A Study of Acetyl Cholinesterase Synthesis in Developing Rat Brain Following Diisopropylphosphofluoridate Treatment as a Model System to Evaluate Macromolecular Synthesis at the Synapse

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A STUDY OF ACETYL CHOLINESTERASE SYNTHESIS IN DEVELOPING RAT BRAIN FOLLOWING DI-ISOPROPYLPHOSPHOFLUORIDATE TREATMENT AS A MODEL SYSTEM TO EVALUATE MACROMOLECULAR SYNTHESIS AT THE SYNAPSE

by

RAJAMMAL SRINIVASAN

A Dissertation Submitted to the Faculty of the Graduate School of LOYOLA UNIVERSITY OF CHICAGO in Partial Fulfillment of the Requirement for the Degree of DOCTOR OF PHILOSOPHY

August, 1974
The protein requirement of synapses poses a unique problem as they are located at some distance from the cell body. The macromolecular requirement of synapses is a problem by itself in normal state, as well as in pathological conditions. The question arises as to whether synapses synthesize all their proteins or do they depend on the cell body for proteins. As a model to study such a mechanism, we chose a system of isolated synaptosomes and microsomes from 20 and 58 day rat brain previously injected with DFP and studied specifically the synthesis of AChE. In 20 day old rats, following DFP, 47% of the inhibited AChE was synthesized at nerve endings, while for the same period 42% of the inhibited enzyme was synthesized at microsomes. Since this period represents a period of rapid growth in the animal, attempts were made to differentiate the enzyme arising due to normal growth and those arising due to enzyme replacement after DFP inhibition. After the appropriate corrections, it was found that AChE synthesized to replace the DFP inhibited enzyme amounted to 43% at the nerve endings and to 16% at the microsomes. In 58 day old rats, 64% of the inhibited nerve ending AChE was synthesized and for the same period microsomes synthesized 45% of the inhibited enzyme. This phenomenon was not an age dependent one as it was found in both age groups. AChE was found to be synthesized at nerve endings without a concomittant
increase or decrease of microsomal enzyme which suggested an independent synthesizing machinery of nerve endings. When Triton X-100 was used to solubilize AChE, the inhibition due to DFP was considerably less in nerve terminals and microsomes compared to DFP inhibition measured in buffer treated fractions. These results suggest that a reservoir of the enzyme is not available to substrate and to inhibitors such as DFP.

We also found differences in the rate of inhibition and recovery of isoenzymes in the microsomal and nerve ending fraction following DFP which further confirmed the independent synthesizing machinery of the nerve endings. On the day of injection, the order of inhibition of the 4 microsomal isoenzymes was 81, 49, 25 and 0% for isoenzymes 4,3,2,1. Fifteen days following DFP, the inhibition of isoenzyme 4 was 36% and isoenzyme 2 was 25%. In the same period, inhibited isoenzyme 3 was completely replaced by newly synthesized AChE. The inhibition and recovery of nerve ending isoenzyme were also studied in a manner similar to those of the microsomal isoenzymes. On the day of injection, the isoenzymes 4,3,2 and 1 were inhibited 83, 30, 4 and 82% respectively. Fifteen days following DFP injection, the inhibition of isoenzyme 4 and 1 was 16 and 25 percent respectively. Inhibited isoenzyme 3 and 2 were completely replaced by newly synthesized enzyme. The isoenzymes of nerve ending demonstrated different order of sensitivity to DFP and of the recovery rate indicating a different turnover rate.
Certain hypothesis were made regarding the function of these isoenzymes. Microsomes might supply the enzyme around the cell body which prevents the proliferation of Ach into cell from other sources. Nerve endings might supply the enzyme at the junctional complex whose main role is to hydrolyze the transmitter following chemical transmission.
VITA

The author, Rajammal Srinivasan, was born in Kolar Gold Fields, Mysore State, India on February 2, 1940.

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Rate of Regeneration of AChE following DFP. (Paper in preparation).
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Angstrom</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>BuChE</td>
<td>Butyrylcholinesterase</td>
</tr>
<tr>
<td>ChAc</td>
<td>Choline acetyltransferase (Choline Acetylase)</td>
</tr>
<tr>
<td>ChE</td>
<td>refers collectively to AChE and BuChE</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CuSO₄⁻</td>
<td>Copper sulfate</td>
</tr>
<tr>
<td>DE</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DFP</td>
<td>Diisopropylphosphofluoridate (Diisopropyl fluorophosphate)</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
</tr>
<tr>
<td>gm</td>
<td>gram</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>i.c.</td>
<td>Intracerebral route of administration</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous route of administration</td>
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<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
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<td>ml</td>
<td>Milliliter</td>
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<td>mm</td>
<td>Millimeter</td>
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<td>mg</td>
<td>Milligram</td>
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<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;S&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Sodium sulfate</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>(NH&lt;sub&gt;4&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;S&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Ammonium sulfate</td>
</tr>
<tr>
<td>P-2AM</td>
<td>Pyridine 2-aldoxime methiodide</td>
</tr>
<tr>
<td>P-4AM</td>
<td>Pyridine 4-aldoxime methiodide</td>
</tr>
<tr>
<td>pH</td>
<td>Negative log of hydrogen ion concentration</td>
</tr>
<tr>
<td>PSP</td>
<td>Postsynaptic potential</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>S.E.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>uc</td>
<td>microcurie</td>
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<tr>
<td>ug</td>
<td>microgram</td>
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<tr>
<td>ul</td>
<td>microliter</td>
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<tr>
<td>tris</td>
<td>Trihydroxy amino methane</td>
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INTRODUCTION

BACKGROUND

The studies of the ultrastructure of the CNS demonstrated that the central nervous system is a highly organized assembly of cells as originally postulated by His (1886), and not a continuous syncytium as originally suggested by the reticularists (Golgi, 1907). Two types of cells are found: the neurons or nerve cells and the neuroglia or supporting cells. Although both types of cells maintain the architecture of nervous system, the transmission and integration of information relayed by the nerve impulses are the unique function of neurons.

STRUCTURE OF THE NEURON AND OF SYNAPSES

The neuron is a complete anatomical unit composed of a nucleus, cytoplasm and a surrounding membrane which protects the cell constituents and forms a boundary with respect to other elements in the nervous tissue. Varieties of neurons may be differentiated on the basis of the differences in size, shape, distribution and on the number and spread of their processes. The typical neuron has a soma, a series of processes called dendrites, and an axon. Dendrites originate at the soma and distribute in all directions; the axons stem from the soma or from a large dendrite and terminate at varying distances in contact with other nerve cells, muscle
fibers or with gland cells.

In the vertebrates, neurons can be monopolar, bipolar or multipolar, depending upon the pattern and characteristics of their processes. Most neurons have a large number of dendrites emerging from cell body and for this reason are said to be multipolar. When a single process arises from cell body, the neuron in question is monopolar; when two processes arise from cell body, the pertinent neurons are bipolar. In mammals the diameter of the neuronal soma varies from few microns, as in the case of granular cells in cerebellar cortex, to close to a tenth of a millimeter for the large vestibular cells of the medulla oblongata and the motor neurons in the anterior horn of the spinal cord.

Dendrites are extensions of the perikaryon of a neuron. They contain the same organelles as the perikaryon and are never ensheathed by myelin. Axons on the other hand may or may not be ensheathed by myelin. Microtubules are the most prominent elements in the cytoplasm of large dendrites arising from multipolar cells. Other cytoplasmic organelles are neurofilaments, endoplasmic reticulum, mitochondria, golgi apparatus, vesicles and multivesicular bodies. A granular endoplasmic reticulum shows distribu-
tion similar to that of perikaryon. In axon, few ribosomes are present in the initial segment, but they do not extend beyond it. The remainder of the axon is devoid of ribosomes, but it contains mitochondria, neurofilaments and multivesicular bodies; it is characterised by absence of two components that are present elsewhere, granular endoplasmic reticulum and free ribosomes.

The morphology of the neuronal contacts is consistent with Ramon y Cajal's neuron theory; this theory states in effect that the transmission of nerve impulses from one cell to another is mediated through functional contacts specialized for transmission, which were defined originally by Sherrington (1897) as synapses. Electron microscopy and subcellular neurochemistry has enormously advanced our knowledge of synapses in the central nervous system; the pertinent studies were particularly advanced by three modern investigators and their associates: De Robertis (1958, 1959), Palay (1958) and Whittaker (with Gray, 1962). As can also be seen in our own microphotographs (cf. Results), the presynaptic fiber ends in an expanded terminal, the synaptic knob which is intimately associated with a portion of the membrane of the postsynaptic neuron. The subsynaptic membrane is separated from the synaptic knob by a very narrow and remarkably uniform space, the synaptic cleft.
The synaptic knob contains several mitochondria and, most characteristically, a large number of synaptic vesicles, 300 to 600 Å in diameter. Synaptic vesicles were independently recognized by two early investigators who employed electron microscopy (De Robertis and Bennett 1954, 1955; Palade and Palay, 1954); the synaptic vesicles are of particular significance because there is much evidence that they contain preformed packets of the transmitter.

FUNCTION OF THE NEURON

In the brain, transmission across relatively significant distances is carried by axons; this type of transmission may be referred as conduction or propagation of nerve impulses which occurs in an all or none fashion. The axons are specialized for conducting information from one place to another. The impulse discharged travels along the various branches (10 to hundreds) of an axon, to activate synapses thereon. According to the principle of convergence, neurons receive synapses from many neurons; these synapses can be excitatory or inhibitory. The synapses are the locus of the so-called synaptic transmission. The number of synapses at a neuron vary, the highest recorded being 80,000. A neuron receives information through synapses located on its dendrites and soma, integrates this information and propagates it over its axon to other nerve
We turn now to the question of how nerve impulses spread down the axon. The key to the mechanism of this impulse propagation are the all-or-none principle of propagation, as well as the phenomenon of passive spread. At any point in the axon, the action potential spreads to the neighboring regions where the depolarization acts as a stimulus to produce another action potential and so on down the axon. The all-or-none method of propagation assures that the action potential will always be full-sized, thereby minimizing the chances that it will get lost along the way. In unmyelinated nerve and in muscle, the spread of current is continuous and conduction appears to be a uniform process. The propagated action potential is further accelerated by glial myelin sheaths causing the action potential in myelinated axon to leap from node to node. The conduction is discontinuous or saltatory and the impulse skips from one node to node in myelinated nerve fibers as proposed originally by Lillie (1925). The active generation of current (generation of action potential) is confined to the nodes of Ranvier. The myelin acts as an insulator with a low electrical capacity which increases conduction velocity by making the local circuit act at a considerable distance ahead of the active region. Applying
the sodium permeability theory to saltatory conduction, it can be said that sodium ions enter myelinated axons at the nodes but depolarize the whole fiber by local circuit action. No one has yet been able to test this particular point directly but there is now a large body of indirect evidence in favor of saltatory theory (Tasaki, 1939, 1955).

When the nerve impulses reach the axon's synaptic terminations on a dendrite, axon or soma, nerve impulse frequency appears to be transduced into a postsynaptic depolarization or hyperpolarisation similar in magnitude electrically to the one which originally generated the axon nerve impulse. It has been shown that the arriving nerve impulse causes the release of a small quantity of a chemical from presynaptic terminal generically known as a transmitter substance which diffuses across the synaptic cleft and results in the dendritic electrical response (depolarisation or hyperpolarisation). Unlike the axonal membrane, the dendritic membrane is said to be only chemically excitable. Ions may depolarise a dendritic membrane, but the release has to be induced by a chemical transmitter.

SYNAPTIC TRANSMISSION

There is sufficient evidence to indicate that the chemical transmitter acetylcholine (Ach) is released in uniform packets at nerve muscle junction (quanta) consisting of a relatively large number of molecules (Hubbard, 1970). The
quantal nature of transmission is currently thought to be consequence of the storage of Ach in synaptic vesicles found in motor nerve terminal, ganglia and central neurons, the contents of each vesicle forming a quantum.

Quantal release is thus equivalent to release of the contents of a synaptic vesicle into a synaptic cleft. Results accumulated indicate that basically the same process is present at many peripheral and central synapses where various chemical mediators, including Ach, are involved in the transmission of impulses.

The transmitter substance parcelled inside a vesicular bag is separated from the postsynaptic target by two barriers, the vesicular membrane itself and the membrane of axon terminal. Release probably occurs upon the collision between vesicular and axonal membranes. As proposed by Katz (1966), the release of transmitter occurs by exocytosis (collision between vesicle and axonal membranes). Ultrastructural evidence to support this hypothesis was obtained in several instances as in the case of secretion of zymogen granules (Palade, 1959) and in that of neurosecretory activity (Smith and Smith, 1966). There are suggestions that this process is rapidly reversible so that a vesicle having discharged its contents would quickly detach itself from
the membrane and start reaccumulating the transmitter substance. Similar processes occur for various transmitters centrally and peripherally.

After the transmitter is released from its storage site by presynaptic action potential, it impinges upon the postsynaptic side and combines with specific macromolecules (the receptors).

The receptors control channels in the subsynaptic membrane for the passage of Na+, K+ and Cl−. By altering the conformation of the receptor protein, the transmitter induce the postsynaptic potential (PSP). The postsynaptic potential could either depolarize or hyperpolarize the cell. Since depolarization of the neuron results in the production of nerve impulses, the depolarizing postsynaptic potential is termed excitatory postsynaptic potential (EPSP). The hyperpolarizing postsynaptic potential is termed inhibitory postsynaptic potential (IPSP). IPSP opposes the action of EPSP's and tends to prevent the generation of action potential. The generation of EPSP or IPSP depends upon the nature of postsynaptic cell receptor and of the particular transmitter agent involved. They can occur in the same cell.
EPSP's and IPSP's arriving over different synapses upon the neuron are summated approximating simple algebraic addition. The effect of IPSP is subtracted from total EPSP's. This property is known as spatial summation. Thus, various inputs to a neuron combine by summation to determine the single output. As the dendrites are electrically unexcitable, there must be some region of the cell closest to the dendrites which is electrically excitable. This region is believed to be axon hillock, the junction between soma and axon. Thus, a depolarization arising in a dendrite spreads passively down the dendrite through the soma and finally to the axon hillock where nerve impulses can be generated. The postsynaptic cell integrates and summates these potential; when the threshold is reached it gives off in terms of the all-or-none principle action potential at the axon hillock which is then propagated to each of its own axon terminals providing for the continuation of the impulse. Summation of the impulse is carried out by the postsynaptic cell.

