thalamo-cortical recruited potentials are readily evoked by the same stimulus parameters as used earlier (arrows). These tracings were made 5 minutes after those seen in figure 37.
Figure 39

ESERINE ATTENUATION OF RECRUITMENT ANTAGONIZED BY CHLORPROMAZINE

RF-BOA
L-RPC
RAC-OC
LH-BOA
RH-RF
L-RAC
EKG

100μV
SEC
Legend for Figure 39

Chlorpromazine Reversal of Eserine Desynchronization and Blockade of Recruitment: Chlorpromazine (2.0 mg/Kg i.v.) administered two minutes after the intravenous administration of Eserine (100 mcgm/Kg i.v.) prevents the EEG alert pattern and antagonizes the depression of recruitment seen at this dose of the anticholinesterase. Stimulation was applied between the arrows and stimulus values are 8.0 c/s, 0.8 msec and a voltage greater than threshold. A previous dose of Eserine had been administered as a control and was effective in desynchronizing the EEG and in attenuating the thalamo-cortical recruited potentials. This figure was taken at eight minutes after the injection of Eserine.
Failure of Atropine to Block Recruitment: Atropine sulfate (3.0 mg/Kg i.v.) fails to markedly attenuate the thalamo-cortical recruited potentials presented between the arrows. Stimulus parameters are 8.0 c/s, 0.6 msec with a voltage greater than threshold. Tracings were made 20 minutes after the atropine had been given.
Sarin Reverses Atropine EEG Sleep and Attenuates Recruitment: Sarin (15.0 mcgm/Kg i.v.) given subsequent to the atropine from the previous record reverses the atropine induced synchronization and elicits a persistent desynchronized pattern. Note that thalamic stimulation to evoke cortical recruitment, with the same stimulus values, is markedly attenuated. The stimuli were effected between the arrows. Tracings were made 15 minutes after the injection of sarin.
Rabbit EEG after Two Days Pretreatment with Reserpine 2.0 mg/Kg per Day: Note the predominant slow wave sleep pattern apparent in all leads, with random 8‐14 c/s EEG sleep spindles. Catecholamines are at markedly low levels (cf. Table III).
Recruitment in the Chronically Reserpinized Rabbit: Thalamo-cortical recruitment is evoked (channel 3, RAC-OC) by stimulation of the thalamic nuclei 8.0 c/s, 0.9 msec at a voltage greater than threshold (arrows). Note the synchronized EEG background activity (cf. Table for depleted levels of norepinephrine).
Effect of Eserine on Chronically Reserpinized Rabbit: (cont) Eserine 100 mcgm/Kg. i.v. fails to attenuate the recruited thalamo-cortical potentials approximately eight minutes after the administration of the anticholinesterase. A decrease in recruitment was not observed at this dose. Note the desynchronized EEG pattern. The stimulus was 7.5 c/s, 0.8 msec, at voltages greater than threshold and was applied as indicated by the arrows.
Effect of Eserine on Chronically Reserpinized Rabbit: (cont) Eserine 200 mcgm/Kg i.v. fails to alter the recruited thalamo-cortical potentials at approximately eight minutes after the administration of the cholinesterase inhibitor. Stimulus parameters as in the previous figure. This figure represents data from the same animal.
Effect of DFP on Chronically Reserpinized Rabbit:  DFP 2.0 mg/Kg i.v. fails to alter the recruited potentials after two days of reserpine pretreatment. Stimulus parameters are the same as in the previous figures. The desynchronous EEG pattern was persistent for greater than two hours. This figure was taken at ten minutes after the administration of the anticholinesterase.
Pargyline Fails to Block Recruitment: Pargyline 12.5 mg/Kg i.v. fails to attenuate the recruited potentials at 40 minutes after injection. Stimulus unaltered from the previous figure and was presented between the arrows. Note the artifact on the right side of the tracings due to movement of the unanesthetized animal.
Figure 48

ESERINE ATTENUATION OF RECRUITMENT AFTER MAOI AND DOPA

RF-BOA
L-RPC
RAC-OC
LH-BOA
RH-RF
L-RAC
EKG

100 μV
Effect of Eserine with Restored Catecholamines: Eserine 100 mcg/m/Kg i.v. is effective in attenuation of recruitment in the chronically reserpinized rabbit after the catecholamine stores have been restored by the administration of pargyline and DOPA (100 mg/Kg i.v.). The norepinephrine precursor was given 40 minutes prior to the administration of the anticholinesterase. The figure was taken at approximately eight minutes after the administration of Eserine. Stimulus parameters are unchanged from the previous figures and are presented as indicated by the arrows in the figure (cf. norepinephrine values in brain tissue Table III).
EEG Activity in Presence of Low Levels of Norepinephrine: The ongoing EEG activity of the rabbit after markedly low levels of norepinephrine are established following the administration of alpha methyl para tyrosine. Note the slow wave EEG sleep pattern with 8-14 c/s activity but this can be interrupted by a sound stimulus resulting in a desynchronization (left center of figure) of the tracings.
Recruitment in the Presence of Low Levels of Norepinephrine: Cortical recruitment following stimulation of the thalamus is evident. Note the response in the lead from RAC-OC, as the cortical potentials wax and wane in a typical recruited pattern. The rabbit had received αMPT (200 mg/Kg i.p.) for two days prior to these tracings. See Table III for brain NE concentrations (cf. also Table II).
Eserine Fails to Block Recruitment in the Presence of Depleted Norepinephrine:

Physostigmine (100 mcgm/Kg i.v.) fails to attenuate the recruited cortical potentials while inducing a desynchronous EEG pattern (note, lead RAC-OC).

Tracings were made 8.0 minutes after the administration of Eserine.
Reserpine (2.0 mg/Kg/day i.v.) administered for two days evoked an EEG pattern that was predominantly slow wave sleep (fig. 42) and did not alter thalamocortical recruitment (fig. 43). Eserine (100.0 mcgm/Kg i.v.) then failed to attenuate the evoked cortical response (fig. 44) and induced an EEG arousal pattern (cf. fig. 34). Doubling the dose of Eserine (200.0 mcgm/Kg i.v.) also failed to block the recruited potentials (fig. 45). Finally, DFP (fig. 46) as well was ineffective in antagonizing thalamocortical recruitment. However, restoration of brain NE levels to control levels by the administration of a combination of MAOI and L-DOPA (figs. 47 and 48) did not alter recruited responses per se, but the effectiveness of Eserine to attenuate recruitment was restored (fig. 48, cf. fig. 34).

The chronic administration of alpha methyl para-tyrosine resulted in the appearance of a predominantly slow wave EEG sleep pattern, during which time peripheral stimulation (see Part I), could evoke an arousal response (fig. 49). Recruitment was not attenuated by alpha methyl para-tyrosine (fig. 50), and Eserine (150 mcgm/Kg i.v.) failed to attenuate the evoked recruited potentials (fig. 51). If brain NE levels were restored by the administration of pargyline and L-DOPA (Table III), Eserine was again
effective and attenuated the thalamocortical recruited potentials in the same manner as was seen in the reserpine sequence (vide supra) (cf. fig. 48).

The results of the effects of increased brain NE on the Eserine induced attenuation of thalamocortical recruitment are shown in figures 52, 53, 54, and 55. Figure 52 reveals that 50.0 mcgm/Kg i.v. of Eserine was insufficient to antagonize recruitment. The evoked responses were most evident in channel number two (RAC-LN) and served as a control. Inhibition of MAO with pargyline (25 mg/Kg i.v.) failed to alter the effectiveness of this low dose of Eserine (fig. 53). However, L-DOPA, (100 mg/Kg i.p.) in combination with the MAOI, increased the effectiveness of the low dose of Eserine and the recruited potentials were attenuated (fig. 54, RAC-LN). Partial recovery of recruitment occurred 40 minutes to one hour after the Eserine injection (fig. 55) at a time when NE levels were being increased (cf. Table III).

Adrenergic blockade affected EEG activity, thalamocortical recruitment, and the effects of AcChE's on these potentials as indicated in figures 56 through 65. Integration of the evoked potentials was carried out and control
Figure 52

RECRUITMENT WITH 50 mcgm/Kg ESERINE

LN-BOA
RAC-LN
LAC-BOA
L-RAC
LH-BOA
L-RPC

100μV
sec
Legend for Figure 52

Low Dose of Eserine Elicits Desynchronization of EEG but Fails to Attenuate Recruitment: Eserine (50 mcgm/Kg i.v.) induces an EEG alert pattern but does not depress thalamo-cortical recruitment. The stimulus is 8 c/s, 0.8 msec, and a voltage greater than control. The stimulus is once every ten seconds for a duration of two seconds as indicated by the solid bars. Note the recruited potentials from the second channel (RAC-LN). This tracing was taken at approximately seven minutes after the administration of the cholinesterase inhibitor.
Figure 53

RECRUITMENT WITH 50 mcgm/Kg ESERINE IN PRESENCE OF MAOI

L-N-BOA
RAC-LN
LAC-BOA
L-RAC
LH-BOA
L-RPC

Pargyline Fails to Enhance the Effect of Eserine: Pargyline (25.0 mg/Kg i.v.) given one hour prior to the administration of 50 mcgm/Kg of Eserine does not provoke a marked attenuation of recruitment at seven minutes after the administration of the latter drug. Stimuli are unchanged from the previous Figure 52 and are presented as indicated by the solid bars.
Figure 54

ESERINE ATTENUATION OF RECRUITMENT
IN PRESENCE OF MAOI & DOPA

LN-BOA
RAC-LN
LAC-BOA
L-RAC
LH-BOA
L-RPC

100 μV
Increased Brain Level of Norepinephrine Enhances Effect of Eserine: DOPA (100 mg/Kg i.v.) administered one hour after pargyline and 40 minutes prior to the time of these tracings reveals an enhancement of the effect of 50 mcgm/Kg i.v. of Eserine on the thalamo-cortical evoked recruitment at eight minutes after injection. While the potentials are still present, note the attenuation most evident in the second channel (RAC-LN). Note the movement artifacts appearing as slow wave excursions particularly in the first four channels. Stimulus is unaltered from the previous figure.
Decrease of Attenuation of Recruitment as the Duration of Action of Eserine Elapses:

Forty minutes after the administration of 50 mcgm/Kg i.v. of Eserine the thalamo-cortical recruited potentials begin to reappear (note channel 2 RAC-LN). Stimulus parameters are the same as previously shown in the preceding figures.
Figure 56

CONTROL RECRUITMENT – SYNCHRONIZED EEG

LN-BOA
LAC-BOA
RAC-LN
LH-LN
RH-BOA
L-RPC

INTGR.