NATURE AND LOCALIZATION OF THE PATHWAYS IN CENTRAL NERVOUS SYSTEM

Due to difficulties involved in studying the various pathway in central nervous system, their nature and localization have not yet been fully determined. Various approaches have been utilized. Macro- and micro-electrodes were employed
to study the neuronal responses to pertinent drugs and putative neuro transmitters. It was also attempted to demonstrate the release of transmitter upon the stimulation of nerve tracts and neurons. In the case of the central nervous system, several criteria have to be met to establish a substance as a neuro transmitter.

(a) The appropriate nerve soma or set of nerve axons is selectively stimulated and the release of the transmitter detected upon passage of action potentials. In the case of the brain it is however impossible today to detect and chemically analyze the release without destroying the functional and structural integrity of the region of the brain being analyzed. Furthermore, it is difficult or impossible to detect such a release upon the stimulation of a single neuron or of a homogeneous group of neurons. However, the release was studied in brain slices incubated in physiological buffer solutions in vivo. They contain, of course, heterogeneous neuronal populations.

(b) Indirect evidence is derived from the study of the distribution of the putative transmitters and of the enzymes involved in the synthesis and in the breakdown of the transmitter.

(c) Furthermore the study of the pharmacology of responses to presynaptic stimulation as compared to that of the responses to applied putative neurotransmitter may yield indirect evidence for the role of the latter in synaptic transmission.
ROLE OF Ach AS A TRANSMITTER IN THE CENTRAL NERVOUS SYSTEM

In 1954, Eccles and his associates provided pharmacological evidence for the cholinergic nature of spinal synapses. Acetylcholine was shown to be the transmitter in the motor neuron collaterals to the Renshaw cell (Eccles et al., 1954, 1956). In the subsequent work of Eccles, Curtis and Ryall (1958) a multimicro electrode system with a multibarrel micropipette was employed for iontophoretic release of drugs and for unit fire recording. Ach, nicotine and cholinomimetic substance were effective in increasing the rate of or initiating discharge from the Renshaw cell.

Searching for cholinoceptive neurons in other central areas, Curtis and Koizumi (1961) and Bradley and Wolstencroft (1965), Curtis and Crawford (1969) studied medullary and mesencephalic reticular formation and the inferior colliculus. Cells responding to Ach were found only in the latter region. The response to Ach were not affected by dihydro e-erythroidine, d-tubocurarine and physostigmine. Parenthetically, inferior colliculus contains moderate amount of ChAc and ChE.

Further studies in which iontophoretic methods were employed demonstrated that the following sites are cholinoceptive: Caudate nucleus, ventral basal thalamus, lateral geniculate body, the supraoptic nucleus and postomedial parts of the thalamus, cochlear nucleus, brain stem, pyramidal cells

Furthermore, Krnjevic and Phillis (1963) suggested that Ach may act as a sensory transmitter at the thermal receptors. Similarly, taste fibre endings and chemoreceptors also seem to be Ach sensitive and excitable upon application of Ach.

Two additional lines of evidence should be added. Synaptic vesicles were found in association with Ach and miniature postsynaptic potentials in the mammalian and amphibian brain (Katz and Miledi, 1963; Brock et al., 1951). These miniature potentials were not analysed pharmacologically and their presence, as well as that of vesicles, cannot as yet be considered as direct evidence of the cholinergic nature of pertinent central synapses (Eccles, 1964).

Second, several investigators have shown that in the presence of anti-ChE's, Ach is continually released from the brain (Elliott et al., 1950; MacIntosh and Oborin, 1953) and that administration of anti-ChE agents increases brain levels of Ach (Michaelis et al., 1957).
It should be stated that pharmacological data are conclusive only with regard to cholinergicity of the synapse at the Renshaw cell, and perhaps with certain synapses in the cortex, thalamus and in the cerebellum, and in the supra optic nuclei. It is possible that central cholinergic cells respond differentially to muscarinic and nicotinic substances. The muscarinic response may be modulatory and facilitatory in character, as proposed by Krnjevic (1969), this can be also argued on the basis of analogy between the central and ganglionic muscarinic responses (Karczmar, et al., 1969). It should be added that Bradley and Wolstencroft (1965) described a fast, depressant responses of certain cortical and subcortical neurons. Again, this response may illustrate the modulatory rather than synaptic role of central Ach. While otherwise central cholinergic synapses may be scattered, they are presumably important in the reticular formation and limbic system or diffuse mesendiencephalic system.

**MEMBRANE EFFECTS OF Ach:**

Ach has been found to have various membrane effects other than the neuro transmitter activity.

Ach stimulates the incorporation of inorganic phosphate into phospholipid (Hokin, 1966). It has been shown that Ach
at a concentration of $10^{-4}$ M stimulates the incorporation of $^{32}$P into phospholipid, especially phosphoinositol and phosphatidic acid, as demonstrated in the case of the salt gland, sympathetic ganglia, brain slices and brain homogenates. Current evidence suggests that Ach stimulates the hydrolysis of phosphoinositides in synaptosomes to form membrane bound diglyceride; it is thought that diglyceride is rate limiting in the synthesis of both phosphatidic acid and phosphoinositide.

Ach seems to be involved in the liberation of bioactive substances. Itokawa and Cooper (1970) have shown that Ach will release thiamine from perfused nerve preparations; it has also been shown to release catecholamine from chromaffin cells and possibly at other sites (Glisson and Karczmar, 1971; Burn, et al., 1963). Thus, Ach may also participate in the central phenomenon by releasing active bioamines such as norepinephrine (NE) or dopamine (DE). Ciliary movement in gill plates of Mytilus edulis and ciliary motility in respiratory and esophageal tracts in mammals are supposed to be controlled by Ach (cf. Karczmar, 1963).

**ROLE OF Ach IN BEHAVIOR:**

A short description of the role of Ach in behavior was included to show the importance of cholinergic system and to justify our work on AChE. An attempt has been made to correlate behavioral changes with the Ach content of the brain.
(Stein, 1970; Karczmar, 1970). Ach level seems to be decreased during convulsions. There was an increase in Ach content during sedation or anesthesia. Furthermore, it was hypothesized that the relation between the adrenergic and cholinergic system is significant in the control of behavior. Earlier investigators suggested that behaviorally, cholinergic system is opposed to adrenergic system (Frommel et al., 1947\textsuperscript{a}, 1947\textsuperscript{b}, 1951; Aprison, 1969). Carlton (1963, 1964) advanced this hypothesis on a more sophisticated level. He suggested that cholinergic system preferentially antagonizes adrenergic activation of unrewarded behavior and minimizes the impact of irrelevant stimuli. Therefore, blockade of unrewarded behavior by the cholinergic system should lead to better learning. This is confirmed by data of some (Whitehouse, 1959) but not all investigators (Bignami and Rosic, 1971).

Another generalization was proposed by Rosenzweig and his collaborators. They associated very small differences in AChE and Ach activity of rat cortex with "flexible" behavior. Rats kept in complex environment showed 2-6\% more cerebral AChE activity and cortical weight than rats bred in isolation.

Altogether, it has been suggested that cholinergic system is responsible for suppression of avoidance and of
non-rewarded operant behavior, for depressed overt behavior and sleep, and for flexible response to environmental changes; more specifically, it was shown that the cholinergic system is involved in a number of appetitive and emotional behavior including aggression and sexual function, temperature control, etc. (for references, cf. Karcamar, 1975). Finally, behavior pattern must involve most complex circuitry, in which the cholinergic system plays a limited if not a minor part (Eccles, 1964a, 1964b; Karczmar, et al., 1971). Thus, this system may have simple or key role and it is impossible to assign it to specific neurophysiological substrates of behaviors in question.

**ANABOLISM AND CATABOLISM OF ACETYLCHOLINE: SYNTHESIS:**

Acetylcholine is synthesized in a reaction catalyzed by choline acetyltransferase (choline acetylase):

\[
\text{ATP + acetate} \rightarrow \text{Adenyl acetate + pyrophosphate}
\]

\[
\text{Adenylacetate + CoA} \rightarrow \text{Acetyl CoA + AMP}
\]

\[
\text{Acetyl CoA + Choline} \rightarrow \text{Ach + CoA}
\]

Choline acetylase

Thus, the overall reaction is

\[
\text{Acetate + ATP + Choline} \rightarrow \text{Ach + AMP + pyrophosphate}
\]

From the experiments on the superior cervical ganglion both in Feldberg's and in MacIntosh's laboratories and from the studies of Fitzgerald (1968), it appears that choline is rate limiting in the synthesis of acetylcholine.
Pathways involved in the anabolism and catabolism of acetylcholine are as follows:

**Phosphatidyl ethanolamine**

Sadenosyl methionine  
\[ \text{Phosphatidyl} \]

Phosphatidyl choline

Patty acid

Glycerophosphocholine

**Citrate**  
**Glucose**  
**Choline**  
**Phosphoryl**

**CTP**

**HYDROLYSIS:**

Enzymatic hydrolysis of Acetylcholine is performed by cholinesterases which hydrolyse acetylcholine into choline and acetic acid.

\[ E + \text{Ach} \rightarrow E \text{ Ach} \]

\[ E \text{ Ach} \rightarrow \text{Acetyl E + Choline} \]

\[ \text{Acetyl E + H}_2\text{O} \rightarrow E + \text{Acetate} \]

It is universally agreed that cholinesterases form a family of enzymes, isozymes and variants; in fact, we cannot be
certain how many cholinesterases exist in the body and what are their specific roles. This subject will be discussed now.

**PROPERTIES OF CHOLINESTERASES: TYPE OF CHOLINESTERASES**

Augustinsson (1963) defined cholinesterases as a group of esterases which hydrolyse choline esters at higher rates than non-choline esters, when hydrolysis rates are compared at optimum conditions as regards substrate concentration, pH, ionic strength, etc. Two types of cholinesterases most frequently encountered in mammals are the serum enzyme designated at various times as pseudo or non-specific S-type ChE and at present as cholinesterase (EC, 3.1.1.8) and the true, specific or C-type ChE, at present designated as acetylcholinesterase (AChE) (EC, 3.1.1.7).

Other related esterases are known. Ali- and aryl-esterases do not hydrolyse choline esters while they are effective against alkyl and aryl esters respectively. These and other ChE's may be distinguished by their inhibitory characteristics; as shown early by Mounter and Whittaker (1950). ChE's are inhibited by organophosphorus compounds and carbamates, aliesterases are inhibited by organophosphorus compounds but not by carbamates, while aryl esterases are inhibited by neither organophosphorus compounds nor carbamates. The organophosphorus anticholinesterases such as
diisopropyl phosphofluoridate (DFP) are generally more potent inhibitors of cholinesterases than of acetylcholinesterase, whereas oxamide derivatives (Ambenonium) are about two thousand times more potent as an inhibitor of acetylcholinesterase than of cholinesterase (Lands et al., 1955).

In the further discussions, AchE will refer to true or specific cholinesterase, BuChE will refer to pseudo or cholinesterase.

The two groups AchE and ChE may be differentiated generally not only by means of selective inhibitors, but also by means of specific substrates. AchE hydrolyses Ach most rapidly, but the other choline esters very slowly. AchE is inhibited by high substrate concentration; when the hydrolysis rate obtained with pure AchE is plotted against the logarithm of substrate concentration, bell shaped curves are obtained. The optimum substrate concentration for the AchE - Ach system is at about 3x10^{-3}M and the dissociation constant (Ks) for the AchE-Ach complex is about 5x10^{-4}M. All ChE's so far studied split butyryl choline at a higher rate than other esters of choline and give the usual dissociation curves (Ks=2x10^{-3}) with butyryl choline, propionycholine and Ach. The esterase activity is not inhibited by high concentrations of these substrates.
**ACETYLCHOLINESTERASE:**

Acetylcholinesterase is of particular importance because of its role in synaptic and neuroeffector transmission. AchE has been analyzed thoroughly at the molecular level by various workers, beginning with Nachmansohn and Wilson (1951). Acetylcholinesterase has been crystallized from electric eel (Kremzner and Wilson, 1964). An electrophoretically homogeneous moiety was found to have a molecular weight of 240,000 (Leuzinger and Baker, 1967).

Nachmansohn and Wilson (1951) found the two main subsites at the active centre of AchE, one being the anionic site which attracts the positive charge in Ach; second, about 5 Å distant from the anionic site, the esteratic site which binds the carboxyl carbon atom of Ach. It has been shown that the anionic site contains at least one carboxyl group present probably in the glutamate component (Cohen et al., 1959). The esteratic site involves a histidine residue adjacent to serine (Bergmann et al., 1956).

Most of the information on the active centre has been obtained using appropriate anticholinesterases. In the study of the esteratic site, radioisotopes (32P or 14C) of organophosphorus reagents were reacted with the purified enzyme, the labelled protein was hydrolysed and the radioactive products were isolated and identified (Michel and Krop, 1951). The sequence glycine-glutamine-serine-alanine-
glycine-glycine (Jansz et al., 1959) established for the esteratic site of ChE is typical and Cohen et al., (1955) hypothesized that a number of DFP-sensitive enzymes carry a similar structure which react with DFP, the radioactive organophosphorus residue being coupled with serine.

Several models were suggested to explain hydrolytic activity of AchE's as well as their inhibition. Bergmann et al., (1958) suggested that the acidic group in the esteratic site forms a hydrogen bridge with ethereal oxygen of Ach and is active in the hydrolysis of enzyme-substrate complex. Other model systems of Ach hydrolysis stresses the significance of serine hydroxyl activated perhaps by imidazole and of the carboxyl grouping (Cunningham, 1957; Fahrney and Gold, 1963). The serine hydroxyl and the carboxyl group may form an internal ester or carboxylate ion. This carboxylate nucleophilic center is capable of intra molecular attack on the carboxyl atom of the ester to produce rapidly hydrolysed anhydride.

Anionic sites may be necessary with regard to interaction between the substrate molecule and ChE. Results obtained with bisquaternary inhibitors suggest (Bergmann, 1955) that anionic sites occur in pairs in the vicinity of the esteratic site and that they consist of the Y carboxylic groups of an Y-glutamyl peptide. It is by no means certain that quaternary
nitrogen of these inhibitors react with the same site as the cationic head of Ach; thus, the enzyme may contain several anionic sites.

ISOZYMES OF ACETYLCHOLINESTERASE AND CHOLINESTERASE:

According to Markert and Moeller (1959), isoenzymes are enzymes which have the same catalytic action and are derived from the same organ, but have different chemical and/or physical properties. Many authors have pointed out (eg. Wieland and Pfeiderer, 1962; Kaplan, 1963) that the multiple molecular forms of an enzyme are only of significance when they do not arise as artifacts of purification. Dubbs (1966) disagrees with their viewpoint; he believes that a ChE isoenzyme which is observable after ultrasonic treatment of serum and not observable without such treatment is not a mere artifact but a significant entity. However, it is probably that an isoenzyme arising after ultrasonic treatment may be an artifact.

Human serum cholinesterase has been separated electrophoretically (Dubbs et al., 1960; Bernsohn et al., 1961, 1966) into at least seven distinct bands, each of which may be referred to as an isoenzyme (Webb, 1964). Juul (1968) employed disc electrophoresis of human serum ChE; he identified twelve isozymes. Almost 80% of the total ChE activity was present in one isozyme (ChE7). In addition to electrophoretic separation of ChE isozymes, it has been possible to fractionate human serum
ChE into component isozymes by gel filtration on sephadex G-200 columns (Harris and Robson, 1963) and also by chromatography on DEAE cellulose columns (Liddell et al., 1963).

In a study of properties of ChE isozymes, Ecobichon and Kalow (1963) removed the sialic acid residues of human serum ChE and found that although electrophoretic mobility was affected, the kinetic properties of the product remain unchanged. La Motta et al., (1965) demonstrated that five of the isozymes of human serum ChE are interconvertible and represent different stages of polymerization. La Motta et al., (1966) later were able to show that all seven serum ChE isomers are interconvertible polymers, each containing the same polypeptide unit.

Barron et al., (1962) have separated the AchE of human brain into isozymes. Ecobichon and Israel (1967) found not only four isozymes of AchE but also two weak bands of ChE and one band of nonspecific carboxylesterase in the electric organ of electrophorus electricus. Massoulie and Rieger (1969) found three main molecular species (A, C and D) exhibited acetyl cholinesterase activity in the extract of torpedo electric organ. This was observed in fresh frozen organ extracts, toluene-autolyzed organ extracts, or autolysis exudates. The fourth species, B, appeared during autolysis.
in the exudate. These species were found to be stable even after treatment with various drugs. Molecular species C and D show the phenomenon of aggregation in low salt concentration whereas A and B do not.

Rieger et al., (1973) differentiated the several forms of AchE by their sedimentation constant and by their structure. They separated them into globular and elongated forms. Elongated forms were in turn separated into forms of various sedimentation coefficient A (8.55), C (14.25), and D (18.45). By using different antibodies they found that the elongated forms were more reactive than the globular forms with each antiserum tested. Neuraminidase treatment decreased the affinity of antiserum for the D and C forms but not for the globular forms following treatment with neuraminidase. This would imply that polysaccharides are important for the structure of elongated forms.

**INTERACTION OF CHOLINESTERASE AND ACETYLCHOLINESTERASE WITH IRREVERSIBLE INHIBITORS:**

Enzyme inhibitors may be classified as reversible or irreversible. As used by enzymologist, the term reversible means that the inhibitory action may be reversed easily, e.g. by removing the inhibitor by dialysis. In the case of irreversible inhibitor, the inhibitory action is not easily reversed and the amount of inhibition will increase progressively until
the enzymatic activity is nullified, provided large amount of inhibitor is present.

The most potent and well characterized irreversible inhibitors of ChE fall into three classes: Organophosphorus inhibitors, carbamates and organosulfonates.

**MECHANISM OF IRREVERSIBLE INHIBITION**

The degree of inhibition observed with an irreversible inhibitor depends on the rate constant of inhibition \( (K_1) \)

\[ \text{--- } E + I \xrightarrow{K_1} EI \]

The inhibitor cannot be recovered intact from an enzyme inhibited by an irreversible inhibitor. Irreversible inhibition has been shown to occur as a multistage process: a reversible formation of an enzyme-inhibitor complex is followed by an irreversible phosphorylation of the enzyme ("aging", cf. below). It is not true that enzymatic activity can never be recovered from enzymes inhibited with irreversible inhibitors. However, even though the enzyme may be recoverable (cf. below) the inhibitor is not recoverable. Thus, in the case of inhibition of ChE by the organophosphorus compound sarine followed by reactivation by pyridine-2-aldoxine methiodide (P-2AM), ChE activity is restored but the sarin moiety is removed from the E+I compound as phosphoric acid rather than as the phosphonofluoride. The acid is not a ChE inhibitor.
REACTIVATION:

Reactivation of irreversibly inhibited ChE is the process by which enzymatic activity is restored to the inhibited enzyme. This process may occur as the result of interaction with the solvent in which case it is referred to as spontaneous. Reactivation may also occur as the result of the action of some compound added to the reaction mixture for the purpose. For reactivation to be possible, the native structure of the enzyme should be retained through the inhibition and reactivation steps (Berendes, 1964). The phenomenon of aging in which the ability to reactivate the inhibited enzyme is lost as the result of a chemical or physical reaction, is discussed later.

REACTIVATORS:

Almost all of the presently known effective reactivators contain a quaternary nitrogen group separated from a reactive group by a distance which is approximately the same as the distance between the anionic and esteratic sites of ChE. The discovery of reactivators may be traced to the finding of Hestrin (1949) that intact AchE and hydroxamate are released upon incubation of acetylated enzyme with hydroxylamine. It was shown that, just as it can be acetylated, hydroxylamine can be phosphorylated by organophosphorus agents (Green and Saville, 1956) and that phosphorylation of reactivators follows their contact with phosphorylated enzyme which is
then reactivated (Wilson 1951, 1952).

Among important factors in reactivation are the extent of the formation of protonated acidic sites, as indicated by relevant pH studies (Wilson et al., 1958) and the degree of nucleophilicity of the reactivator which depends upon the substituents on the hydroxamate or oxime anion. In 1959, Wilson suggested that a good reactivator could be obtained by combining a nucleophilic group with suitably placed quaternary nitrogen atom. These and previous findings led Wilson to development of monoquaternary pyridine aldoximes such as pyridine 2 aldoxime methiodide and pyridine 4 aldoxime methiodide (P-2AM and P-4AM). Wilson (1958, 1959) suggested that an ideal reactivator should be bound to the organophosphorus inhibited enzyme so that a nucleophilic grouping such as the oxygen should fall one bond length from the phosphorus atom.

Reactivators are important not only for theoretical studies as they can be used in vitro but are also very practical in their applicability for the treatment of poisoning by anti ChE agents and for prophylaxis against such poisoning (Wills, 1963).

AGING:

Aging is a process in which inhibited enzymes become
refractory to reactivation. In the case of acetyl and cholinesterase an irreversibly inhibited (e.g. phosphorylated) enzyme which can be at first reactivated by high concentrations of reactivator is transformed subsequently to a form that cannot be reactivated (Heilbronn-Wikstrom, 1965). At present, it is generally thought that aging depends on the change in the phosphoryl moiety (Jansz et al., 1959). Berendes (1968) postulated that the reaction involved in aging is due to hydrolysis of one alkoxy group from phosphorylated enzyme:

\[(OR) PO - E + HO \rightarrow (OR) (OH) PO E + ROH\]

The evidence for this is as follows:

In a normal reactivation process of DFP inhibited enzyme, \(^{32}\)P was released as diisopropyl phosphate, whereas in an aged sample it was released as monoisopropyl phosphate by alkaline hydrolysis. The refractory portion of the phosphorylated enzyme was a hydrogen phosphate known to be resistant to nucleophiles (Heath 1961).

Inhibition followed by aging is an important and an useful mechanism which can be used to study the synthesis of AChE. The enzyme which arises following inhibition with DFP represents the newly synthesized enzyme. The irreversible inhibition can, therefore, be used to study the turnover rate of AChE. From the amount of inhibited enzyme, time taken for 50% of the enzyme to be synthesised is determined. From the value, turnover rate of the enzyme was calculated.
INHIBITION OF ACETYLCHOLINESTERASE BY DFP
WITH REVERSAL OF THE INHIBITION BY 2 PYRIDINE
ALDOXIME METHIODIDE

This diagram illustrates the irreversible inhibition of AchE by diisopropyl fluorophosphate as due to phosphorylation of AchE through the serine hydroxyl present at the esteratic site.

Cohen et al., (1955)
This diagram illustrates the reactivation of DFP inhibited AchE by pyridine 2 aldoxime methiodide. As a result of this process, the reactivator becomes phosphorylated.

WILSON (1959)
DISTRIBUTION OF AChE IN THE CENTRAL NERVOUS SYSTEM:

1) Subcellular Distribution:

The cytological distribution of AChE in the central nervous system appears to resemble the pattern observed in their peripheral counterparts, in that the concentration is greatest at the membrane, while lesser concentrations of AChE are also present in the cytoplasm as well as along the length of the processes insofar as they can be traced. This could be demonstrated histochemically (Fukada and Koelle, 1959). In frog nerve and brain, the highest activity of AChE was found in the particulate fractions especially in the microsomes, in the membranous materials, less amounts of AChE were found in the mitochondria (Clouet and Waelisch, 1961a,b).

The type of cholinesterase found in tissue is often a reflection of the tissue. Neural tissue contains AChE while non-neural tissue contains ChE. In the blood of most mammals, erythrocytes contain only AChE while plasma contains ChE (Zajicek and Datta, 1953).

2) Tissue Distribution:

Histochemical studies indicate that medulla, reticular formation, midbrain and caudate nucleus were those brain areas which exhibited highest concentrations of AChE (Holmes and Wolstencroft, 1964; Koelle, 1954 and 1963; Shute and Lewis,
1963). Primary efferent neurons were low in AChE. However, activity of AChE increased progressively in the tertiary and secondary sensory relay neurons. The cerebellar cortex of several mammalian species and of the duck also contained AChE (Phillis, 1965). Many AChE-containing fibers were found in cerebellar peduncle (cf. Karczmar, 1969). In tissue culture, cortical AChE appeared to be presynaptic while glial cells from cortex and elsewhere in the brain contained ChE (Geiger and Stone, 1962).

In the cat retina, high AChE activity was confined to the innermost region of the inner nuclear layer and to a band extending from the inner zone of the inner plexiform to the outer part of the ganglion cells (Koelle et al., 1952). The high staining in the cytoplasm of amacrine and ganglion cells seems to indicate that these cells are cholinergic. The four stained synaptic bands represent regions where the axons of amacrine and bipolar cells synapse with the dendrites of ganglion cells which might contain ACh as transmitter. Cells which are capable of responding to ACh and contain AChE, ChAC are considered cholinergic.

In the auditory system highest concentration of AChE were noted in the neighborhood of the nerve chalices terminating on the inner rows of hair cells and in the efferent nerve fibers of the olivochochlear bundle, oriented towards
the organ of Corti (Rossi and Cortesina, 1961).

As this study deals with the properties of the nerve endings in the synaptosomal preparations, certain properties of the synaptosomes will be discussed below.

**PROPERTIES OF SYNAPTOSOMES:**

**Preparation:** During homogenization of the brain tissue in isosmotic aqueous sucrose, the central presynaptic nerve terminals seal off and form detached particles away from axons (Gray and Whittaker, 1960, 1962). Whittaker et al., (1964) called these detached particles "synaptosomes". These synaptosomes retain the morphological nature and chemical content of the intact presynaptic terminal (including the transmitter). By differential centrifugation they can be separated from other organelles (mitochondria, myelin) and provide a new type in vitro preparation particularly appropriate for neurochemical studies of neuronal function.

Various types of central nerve terminals from various parts of the brain have been observed to form synaptosomes during homogenization; for instance, the mossy fiber endings of the cerebellar cortex are a good source of synaptosomes. The following discrete regions were shown to form synaptosomes; cerebral neocortex, hippocampus, caudate nucleus (Laverty et al.,
1963), spinal cord, hypothalamus (Michaelson et al., 1963), and hypophysis (Labella and Sanwal (1965). Brain parts of various species such as the mouse (Weinstein et al., 1963), rat, guinea pig, rabbit, cat, dog and man (Siakotos) form synaptosomes during homogenization. However, the richly innervated peripheral tissues show a very low yield of synaptosomes. On subjecting the synaptosomes to osmotic shock, they may be further fractionated into soluble cytoplasm, synaptic vesicles, external synaptosomal membrane and intraterminal mitochondria. Morphological and chemical analysis of these fractions give a good picture of organization of the pre-synaptic terminal. Normally synaptosomes should be free of microsomes except those which arise during preparation.

METHODS OF PREPARATION:

The methodology of the synaptosomal preparation is described below (cf. Methods); a general outline will be presented at this time. Synaptosomes are osmotically sensitive structures. In isosmotic media, any kind of chopping, mincing or dispersion of the tissue probably permits the survival of a considerable proportion of them as organized structures. Synaptosomes have sedimentation properties which are broadly similar to those of mitochondria and of the smaller fragments of myelinated axons, dendrites and glial processes formed during homogenization.
The mitochondrial fraction obtained from sucrose homogenates of brain tissue by differential centrifugation is a mixture of all these components. Synaptosomes can be separated from myelin and mitochondria by making use of differences in equilibrium density in a sucrose density gradient. Myelin floats on 0.8M sucrose, whereas synaptosomes have an intermediate density (between mitochondria and myelin). In our study we used gradients of Ficoll made isosmotic with sucrose (Kurokawa et al., 1965). Electron microscopy analysis indicates that these preparations are as intact as those obtained using sucrose as a density gradient; in some instances better respiratory activity of mitochondria was retained. Thus, a fraction rich in synaptosomes and essentially free from myelin and somatic mitochondria may be obtained by centrifuging a crude mitochondrial fraction in a five step discontinuous ficoll gradient consisting of 20%, 13%, 10%, 7.5% and 5% layers that have been allowed to diffuse into each other 1½ hours before use. Flow chart for this isolation is shown in the Method Section.

Once formed the synaptosomes are relatively labile structures; they are readily disrupted by variety of treatments, e.g. warming, vigorous mechanical agitation, supersonic vibrations, detergents, freezing or thawing and hypo-osmotic treat-
ment. Most of these treatments result in the prompt liberation of soluble cytoplasmic components. Hypo-osmotic disruption provides the most suitable procedure so far found for the isolation of the constituent organelles of the synaptosome. Large number of synaptic vesicles survive intact, external membrane and intraterminal mitochondria are not unduly fragmented and can be separated from vesicles by centrifuging.