100 μV
sec
Legend for Figure 56

Integrator Analysis of Thalamo-Cortical Recruitment: Control period of thalamo-cortical recruitment on a background of synchronous EEG activity. Channel 2 (LAC-BOA) is being analysed by integration and the result appears in channel 7 in this and in subsequent figures (INTGR.). Also, the dotted line has been drawn to indicate the peaks during the control period, and is the reference level in the subsequent figures utilizing integration analysis of recruited potentials. Stimulation parameters are: 8.0 c/s, 0.8 msec, for two seconds at a voltage greater than the threshold. The stimulus was presented once every ten seconds. Note the relative constancy of the amplitude of the response as reflected by the integrator.
Integrator Analysis of Thalamo-Cortical Recruitment: Control period of thalamo-cortical recruitment during a phase of desynchronous EEG activity. Note that the integrated response from channel 2 appears slightly greater than that seen during EEG sleep patterns. Stimulus parameters are as previously stated.
Drug Attenuation of Recruited Potentials: The effect of 100 mcgm/Kg i.v. of eserine on thalamo-cortical recruitment shows a marked attenuation of the integrated potentials. Control level is indicated by the dotted line. Stimulation parameters as presented in the previous tracings. Tracings were made approximately 7.0 minutes after injection.
Eserine Attenuation of Recruitment in the Presence of Atropine Methyl Nitrate:
Eserine (100 mcgm/Kg i.v.) markedly attenuates thalamo-cortical recruitment when given after the quaternary atropinic agent. Note the marked decrease of integrated data below the control levels (dotted line). Stimulus parameters are unchanged from the previous recordings.
Initial Effect of Phenoxybenzamine on EEG and Thalamo-Cortical Recruited Potentials:
Phenoxybenzamine, 5.0 mg/Kg i.v. induces an initial transient EEG arousal lasting a few minutes that also attenuates the evoked thalamo-cortical recruitment response. The pre-drug control level of integrated signals is indicated by the dotted line. Stimulus parameters are 8.0 c/s, 0.8 msec, two seconds duration and the stimulus is presented once every ten seconds. Again, channel 2 is being utilized for integration.
Persistent Effect of Phenoxybenzamine on EEG and Thalamo-Cortical Recruited Potentials: Following the initial transient effects as seen in the previous figure for phenoxybenzamine 5.0 mg/Kg i.v., a persistent synchronous EEG pattern ensues and the attenuation of recruitment is reversed as indicated by the integrated data in channel 7. Note that the amplitudes of the signals are greater than those seen during the control period (dotted line) of synchronous activity. Stimulation parameters as in the previous phenoxybenzamine recording.
Eserine Reversal of the Phenoxybenzamine Enhancement of Thalamo-Cortical Recruitment:

Eserine (100 mcg/mKg i.v.) reverses the synchronous EEG pattern elicited by the administration of phenoxybenzamine and induces a pronounced attenuation of the evoked recruitment. Note the desynchronized EEG pattern and the integrated potentials values below control levels (dotted line). Stimulation parameters are the same as in the previous tracings. Recordings were made 7.0 minutes after Eserine.
data during synchronized and desynchronized EEG background activity is shown in figures 56 and 57. Amplitudes of the integrated responses were relatively constant and recruitment evoked during the EEG alert appeared to be somewhat greater than that seen during the slow wave EEG sleep pattern. Eserine (100 mcgm/Kg i.v.) antagonism of recruitment was reflected by a marked transient reduction in integrated response that was at a maximal stage 7 minutes after injection (fig. 58). Atropine methyl nitrate (1.0 mg/Kg i.v.) failed to antagonize the effect of Eserine (100 mcgm/Kg i.v.) on EEG activity as well as on the evoked potentials (fig. 59, cf. fig. 58).

Phenoxybenzamine elicited a biphasic response on recruitment and EEG activity (figs. 60 and 61). The initial effect (fig. 60) was an EEG desynchronization that lasted approximately 2 minutes during which time thalamocortical recruitment was attenuated. Synchronized EEG patterns followed (fig. 61) and the integrated response to recruitment exceeded control levels. This alpha adrenergic blockade was readily antagonized by Eserine (100 mcgm/Kg i.v.) and EEG desynchronization as well as attenuation of recruitment occurred (fig. 62). The subsequent adminis-
tration of phenoxybenzamine in a lower dose (2.5 mg/Kg i.v.) elicited antagonism of the AntiChE-attenuated recruitment, but not the EEG alert pattern of Eserine (fig. 63).

Control responses prior to beta blockade are shown in figure 64. After the administration of 2.0 mg/Kg i.v. of D(-) INPEA an EEG arousal was evoked and recruitment was attenuated as is shown in figure 65.

Proposed intracellular pathways for the biosynthesis and fate of NE are included for reference in figures 66 and 67 (cf. Wurtman 1966).
Phenoxybenzamine Reversal of the Eserine Antagonism of Thalamo-Cortical Recruitment: Phenoxybenzamine (2.5 mg/Kg i.v.) antagonizes the attenuation of recruitment induced by Eserine but does not evoke a synchronous EEG pattern. Note that the amplitude of the integrated potentials (channel 7) is somewhat greater than the control levels as indicated by the dotted line. Stimulus parameters are unchanged. Tracings were made 10 minutes after injection.
Control Thalamo-Cortical Recruitment: Evoked potentials during a phase of desynchronous EEG activity prior to the administration of $D(-)$-INPEA. Stimulus parameters are 8.5 c/s, 0.9 msec for two seconds at voltage greater than threshold. The stimulus was presented once every ten seconds.
Figure 65

D(-)-INPEA EVOKED EEG DESYNCHRONIZATION AND ANTAGONISM OF RECRUITMENT

Effect of D(-) - INPEA on EEG and Recruitment: Marked EEG arousal patterns are evoked by the intravenous administration of 2.0 mg/Kg of D(-) - INPEA. Note the attenuation of the recruited potentials in the data integrated from channel 2 and recorded in channel 7. The dotted line represents the control level from the previous record. Stimulus parameters are unaltered. Tracings were made 15 minutes after injection.
Figure 66

**NE BIOSYNTHESIS — INTRACELLULAR PATH**

**Circulation**

**TYROSINE**

**Brain or Sympathetic Nerve Ending**

Tyrosine Concentrating Mechanism — (Active Transport)

**Mitochondria**

**TYROSINE HYDROXYLASE**

**DOPA**

— **DOPA DECARBOXYLASE**

**DOPAMINE**

**Granule**

— **DOPAMINE Beta OXIDASE**

**NE**
Legend for Figure 66

Proposed Intracellular Pathway of Biosynthesis of Norepinephrine in Brain and Peripheral Nerve Tissue: Circulating tyrosine is proposed to be picked up by an active transport mechanism into the neuron or sympathetic nerve ending. After entry into the mitochondria the tyrosine is hydroxylated by tyrosine hydroxylase and the DOPA thus formed is decarboxylated to form dopamine. Dopamine is then oxidized by dopamine beta oxidase to form the postulated neurotransmitter norepinephrine.
Figure 67

**FATE OF NE — INTRACELLULAR PATH**

Brain or Sympathetic Nerve Ending

- Storage Granule
  - NE
  - NE (released)

- Mitochondria
  - MAO
  - DHMA
  - DHPG

- Parenchymal Cell
  - COMT

- Circulation
  - DHMA
  - VMA
  - NE
  - NM
  - DHPG
  - MHPG

- Parenchymal Cell
  - COMT

- Receptor
Legend for Figure 67

Proposed Intracellular Pathway of Fate of Norepinephrine in Brain and Peripheral Nerve Tissue: Within the brain or sympathetic nerve ending, norepinephrine exists in the bound state (NE) which is in equilibrium with the free state (NE). Also an equilibrium exists between the free norepinephrine and the oxidative enzyme within the mitochondria, MAO. The release of NE follows the arrival an impulse and this released NE can be either rebound back into the nervous tissues; react with the receptor; be converted to normetanephrine by COMT of the parenchymal cells and then picked up by the circulation; or incorporated directly into the circulation. If the NE is shunted into the MAO pathway, the DHMA or DHPG thus formed may also be acted upon by COMT of the parenchymal cells and then incorporated into the circulation as DHMA, DHPG VMA or MHPG.
IV. DISCUSSION

The increased parasympathetic activity in rabbits after chronic biogenic amine depletion included miosis, ptosis, diarrhea and decreased spontaneous motor activity. Similar effects have been noted by Malhotra and Pundlik (1959) and Häggendal et al. (1967). Bhargava (1967) suggested that alpha methyl DOPA and reserpine promoted the release of ACh and facilitated parasympathetic activity. In the present studies, hypotension, bradycardia, and death appeared in response to doses of Eserine that were not lethal in the presence of normal autonomic nervous system balance. Therefore, an evaluation of the effects of anticholinesterases in the CNS of animals with depleted biogenic amines required protection of the cardiovascular system. The antimuscarinic properties of atropine methyl nitrate were effective as a prophylactic measure for the potentiated cardiovascular responses subsequent to intravenously administered anticholinesterases. However, attenuated responses to peripheral and direct CNS stimulation have been attributed to methyl atropine (Riehl et al. 1960). An increased EEG arousal threshold to reticular formation stimulation in the presence of atropine methyl nitrate was observed. Unlike
Riehl et al. (1960), the present experiments showed atropine methyl nitrate did not attenuate EEG arousal responses nor thalamocortical recruitment. Quaternary amines of poor lipid solubility have restricted access to the CNS due to the blood brain barrier (BBB) (Weil-Malherbe 1961). Methyl atropine was selected as a prophylactic agent since its antimuscarinic properties are primarily restricted to the periphery. Moreover, Hance et al. (1963) noted that atropine methyl nitrate (0.05-5.0 mg/Kg i.v.) had no consistent effect on EEG activity of cats and had minimal, if any effects on cortical evoked responses. Rosecrans et al. (1968) found hyoscine but not atropine methyl nitrate effective in depression of conditioned response pole jumping in rats. Also, methyl atropine partially antagonized the effect of Eserine on the conditioned response. They concluded that drug action occurred in the periphery and suggested that peripheral cholinergic blockade may influence CNS responses and must be considered in the evaluation of central effects of anticholinesterases.