3) Morphological characteristics of synaptosomes:

Synaptosome fractions can be prepared for electron microscopy by any of the standard methods of fixing, staining and embedding nervous tissue that gives a good preservation of nerve terminals in whole tissue block. Electron microscopy method which we employed are discussed in detail in Method Section.

In section, synaptosomes appear as membrane-bound, oval shaped bodies containing numerous vesicles and sometimes small mitochondria. Generally, sections of thickened membrane can be seen which are attached to the extra synaptic membrane; a cleft at about 200Å can be seen between the membranes. In addition, most synaptosomes contain a few larger vesicles and dense core vesicles. It is assumed that on homogenization the terminal region of the axon is pinched off and seals up to form a subcellular particle which carries with it a length
of the characteristically thickened post-synaptic membrane of next cell. In viewing the EM picture, it must be kept in mind that even if all synaptosomes possess post-synaptic attachments and mitochondria as well as synaptic vesicles, only certain planes of section will contain all three structural components (Whittaker, 1963).

**IN VITRO USE OF SYNAPTOSOMES:**

The synaptosome as a pinched off cell process has within its external membrane, alcytoplasm containing a normal complement of glycolytic enzymes and one or more mitochondria. It thus may be regarded as a small, non-nucleated cell. Synaptosomes have been used in vitro to study the synthesis, storage and release of transmitter (Krnjevic and Whittaker, 1965) and for sampling terminal axoplasm in studies of axonal flow (Barondes, 1966). Various workers (Austin and Morgan, 1967a) have used the synaptosomes for the investigation of the problem of the site of synthesis of axonal proteins, particularly those contained within membranous structures.

Synaptosomes do in fact actively respire when warmed and provided with substrate. They also synthesize high energy substrate, take up choline by a Na\(^+\) dependent Hemicholinium sensitive mechanism and possess a membrane with passive permeability properties similar to those of other non-myelinated neurons. Active transport have not been shown in synaptosomes
so also depolarisation. DNA has never been found in synaptosomes. Austin and Morgan (1967) found RNA in unfractionated and fractionated synaptosomes.

**ENZYMES:**

Synaptosomes in general host a number of enzyme systems, including enzymes responsible for synthesis and degradation of various transmitters, as well as enzymes concerned with protein synthesis. Synaptosome cytoplasm is believed to contain a full complement of glycolytic enzymes. Enzymes of external synaptic membrane include cholinesterases and Na+K+-activated ATPase. Synaptic vesicles contain only one enzyme Mg++-activated ATPase in low activity (Hosie, 1965).

**TRANSMITTER CONTENT:**

Acetylcholine, noradrenaline, dopamine, hydroxytryptamine, Y-amino butyrate and glycine are found in appreciable concentrations in any synaptosomal fraction. If the synaptosomes were prepared from specific areas in the brain, they can be shown to be rich in one or two specific transmitters. It is now known that most cholinergic neurons in mammalian brain form part of great ascending system, the reticular activation system which terminates on cortical and striate neurons (Lewis and Shute, 1966). Thus 70% of the total acetylcholine of the
forebrain remains in a bound form on homogenization and much of it is recovered in the synaptosomal fraction. Glycine in the spinal cord and gamma amino butyrate in the cortex are strong contenders for the role of inhibitors. In the forebrain, they both show the type of distribution predicted for the transmitter of an inhibitory neuron (by electrophysiological studies). Further the amount of gamma amino butyrate in cortical synaptosome preparations is adequate to sustain a transmitter role (Whittaker, 1968).

**PROTEIN SYNTHESIS IN ISOLATED SYNAPTOSOMES: In vitro studies:**

The synaptosomal fraction of a mammalian brain is a useful *in vitro* system to study neuronal and synaptic function, since morphological and biochemical evidences have shown their intactness and relative purity (De Robertis, et al., 1963; Whittaker et al., 1964). They are intact and pure without contamination from other organelles. They are intact and pure, the way a mixture of cells are.

Synaptosomal preparations have been used *in vitro* to study protein synthesis. In studying protein synthesis in such system, the misinterpretation of the results due to contamination from subcellular particles are microsomal, mitochondrial and bacterial components capable of protein synthesis should be eliminated. For this reason, specific protein synthesis inhibitors are used.
<table>
<thead>
<tr>
<th>Inhibitor or enzyme</th>
<th>Mode of action</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>DNAse</td>
<td>Hydrolyses DNA 3'5'-phosphate does not affect mitochondrial DNA</td>
<td>Wintersberger (1966)</td>
</tr>
<tr>
<td>RNAse</td>
<td>Hydrolyses ribosomal RNA but does not affect mitochondrial RNA</td>
<td>Roodyn et al. (1961)</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Specific for eukaryotic ribosomal systems, but not mitochondria</td>
<td>Ennis and Lubin (1964), Siegel and Sisler (1965)</td>
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<tr>
<td>Chloramphenicol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Affects bacterial and mitochondrial systems</td>
<td>Clark-Walker and Linnane (1967)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
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<tr>
<td>Lincomycin</td>
<td></td>
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<tr>
<td>Actinomycin-D</td>
<td>Inhibits DNA-dependent RNA polymerase including mitochondrial polymerase</td>
<td>Wintersberger (1966)</td>
</tr>
<tr>
<td>Puromycin</td>
<td>Inhibits brain ribosomal system</td>
<td>Campbell et al. (1966)</td>
</tr>
<tr>
<td></td>
<td>Inhibits mitochondrial system</td>
<td>Kroon (1964)</td>
</tr>
</tbody>
</table>

Specific inhibitors added to the incubation medium which contains the labelled amino acid, inhibits protein synthesis arising due to specific organelles. For instance, cycloheximide inhibits the protein synthesis arising from the ribosomal system whereas chloramphenicol will inhibit the protein synthesis of the bacterial and mitochondrial system.

The work of Autilio et al (1968) showed that brain synaptosomal preparations are capable of incorporating amino acid into proteins in vitro. They ruled out the contamination by
bacteria, microsomes and mitochondria that may have been present outside the nerve endings by using specific inhibitors. Bacterial contribution to protein synthesis was excluded from the limited inhibition they found with chloramphenicol (25%) and also the short period of linear protein synthesis in synaptosomes compared with exponential rate of synthesis in bacteria. By using ribonuclease or by adding supernatant enzymes or microsomes, no alteration was found in the rate of synaptosomal protein synthesis. This excluded the contribution of extra synaptosomal ribosomes or microsomes to the protein synthesis. Austin and Morgan (1967) demonstrate that labelled microsomes added during homogenization of brain tissue are not incorporated into the synaptosomal fraction. In addition, protein synthesized in synaptosomes sedimented differently in sucrose gradients from those synthesized in microsomes. As regards to elements within synaptosomes responsible for protein synthesis, they suggested that within the nerve ending particles, 75% of the radioactive protein may be synthesized outside mitochondria and 25% within mitochondria. These conclusions were reached following use of various inhibitors.

Austin and Morgan (1967) studying a cell free synaptosomal preparation found that the rate of incorporation of $^{14}$C leucine was linear and decreased with age.
Rate of incorporation of leucine with animal age in months

On the basis of the results obtained with protein synthesis inhibitors, Morgan and Austin (1968) suggested that synaptosomes can be considered as an anucleated mammalian cell which exhibits two protein synthesizing systems. The mitochondrial system is sensitive to chloramphenicol and chloramphenicol-like antibiotics tetracycline, erythromycin and lincomycin whereas the membrane system is sensitive to cycloheximide. Morgan and Austin (1968) found release of labelled fragments (following incorporation of $^{14}$C leucine) upon subjecting preparations containing synaptosomes to osmotic shock. The only osmotically-sensitive structures in their preparation were the synaptosomes. The release of material upon osmotic shock is therefore a good indication that the material originates from the synaptosomes.

Bachelard (1966) suggested that protein synthesis in synaptosomes might be entirely due to the presence of synaptosomal mitochondria which presumably transport protein via the soluble fraction to the membrane. Normally chloramphenicol should inhibit mitochondrial protein synthesis and should
Goldberg (1971) studied protein synthesis in mitochondria and nerve endings and compared the incorporation of $^{14}$C-leucine under identical incubation conditions. He found that both fractions had similar requirements for sodium and potassium activation, with concentrations of 100mM and 10mM respectively producing maximum levels of incorporation. He suggested that the mechanism of protein synthesis in nerve endings and brain mitochondria was similar and similar synthetic systems are present in the mitochondria and nerve ending fractions.

EVIDENCE FOR AXONAL RNA:

Despite the fact that the electron microscope has failed to reveal the presence of ribosomes within the axonal and synaptic regions of neurons, (Palay and Palade, 1955) there is evidence indicating that protein synthesis proceeds in isolated synaptosomes. There must therefore be a specialized site and mechanism concerned with protein synthesis at the nerve endings.

Evidence for the presence of RNA in the axon is readily available today. By using ultra microanalytical procedures,
Edstrom (1964) demonstrated RNA in the Mauthner axon of gold fish and in the lobster stretch receptor neuron at a concentration of 0.05% (wt/volume). This concentration paralleled that found for the myelin sheath; high concentration of RNA were present both in the proximal and distal regions of the axon (cf. Table below).

Content of RNA in 200 μl pieces of Mauthner axons and myelin sheaths from the gold fish (Edstrom et al., 1962)

Method of extraction:

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formalin Fixation</td>
<td>Carnoy fixation</td>
</tr>
<tr>
<td>Myelin Axons</td>
<td>Axons</td>
</tr>
<tr>
<td>0.4mg RNase/ml</td>
<td></td>
</tr>
<tr>
<td>dist. water pH-6.0 3 hours, 37°C</td>
<td>50±8</td>
</tr>
<tr>
<td>Control dist. water pH6.0, 3hrs. 37°C*</td>
<td>15.9</td>
</tr>
<tr>
<td>Difference</td>
<td>38</td>
</tr>
</tbody>
</table>

Mean values in μg± S E. of the mean

*Either without any added enzyme or with 0.4mg of ultraviolet inactivated RNase.

The base composition of RNA in the axon and myelin sheath although variable differed distinctly from that of ribosomal RNA. On the other hand, the base composition of RNA in the cell body is similar to that of ribosomal RNA i.e. it is rich in guanine and cytosine. Edstrom et al., (1962) pointed out
that this is compatible with the absence of ribosomes from the axons as determined by electron microscopy (Palay and Palade, 1955).

The long period of time that a messenger RNA would need to traverse the length of long axons and reach the terminals would require a highly stable messenger to survive the trip. Thus Edstrom (1962) believes that protein synthesis in distant portions of the axon is not directed by messenger RNA which was made in the perikaryon and that there is evidence for local synthesis of RNA in axons.

Koenig (1965) reported that denuded myelin free axons from the spinal accessory nerve of the rabbit contain RNA in concentrations far smaller than that found in Mauthner axon, that is, about 1/1000 as great as that of perikaryon. Koenig (1967) found also in vivo incorporation of $^3$H leucine into axonal protein and of $^3$H-orotic acid and $^3$H-adenine into what he believes to be axonal RNA. Some of the latter material is degraded by RNase. Actinomycin did not inhibit leucine incorporation into protein while it inhibited precursor incorporation into RNA; this suggests local RNA synthesis (Koenig, 1967). These results indicate that RNA and protein synthesis in axons might be a general phenomenon (Edstrom, 1962).
This concept is supported by the data of Austin and Morgan (1967). They analyzed RNA in purified subfractions from rat cortex synaptosomes and found in unfractionated synaptosomes 12 µg of RNA/mg protein; in synaptosome ghosts 20 µg/mg protein; in the membrane subfraction E 14 µg/mg protein (subfraction E contains membrane fragments of microsomal dimension and some synaptic vesicles); in the subfraction F 21 µg/mg protein (fraction F is believed to consist mainly of extra synaptic membrane and synaptosomal ghosts). The microsomal fraction contained 40 µg/mg protein.

Koenig (1968) hypothesized that axonal membrane is omni-potential or omnivalent in terms of its functional properties as the information specifying the characteristics of its protein is found throughout the membrane. It was postulated that translation of encoded information is controlled by local extra membrane DNA dependent mechanisms which could either be extra or intracellular. According to Koenig, it should be possible to bring about a transduction of a given membrane region under proper conditions from one functional type to another. It appears altogether that there is strong evidence regarding the protein synthesizing capacity of synaptosomes and that it is likely that synaptic membranes synthesize their own protein and including the enzymes such as AChE.

**THE AIM OF THE RESEARCH PROBLEM**

One important element in the organization and functioning of the central nervous system is determined by the pattern
of synaptic connection between cells and the efficiency of such synapses in interneuronal communication. Many of the electrical characteristics of synapses have been elegantly evaluated by electrophysiological techniques (Eccles, 1964). Yet, our understanding is still extremely limited as regards the biochemical events which may be rate-limiting in the intracellular transfer of information. Proteins are obviously important with respect to the metabolism of neuro-transmitters, the structure of membranes of the nerve terminals and the synapses as well as with respect to other vital components of conduction as well as of the chemical transmission from one cell to another. An understanding of macromolecular metabolism, therefore, becomes critical for an understanding of synaptic function. To understand the metabolism of an important macromolecule like AChE at the synaptic junction is crucially important due to the specific role of this enzyme in transmission and as this knowledge may explain the general phenomenon involved in the structurization of the synapse.

The synapse may be considerably removed from the cell body and its nucleus. To meet its macromolecular requirements, the appropriate proteins must either be synthesized at the synapse or migrate from cell body to the synaptic junction.
It was shown in the classic study of Weiss and Hiscoe (1948) that after an application of a ligature around a nerve, the diameter of the nerve on the cell body side of the ligature increased, whereas it decreased on the peripheral side of the nerve. Following the removal of the ligature the diameter of peripheral segment returned to normal at the rate 1mm per day. The conclusion from those experiments was inescapably that there was a constant movement in a proximo distal direction and that any local throttling of the progress of that movement resulted in the piling up of neuronal content.

Weiss and Hiscoe (1948) proposed the phenomenon of axoplasmic flow or axoplasmic transport as the mechanism by means of which the nerve tissue located at a distance from the cell body receives various substances such as proteins and enzymes from the cell body following the synthesis at that site.

The biochemical study of axonal flow by means of isotopes has become widespread and a wide body of information has been obtained with the use of labelled compounds. Following the injection of labelled amino acid Miani (1964) and Ochs (1966) examined a series of segments of a nerve at various intervals of time; progressive migration of the labelled amino acid was demonstrated in these experiments.
Using labelled amino acid, a rate of 1mm per day has been observed for migration of labelled proteins down the axon (Droz, 1965; Lasek, 1966; Weiss, 1967). These experiments involved the peripheral nervous system. Barondes (1964) has postulated a similar axonal flow within the central nervous system. On the other hand, there are data suggesting that proteins may also be synthesized at synaptosomal sites (cf. above), and it is possible to infer that protein synthesis occurs in synapses from the observation that brain slices incorporate radioactive amino acids into synaptosomal protein without any appreciable time lag (Austin and Morgan, 1967).

We undertook to study specifically the genesis of AChE in synapses in the central nervous system. As AChE is localized at the external membrane of synaptic endings in cholinergic neurons, a mechanism must exist by means of which AChE could be either transported to or formed at this region without hydrolyzing cytoplasmic ACh present presynaptically. Preliminary evidence was obtained in the peripheral system suggesting that the enzyme may be formed in the cell body and then move down an axon; it may be possibly attached to neurotubules acting as a carrier, (Ochs, 1963). On the other hand, evidence for local synthesis of the enzyme in axons has also been adduced (cf. above). These experiments were carried out with respect to peripheral nerve employing sectioning tech-
niques as well as anticholinesterases. Koenig (1965a,b) has been actively pursuing the study of axonal AChE synthesis. Cat hypoglossal and cervical sympathetic nerves and the hypoglossal nucleus were assayed for AChE on day 1, 3, 5 and 10 after the administration of a high dose (40μ moles/kg I.V) of DFP; the resynthesis of AChE following its inhibition by DFP with and without axotomy was studied. Resynthesis of AChE was constant all along the various segments of peripheral nerve studied at each time interval. Koenig noted a loss of AChE from the perikaryon with a corresponding suppression of the somal synthesis during outgrowth of regenerating axon while the enzyme continued to be synthesized locally in the axon. The restoration rate of AChE activity in hypoglossal nerve following DFP was of order 7% per day. On the basis of these data, they proposed that axonal proteins which are synthesized in perikaryon remain as constituents of axoplasm whereas axolemma-bound structural proteins and enzymes such as AChE are synthesized by a local axonal mechanism.

We undertook to study the genesis of AChE in synapses in the central nervous system. The problem to be investigated was whether AChE arrives at the terminal by axoplasmic transport following its synthesis in the microsomes in the cell body or whether it is synthesized at the nerve endings. This study was therefore carried out to understand the mechanism of
AChE synthesis in the nerve ending fraction. We pursued this study in rat brain by following the resynthesis of AChE after irreversible inhibition of the enzyme by DFP. Following the intracerebral injection of DFP, time was given to allow the DFP inhibited enzyme to age causing irreversible inhibition and new AChE synthesis was studied at various intervals in various subcellular fractions. As *in vitro* model to study such a system, isolated synaptosomes (nerve endings) and microsomes from 20 and 58 day old rat brain previously injected with DFP was chosen.

If AChE is transported from the cell body following its synthesis at microsomes, there should be time lag following DFP in the appearance of the enzyme at nerve endings as compared to the appearance in the microsomes of the cell body. If the nerve endings synthesized their own AChE, AChE activity in microsomes of the soma and in nerve endings would appear either simultaneously or it might be seen earlier at nerve endings than in the microsomes. Electron microscopy has failed to reveal the presence of ribosomes within the axonal and synaptic regions of neurons (Palay and Palade, 1955). The axon contains no morphologically identifiable protein synthesizing system. The nerve terminals at the end of axons are also devoid of identifiable ribosomes RNA have been shown to be present in the synaptosomal fraction (Austin and Morgan, 1967).
Normally synaptosomes do not contain microsomes except as a contaminant. By making proper corrections, effect arising due to this was eliminated. Our data provide evidence for the synthesis of AChE at nerve endings in 20 and 58 day old rat brain following inhibition of the enzyme with DFP.

The effect of Triton X-100 on the nerve ending and microsomal AChE was also studied. As the Triton X-100 released the membrane-bound enzyme, this would give some results as to the effect of DFP on the membrane-bound enzyme AChE.

The turnover of microsomal and nerve ending AChE isoenzymes was studied following inhibition with DFP. From the percent of enzyme synthesized over a period of days following DFP, the percent of enzyme turned over per day was calculated (i.e. turnover rate). The gel electrophoretic pattern of isoenzymes following DFP was scanned and the individual isoenzyme value was compared against the scanned control value. From these values, turnover rate of each isoenzyme was determined. The isoenzymes may be localized in different cell membrane and structures. The turnover study would indicate their localization and functional role of these molecular species.
In understanding studies of this nature, the misinterpretation of results due to various contaminants (from different organelles) should be eliminated. It is difficult to assess for contamination as there are no specific markers for synaptosomes; however, contamination can be evaluated by studying possible contaminating components of the preparation (cf. above) the only contamination encountered was due to microsomes (primary protein synthesizing machinery of the cell). The synaptosomes isolated at various time intervals were assayed for NADPH dependent cytochrome C reductase (microsomal marker enzyme) and corrections were made relative to this marker.

Another misinterpretation of the results might arise because of the contamination of the synaptosomes by non-neuronal elements such as glial cells. As glial cells contain mostly cholinesterase, the values were corrected using suitable inhibitors.

There is 20 percent contamination from membranes of unknown origin. As results are obtained by comparing the values with control group (prepared in a similar way to drug treated group), the error due to membranes of unknown origin was reduced. The difficulty in using Ficoll is its variability from batch to batch which varies depending upon the ion concentration and particle flux. To study ion flux, it
was necessary to remove the high salt content by dissolving the Ficoll in a small amount of water and reprecipitating it from 95% ethanol. The ethanol was removed by lyophilization for prolonged periods of time. In this case also, differences were eliminated by comparing the values of drug treated animals with those of control animals.
MATERIALS AND METHODS:

Male Sprague Dawley derived rats, 16, 20 and 58 days of age were used in these experiments. All animals were bred and raised at the appropriate facilities of the animal house at Veterans Administration Hospital, Hines, Illinois.

Diisopropyl phosphofluoridate (DFP) was obtained from Sigma Chemicals. $^{14}C$ labelled DFP was obtained from New England Nuclear Corporation ($1\mu$C/$1\mu$ mole). Working standard was prepared fresh in propylene glycol for each group of injections.

In early experiments with $^{14}C$ labelled DFP, 10$\mu$l DFP containing 2$\mu$c was administered intracerebrally under ether anesthesia to 20 day old rats. A 10$\mu$l Hamilton microsyringe filled with known volume of labelled DFP was injected at the predetermined positions. Each time the needle was inserted to a marked region ($\frac{1}{4}"$). Prior to all DFP injections, the rats received 6mg/kg of atropine methylnitrate intraperitoneally.

At various time intervals, the rats were killed by decapitation. A 10$\%$ homogenate of the brain was prepared in 0.32M sucrose in 25mM Tris, pH 7.5. A known amount of homogenate was solubilized with a solubilizer (phase combining
system, PCS™, obtained from G.D. Searle and Co., Arlington Heights, Illinois) and was transferred to a scintillation vial containing 15ml solution of premix P (98% PPO 2:5 diphenyl oxazole + 2% PoPoP 1:4 bis - 2 (5 phenyloxazoly1) Benzene). Solution of premix P was made at a concentration of 5gms/litre of toluene. Premix P was purchased from Packard Co., Desplaines, Illinois. The amount of radioactivity in the vials were determined on a Packard Tricarb liquid scintillation spectrometer model 3003. Two aliquots from the same fraction were used for AChE and protein determinations. To determine the amount of radioactivity bound to the protein, homogenate was precipitated with 5 vols. of trichloracetic acid. After drying, the precipitate was dissolved in NAOH. Toluene was added to solubilize the precipitate and the resulting solution was transferred to a vial containing 15 ml of premix P solution and radioactivity was determined as described above.

When a dose of 1.5mg/kg DFP was used, the inhibition of AChE at various subcellular fractions was found to be insufficient (cf. results, Table III). A dose of 3.5mg/kg was used in subsequent studies in 20 day old rats. Control animals were given propylene glycol intracerebrally; the volume (10µl) was identical to that employed for the injection of DFP.
58 day old rats were anesthetized with pentobarbital (20mg/kg) 30 minutes following i.p. administration of atropine methyl nitrate (6mg/kg). Animals were held to the stand with the help of strings and a longitudinal incision was made down the center of the scalp. Using a scalpel blade, skull was cleared of muscle and connective tissue. With predetermined coordinates, a small hole was drilled using a hand drill containing 1/32" drill bit. 10μl Hamilton Microsyringe filled with DFP at a dose of 2mg/kg were lowered through wax filled hole. The needle was inserted to a depth indicated by a mark, DFP injected in the volume of the hole immediately filled with bone wax and the skin incision sutured with nylon. No antibiotic was administered. Similar surgical procedures were performed in control animals which received propylene glycol instead of DFP. Due to survival problem, the dose of 2mg/kg of DFP was used rather than 3.5mg/kg. Following the DFP injections, animals were sacrificed at various intervals of time. The cerebral hemispheres were excised and bathed in 0.32M sucrose buffer prior to homogenization. Excision was completed within 20 seconds following sacrifice of the animal.

FRACTIONATION PROCEDURE: The isolation of subcellular fractions. What follows is a diagram of the general procedure of isolation of synaptosomes and microsomes (Autilio et al., 1968).
FLOW CHART FOR ISOLATION OF SUB-CELLULAR FRACTIONS

10% Homogenate (0.32M sucrose in 25mm Tris adjusted to pH 7.5)

Centrifuge
1000xg for 10 minutes

Pellet

Supernatant washed with 10ml of sucrose buffer

Re-centrifuged at
1000xg for 10 minutes

Supernatant + Supernatant

1

2

(Nuclei, unbroken tissue fragment)

Combined Centrifuge
14,000xg for 15 minutes

Supernatant Pellet

3

(Crude mitochondria)
(Resuspended in 0.32M sucrose in 25mm Tris adjusted to pH 7.5)

Centrifuge
15,000xg for 20 minutes

Supernatant + Supernatant

3

4

Pellet (mitochondria)

Combines and centrifuged
110,000xg for 75 minutes

Suspended in 0.32M sucrose in 25mm Tris pH 7.5 3ml/gm of original tissue (used for synaptosome isolation)

Supernatant Pellet Microsomes

This fraction was layered on a Ficoll gradient
The patterns of Ficoll gradient were as follows:

5 ml of mitochondria in 0.32M sucrose in 25mm Tris (pH 7.5)
5 ml of 5% Ficoll in 0.32M sucrose in 25mm Tris (pH 7.5)
5 ml of 7.5% Ficoll in 0.32M sucrose in 25mm Tris (pH 7.5)
5 ml of 10% Ficoll in 0.32M sucrose in 25mm Tris (pH 7.5)
5 ml of 13% Ficoll in 0.32M sucrose in 25mm Tris (pH 7.5)
5 ml of 20% Ficoll in 0.32M sucrose in 25mm Tris (pH 7.5)

The crude mitochondrial fraction on a Ficoll gradient was centrifuged at 25,000xg for 45 minutes in an IEC head SB-100. Five layers were visible after centrifugation; myelin being lighter was in the top layer and the denser mitochondria formed the lower layers. Synaptosomal fraction of intermediate density was formed as two separate layers between 7.5% and 13% Ficoll. As these two layers between 7.5% and 13% Ficoll represented a good source of nerve ending fraction (demonstrated by electron microscopy) they were pooled and analyzed. Synaptosomal layers were collected, diluted in sucrose buffer and centrifuged at 40,000xg for 30 minutes. The pellet obtained in the wash was rewarshed in sucrose buffer to remove Ficoll. Each final pellet obtained from the various fractions (microsomes, mitochondria and synaptosomes) was homogenized in sucrose buffer (0.4ml/g of original tissue) and stored at -40°C until use. A known amount of these fractions were used for AChE assay, protein determination, electrophoretic separation, triton extraction and electron microscopy.
ELECTRON MICROSCOPY:

The synaptosomal pellets were fixed for electron microscopy in 0.1M phosphate buffered glutaraldehyde (3.125%). After overnight washing in 0.1M phosphate, the sample was osmicated for 1 hour in 1% buffered osmium tetroxide in the dark, which was followed by dehydration in 35%, 50%, 70%, 80%, 95% and absolute ethanol (10 min. each) at approximately 4°C. The sample was allowed to stand in propylene oxide for 20 minutes, in propylene oxide: Epon resin 1) (1:1) for 45 minutes, in propylene oxide: Epon resin (1:3) for 45 minutes, epon for 1 hour and finally in epon for 8 hours. Finally, it was embedded in fresh epon in electron microscopy capsules and heated at 65°C for 48-72 hours.

1) Epon employed for embedding had the following components: 16 volumes Epon (Epon is a glycerol based aliphatic epoxy resin of almost water white color and low viscosity.) 10 Volumes dodecenyl succinic anhydride. 9 volumes methyl nadic anhydride. 0.6 volumes dimethyl amino methyl phenol (DMP 30).

When Epon is cured with dodecenyl succinic anhydride, a rather soft block results, whereas when cured with methyl nadic anhydride and dimethyl amino methyl phenol, a very hard block is formed. To produce blocks of desired hardness, various proportions of stock mixture was used.
PROCEDURE FOR ELECTRON MICROSCOPY:

The Epon embedded pellets were sectioned with a Du Pont diamond knife on the servall Porter-Blum ultra microtome MT-2. After the sections were placed on 300 mesh specimen grids, they were stained with 10\% ethanolic solution of uranyl acetate and 0.28\% of alkaline solution of lead citrate for two minutes in each solution with several rinses in between. Electron microscopic study was performed on RCA EMU-3H instrument.

TRITON X-100 EXTRACTION:

Known amounts (mg/ml) of synaptosomal and microsomal fractions were homogenized with equal volumes of 2\% Triton in sucrose buffer, thus making the final concentration to 1\% Triton. Solutions were stored at 4°C for 6 hours and then centrifuged at 100,000xg for 30 minutes. The sediment was suspended in 1ml H₂O. Supernatant and sediment were analyzed for enzymatic activity.