Although quaternary anticholinesterases such as neostigmine fail to penetrate the BBB and reveal little or no direct CNS activity (Longo 1962), Paulet et al. (1957) suggested that neostigmine along with parathion and
octamethylpyrophosphoramide (OMPA) increased the permeability of the BBB to another drug, sulfanilamide. However, this effect was not attributable to inhibition of cholinesterase. Aaseth and Barstad (1968) revealed that quaternary anticholinesterases did not increase existing brain cholinesterase inhibition. Mazel and Bush (1969) concluded that the Eserine-induced increased onset and duration of barbital sleep was not due to an increased permeability of the BBB but was mediated through the peripheral nervous system. Finally, Koelle and Steiner (1956) demonstrated that tertiary cholinesterase inhibitors did not enhance the penetration of quaternary anticholinesterases through the BBB.

Reserpine depletion of brain NE occurred rapidly and was maintained by chronic administration (Table II, cf. Table III, Pletscher et al. 1956, Weil-Malherbe and Bone 1959, Costa et al. 1962, Brodie et al. 1966, Håggendal et al. 1967, and Kety 1967). The values for depletion of brain NE seen in Table II served as control references for subsequent experiments in which reserpine-induced NE depletion was desired (cf. figs. 42 through 46). The action of reserpine has been described as "hit and run". It is
metabolized and disappears from brain tissue in 1-2 hours (in rabbit) while a marked depletion of biogenic amines continues for up to 36 hours (Hess et al. 1956). Costa et al. (1962) reported that rabbit brain NE was depleted to 12 per cent of control value at 4 hours after 1.5 mg/Kg i.v. However, Andén (1967) observed a differential depletion of rat brain monoamines after acute reserpine treatment. Low doses of reserpine (1.0 mg/Kg i.p.) resulted in resistance to depletion in some brain areas while a high dose (10.0 mg/Kg i.p.) uniformly depleted brain NE. Furthermore, Liebmann and Mattheis (1964) in a study on the influence of reserpine on cholinomimetic toxicity observed an increased toxicity at 1-2 hours after reserpine and maximal toxicity at 18-24 hours. This fluctuation in toxicity may be related to the circadian rhythm of the endogenous biogenic amines (Sterman et al. 1965, Reis et al. 1968, Friedman and Walker 1968). Therefore, chronic administration of reserpine was employed in the present study and a uniform depletion was obtained.

Depletion of brain NE by αMPT was linear with time and was maintained by chronic administration (Table III, cf. Table II). These findings are also in agreement with current literature (Spector et al. 1965, Brodie et al. 1968).
1966, Kety 1967). The values shown in Table II served as control references for subsequent experiments in which brain NE was depleted by αMPT (cf. figs. 49 through 51). The linear decrease of brain NE by αMPT is dependent on maintaining an adequate tissue level of the rate-limiting enzyme inhibitor (Brodie et al. 1966a) compared with reserpine-induced depletion of biogenic amines which was rapid and associated with a rapid disappearance of the drug from the tissues. Single large doses of αMPT have been reported to cause renal toxicity but repeated lower doses resulted in no toxicity and a decreased catecholamine concentration (Moore et al. 1967). Andén (1967) observed that αMPT uniformly depleted brain NE compared with reserpine and that the level of nerve activity had a greater influence on αMPT-NE depletion than on reserpine-NE depletion. In addition, Smookler and Buckley (1969) found that αMPT depleted NE 85% in male rats stressed by light, sound, and oscillation while non-stressed animals were depleted 45%. Stress also markedly increased NE turnover rates. Moreover, concentrations of endogenous amines have been implicated as factors in NE depletion. Nagatsu and Takeuchi (1967) showed that high concentrations of phenylalanine inhibited NE synthesis 80% in vitro. It was
concluded that since tyrosine hydroxylase catalyzed the hydroxylation of phenylalanine to tyrosine as well as tyrosine to DOPA, abnormally high concentrations of phenylalanine would compete with tyrosine for tyrosine hydroxylase, thus decreasing the amount of NE being synthesized.

Restoration of depleted brain biogenic amines necessitated consideration of mechanisms of biogenic amine depletion in the CNS. The mechanisms by which reserpine depleted endogenous NE (Holzbauer and Vogt 1956) throughout the neuron (Dahlström and Fuxe 1964) and blocked reuptake of NE (Glowinski and Axelrod 1965, 1966) differ from αMPT-NE depletion (Spector et al. 1965, Brodie et al. 1966a, Corrodi and Fuxe 1967). Depletion of the catecholamines, DOPA, dopamine (DA), NE and epinephrine (EPI), occurred after αMPT while reserpine depleted both catecholamines and indoleamines to include serotonin (Hess et al. 1956, Pletscher et al. 1956, Brodie et al. 1957, Shore et al. 1958, Weil-Malherbe and Bone 1959, and Kety 1967). Reserpine-induced sedation has been correlated with the initial rate of release of serotonin but not final concentrations (Brodie et al. 1966). Koella and Czicman (1966) and Koella et al. (1968) suggested that serotonin was the mediator associated with
EEG synchronization and behavioral sedation (cf. also Marczynski 1967 for additional references). Jouvet (1967) postulated that serotonin-containing neurons supported slow wave sleep while paradoxical sleep was a function of NE neurons of the locus coeruleus. Pich and Rech (1968), on the other hand, concluded that the depletion of brain NE or DA was a critical factor in behavioral sleep and electrocortical responses to αMPT and reserpine. Both catechol- and indoleamines may be involved in the mechanism of action of reserpine. At present the issue is unresolved (Kety 1967).

Restoration of brain biogenic amines by the parenteral administration of the putative transmitters DA, NE, or EPI failed to yield consistent results, and because of the BBB it is doubtful that direct CNS effects are observed (Longo 1962, Baust and Nieczyk 1964, Wurtman 1966, Barchas et al. 1968). Intraventricular administration has been used to circumvent the BBB (Feldberg and Sherwood 1954) but results are difficult to interpret. The CNS stimulant effects of sympathomimetic amines such as amphetamine as well as other phenylethanolamines that penetrate the BBB, revealed desynchronization of the EEG and behavioral arousal when given by different routes of administration (Van Meter and
Ayala 1961, Bradley 1968). Serotonin, which penetrates the BBB only to a slight extent, evoked an initial EEG arousal followed by an EEG synchronization while its precursor 5-hydroxytryptophan (5-HTP) readily gained access to the brain and caused an increased EEG synchronization, behavioral sleep and an associated rise in brain serotonin concentrations (Longo 1962, Koella 1966, Brodie et al. 1966, Bradley 1968). Bradley (1968) noted that the action of amphetamine when applied iontophoretically to CNS neurons, resembled NE but not ACh, serotonin or histamine. He concluded that amphetamine induced central excitation by releasing endogenous NE but pointed out that catecholamines injected into immature animals with poorly developed BBB as well as intraventricular injections, resulted in sedation or sleep. Matsuda (1968) found similar responses to ventricular administration of EPI in rabbits where EEG and behavioral sleep appeared without changes in threshold of thalamocortical recruitment.

There is evidence that the uptake-storage mechanism for catecholamines may be altered by reserpine. Häggendal and Malmfors (1969) observed that adrenergic nerves of rats failed to take up exogenous NE and failed to show functional responses when stimulated 16 hours after reserpine-NE
depletion and monoamineoxidase inhibition. However, nerve stimulation in the presence of exogenous DA revealed functional responses and synthesis of NE from that precursor. Furthermore, DA uptake has been shown to exceed that of NE in rat brain homogenates and there was evidence for a common uptake path for NE and DA (Snyder and Coyle 1969). In fact, dopaminergic transmission and pathways have been postulated in both the periphery and CNS (Hornykiewicz 1966, and Takagi et al. 1968). On the other hand, L-DOPA restored NE to αMPT-NE depleted but not reserpine-NE depleted rats unless a MAOI was used (Corrodi and Fuxe 1967). These authors observed that inhibition of MAO also increased the recovery level of NE in the αMPT-DOPA treated animals and they concluded that the uptake-storage mechanism was intact after αMPT-NE depletion but that the mechanism may have been altered by reserpine. Administration of the monoamine precursor L-DOPA in the presence of MAO inhibition effectively restored depleted brain biogenic amines and reversed reserpine depression of behavior (Everett 1961, Everett et al. 1963). In the present experiments, pargyline plus L-DOPA successfully reestablished depleted NE levels in brain and increased NE levels in non-depleted animals (cf. Table III and figs. 48,
Background EEG activity alters the stimulus threshold for evoking thalamocortical recruitment. Longo (1962) and Giaquinto (1968) showed that desynchronized EEG activity raised the threshold required for elicitation of thalamocortical recruitment. This was also noted during preliminary studies in the present research. Therefore, stimulus voltage for recruitment was established with background of desynchronized EEG activity (figs. 33, 57). Threshold values for thalamocortical recruitment established prior to the administration of the particular drug in question were maintained during post-drug observations. The stimulus threshold required to evoke recruitment during periods of EEG arousal was effective in the presence of synchronized EEG activity (figs. 33, 57, 64).

Eserine (100-150 mcgm/Kg i.v.) induced an initial, slow wave, EEG sleep pattern of approximately 2 minutes duration followed by a transient desynchronized EEG pattern. The greatest intensity of the desynchronous EEG response occurred between 5 to 10 minutes after the injection. The same sequence of synchronized EEG activity followed by desynchronized activity was noted after the intracarotid administration of Eserine (vide supra, Part I) with the
exception that the slow wave sleep pattern was of much shorter duration and lasted only a few seconds. Thalamocortical recruitment was attenuated during the maximal intensity of desynchronization (fig. 34) but reappeared about 20 minutes later during recovery from the Eserine injection. The recovery of thalamocortical recruitment occurred prior to the reappearance of a synchronized EEG pattern (fig. 35). The EEG pattern resumed pre-injection characteristics 30-40 minutes after the cholinesterase inhibitor had been given. The initial slow wave EEG pattern was not reported previously in descriptions of the reversible effects of Eserine on the EEG of rabbits (Longo and Silvestrini 1957, Longo 1962). In addition, the present work revealed the reappearance of thalamocortical recruitment prior to the recovery of the synchronized EEG activity after Eserine. Similarly, DFP also evoked a desynchronized EEG pattern and attenuated thalamocortical recruitment (fig. 36) at least two hours at which time experiments were terminated. These events are probably closely associated with the inhibition of cholinesterase and the increased brain ACh concentrations. In an evaluation of the interrelationship of inhibition of cholinesterases, increases in brain ACh concentrations and cholinomimetic toxicity, Holmstedt et al.
(1967), noted lethal doses of organophosphates were not required to induce marked increases in brain ACh concentrations. Classical toxic responses to organophosphate poisoning occurred only when cholinesterases were inhibited and after ACh levels in the brain attained values of 60 per cent or greater than controls. A similar relationship was noted with Eserine but the inhibition of enzyme was reversible. On the other hand, oxotremorine and arecoline provoked salivation, lacrimation, and tremor at brain ACh levels that were elevated only 20 per cent above control values. Leslie (1965) observed that oxotremorine and arecoline were the most potent antagonists of drug-induced sedation in rabbits. Oxotremorine was the only cholinomimetic that produced prolonged excitation when iontophoretically applied to ACh-excitible neurons of the pons-medulla of the cat (Bradley et al. 1966). The effects of Eserine on the EEG and thalamocortical recruitment in cats have been described as muscarinic (Hance et al. 1963). They reported that atropine sulfate (1.0 mg/Kg i.v.) was an effective antagonist of the EEG response to Eserine but had minimal action on evoked cortical potentials. While an accumulation of ACh may result from the inhibition of cholinesterases (as pointed out in Part I), other actions of
AntiChE's aside from enzyme inhibition should be considered, particularly during states of pronounced drug intoxication (cf. figs. 11 through 16). Moreover, as pointed out by Friedman (1967), oxotremorine was only one of a chemically and pharmacologically diverse group of compounds that promoted the release of ACh and other endogenous amines including DA as well as NE and EPI. In fact, low doses of nicotine (5.0-20.0 mcgm/Kg i.v.) have been shown by Domino (1967) to evoke an EEG arousal pattern in rabbits with midpontine brainstem transections. Nicotine induced an EEG desynchronization that was concomitant with a behavioral arousal. However, Domino suggested that because nicotine released ACh, catecholamines, serotonin and vasopressin and evoked a cholinergic blockade in higher doses, its mechanism of action was obscured. Furthermore, in an earlier study, Monnier and Romanowski (1962) found that ACh, Eserine, and pilocarpine all evoked an EEG arousal in rabbits, induced an increased arousal reaction to sensory and reticular formation stimulation and increased the cortically evoked potential from caudate or hippocampal stimulation, but decreased thalamocortical recruitment. These drug effects were blocked by atropine. Also, Domino (1968) revealed that
the muscarinic receptors had a greater sensitivity to evoked EEG desynchronization than did nicotinic cholinergic receptors and he concluded that the cholinergic mechanisms for inducing arousal were more muscarinic than nicotinic.

Reciprocal antagonism between anticholinesterases and anticholinergics was demonstrated in Part I and is reiterated in figure 37 that shows the scopolamine antagonism of DFP-induced desynchronization (cf. Longo 1962). Antagonism of the EEG arousal by scopolamine did not attenuate thalamo-cortical recruitment per se (fig. 37, Hance et al. 1963) but antagonized the attenuation of recruitment by DFP (cf. Longo 1962).

Chlorpromazine antagonized the transient attenuation of recruitment seen after Eserine (fig. 39) and the antagonism may be the result of alpha adrenergic blocking properties. Boakes et al. (1968) found that chlorpromazine was the most effective blocker of the excitatory effect of iontophoretically applied (-) norepinephrine on brainstem neurons. Rinaldi and Himwich (1955a, 1955b) have proposed anticholinergic properties for the chlorpromazine block of anticholinesterase evoked EEG desynchronization. Imipramine has properties similar to those of chlorpromazine
and Monnier and Krupp (1959) observed an increased threshold to electrocortical arousal following the administration of either drug while Rinaldi (1967) noted convulsive activity subsequent to administration of high doses. Dasberg and Feldman (1968) investigated the EEG arousal blocking effect of imipramine. These authors found that imipramine raised the threshold for EEG arousal evoked by the i.v. injection of Eserine and that the threshold for EEG arousal evoked by amphetamine was lowered. They concluded that imipramine acted by a cholinergic blocking mechanism. If a cholinergic-link concept is considered, Dasberg and Feldman's results could be interpreted to mean that Eserine promoted the release of biogenic amines in the CNS (Rosecrans et al. 1968, cf. Marczynski 1967) which elicited the EEG arousal. The anticholinergic effect of imipramine antagonized the EEG alert by blocking the action of Eserine and ACh on the adrenergic component. Amphetamine would still act at the CNS by releasing NE (Bradley 1961, Bradley 1968). The decreased threshold for amphetamine-induced desynchronization seen after imipramine could either be the result of cholinergic blockade or inhibition of catecholamine uptake (Glowinski and Axelrod 1966), by blockade of membrane
transport of amines (Giachetti and Shore 1966). Imipramine antagonism of Eserine-induced EEG arousal could also be attributed to the later adrenergic blocking effect. Furthermore, imipramine-antagonism of reserpine-induced sedation depended on the presence of catecholamine stores in the CNS. Depletion of brain catecholamines resulted in failure of imipramine-antagonism of benzoquinolazaine sedation (Sulser et al. 1964). Chlorpromazine, similar to imipramine, probably antagonized the effects of Eserine by a combination of cholinergic and adrenergic blocking effects. The brain stem reticular formation is a site of cholinergic activity (Rinaldi and Himwich 1955a, Rinaldi and Himwich 1955b). Cholinergic mechanisms are also implicated in EEG desynchronization at the level of the cerebral cortex, particularly with slow and prolonged excitatory effects (Krnjevic 1967). Krnjevic (1967) also observed that repetitive responses of cortical neurons to thalamic stimulation were markedly enhanced by ACh or Eserine. Therefore, the antagonism of Eserine by chlorpromazine occurs in the cortex as well as brain stem and there is probably not a specific locus of action.

Atropine evoked a synchronized EEG (fig. 40) and this has been reported to be dissociated from behavioral sleep
(Wikler 1952). However, thalamocortical recruitment was not attenuated. This finding with the observation that atropine also failed to alter caudatocortical recruitment has been shown in rabbits (Longo 1962). On the other hand, Domino et al. (1968) studied cholinergic mechanisms in sleep and wakefulness in cats. They found that physostigmine evoked an initial EEG desynchronization in both neocortical and limbic areas and that this response was associated with a behavioral arousal. These authors suggested that early findings of dissociation between EEG responses and behavior attendant to the administration of cholinergic agonists and antagonists (Wikler 1952, Bradley and Elkes 1957, Bradley and Nicholson 1962) were noted before the concepts of paradoxical or fast wave sleep cycles in cat were well established (Dement 1958, Jouvet 1961). The observation of a fast wave sleep pattern then would be acceptable as a concurrent event with behavioral sleep. Moreover, in an earlier study, Yamamoto and Domino (1967) had shown in cats that the initial EEG alert after Eserine was associated with a behavioral arousal and that paradoxical sleep appeared after a delay of 30 minutes or longer, during the recovery from the injection. Pilocarpine also showed the same result. Furthermore, denial of
paradoxical sleep in rats has been noted to evoke a rebound increase in paradoxical sleep after the period of denial (Pujol et al. 1968). Turnover of cerebral NE was increased during the rebound phase of paradoxical sleep. Jouvet (1967) proposed that slow wave sleep depended on serotonergically-mediated events while paradoxical sleep appeared to be related to catecholaminergic mechanisms. The atony associated with fast wave sleep could be blocked by atropine and yet DOPA induced normal paradoxical sleep in cats treated with reserpine. Jouvet (1967) concluded that both cholinergic and adrenergic mechanisms were required to produce paradoxical sleep with total atony.

A subconvulsant dose of sarin antagonized the EEG effects of atropine sulfate (3.0 mg/Kg i.v., fig. 41, cf. fig. 14 Part I). Thalamocortical recruitment was markedly attenuated (arrows) and this blockade persisted for two hours at which time the experiments were terminated. These observations are in agreement with Longo (1962) but not White (1966). The latter worker noted that 100 mcgm/Kg i.v. of Eserine in rabbits failed to evoke an EEG desynchronization if given after 1.0 mg/Kg i.v. of scopolamine. The lack of agreement of the effects of an anticholinesterase in the presence of an atropinic anticholinergic blockade
undoubtedly lies in the doses used. Scopolamine is approximately 10-15 times more potent than atropine as an antagonist of EEG arousal in the rabbit and the effective dose for total inhibition of the EEG arousal response elicited by direct electrical stimulation of the reticular formation was found to be 0.04-0.05 mg/Kg i.v. (Longo 1956). Furthermore, the reciprocal and competitive nature of the interaction between antimuscarinic and anticholinesterase drugs necessitates a consideration of the doses used, as well as the duration of inhibition of cholinesterases.

Chronic administration of reserpine effected (1) EEG activity (fig. 42), (2) brain levels of biogenic amines (Tables II, III), and (3) behavior (vide supra). EEG activity changed from alternating slow wave sleep and alert tracings to prolonged synchronization during which EEG arousal and thalamocortical recruitment were evoked (figs. 42, 43). Initial disagreement on the EEG response of laboratory animals to reserpine (Longo 1962) resulted from observations taken under markedly different conditions. Reserpine like amphetamine initially promoted the release of stored NE from nerve granules and brain (Vogt 1954). During this time there was a brief period of continuous
EEG desynchronization that began about 45 minutes after intravenous administration and persisted for about 2 hours (Rinaldi and Himwich 1955). The pattern of behavior was one of depression with the animal in a sleep-like state, attributable to paradoxical sleep (Jouvet 1967a) or perhaps due to interneuronal deamination of reserpine-liberated catecholamines which rendered them physiologically less active (Glowinski and Axelrod 1965, 1966). Only after 4-6 hours, the synchronized EEG predominated and the brain catecholamines were beginning to show marked depletion (fig. 42, Table III). Häggendal et al. (1967), in a recent study of the effects of chronic reserpinization on monoamine metabolism and behavior, noted that the first signs of reserpine activity appeared approximately one hour after injection at a time when the major part of the monoamines were still present. In the current work (Table III), long term treatment with reserpine revealed brain levels of monoamines similar to those seen at 4 hours through 24 hours (Table II). Häggendal and co-workers (1967) were unable to restore brain biogenic amine levels to control values by DOPA alone. Everett (1961) and Everett et al. (1963) showed that DOPA plus a MAOI rapidly restored biogenic amine levels in brain tissue. Bueno et al.
(1968) found that the appropriate combination of a MAOI and reserpine could evoke prolonged periods of alert EEG activity in rabbits. Brain concentrations of biogenic amines per se, could not be correlated with the EEG response but an increased ratio of concentration of serotonin to NE was seen with behavioral excitement and EEG desynchronization. Furthermore, as pointed out by Costa (1966) and Brodie et al. (1966a), synthesis rate and turnover time of tissue biogenic amines may correlate with physiological and behavioral activity more significantly than does actual tissue concentration. Moreover, turnover time as well as synthesis rate can be influenced by a variety of factors to include gonadectomy which increased brain NE turnover in rats (Anton-Tay and Wurtman 1968) and physical stress (Smookler and Buckley 1969). Gal (1961) found that reserpine evoked a greater prolonged depletion of serotonin and NE in rats fed on a tryptophan deficient diet than with animals fed a normal diet. Therefore, while changes in turnover and synthesis rate may be of value in correlation of experimental observations, the experimental conditions are of critical importance.