ENZYMATIC ASSAYS:

Acetyl cholinesterase (AChE) assays were performed by the method of Ellmann et al., (1961) using acetyl thiocholine iodide (2.6mM) as substrate (Sigma Chemical Company). The enzyme activity is measured by following the increase of yellow color produced from thiocholine when it reacts with dithio-bis-nitrobenzoate ion. It is based on coupling of
the following reactions:

Acetyl Thiocholine Enzyme Thiocholine + acetate

Thiocholine + Dithiobisnitrobenzoate → yellow color.

The yellow color is measured in a spectrophotometer at 412mu.

The reaction with the thiol has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the enzyme and the concentrations used do not inhibit the enzymic hydrolysis (Ellmann et al., 1961). Corrections were made for non-enzymatic substrate hydrolysis. A linear rate of hydrolysis was obtained for first 10 minutes. The slope of the curve expresses the rate in absorbance units/minute. From the extinction coefficient of the yellow anion moles of substrate hydrolyzed were determined Enzyme activity was expressed as specific activity; specific activity is equivalent to umoles of acetyl thiocholine hydrolyzed per minute per mg of protein. Quinidine sulphate (1%) was added at the beginning of the reaction to inhibit cholinesterase (Ellmann et al., 1961).

Cytochrome C reductase was assayed by the spectrometric method of Sottocosa et al., (1967). Essentially, the method involves the measurement of the formation of reduced cytochrome C in terms of the increase in optical density at 550mu. The final concentration of cytochrome C was 1 mg /ml. Rate was linear for the first minute and declined after 40% cytochrome C was reduced. Rate of cytochrome C reduction was calculated
from linear portion of the curve. Increase in O.D. at 550µm per mg of protein per minute was used for calculating the activity.

The protein was determined by the method of Lowry et al., (1951) using bovine serum albumin (Pentex, Kankakee, Ill.) as standard.

**ACRYLAMIDE GEL ELECTROPHORESIS:**

When subjected to electrophoresis in acrylamide gels, a complex mixture of proteins divides up into number of well defined zones or bands (Ornstein and Davies, 1964). Acrylamide gels have the great advantage of providing an uncharged matrix in which separation based on molecular sieving and mobility differences can be carried out. The ability of molecules to pass through a sieve depends on the size and shape of the holes in the gel, the size and shape of the molecules being sieved and interactions such as ion exchange which may occur between the molecules and matrix of the gel.

Acrylamide gels used in these experiments are long chain molecules with varying proportion of cross linkages resulting from the addition of a proportion of bisacrylamide before polymerization.
A set of three gels were used in the separation, the sample, the spacer and the separating gel. These three gels provided a better separation of the sample. In the case of sample and spacer gel, the polymerization of acrylamide was achieved by the addition of riboflavin. Solution of acrylamide and riboflavin illuminated by an ultra violet light brought about the gel formation. Gels were formed after exposing the solution (acrylamide + riboflavin) to ultra violet light to a pre-determined time.

In the case of separating gel, polymerization of acrylamide was brought about by the addition of ammonium persulphate. The sample and spacer gel are characterized by a large pore size and different buffer system as compared to the separating gel with a small pore size. The sample applied on the sample gel concentrates sharply at the gel interface due to difference in buffer system and pore size (between the separating and sample gel). After this process is completed, the concentrated sample separates into the isoenzymes as they migrate through the small pore gel. The greatest advantage of this system is that it permits the concentration of the sample from a relatively large volume.

PROCEDURE FOR POLYACRYLAMIDE GEL ELECTROPHORESIS:

Polyacrylamide disc electrophoresis was performed in a Canalco apparatus according to the method of Davies (1964)
as modified by Juul (1968) using glass tubes (I.D) 5mm, length 68mm, containing 1ml of separating gel. The composition of the various gels are shown below.

SAMPLE GEL: (100ml) consisted of 2.5gm acrylamide, 0.25gm of N.N. methylene bis acrylamide, 0.063ml of N:N:N:tetramethylene diamine, 20 gm of sucrose, 0.63 mg of riboflavin, 6.3 ml of IN HCl, and 0.69 gm of Tris hydroxyamino methane. The pH of the final solution was adjusted to pH=5.8.

SPACER GEL was identical in composition and pH with sample gel, except that sucrose was added in the amount of 30 rather than 20 gm.

SEPARATING GEL (100ml) was made with 9 gm acrylamide, 0.16 gm N:N methylene bis acrylamide, 0.03 ml N:N:N:N tetramethylene diamine, 10 gm sucrose, 0.075 gm ammonium persulfate, 6.3 ml INHCl, and 3.75 gm tri-hydroxy amino methane. The pH of the final solution was adjusted to 8.7.

All chemicals for electrophoresis were from Canal Industries Corporation, Maryland.

The actual procedure for electrophoresis was as follows:
The separating and spacer gel following polymerization were kept at 4°C for 20 hours. The sample gel carrying AChE-containing sample (synaptosomes or microsomes) was polymerized prior to electrophoresis. Normally 50-75 μg of protein was applied to each gel. Electrophoresis was performed at 3 mA per column for 3 hours at 4°C using Tris-glycine buffer at pH 8.0. The gels were recovered from each glass tube by rinsing individual tube with a jet of water. After removal, each gel was incubated for 30 minutes in 15 ml of 1/15M phosphate buffer, pH=6.1, containing 0.01M glycine, 0.001M CuSO₄, 0.03M Na₂SO₄ at 25°C. Final incubation was performed in the same buffer containing 3x10⁻⁵M acetyl thiocholine iodide for 90 minutes. Both preincubation and incubation solutions included suitable inhibitors when required. The inhibitor was ambenonium mytelase; N:N’Bis, 3 diethylamine ethyl oxamide bis 2 chlorobenzyl chloride which inhibited AChE at a concentration of 5x10⁻⁶M; this inhibitor was used to differentiate the acetyl- and cholinesterases. After the enzymic reaction had proceeded sufficiently, the gels were then transferred into 3M (NH₄)₂SO₄ saturated with dithiooxamide to fix the enzyme and were allowed to stand at 4°C for 24 hours. Destaining was accomplished with 7% acetic acid. The gels containing the stained isoenzymes of microsomes and synaptosomes were scanned in a Canalco model T microdensitometer without a filter to determine relative distribution of AChE activity in the various isoenzymes.
For heavier and weaker bands, the settings were 20 mm$^2$ and 4 mm$^2$ per integration respectively. The base line was established at anodal end. The percent of each peak was calculated from the total integration of all peaks.

From the specific activity (AChE) of the applied sample and from the percentage of each peak, relative specific activity of each isoenzyme band was calculated. Microsomes and synaptosomes from control and DFP treated rats were analyzed for isoenzymes in this manner. By comparing the relative specific activity of the isoenzymes of the control group with that of DFP treated group (rats), the percent inhibitions of microsomal and synaptosomal isoenzymes were calculated. The relative rate of the return of enzyme activity of microsomal and synaptosomal isoenzyme with time was measured by analyzing and comparing the values of various groups of DFP treated rats with control rats. By subjecting the values from various animals to computer analysis, the standard error of the mean was determined.
RESULTS:

The purified rat brain synaptosomes or nerve endings obtained by using a discontinuous Ficoll gradient are shown in Figure 1. Bands B and C represent a compact mass of intact nerve endings.

The combined B and C fractions layered between 7.5 and 13% Ficoll in a Ficoll gradient represent the synaptosomal or nerve ending fraction. The combined fractions were analyzed by electron microscopy; a typical electron micrograph of these fractions is shown in Figure 2 and 3. Synaptosomes can be expected to contain the organelles present presynaptically (intraterminal mitochondria and vesicles) and their post synaptic attachments. As demonstrated by electron microscopy our synaptosomal fractions were found to be relatively pure; in most cases, the intact synaptosomes with their vesicles, intraterminal mitochondria and post junctional complexes could be seen. Dark, ill defined fragments were also present; according to many investigators (cf. for instance De Robertis et al., (1963)) these fragments are the so-called synaptosomal ghosts and represent the synaptosomal junctions. This specialized junction associated with its organelles and particularly with the clumped presynaptic vesicles has been called by Palay (1958) "The synaptic complex". There is no direct evidence to prove that the
FIGURE 1 Final stage of purification of 20 day old rat brain synaptosomes: the discontinuous Ficoll gradient, synaptosomal fraction sediments between 7.5% and 13% Ficoll.
Figure 2: Electron Micrograph of 20 day old rat brain

Synaptosomal preparation 44,900 x magnification.

1. Synaptic vesicles
2. Large vesicles
3. Coiled elongated mitochondria
4. Dark Fragments (Junctional complex)

Specimen fixed in glutaraldehyde and OSO₄ as described in methods.
Figure 3: Electron Micrographs of 20 day old rat brain synaptosomal preparation. 80,820 x magnification.
synaptic complex corresponds to the site where chemical transmission takes place. This conclusion can be however extrapolated from neurophysiological studies of intact synapses; moreover, indirect evidence indicates that the synaptic complexes are the specialized zones of the neuronal membrane where the neurotransmitter is released at the presynaptic site and where the substance exerts the effects at the post synaptic site. Figure 3 represents the electron micrograph of the synaptosomal fraction under higher magnification. The elongated mitochondria, vesicles and post-synaptic attachment can be seen at higher magnification.

STUDIES USING (C\(^{14}\))-LABELLED DFP:

Initially it was intended to study the binding of (C\(^{14}\))-DFP to various subcellular fractions and the turnover of radioactivity at various intervals of time. For this purpose, DFP was administered by intracerebral injection so as to avoid its accumulation in fat depot which would occur following peripheral (i.v. or s.c.) administration. In the initial experiments, varying amounts of (C\(^{14}\))-DFP (1μc/lumole) were injected intracerebrally in 20 day old rats. The animals were sacrificed 24 hours following the injection. A 10% homogenate of the rat brain in 0.32M sucrose buffer was made. Counts per minute per total homogenate were determined. As can be seen in Table I, the radioactivity of the rat brain homogenate was increased with increasing amount of labelled compound.
RECOVERY OF RADIOACTIVITY IN THE RAT BRAIN HOMOGENATE FOLLOWING INTRACEREBRAL INJECTION OF Cl\textsubscript{4} DFP

<table>
<thead>
<tr>
<th>Amount of Cl\textsubscript{4} DFP used</th>
<th>Number of rats</th>
<th>Time of sacrifice following the injection</th>
<th>Counts per minute per total homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 2.0\mu C</td>
<td>3</td>
<td>3 hours</td>
<td>63,720±2580</td>
</tr>
<tr>
<td>0 1.4\mu C</td>
<td>3</td>
<td>24 hours</td>
<td>41,985±2095</td>
</tr>
<tr>
<td>0 0.8\mu C</td>
<td>6</td>
<td>24 hours</td>
<td>19,760±720</td>
</tr>
<tr>
<td>counts in the liver</td>
<td>6</td>
<td>24 hours</td>
<td>22,270±1020</td>
</tr>
<tr>
<td>0 0.4\mu C</td>
<td>4</td>
<td>24 hours</td>
<td>11,340±450</td>
</tr>
<tr>
<td>0 0.1\mu C</td>
<td>3</td>
<td>24 hours</td>
<td>1,708±100</td>
</tr>
</tbody>
</table>

Table I: Varying amounts of Cl\textsubscript{4} DFP were injected by intracerebral injection. Animals were sacrificed at various time intervals.
(1708 cpm for 0.1µc to 41985 cpm for 1.4µc). Total counts were also determined in the liver in one set of experiment, the purpose of which will be discussed later.

The recovery of radioactivity in the homogenate following the injection of \((C^{14})\)-DFP could be calculated from the values in Table I. The recovery of radioactivity was approximately 1% following various injections. Various attempts were made to improve the recovery. \((C^{14})\)-DFP used in the experiment depicted in Table I was prepared in propylene glycol, emulsions were prepared in a number of different media so as to facilitate better binding of \((C^{14})\)-DFP. The media used were peanut oil, saline (0.9%), albumin (10%) and unlabelled DFP (60µg). Varying amounts of \((C^{14})\)-DFP were mixed with 10µl of the media described above and injected intracerebrally into 20-day old rats. Rats were sacrificed 24 hours following the injection. Counts per minute in the total homogenate were determined (Table II). The percent recovery of radioactivity using various media were as follows: \((C^{14})\)-DFP in propylene glycol 1.0% (calculated from the values in Table I) with unlabelled DFP 0.00019%, peanut oil 0.00054%, saline 0.0023% and albumin 0.0093%. It can be seen that using unlabelled DFP or peanut oil as a media reduced the recovery of counts in the homogenate following the injection.
EFFECT OF VARIOUS CARRIERS USED WITH C\(^{14}\) DFP IN THE RECOVERY OF RADIOACTIVITY FOLLOWING INTRACEREBRAL INJECTIONS

<table>
<thead>
<tr>
<th>Amount of C(^{14}) DFP used</th>
<th>Carrier</th>
<th>Time of Sacrifice</th>
<th>Counts per minute in total homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8(\mu)c (6)</td>
<td>Unlabelled DFP</td>
<td>24 hrs.</td>
<td>7690 ± 1200</td>
</tr>
<tr>
<td>1.7(\mu)c (3)</td>
<td>Peanut oil</td>
<td>24 hrs.</td>
<td>2028 ± 200</td>
</tr>
<tr>
<td>1.4(\mu)c (3)</td>
<td>Saline</td>
<td>24 hrs.</td>
<td>7326 ± 800</td>
</tr>
<tr>
<td>1.0(\mu)c (3)</td>
<td>Albumin</td>
<td>24 hrs.</td>
<td>20,472 ± 2000</td>
</tr>
</tbody>
</table>

Table II: C\(^{14}\) DFP was injected intracerebrally with various carriers. Rats were sacrificed 24 hours following the injection. Counts per minute in total homogenate was determined. Number in parenthesis indicates the number of animals used. Values are expressed as mean ± S.E.M.
It was critical at this point to examine the distribution of \((\text{C}^{14})\)-DFP (1.0 μc) in the various subcellular fractions following the injections. Twenty-four hours following the injection, rats were sacrificed, brains from three experiments were pooled and a 10% homogenate in sucrose buffer was made. Subcellular fractions were obtained from the homogenate by differential centrifugation. Distribution of \((\text{C}^{14})\)-DFP with respect to total homogenate was determined. As can be seen in Table III microsomes, mitochondria and nerve endings carried 1.12, 1.07 and 0.004 percent of the counts respectively. The rest about 98% of the count was found in nuclear and soluble fractions. To ascertain the nature of these counts and whether they were protein bound or not, these fractions were precipitated with TCA. Microsomal TCA precipitate exhibited 13% of the original counts whereas mitochondrial TCA precipitate exhibited 13% of the original counts whereas mitochondrial TCA precipitate exhibited 23% of the original mitochondrial counts.