Reserpine per se did not depress thalamocortical recruitment (fig. 42). Gal et al. (1961) observed that
reserpine did not alter arousal threshold nor recruitment threshold in rats. White (1966) observed that reserpine facilitated the indirect action of mephenteramine and induced a desynchronized EEG but failed to depress evoked potentials in the reticular formation. In the same study, pretreatment with reserpine followed by mephenteramine failed to alter the Eserine-induced EEG desynchronization and blockade of reticular evoked potentials, while scopolamine in an extremely high dose (1.0 mg/Kg i.v.) blocked the effects of Eserine (vide supra). In the present studies, brain biogenic amine depletion by chronic administration of reserpine failed to alter the desynchronized EEG and attenuation of thalamocortical recruitment evoked by Eserine at doses of 100 mcgm/Kg i.v. (fig. 44) or 200 mcgm/Kg i.v. (fig. 45). DFP (2.0 mg/Kg i.v.) also failed to block thalamocortical recruitment but evoked an EEG desynchronization (fig. 46). Restoration of brain biogenic amines by the combination of a MAOI, which by itself did not attenuate recruitment, and L-DOPA restored Eserine-blockade of recruitment. While Everett (1961) pointed out that MAOI and DOPA combinations could evoke an EEG alert, the current study showed that thalamocortical recruitment could still be evoked and that Eserine could block this
response. In fact, increased brain concentrations of NE by the administration of the MAOI and L-DOPA reduced the dose of Eserine required to attenuate recruitment, to 50 mcgm/Kg (vide infra).

Depletion of brain NE by chronic inhibition of synthesis (αMPT) also evoked a marked increase in synchronized EEG activity with evoked EEG arousal and recruitment responses (figs. 49 and 50). Eserine also failed to attenuate thalamocortical recruitment though an EEG desynchronization was evoked (fig. 51). Restoration of brain amines by MAOI and DOPA resulted in the same response as seen above for reserpine-NE depletion and restoration. While depletion of brain biogenic amines following the chronic administration of either reserpine or αMPT is through different mechanisms, results were similar. EEG patterns showed a marked increase in slow wave sleep activity. Rats have also demonstrated an increase in integrated voltage of the electrocorticogram after multiple doses of αMPT and this response was antagonized by MAOI (Pirch and Rech 1968). These latter authors concluded that the effect of αMPT was similar to reserpine and that a decrease in brain-NE concentration was an important factor in the behavioral and EEG response induced by either drug.
Depletion of brain biogenic amines altered the response to cholinomimetics. In particular, the depletion of the catecholamines, DOPA, DA, NE, and EPI appears to be implicated. Reserpine depleted both catecholamines and indoleamines and thus either serotonin or the catecholamines may be involved. However, αMPT-NE depletion provides evidence that the catecholamines are an essential factor in Eserine-attenuation of thalamocortical evoked potentials. Electrocortical activity of sleep may be associated with serotonin (Koella 1966, Koella et al. 1968, cf. Marczynski 1967), but it is unlikely that serotonin is an essential factor for the Eserine-attenuation of recruitment. In fact, with the marked depletion of serotonin that follows the administration of reserpine (Hess et al. 1956), recruitment can still be evoked and EEG sleep patterns are enhanced (cf. figs. 42 and 43).

In addition to the observation of responses in the presence of depleted brain NE and its subsequent restoration, the responses of drugs and stimuli in the presence of increased brain NE in non-depleted animals were observed. Recruitment was not attenuated by 50 mcgm/Kg i.v. of Eserine, a dose which evoked an EEG desynchronization (fig. 52, cf. Longo 1962). Inhibition of MAO alone did not facilitate
Eserine-attenuation of recruitment (fig. 53). However, L-DOPA in the presence of inhibited MAO increased brain NE (cf. Table III, and Everett 1961), and enhanced Eserine-attenuation of recruitment (fig. 54). Moreover, a transient blockade of thalamocortical recruitment was seen (fig. 55) which was attributable to the reversible nature of the anti-cholinesterase (Longo and Silvestrini 1957, Longo 1962). While complete recovery of the evoked potential was not observed, levels of brain NE were increasing and contributed to the decrease in EEG arousal threshold (Everett 1961, Kety 1967) and to the increased threshold of recruitment. Therefore, instead of depletion of brain NE with subsequent restoration of levels by MAOI plus L-DOPA, brain NE raised above control values in non-depleted animals (Table III, cf. Table II), enhanced behavioral and EEG arousal and lowered the dose of Eserine necessary to attenuate thalamocortical recruitment.

Phenoxybenzamine evoked a biphasic EEG response consisting of a transient EEG desynchronization of less than two minutes followed by a continuous EEG synchronization (figs. 60 and 61). The initial desynchronization (fig. 60) was probably an alpha response and the EEG synchronization was the result of a beta response unmasked by alpha
blockade (Goldstein and Muñoz 1961, Muñoz and Goldstein 1961). On the other hand, beta blockade by INPEA (Teotino et al. 1963) (fig. 65) unmasked only the alpha EEG response of desynchronization (Goldstein and Muñoz 1961, Muñoz and Goldstein 1961), and blocked recruitment.

Integration of thalamocortical recruitment was carried out by a stimulator-controlled, relay operated unit. Inter-recruitment EEG background activity was excluded since the integration unit was in operation only during the presentation of stimuli (cf. Koella and Czicman 1966). Supra-threshold stimuli evoked potentials that were relatively constant in the presence of synchronized or desynchronized EEG background activity (figs. 56 and 57). The amplitudes of the integrated potentials appeared constant but there was no determination made of the area under the curve and this may have varied. Eserine markedly attenuated the integrated thalamocortical potentials (fig. 58) which atropine methyl nitrate failed to antagonize (fig. 59). Eserine antagonized the EEG synchronization induced by phenoxybenzamine and blocked recruitment as well (fig. 62). An increase in the dose of phenoxybenzamine also blocked the EEG desynchronization evoked by Eserine. On the other
hand, beta blockade, attenuated the integrated thalamocortical potentials similar to Eserine (fig. 65, cf. fig. 58). White et al. (1965) noted that Eserine, catechol and amphetamine induced EEG arousal patterns and reticular evoked responses in rabbits. They found that phenoxybenzamine induced EEG synchronization but did not antagonize the reticular evoked potential nor the responses to Eserine. It was concluded that the reticular formation was not pharmacologically homogenous and there were examples of a drug-induced "dissociation" of responses. In an earlier study, White and Daigneault (1959) found that atropine antagonized adrenomimetic CNS stimulants and concluded that an interaction between cholinergic and adrenergic elements was involved in the action of the drug.

Attenuation of thalamocortical recruitment by an anticholinesterase is dependent on the maintenance of brain catecholamine levels but the mechanism is not clear. Eserine evoked an EEG desynchronization that enhanced the duration of paradoxical sleep in decerebrate or in pontine transected animals, yet evoked behavioral arousal in intact preparations (Jouvet 1967a). Lederbur and Tissot (1966) noted that local injections of 5HTP or L-DOPA into the bulbo-pontine somnogenic structures described by Jouvet
(1962) and Moruzzi (Magni et al. 1962) evoked a synchronized EEG when 5HTP was given into the "center" described by Moruzzi, but EEG desynchronization when administered into the "center" described by Jouvet. DOPA evoked an EEG alert pattern when given into either center, and the response was attended by a marked decrease in muscular activity. It is of interest that 5HTP could evoke either slow wave sleep or paradoxical sleep patterns. Jouvet (1967, cf. Marczynski 1967) suggested that serotonergic mechanisms were involved in the processes of falling asleep while noradrenergic mechanisms supported paradoxical sleep. Furthermore, sleep spindles were non-existent during paradoxical sleep and the elicitation of recruitment during activated sleep was markedly attenuated (Yamaguchi et al. 1964). Early descriptions of thalamo-cortical recruitment led to the conclusion that recruitment and rhythmical 8-14 c/s spindle bursts involved the same structures and were the same events (Morison and Dempsey 1941-1942, Dempsey and Morison 1941-1942). Cortical and subcortical sites for the mechanism of recruitment were described by Verzeano et al. (1953). Ajmone-Marsan (1958) concluded that recruitment resulted from activation of
longer or shorter chains of neurons and that the latency characteristic of recruited potentials was not due to conduction times between thalamus and cortex. Latency was a result of events occurring at the level of the thalamus. On the other hand, Velasco and Lindsley (1965) observed that only ablation of the orbital cortex abolished recruitment in thalamus and cortex and they concluded that orbital cortex played a crucial role in the regulation of thalamocortical recruitment in cats. In a later study, Velasco et al. (1968) showed that thalamocortical recruitment and spindle bursts after mesencephalic reticular formation lesions, were abolished only by orbital-frontal cortical ablations. It was concluded that the orbito-frontal cortex was an essential link in the synchronizing systems of thalamocortical nature responsible for spindle bursts and recruitment. On the other hand, Demetrescu and Demetrescu (1962a) proposed an active ascending inhibitory pathway from the pontine reticular formation to the caudate nucleus and then to the cortex. The ascending inhibitory pathway paralleled and was ventral to the ascending excitatory reticular formation pathway and operated in a feedback servo manner. Rocha-Miranda (1965) showed caudate-cortical
connections of a direct nature not dependent on collateral or corticopetal pathways from lower levels. Furthermore, limbic projections to the anterior thalamic nuclei have been demonstrated that altered their excitability and that of the cortex (Sparks and Powell 1966). It has been shown that septal neurons may act as pacemakers for rhythmic activity of the hippocampus (Gogolak et al. 1967), and the latter structure has been proposed as a pacemaker for thalamic neurons (Manzoni and Parmeggiani 1965). Furthermore, Abeles (1967, 1967a, 1967b) in a series of studies on the excitability of thalamic and brain-stem neurons associated with EEG, synchronization and desynchronization, observed that desynchronizing cells had lower chronaxies. He concluded that there were not two sets of neurons; one for synchronization and another for desynchronization but rather one set that responded to both kinds of stimuli with desynchronization being more readily obtainable. Yamaguchi et al. (1964) also had noted that low frequency stimulation of the thalamic nuclei evoked recruitment while high frequency stimulation evoked both EEG desynchronization and behavioral arousal (cf. Longo 1962).

Sleep-wakefulness cycles have been categorized into 4 groups: (1) strong arousal with desynchronized EEG, a
high level of EMG (electromyographic) activity and a high threshold for recruitment, (2) relaxed wakefulness with desynchronized EEG, no paradoxical sleep and a slightly elevated threshold for recruitment, (3) slow wave sleep with synchronized EEG activity, low EMG activity, and a low threshold for recruitment, and (4) paradoxical sleep with a desynchronized EEG, low EMG activity and a threshold for recruitment similar to that seen during relaxed wakefulness. An evaluation of the multiple unit activity of thalamic and reticular neurons during wakefulness, sleep and stages of anesthesia led Goodman and Mann (1967) to conclude that there were no unique differences in levels of activity among them and that characteristics of spontaneous unit firing from the thalamus and reticular formation could not be used to differentiate between waking states, sleep or anesthesia. However, Phillis and Tebecis (1967) found that pentobarbitone blocked the ACh excitatory and the NE inhibitory effect on thalamic neurons without altering the level of excitation of the neuron, which remained responsive. Findlay and Hayward (1966) observed that unit activity from the massa intermedia of the thalamus of rabbits fired biomodally during slow wave sleep but was silent during EEG desynchronization. Pharmacolog-
ical studies on thalamic neurons have revealed a mixed muscarinic-nicotinic type of excitation (Andersen and Curtis 1964) in ventrobasal nuclei of cats. However, McCance et al. (1966) found that muscarinic agonists were the most potent and that atropine sulfate blocked more readily than dihydro beta-erythroidine.

The role of the hippocampus as a pacemaker for the rhythmic activity of the anterior dorsal nuclei of the thalamus has been suggested (Manzoni and Parmeggiani 1965). Intrathalamic pacemaker function has also been proposed by Andersen et al. (1967, 1967a). These authors concluded that the rhythm of the cortical spontaneous barbiturate spindles was under thalamic control while voltage was a function of cortex. And, that during barbiturate anesthesia small assemblages of thalamic neurons generated independent rhythmic discharges that paced the rhythm of the cortexes via thalamocortical columns. It has been reported that rhythmic thalamic unit activity was blocked by electrophoretic application of serotonin and initially by ACh (Andersen and Andersson 1968). However, a secondary excitatory response to ACh appeared during which rhythmic activity was increased. Also, these authors observed that single shocks in the
thalamus as well as electrophoretic application of glutamic acid, evoked rhythmic discharges in the ipsilateral nucleus caudatus. The caudate neurons are depressed by the electrophoretic application of NE, (Bloom et al. 1964). Herz and Ziegelgansberger (1968) observed that DA blocked firing of neurons in the caudate and putamen and that glutamate antagonized DA. Serotonin, NE, and oxotremorine inhibited unit discharges and ACh showed both excitation and inhibition. It was concluded that since the effect of DA and hyperpolarizing currents were the same, that the action of DA on the cell membrane was one of hyperpolarization. ACh content of the caudate has been shown to be increased during periods of increased sleep spindle activity and to be decreased during EEG desynchronization (Traczyk and Sadowski 1964). Chlorpromazine lowered the threshold to single shock-caudate spindles (Tokizane et al 1957), as well as, pentobarbital and synchronized EEG activity (Buchwald et al. 1961). On the other hand, stimulation of the reticular formation, or ablation of the nucleus ventralis anterialis of the thalamus blocked the caudate spindles. A "caudate loop" has been described in the pathway from reticular formation to ventralis anterialis, to caudate, back to ventralis anterialis and then to cortex (Heuser et
Caudate spindles as well as caudate induced EEG arousal depended on the integrity of the thalamic nucleus. Cortical sites that showed spindling in response to caudate stimulation were the same as recruitment sites (Buchwald et al. 1961a), and Buchwald et al. (1961b) proposed an inhibitory caudate circuit to balance the mesodiencephalic activating system. Caudate recruitment has been shown to be dependent on cortical activity and anatomical connections from the anterior thalamic nuclei to the caudate have been established (Kaji et al. 1968). However, Goldring et al. (1963) found that caudato-cortical recruitment differed between monkey and cat. In the macaque monkey, caudate stimulation failed to evoke cortical recruitment whereas stimulation of the internal capsule was successful. It was suggested that caudato-cortical recruitment in the cat was due to spread of the electrical stimulation of the caudate to the afferent sensorimotor cortical fibers in the internal capsule. These fibers lie adjacent to the caudate nucleus in the cat but are located more caudally in the monkey. While the work of Goldring et al. (1963) may explain neuroanatomical relationships in monkey it does not exclude the existence of caudato-thalamic fibers in the cats.
Cholinergic stimulation of the caudate nucleus evoked responses similar to those seen after stimulation of the ventralis anterialis of the thalamus both of which were blocked by atropine (McLennan and York 1966). These workers had also found that stimulation of the ventralis anterialis evoked a release of ACh from the caudate nucleus and concluded that a cholinergic link existed between the thalamus and caudate. While a cholinergic link hypothesis is highly controversial (Burn 1966, Ferry 1966, Boura and Green 1965, Burn 1968), a cholinergic-adrenergic inter-relationship may be involved in the mechanism of action of anticholinesterases on thalamocortical recruitment, with resulting implications on mechanisms of sleep and wakefulness. The presence of cholinergic pathways ascending from (1) the dorsal mesencephalic reticular formation, through the dorsal tegmentum to specific and non-specific nuclei of the thalamus, to cortex and from the thalamus to caudate nucleus and then to cortex and (2) from the substantia nigra through the ventral tegmentum, hypo- and sub-thalamus to basal forebrain area, from which fibers project through the olfactory bulb and cerebral cortex, has been established in rats (Shute and Lewis 1963, 1965, 1966). Krmjević and Silver (1965) confirmed these pathways in primates and Kania
and Szerb (1965) showed that ACh was released from the cortex during arousal evoked by stimulation of the midbrain reticular formation. The cholinergic nature of the mesodiencephalic activating system was described in early papers of Rinaldi and Himwich (1955a, 1955b). Frazier and Boyarski (1967) observed that DFP or Eserine first stimulated and then blocked evoked potentials in the thalamus and cortex. While atropine antagonized these actions, its administration prior to either anticholinesterase did not prevent these effects. It was concluded that cholinergic responses were present at junctional sites of the primary afferent pathway.

Monoaminergic pathways have also been established in the CNS. Andén et al. (1966) described 4 different ascending monoamine neuron systems beginning from the lower brain stem and ascending to the diencephalon and telencephalon: (1) Nigro-neostriatal DA system, (2) Mesencephalic to median forebrain bundle to olfactory tuberculum DA pathway, (3) NE pathway from bulbo-pontine reticular formation to limbic forebrain structures, neocortex and hypothalamus via the median forebrain bundle, and (4) a serotonergic pathway from the mesencephalon to the limbic forebrain and hypothalamus via the median forebrain bundle. Histochemical
evidence has also been presented that demonstrates the presence of AChE and catecholamines in the same nerve trunk in guinea pig vas deferens (Jacobowitz 1965). Walker (1969) has demonstrated the presence of possible ACh vesicles at synaptic sites and adrenergic granules in the same terminal of hypothalamic neurons.

Norepinephrine has been implicated in the mechanism underlying paradoxical sleep (*vide supra*). In addition, oxotremorine or carbachol administered by microinjection into the ponto-mesencephalic reticular formation induced paradoxical sleep and was antagonized by atropine (George et al. 1964). Precollicular decerebration in cats induced subcortical paradoxical sleep and this activity appeared at random intervals. Eserine evoked the same pattern of paradoxical sleep and this was antagonized by atropine (Matsuzaki et al. 1967).

Malcolm et al. (1967) showed an interrelationship between adrenergic and cholinergic mechanisms in the cerebral cortex of rats and interpreted it to be similar to the Eccles-Libet scheme of transmission at the superior and cervical ganglion of the rabbit (Eccles, R. M. and Libet, B. cited in Eccles 1964). Phenoxybenzamine blocked the effect of NE, carbachol, Eserine, and ACh on the P wave
of evoked cortical potentials arising from stimulation of the fore paw of the rat, whereas atropine did not block effects of NE. Furthermore, both atropine and phenoxybenzamine enhanced the evoked potential, but phenoxybenzamine did not increase the effect of atropine while atropine increased the effect of phenoxybenzamine. Therefore, the effect of adrenergic blocking agent was enhanced by the presence of an anticholinergic while the reverse condition did not obtain.