We further explored the poor recovery problem, by analyzing the various subcellular fractions electrophoretically. Isoenzymes were cut from the gel and extracted with buffer. Counts per minute per each isoenzyme band were found to be few in numbers (approximately 20 cpm per band). It was clear from these results that DFP of higher specific
PERCENT DISTRIBUTION OF $^{14}$C IN VARIOUS SUBCELLULAR FRACTIONS FOLLOWING INTRACEREBRAL INJECTION OF $^{14}$DFP

percent compared to total homogenate

Microsomes 1.12%
Mitochondria 1.07%
Nerve endings 0.004%
Nuclear Fraction 29.3%

Table III: Following $^{14}$DFP injection, brains were pooled. Various subcellular fractions were isolated following differential centrifugation. Total counts in various fractions were calculated against the total count in the homogenate and percent distribution was calculated.
activity was needed to provide greater absolute recovery and data needed for subsequent experiments. As we could not obtain DFP of the high specific activity needed and thus the problem of recovering sufficient counts appeared insoluble, our initial experimental design was altered.

Initially, it was intended to study the loss of radioactivity at various intervals in microsomal and in nerve ending fraction following \((\text{C}^{14})\)-DFP injection. It was also intended to study the loss of radioactivity in various microsomal and nerve ending isoenzymes. Loss of radioactivity of microsomal and nerve ending fraction at various intervals would give an indication of the disappearance of \((\text{C}^{14})\)-DFP or the new synthesis of AChE. \((\text{C}^{14})\)-DFP was employed in these experiments at a pharmacological inactive dose, so that inhibition sufficient to establish the new synthesis was not produced. Our subsequent plan was therefore to use a high dose of non-labelled DFP and produce sufficient inhibition of AChE in various subcellular fractions.

INHIBITION OF RAT BRAIN AChE BY DI-ISOPROPYLFLUOROPHOSPHATE IN VITRO:

To estimate the dose of DFP needed to obtain the necessary inhibition, preliminary studies were carried out in vitro with rat brain homogenate. Equal amounts (5 ml) of
rat brain (20 day old) homogenate (1:10) were incubated with various concentrations (\(10^{-3}\) M to \(10^{-8}\) M) of DFP in propylene glycol for an hour at room temperature (Figure 4). AChE activity was determined at the end of the incubation. Percent inhibition of AChE was determined from the enzyme activity of control incubated with propylene glycol. The curve was linear for the \(10^{-5}\) M - \(10^{-7}\) M concentrations; \(10^{-5}\) M concentration induced almost complete inhibition (92%) and not much additional inhibition was obtained with higher concentrations.

The in vitro inhibition of rat brain AChE was analyzed electrophoretically (Figure 5). The normal AChE isoenzyme pattern of control homogenate is shown in A. Four isoenzymes were found in the control homogenate following incubation with the substrate (acetyl thioccholine iodide). At a concentration of \(10^{-6}\) M DFP, Band 4 was inhibited partially and a small amount of AChE was found in the region of Band 2. Bands 3 and 1 were inhibited almost completely. At the concentration of \(10^{-5}\) M, the isoenzymes were almost completely inhibited except for the presence of a light band at region 4. The concentrations of \(10^{-4}\) M and \(10^{-3}\) M caused complete inhibition of all the molecular species of the homogenate (Figure 5). These results indicated that a \(10^{-5}\) M concentration of DFP causes a 92% inhibition of the AChE of the rat brain homogenate and almost a complete inhibition of the four iso-
Figure 4: Equal amounts of rat brain homogenates were incubated with various concentrations of DFP for an hour at room temperature. Percent inhibition was determined from the enzyme activity of the control, incubated similarly.
Figure 5: Acrylamide gel electrophoretic pattern of rat brain AChE, when the homogenate was incubated with DFP in vitro. Conditions were the same as described for Figure 4.

Set A: Gel 1 - Control homogenate incubated with substrate
Gel 2 - Incubated with substrate and myetalase

Set B: Homogenate incubated with $10^{-6}$M DFP. In all cases 1st gel was incubated with substrate, 2nd gel with substrate and ambenonium (Myetalase).

Set C: Homogenate incubated with $10^{-3}$M DFP
Set D: Homogenate incubated with $10^{-4}$M DFP
Set E: Homogenate incubated with $10^{-3}$M DFP

All incubations were carried out at room temperature for an hour.

Set F: Homogenate incubated with $10^{-3}$M DFP for 4 hours at room temperature.
enzymes separated electrophoretically.

**IN VIVO INHIBITION OF RAT BRAIN AChE BY DFP:**

*In vitro,* $10^{-5}$M DFP caused 92% inhibition of rat brain AChE. We chose a dose that by calculation - in terms of equi-distribution of DFP - provided three times this concentration as the lower dose for the *in vivo* studies (1.5mg/kg). Twenty-day old rats were injected intracerebrally with various doses (1.5 - 4.0mg/kg) of DFP. Four hours later, the animals were sacrificed. AChE activity was determined in the 10% homogenate. Percent inhibition was determined from the enzyme activity of control animal injected intracerebrally with propylene glycol. As shown in Figure 6, inhibition varied from 76% at 1.5mg/kg to 95% at 4.0mg/kg.

*In vivo,* 120μg of DFP (4.0mg/kg) was required to inhibit 95% of brain AChE in a rat with an average weight of 30gms. *In vitro,* 18.4μg of DFP ($10^{-5}$M) inhibited 92% of brain AChE in a 10ml brain homogenate (equivalent to 1 brain). These results show that to inhibit the enzyme *in vivo,* six times the *in vitro* concentration of DFP is needed. These results indicate the difficulties involved in inhibiting the AChE *in vivo* following injections at various doses.

**THE EFFECT OF DFP (1.5mg/kg) ON MICROSOMAL AND SYNAPTOSOMAL AChE IN 16-DAY OLD RATS:**
Figure 6: 20-day old rats were injected with various doses of DFP intracerebrally. Four hours following the injection, animals were sacrificed. 10% brain homogenate in 0.32M sucrose (in 25mM Tris pH 7.5) was made and analyzed for AChE. Percent inhibition was determined from the enzyme activity of control animal injected in a similar kind of injection with propylene glycol. Values are expressed ± S.E.M. (Three experiments, 6 rats).
THE EFFECT OF DFP (1.5mg/kg) ON MICROSOMAL AND SYNAPTOSOMAL AChE IN 16-DAY OLD RATS:

The inhibition found with homogenates in vitro following incubation with DFP, does not indicate the degree of inhibition in the various subcellular fractions, so a series of experiments to determine this was carried out. Control animals received propylene glycol. AChE specific activity of various subcellular fractions of control and DFP treated rats was determined at various intervals of time.

Table 4 lists the specific activity of AChE of microsomal and nerve ending fractions of control and DFP treated rats at various intervals following the injection. From the specific activity of control animals, the percent inhibition was calculated for the various intervals after the DFP injection. The nerve ending and microsomal AChE were inhibited 33.4 and 54.6 percent respectively 4 hours following the administration of DFP. Twenty-two days following the injection, i.e. in 38-day old rats (Table IV), the percent inhibition in nerve ending and microsomal enzyme was reduced to 1.6 and 32.3 percent respectively. As DFP causes irreversible inhibition of AChE, the enzymatic activity after DFP treatment could be accomplished only by the synthesis of new AChE. This percent activity thus represents the amount of newly synthesized AChE. Thus, for a period of 22 days, AChE activity was found to be increased by 31.8% and 22.3% of nerve
Table IV: Sixteen-day old rats were injected with DFP (1.5 mg/kg) intracerebrally and sacrificed at various time intervals. Acetylcholinesterase activity was determined in synaptosomes and microsomes which were obtained by differential centrifugation as described in the text.

Specific activity is expressed as micromoles of acetylthiocholine iodide hydrolyzed /min/mg protein.
endings and microsomes respectively over the initial 4 hour inhibition. As the recovery of synaptosomal enzyme activity was faster than that of microsomal enzyme, it follows that nerve ending synthesis of AChE following DFP occurred faster than the microsomal synthesis.

As shown in the above experiment, the inhibition of subcellular fraction with a dose of 1.5mg/kg was incomplete. This may have been due to several reasons, most likely to the multiple binding of DFP to various proteins other than AChE and proteolipids. It was decided therefore to saturate the system with a higher dose, so that a better inhibition of subcellular fraction could be obtained.

In the pertinent set of experiments, a dose of 3.5mg/kg was administered through the intracerebral routes. Control rats were given appropriate volume of propylene glycol intracerebrally. Both control and DFP treated rats were sacrificed at various intervals of time. Specific activity of AChE in various subcellular fraction was determined. Table V summarizes the microsomal and nerve ending specific activity of AChE of control and DFP treated rats at various intervals of time. The percent enzyme (AChE) inhibition of DFP treated rats was determined on various days with respect to the enzyme activity of control rats obtained on the same days. On the day of its
# REGENERATION OF ACETYLCHOLINESTERASE IN SUBCELLULAR FRACTIONS OF RAT BRAIN FOLLOWING INHIBITION OF THE ENZYME BY DFP (3.5 mg/kg) INJECTED INTRACEREBRALLY IN 20 DAY OLD RATS

Table V: 20-day old rats were injected with DFP (3.5 mg/kg) intracerebrally and sacrificed at various time intervals after treatment with DFP. Control rats received propylene glycol.

Subcellular fractions of brain were obtained by differential centrifugation and assayed for Acetylcholinesterase activity.

Specific activity is expressed as defined in Table IV.
administration (4 hours following injection), DFP caused an initial inhibition of 76 and 60 percent in the microsomal and nerve ending AChE activity respectively. With time (15 days), the enzymic inhibition in microsomes and in nerve ending was reduced to 34.0% and 13.0% respectively. Thus, after a period of 15 days, AChE activity was found to be increased by 47.0% and 42.0% of nerve endings and microsomes respectively over the initial 4 hour inhibition value. Again, this reduction in inhibition could be accomplished only by new synthesis of the enzyme.

Synthesis of nerve ending AChE versus microsomal AChE following DFP is shown in Figure 7. In this figure, the enzyme activity in various subcellular fractions following treatment with DFP was plotted against time. For each day, the percent of new synthesis was determined in the DFP treated rats from the AChE activity of control rats determined on the same day. It is clear from Figure 7 that the recovery of enzyme activity in nerve ending fraction was always higher than the recovery in microsomal fractions.

In the course of the above experiments, DFP was given during the period of rapid growth (myelin formation) at which time the enzymes are being formed rapidly. It was critical to differentiate the new AChE that occurs in normal growth
Figure 7: 20-day old rats were injected with DFP at a dose of 3.5mg/kg. After decapitation, subcellular fractions were isolated from the cerebral cortex at various intervals of time by differential centrifugation. Percentage of enzyme activity in microsomes and nerve endings representing the new AChE synthesis were determined on the basis of AChE activity of fractions from control rats isolated at similar time periods. The first point shows the enzyme activity 4 hours following DFP. (Values with S.E.M in Table V.)
from the AChE which is replacing the old inhibited AChE. The enzyme activity arising due to normal development was determined from AChE values of control rats (Table V). The values for AChE following DFP was corrected on the basis of the developmental data and the results are presented graphically in Figure 8. In this case the developmental AChE increment was subtracted from the enzyme activity of DFP treated rats at various intervals of time and the difference represents AChE synthesized to replace specifically the DFP inhibited AChE. As already stated, in 20-day old rats, DFP caused on the day of injection, an inhibition of 76% in the microsomal AChE activity as compared to control; after 15 days, inhibition was reduced to 34% as a result of replacement by newly synthesized AChE. For a period of 15 days, therefore, the new synthesis of AChE in microsomes constituted 42% of normal values; finally, after correcting this latter figure for the ontogenic synthesis of AChE, the amount of AChE synthesized for a period of 15 days to replace the inhibited enzyme was 16% $\pm$ 2% (Figure 8).

Analogous data for the synaptosomal AChE was as follows: DFP caused an initial inhibition of 60% in the nerve ending AChE activity four hours following the injection; 15 days later, the enzymic activity was only 13% inhibited as com-
RECOVERY OF AChE ACTIVITY AFTER INTRACEREBRAL DFP ADMINISTRATION (3.5mg/Kg) IN 20-DAY RATS. VALUES ARE CORRECTED FOR NORMAL DEVELOPMENTAL RATE.

Figure 8: Conditions as described in Figure 7. To differentiate the synthesis of enzyme arising due to normal development and the one arising due to inhibition with DFP. Corrections for normal developmental rise were made in the DFP treated rats and the initial point was extrapolated to Zero.
pared to controls (cf. Table V). After the corrections for normal developmental synthesis was made, it appeared that during a period of 15 days the synthesis of AChE arising to replace the DFP inhibited enzyme was 43% of the control values (Figure 8). Two pools of AChE were formed in DFP treated rats; one was developmental enzyme, another the enzyme synthesized to replace inhibited AChE. In control rats, only one pool of enzyme, that is the developmental AChE is being synthesized. As the two groups of rats were of same age, the increase in AChE synthesis in DFP treated rats seems to be related to the inhibition of AChE by DFP. This inhibition caused by DFP might have repressed the feedback inhibition, normally due to the accumulation of AChE thus resulting in an increased AChE synthesis. This increase may be regulated either at the nuclear or at the microsomal level. There could be an increase in the production of mRNA or protein. The increase in AChE in DFP treated rats might represent the developmental enzyme. At this point with results available, we are not able to distinguish clearly as to which pool of enzyme activity was increased.