In the present studies in the CNS, the effect of the catecholaminergic system on the AntichE-induced attenuation of recruitment could be interpreted as in figures 68 and 69. Eserine initially induces synchronization followed by a desynchronized pattern which is enhanced by NE. A more intense EEG alert is evoked and finally thalamocortical recruitment is attenuated. The direct effects of Eserine may also be contributing by acting on thalamic neurons (Bradley et al. 1966). The effect of Eserine is transient due to its rapid metabolism while that of an irreversible inhibitor such as DFP or sarin maintains an attenuation of recruitment (figs. 68 and 69). Adrenolytics, and anticholinergic drugs antagonize this effect of anticholin-
esterases as does the marked depletion of catecholamines by reserpine of oMPT. Restoration of depleted biogenic amines by MAOI plus L-DOPA restores the action of Eserine on recruitment. Finally, the effective dose of Eserine required for attenuation of thalamocortical recruitment is lowered by increases in brain levels of catecholamines. The Burn-Rand hypothesis (Burn and Rand 1959, 1962, 1965) suggested that the presence of unbound acetylcholine released, for example by nerve stimulation, promoted an increased permeability of the presynaptic neural membrane to calcium ions and this in turn promoted the release of bound norepinephrine. Moreover, this hypothesis may be viewed as an "adrenergic-link" (Van Meter and Karczmar 1967) as well, in which case the action of acetylcholine may be contingent upon the availability of NE or even a monoaminergic substrate such as DA (Takagi et al. 1968). If the Burn-Rand concept as proposed for sympathetic postganglionic nerves is supported by further experimentation, a similar concept could apply to the CNS as well, and explain the results presented here. However, the Burn-Rand theory may not apply to all systems (Thoenen et al. 1966, Pscheidt et al. 1967). Moreover, it is apparent that the interaction of the systems is not a simple two-step "acetylcholine-
norepinephrine" process. Depolarization of neurons, ionic dependent release of NE (Burn 1966) as well as the possibility of a dopaminergic system (Takagi et al. 1968) may be only a few of the additional factors to be taken into consideration. Recent observations of Pohorecky et al. (1969) have shown that phenylethanolamine-N-methyl transferase activity in the brains of rats, cats, hens, and turtles increased in the presence of the epinephrine substrates, NE and phenylethanolamine. However, the enzyme activity was not homogenous and the greatest activity was seen in the olfactory tubercle and bulb. Hypophysectomy abolished the effects while dexamethasone, which induces the synthesis of phenylethanolamine-N-methyl transferase, resulted in an increased enzyme activity. Intraventricular administration of $^3$HNE resulted in the synthesis and storage of $^3$HEPI. These authors concluded that the activity of phenylethanolamine-N-methyl transferase activity in mammals was the same in brain and in the adrenals though the distribution and activity in the CNS appeared to be concentrated in olfactory areas. In other studies, evaluation of crude pellet fractions disclosed that the rostral brain-stem which consisted of hypothalamus and diencephalon, had the highest synthesis rate of EPI in rats and dogs
REVERSIBLE INHIBITION

Eserine (Active)

Direct Action

AntiChE

Recruitment

Increased ACh

EEG Sleep Increased

NE Released

Desynchronized EEG

Control

Control

Present

Present

Attenuated
Figure 69

**IRREVERSIBLE INHIBITION**

DFP-Sarin (Active)

Direct Action AntiChE Recruitment

Increased ACh

EEG Sleep Increased

NE Released

(Sustained Effect) (Sustained Effect) Desynchronised EEG (Sustained) Attenuated (Sustained)
(Ciaranello et al. 1969). Differential untracentrifugation studies with the crude pellet revealed distribution of enzyme activity throughout subcellular fractions with highest activity in the 22,000g fractions. Ciaranello and co-workers concluded that the mcgm EPI formed per hour per gram tissue in vitro correlated with brain concentrations in vivo and that regulation of endogenous brain EPI could be controlled by an inhibitor found in the particulate fraction as well as by metabolism.

These findings suggest that EPI may also be a factor in the CNS as a neuroregulator or transmitter substance. Alpha blockade by chlorpromazine or phenoxybenzamine would then reflect an unmasked beta activity. Likewise, beta blockade then results in alpha desynchronization with the attenuation of thalamocortical recruitment (cf. fig. 65).

Amphetamine has been shown to evoke an EEG arousal but not attenuate thalamocortical recruitment (Longo and Silvestrini 1967). It has also been reported (Barnes 1966) that while both Eserine and amphetamine evoke EEG alert patterns, the effect of both given together was less than that of either given separately. Either Eserine or amphetamine antagonized barbiturate EEG patterns, but when one was given in the presence of the antagonism induced by
the other, then the degree of antagonism was lessened. Furthermore, in the presence of this attenuated antagonism, atropine always reversed the lessened antagonism and induced a more desynchronized EEG. Barnes concluded that Eserine and amphetamine acted on the ascending reticular activating system on two separate "pools", one adrenoceptive and the other cholinoceptive. The action of amphetamine is probably dependent on the biosynthesis of NE (Bradley 1968), and it has been demonstrated that inhibition of NE synthesis in the CNS blocks its psychomotor stimulant effect (Dingell et al. 1967). Gessa et al. (1969) has recently shown that p-hydroxynorephedrine, a metabolite of amphetamine, replaced NE stores in sympathetic nerve endings and acted as a false transmitter. They also noted that p-hydroxynorephedrine was released in preference to NE by subsequent administration of amphetamine and prevented the entrance of the parent drug into the adrenergic granules. They concluded that amphetamine tolerance was the result of these actions. The presence of a false transmitter, p-hydroxynorephedrine may account for: the less intense desynchronization of the EEG seen with amphetamine as compared with that of beta blockers; increased endogenous NE; and the failure of anticholinester-
ases, as well as the failure of amphetamine to attenuate thalamocortical recruitment.

At this time it is not possible to specify that NE has an absolute role and is the only factor involved in the mechanism of anticholinesterase-induced desynchronization and attenuation of recruitment. But this study does indicate that one of the actions of anticholinesterases that is, the attenuation of recruited potentials from stimulation of the anterior dorsal nuclei of the thalamus, may depend on the maintenance of a catecholaminergic system that includes norepinephrine.
V. SUMMARY

Effects of anticholinesterases on the EEG and thalamocortical recruitment were evaluated in rabbits in which brain biogenic amines had been altered. Chronic reserpine treatment resulted in a marked and maintained depletion of NE which was measured in selected brain parts. αMPT evoked a linear decrease in brain NE that was maintained by chronic administration and also was measured in selected brain parts. Restoration of depleted amines was achieved by the administration of a MAOI and L-DOPA.

EEG activity during states of depleted brain biogenic amines was predominately slow wave sleep with some 8-14 c/s burst activity from the cortices. Peripheral tactile, pain and sound stimuli evoked an EEG desynchronization. Thalamocortical recruitment was present.

Eserine evoked a transient EEG desynchronization but failed to attenuate thalamocortical recruitment. DFP induced an enduring EEG arousal but also failed to attenuate thalamocortical recruitment. The restoration of depleted catecholamines (vide supra) induced an EEG desynchronization during which thalamocortical recruitment could be evoked. Anticholinesterases intensified the desynchroniza-
tion and attenuated recruitment.

Low doses of Eserine (50.0 mcgm/Kg i.v.) that did not attenuate recruitment in the control periods attenuated recruitment after brain biogenic amine levels had been raised by MAOI plus L-DOPA.

Atropine and scopolamine antagonized the EEG effects and blockade of thalamocortical recruitment induced by the anticholinesterases. Alpha receptor blockade by phenoxybenzamine and chlorpromazine antagonized the EEG effects and attenuation of thalamocortical recruitment induced by Eserine. Phenoxybenzamine evoked a biphasic response consisting of an initial brief EEG desynchronization followed by a maintained synchronized pattern. Chlorpromazine induced a prolonged slow wave sleep pattern and also antagonized the effects of the anticholinesterases. Beta receptor blockade by INPEA, evoked an EEG desynchronized pattern with an attenuation of recruitment.

These results are discussed in terms of cholinergic and adrenergic relationships and it is concluded that the attenuation of thalamocortical recruitment by an anticholinesterase may be dependent on a catecholaminergic system.
APPENDIX A

ELECTRODE CONSTRUCTION

Formvar insulated 0.005 inch diameter nichrome wire was used for the preparation of depth electrodes. A two foot length of the wire was formed into a loop, the free ends of which were placed into the jaws of a small table top vise. This loop was straightened by applying a steady upward force with a small rod placed in the loop. The vise was then released from the table and by carefully spinning it, the two sides of the nichrome wire loop were uniformly twisted together and the twisted pair remained linear. Twisted pairs of these wires were cut into 8.0 cm lengths. One end was untwisted for about 1.0 cm and the insulation scraped off so that the bared wires could be soldered to a pair of two foot lengths of #37 gauge stranded cable. To solder the nichrome alloy, a Chromalloy flux was required. Small pieces of 3/64 inch diameter heat shrinkable tubing were used to insulate the solder joints. Electrode assemblies were constructed out of brass hubs and stainless steel tubing shanks according to the dimensions in figure 1 in the text. A short length of
PE# 60 tubing was inserted into the stainless steel shank and trimmed flush with the twisted pair of nichrome wires which was subsequently inserted into the assembly (cf. fig. 2). Prior to the installation of the PE tubing, another two foot of #37 gauge cable had been soldered to the brass hub. The cavity of the hub with the depth electrodes in place was then carefully filled with dental acrylic which was allowed to harden. A single piece of 1/8 inch diameter heat shrinkable tubing was used to cover the completed electrode. Several of these electrodes were prepared in advance and trimmed to the desired stereotaxic coordinates. These electrodes could be used repeatedly for periods of several months in acute experiments.
<table>
<thead>
<tr>
<th>Brain Site</th>
<th>Lateral</th>
<th>Anterior</th>
<th>Posterior</th>
<th>Depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC</td>
<td>6.5</td>
<td>4.0</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td>RAC</td>
<td>6.5</td>
<td>2.0</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td>LPC</td>
<td>6.5</td>
<td>-</td>
<td>4.5</td>
<td>2.0</td>
</tr>
<tr>
<td>RPC</td>
<td>6.5</td>
<td>-</td>
<td>4.5</td>
<td>2.0</td>
</tr>
<tr>
<td>RF</td>
<td>2.0</td>
<td>-</td>
<td>8.5-9.0</td>
<td>12.0</td>
</tr>
<tr>
<td>THAL</td>
<td>1.5</td>
<td>-</td>
<td>3.0</td>
<td>10.0</td>
</tr>
<tr>
<td>OC</td>
<td>2.0</td>
<td>-</td>
<td>8.5-9.0</td>
<td>2.0</td>
</tr>
<tr>
<td>LC</td>
<td>1.5</td>
<td>-</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>BOA</td>
<td>6.5</td>
<td>4.0</td>
<td>-</td>
<td>12.0</td>
</tr>
<tr>
<td>LN</td>
<td>6.5</td>
<td>2.0</td>
<td>-</td>
<td>9.0</td>
</tr>
<tr>
<td>LH</td>
<td>6.5</td>
<td>-</td>
<td>4.5</td>
<td>4.5-5.5</td>
</tr>
<tr>
<td>RH</td>
<td>6.5</td>
<td>-</td>
<td>4.5</td>
<td>4.5-5.5</td>
</tr>
</tbody>
</table>

The coordinates are given in mm and are applicable to New Zealand albino rabbits of 2.5-3.5 Kg body weight.
STAINING SCHEDULE: THIOCHOLINE FOR FROZEN SECTIONS

A. Reagents:

Incubation solutions were prepared from the following reagents.

1. Copper glycine
   glycine 3.75 Gm
   CuSO$_4$$\cdot$5H$_2$O 2.5 Gm
   dist. H$_2$O qs 100.0 ml

2. Maleate buffer
   NaH Maleate 9.6 Gm
   NaOH (N) 51.2 ml
   dist. H$_2$O qs 100.0 ml

3. MgCl$_2$
   MgCl$_2$ 9.52 Gm
   dist. H$_2$O qs 100.0 ml

4. Na$_2$SO$_4$
   Na$_2$SO$_4$ 40% (w/v) adjust to pH 6.0; store at 38°C

5. AThCh
   acetylthiocholine I 46.0 mg
   dist. H$_2$O 2.4 ml
   CuSO$_4$ (0.1 M) 0.8 ml
   Centrifuge for 20 min. Save decanted supernatant.

6. BuThCh
   Butyrylthiocholine I 50.0 mg
   dist. H$_2$O 2.4 ml
   CuSO$_4$ (0.1 M) 0.8 ml
   Centrifuge for 20 min. Save decanted supernatant.

7. Ammonium Sulfide
   NH$_4$OH (con.) 100.0 ml
   dist. H$_2$O 50.0 ml
   H$_2$S saturate
   Store in refrigerator as a stock solution. For use, dilute 1:25 (v/v) with distilled water, and prepare daily prior to use. Test for activity by adding a few drops of 0.1 M CuSO$_4$ solution to a few drops of the (NH$_4$)$_2$S, a brownish black precipitate indicates usable strength.
8. CuThCh  
Copper thiocholine the reaction produce was obtained by initially running control samples through the procedure which saturated the solutions with the reaction product.

B. Solutions:

1. Storage solutions for slides prior to incubation.
   
   (a) DFP  
   DFP (10^{-6} M)  1.5 ml  
   Na_{2}SO_{4} (40%)  9.0 ml  
   dist. H_{2}O  4.5 ml  

   DFP prepared from stock solution of 0.1 M prior to use.

   (b) All cholinesterases (solution "A") and butyrylcholinesterase (solution "C").  

   Na_{2}SO_{4} (40%)  10.5 ml  
   dist. H_{2}O  4.5 ml  

   (c) Acetylcholinesterase (solution "B") and control (solution "D").  

   Na_{2}SO_{4} (40%)  9.0 ml  
   dist. H_{2}O  6.0 ml

2. Incubation solutions for slides after appropriate storage solution treatment.

<table>
<thead>
<tr>
<th>All ChE (solution&quot;A&quot;)</th>
<th>AChE (solution&quot;B&quot;)</th>
<th>BuChE (solution&quot;C&quot;)</th>
<th>Control (solution&quot;D&quot;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagents:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuGlycine</td>
<td>0.6 ml</td>
<td>0.6 ml</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>H_{2}O</td>
<td>0.6 ml</td>
<td>2.1 ml</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>Mal.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Na_{2}SO_{4}</td>
<td>10.5 ml</td>
<td>9.0 ml</td>
<td>10.5 ml</td>
</tr>
<tr>
<td>MgCl_{2}</td>
<td>0.6 ml</td>
<td>0.6 ml</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>CuThCh</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>AThCh</td>
<td>1.2 ml</td>
<td>1.2 ml</td>
<td>-</td>
</tr>
<tr>
<td>BuThCh</td>
<td>-</td>
<td>-</td>
<td>1.2 ml</td>
</tr>
</tbody>
</table>
DFP pretreatment was carried out for solutions B and D where the concentration of $10^{-6}$M specifically inactivated BuChE. Incubation time for brain sections was maintained at 60 minutes.

C. Staining Schedule:

Frozen sections were cut in the cryostat at 16 microns, placed on slides and dried at room temperature for 5-10 minutes prior to staining schedule:

1. Appropriate storage solution for 30 minutes.
2. Incubation solution for 60 minutes at $38^\circ$ C.
3. Rinse solution ($20\% \text{Na}_2\text{SO}_4$ saturated with CuThCh) 5 minutes at $38^\circ$ C.
4. Rinse solution ($10\% \text{Na}_2\text{SO}_4$ saturated with CuThCh) 1 minute at room temperature.
5. Distilled water 1-2 sec.
6. Ammonium sulfide developer solution (20 sec).
7. Rinse solution (distilled water or 15% EtOH).
8. Fixation in 10% formalin if desired.
9. 35% alcohol (15 sec).
10. 50% alcohol (15 sec).
11. 70% alcohol (15 sec).
12. 80% alcohol (15 sec).
13. 95% alcohol (15 sec).
14. abs. alcohol (1 min).
15. abs. alcohol (1 min).
16. Xylol (1 min).
17. Xylol (1 min).
18. Mount in permount cover and read. Store in absence of light.
STAINING SCHEDULE: THIONIN FOR FROZEN SECTIONS

A. Solutions:

1. Thionin (0.5% in 20% alcohol)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thionin</td>
<td>475.0 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>150.0 ml</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>40.0 ml</td>
</tr>
</tbody>
</table>

2. Eosin Y

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosin Y</td>
<td>2.0 Gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>25.0 ml</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>475.0 ml</td>
</tr>
</tbody>
</table>

(Filter into staining dish and use as needed.)

B. Staining Schedule:

Frozen sections were cut in the cryostat at 32 microns thickness, placed on slides and dried at room temperature for 5-10 minutes prior to staining.

1. Thionin (30-60 sec)
2. Distilled water (wash at least 1 min)
3. 35% alcohol (15 sec)
4. 50% alcohol (15 sec)
5. 70% alcohol (15 sec)
6. 80% alcohol (15 sec)

7. 95% alcohol (15 sec)
8. Eosin Y (dip)
9. 95% alcohol (15 sec)
10. Abs. alcohol (1 min)
11. Abs. alcohol (1 min)
12. Xylol (1 min)
13. Xylol (1 min)
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sarin</th>
<th>DFP</th>
<th>Control</th>
<th>Sarin &amp; AMN</th>
<th>Sarin &amp; AtSO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>211.98(192.92-229.36)²</td>
<td>1.69(-1.61-8.92)²</td>
<td>17.75(15.28-20.22)²</td>
<td>176.54(161.53-200.01)³</td>
<td>13.85(10.07-20.20)³</td>
<td>18.71(13.34-24.20)³</td>
</tr>
<tr>
<td></td>
<td>22.57(19.49-25.65)²</td>
<td>6.83(3.58-10.07)²</td>
<td>3.58(3.00-4.70)²</td>
<td>24.53(23.41-25.65)³</td>
<td>4.81(0.0-9.62)³</td>
<td>8.421(-----)³</td>
</tr>
</tbody>
</table>

1. Cholinesterase activity is expressed as microliters of CO₂ of a 1:50 brain to bicarbonate (0.03M) buffer dilutions from one-half brain samples divided at the midsaggital section. AChE determinations were made in triplicate and the BuChE determinations in duplicate.

2. Figures represent the average values at 60 minutes with the range expressed in parenthesis.

3. Figures represent the average values at 40 minutes with the range expressed in parenthesis.
BLOCK DIAGRAM—INTEGRATION OF EVOKED RESPONSES

- Stimulator
- Trigger
- Signal (Input)
- Integrator (Output)
- DC Amplifier
- Read Out
A. Reagents:

1. n-Butanol, purified with HCl and distilled water and the aqueous phase removed with NaCl.
2. n-Heptane, washed with NaOH, HCl and distilled water.
3. Iodine reagent 0.1 N (in abs. alcohol)
4. Sodium thiosulfate 0.05 N
5. Acetate buffer, 2.0 M, pH 5, prepared by 2:1 (v/v) 2.0 M sodium acetate and 2.0 M acetic acid
6. Acetate buffer, 2.0 M, pH 3, prepared by 50:1 (v/v) acetic acid (2.0 M) and sodium acetate (2.0 M)
7. Alkaline ascorbate solution. Prepared just before use by 2:1 5.0 N NaOH and 1% aqueous ascorbate solution.
8. Standard NE solution. A stock solution of 0.005% is prepared and refrigerated in a light tight container. From this concentration of 50.0 mcgm/ml working standards are prepared.
B. Procedure:

1. Chilled homogenization of weighed brain parts in 2.0 ml of 0.01 N HCl.
2. Homogenate transferred to 35 ml reaction vessel containing 2.0 Gm NaCl and 20.0 ml n-Butanol.
3. Shake for one hour.
4. Centrifuge 10 minutes at 1500 rpm.
5. Butanol aliquotes (10.0 ml) are transferred to reaction vessels with 2.0 ml 0.01 N HCl and 15.0 ml of n-Heptane and are shaken for 15 minutes.
6. Centrifuge 15 minutes at 1500 rpm.
7. Transfer 1.5 ml aliquote to test tube.
8. Add 0.5 ml of pH 5.0 acetate buffer for NE.
   Add 0.5 ml of pH 3.0 acetate buffer for E.
9. Add 0.05 ml of 0.1 N iodine reagent.
10. Inactivate iodine with 0.1 ml sodium thiosulfate (0.05 N).
11. Add 0.5 ml of alkaline ascorbate to all test tubes except reagent blank and tissue blank.
12. Add 0.3 ml of 5.0 N NaOH to each of the tissue blanks and reagent blanks.
13. Use 1.0 mcgm NE to determine reagent blank.

15. Add 0.2 ml of ascorbate solution 10 minutes prior to reading.

16. Read in Aminco-Bowman spectrophotofluorometer at 400 u activating wavelength and at 520 u fluorescent wavelength.

17. Calculation:

   Tissue Factor (TF) = 1000/wt (mg)

   Corrected Reading = Initial Reading - Tissue Blank

   Corrected NE Reading = Initial Reading of Standard-Reagent Blank Reading

   Standard NE Constant = $K_{ne} = \text{Total mg NE/total corrected NE readings}$

   NE Concentration = (TF) ($K_{ne}$) (Corrected Reading) = NE mcgm/Gm
LITERATURE CITED


Walker, C. A. (1969) Ph.D. Dissertation; Department of Pharmacology, Stritch School of Medicine, Loyola University, Hines, Illinois.


The dissertation submitted by William G. Van Meter has been read and approved by four members of the faculty of the Graduate School of Loyola University and by Dr. T. J. Marczynski, a faculty member of the Graduate College of the University of Illinois.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

4 March 1970

Date

Signature of Advisor