Altogether, whether corrected for normal development or not, the nerve endings showed higher percent of AChE synthesis as compared to microsomes following DFP treatment (Compare Figures 7 and 8); in fact, the corrected figure shows a more rapid rate of AChE synthesis in the synaptosomes as compared to microsomes.
CHARACTERISTICS OF THE EFFECT OF DFP IN OLDER RATS (58 day):

Various workers have demonstrated that protein synthesis occurs in isolated synaptosomal preparations. Austin and Morgan (1967) studying protein synthesis in synaptosomes showed the rate of incorporation of (C\textsuperscript{14})-leucine as linear and decreased with the age of the rat (cf. Introduction). The question arises as to whether the nerve ending synthesis is associated with the normal development of the animal and ceases at maturity or is a phenomenon which occurs in all age groups. It seemed appropriate therefore to compare the synthesis of AChE after DFP in the nerve ending of older animals. A group of 58 day old rats were used for this study. As it was difficult to keep the older rats alive after the higher dose (3.5 mg/kg) of DFP, a dose of only 2 mg/kg was given via the intracerebral routes. Control rats received intracerebrally the appropriate volume of propylene glycol. Rats were sacrificed at various intervals of time following the administration of DFP or propylene glycol. The specific AChE activities of the microsomal and nerve ending fraction of the brains of both the experimental and control rats were obtained on days 1, 5, 11 and 20 and AChE activity expressed as percent of controls. Figure 9 shows AChE activity of microsomes and nerve endings as compared to control values on days 1-20 following DFP administration. As can be seen, 80% of microsomal AChE and 86% of the nerve ending AChE was inhibited on the day of injection.
Figure 9: 58-day old rats were injected with DFP (2.0 mg/kg) intracerebrally and killed at various intervals of time following the injection. Subcellular fractions were obtained by differential centrifugation and assayed for AChE. Percentage enzyme activity representing the new synthesis was determined from enzyme activity of control animal injected with propylene glycol. Each point in the curve represents the value obtained from 6 rats. Values are expressed as ± S.E.M.
Twenty-two percent of nerve ending AChE and 35% of microsomal AChE remained inhibited on day 20 after DFP; as compared to normal values, 45% of the inhibited microsomal enzyme and 64% of the inhibited nerve ending enzyme was replaced by newly synthesized AChE. As in the case of younger animals, at all intervals after the administration of DFP the nerve endings always showed higher percent of replacement of the inhibited enzyme when compared to microsomes, and particularly between days 1 and 4, AChE of the nerve ending fraction showed a more rapid rate of synthesis as compared to microsomal AChE.

The recovery of AChE following DFP injection was more rapid in 58 day than in 20 day old rats. In the case of 20 day old rate, 47.0 and 42% of DFP inhibited enzyme was replaced by newly synthesized AChE at the nerve ending and at the microsomes, respectively, after a period of 15 days. In the case of 58 day old rats, 64 and 45% of the DFP-inhibited enzyme was replaced at the nerve endings and at the microsomes, respectively, during the same time period. (Figures 7 and 9, Table V).

Furthermore, in 58 day old rats DFP caused on the day of injection an 80% inhibition of microsomal AChE and 86% inhibition of the nerve endings enzyme, whereas in 20 day old rats DFP caused an inhibition of 76 and 60% inhibition in microsomes and nerve endings, respectively. Due to the constant need for AChE, particularly in the adult, marked inhibition
might alter the synthesizing mechanism resulting in a rapid synthesis. Except on the day of injection, in both age groups, the nerve endings always showed higher percent of enzyme recovery when compared to microsomes. The faster rate of nerve ending AChE synthesis following DFP inhibition both in young and older rats demonstrates that at least a fraction of AChE synthesis at the nerve endings is not an age dependent phenomenon.

**EFFECT OF TRITON ON AChE:**

As AChE is a membrane bound enzyme, various procedures must be used to solubilize the enzyme. If the enzyme is bound in such a way that its active centers are directly available to substrate, then permeability limitations with respect to DFP and substrate will not apply. However, as the enzyme is present in intact organelles (microsomes, synaptic cleft), permeability limitations will be evident if the active center of the enzyme resides within such structures. To ensure that the substrate is available to the active site, we decided to solubilize the enzyme, with non-ionic detergent Triton X-100.

Preliminary studies of the effect of Triton X-100 were conducted on human brain AChE. 2 gm of human brain was homogenized with 4 vols. of extraction media (0.9% NaCl or Triton X-100 (0.2-5.0%) in 0.9% saline solution). Following
centrifugation, supernatant and sediment was analyzed for AChE activity and values obtained with and without Triton X-100 were compared. Effect of various concentrations of Triton X-100 on the human brain AChE activity is shown in Table VI. There is redistribution of protein between supernatant and sediment, and solubilization of more than 50% of the protein when the concentration of the Triton X-100 is increased from 0 to 5.0%. Despite the loss of protein in the residue, the total AChE activity remains virtually constant in the residue at Triton X-100 levels of 0.2 to 2% and only at 5% level does some inhibition of activity appears to occur. "Relative" specific activity was expressed as the specific activity which was arbitrarily set at 100. At 1% Triton X-100 levels, the relative specific activity value of the supernatant was increased to 360, with little change in relative specific activity of the residue. At 2.0% and 5% Triton X-100 concentration, the relative specific activities were 230 and 120, respectively. Without a concomitant loss of enzyme activity in the residue, the increase in supernatant enzyme activity indicates the activation by Triton X-100 of AChE or release of bound AChE which was not normally available to the substrate. There was also an increase in the total enzyme activity.

It was important to rule out that the effect (increase in AChE) observed by Triton X-100 was not due to the effect of Triton X-100 on the enzyme substrate reaction rather than
Table VI: Human brain, 2 gm, was homogenized with 4 vol. of extraction media (0.9% NaCl or the appropriate Triton X-100 concentration )0.2-5.0% in a saline solution) and incubated for 30 minutes at 4 °C and centrifuged at 100,000xg for 1 hour. The sediment was dissolved in 12 ml of water. The supernatant and sediment were analyzed for AChE activity as described in Methods.
to the solubilization of the enzyme. The effect of Triton X-100 on the AChE assay system therefore was studied. This system contained soluble saline-extracted enzyme. Various concentrations of Triton X-100 were added to the reaction medium, containing saline extract of human brain ACbE and acetyl thiocholine iodide. With various concentrations of Triton X-100 in the enzyme-substrate medium neither the residue nor the supernatant showed any significant changes in ACbE activity. The specific activity of the residue and supernatant were approximately 2.0 and 1.0 respectively at all detergent (Triton X-100) concentrations. Thus, Triton X-100 per se does not appear to have any effect on enzymic activity after the enzyme is extracted. (Table VII)

The activation of AChE brought about by Triton X-100 may be due to the release of the enzyme from its association with membrane fragments in varying degree or to the unmasking of the hidden catalytic sites. It is possible that Triton X-100 may open up active sites which are not normally available for substrate and are hidden in the membranes.

**EFFECT OF TRITON X-100 ON THE DFP INHIBITED AChE:**

With the background of information obtained as to the effect of Triton X-100 on human brain AChE, we decided to study the effect of Triton X-100 on the DFP-inhibited enzyme. The purpose was to examine the effect of DFP on the
EFFECT OF TRITON X-100 ON ACETYLCHOLINESTERASE ASSAY VALUES

<table>
<thead>
<tr>
<th>Residue</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Spec. act. (x10^2)</td>
</tr>
<tr>
<td>Saline residue</td>
<td>2.0</td>
</tr>
<tr>
<td>+ 0.2% Triton X-100</td>
<td>1.9</td>
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<tr>
<td>+ 0.5% Triton X-100</td>
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<td>2.2</td>
</tr>
<tr>
<td>+ 5.0% Triton X-100</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Specific activity is expressed as µ moles acetylthiocholine iodide hydrolyzed/min per mg protein. Residue contained 7.4 mg protein/ml. Supernatant contained 3.4 mg protein/ml.

Table VII: Conditions as described in Table VI. Various concentrations of Triton X-100 was added to the assay medium and values were compared to the control without Triton X-100.
membrane bound or Triton-extractable enzyme. By studying the effect of Triton X-100 on the DFP-inhibited enzyme, the presence of hidden or bound catalytic site would be revealed.

Subcellular fractions from the brain of DFP (3.5 mg/kg) treated and control rats (20 day old) at a protein concentration of 1 mg/ml were homogenized with and without 1% Triton X-100. The supernatant and sediment obtained following centrifugation was analyzed for AChE activity. The combined specific activity of supernatant and sediment represents the total enzyme activity. The enzyme activity determined in the absence of Triton X-100 represents the soluble AChE. The difference in amount between the total and the soluble enzyme activity represents enzyme released by Triton X-100, i.e. the bound enzyme. The per cent recovery at various times following DFP of the total enzyme activity of various subcellular fractions of brains of rats injected with DFP was determined with respect to the total enzyme activity of the control rat brains. It could be calculated from the values in figure 10 that 4 hours after the inhibition of the total enzyme activity of the nerve ending was 51% while the inhibition of the soluble synaptosomal enzyme was 60%. Fifteen days following the injection, the inhibition of the total enzyme activity was 1% while the inhibition of soluble AChE was 13.0%.
EFFECT OF TRITON X-100 ON THE RECOVERY OF ENZYME ACTIVITY (AChE) IN SUBCELLULAR FRACTIONS AFTER I.C. INJECTIONS OF 3.5 mg/kg DFP

Figure 10: Microsomal and nerve ending fraction from DFP (3.5 mg/kg) treated 20-day old rats at a concentration of 1 mg/ml was homogenized with Triton X-100 to a final concentration of 1%. After incubating for 6 hours at 4°C, mixture was centrifuged, supernatant and sediment were assayed, percent enzyme activity was determined by calculating the values of Triton X-100 treated DFP fractions (activity of Triton X-100 treated sup + sed) against the Triton X-100 treated similar control fractions. Each point in the curve represents the value obtained from 6 rats. Values are expressed as ± S.E.M.
In microsomes on the day of injection the soluble and total AChE were inhibited 76% and 66% respectively. Fifteen days following DFP, the total AChE was found to be inhibited 24% and the soluble AChE to 34%.

On the first day following DFP, the difference in inhibition of the total enzyme as compared to soluble enzyme was around 10-12% in the case of both microsomal and nerve endings. This 12% might represent the AChE which is membrane bound and not accessible to inhibitors or it might represent the membrane bound enzyme which is being newly synthesized. In other words, there was membrane bound AChE which was not inhibited by DFP. When this enzyme was released by Triton X-100, it diluted the inhibited enzyme, consequently, the activity value for the total enzyme was higher (or inhibition lower) than that for soluble enzyme.

**EFFECT OF TRITON X-100 ON THE DFP-INHIBITED AChE AND CONTROL AChE:**

We decided to study the effect of Triton X-100 on the microsomal and nerve ending AChE of control and DFP-treated rats at various time intervals. The effect of Triton X-100 on the microsomal and nerve ending AChE from the DFP-injected rats was compared with the effect on the microsomal and nerve ending AChE of control rats.

Microsomes isolated as described under Methods were obtained from DFP (3.5 mg/kg)-treated and control 20 day old rats.
adjusted to a protein concentration of 1 mg/ml and treated with 1% Triton X-100. The difference in amount between the total enzyme activity and soluble enzyme activity represented the Triton X-100 released AChE or the bound AChE.

It can be seen in figure 11 that in the case of microsomes obtained from control rats, the soluble AChE of control microsomes increased whereas the Triton X-100 released enzyme decreased with the age of the animal. The specific activity of the soluble enzyme of control microsomes increased in 15 days from a specific activity of 0.109 to 0.190 whereas the specific activity of Triton X-100 released enzyme underwent a change of 0.210 to 0.120 in the same time period. In the microsomes of control animal, the increases in soluble enzyme accompanied by a decrease in Triton X-100 released enzyme at various time intervals might reflect the release of the enzyme from the bound state (following its synthesis) to the soluble enzyme.

In the microsomes of DFP-treated rats, the specific activity of soluble enzyme increased markedly from 0.0263 to 0.125 for the period of 15 days. For the same period, the Triton X-100 released enzyme underwent a small or no change in specific activity (from 0.09 to 0.080). It appears that in DFP-treated rats, there is a faster rate of AChE synthesis as compared to control rats. There might exist an equilibrium between the Triton X-100 released enzyme and the soluble enzyme. The steady state of specific activity in Triton X-100 released enzyme (AChE) may be due to occurrence of equilibrium
EFFECT OF TRITON X-100 ON MICROSMAL ACETYL CHOLINESTERASE AT VARIOUS TIME INTERVALS IN CONTROL AND DFP (3.5 MG / KG) - TREATED 20-DAY-OLD RATS

Figure 11: Microsomes from control and DFP (3.5 mg/kg) treated 20-day old rats at a concentration of 1 mg/ml was homogenized with Triton X-100 to a final concentration of 1%. After incubation for 6 hours at 4 °C, the mixture was centrifuged at 100,000xg for 1 hour. The supernatant and sediment (dissolved in 0.5 ml of H₂O) was analyzed for AChE activity. Normal enzyme activity without the Triton X-100 was the soluble enzyme. The difference between the Triton treated and the soluble enzyme was named as the Triton released.
between the rapid synthesis of enzyme and its rapid release into the soluble form.

Nerve endings from DFP (3.5 mg/kg) and control rats (20 day old) at a protein concentration of 1 mg/ml were treated with 1% Triton X-100 in a manner similar to that described for microsomes. The results showed that the amount of soluble and Triton X-100 released enzyme increased both in control and DFP-treated rats. In control rats, the specific activity of soluble enzyme of the nerve endings increased from 0.098 to 0.120 for a period of 15 days (Fig. 12). The Triton X-100 released enzyme for the same period underwent a change in specific activity of 0.110 to 0.18. In DFP-treated rats, the specific activity of the soluble enzyme of the nerve endings increased from 0.04 to 0.10 for a period of 15 days, whereas Triton X-100 released enzyme underwent a change in specific activity of 0.07 to 0.18. The significance of this increase in Triton X-100 released enzyme is not known. This enzyme might act as a reservoir. The main function of AChE is at the nerve endings where it hydrolyzes the released AChE. It seemed appropriate to visualize the existence of a reserve pool of enzyme which could be used in time.

**TURNOVER OF ACETYL CHOLINESTERASE ISOENZYMES:**

Microsomal and nerve ending fraction of DFP (3.5 mg/kg)-treated and control rats (20 day old) were treated with 1% Triton X-100. Supernatant obtained following treatment with
EFFECT OF TRITON X-100 ON NERVE ENDING ACETYL CHOLINESTERASE AT VARIOUS TIME INTERVALS IN CONTROL AND DFP (3.5 MG/KG)-TREATED 20-DAY-OLD RATS

Figure 12: Conditions were same as described for figure 11 except 1 mg/ml of nerve ending (synaptosome) preparation was used.
Triton X-100 was analyzed electrophoretically by polyacrylamide gel electrophoresis. By densitometric scanning, the percent contribution of each isoenzyme to total specific activity was determined in both groups. From the specific activity of AChE of the supernatant applied to each gel, relative specific activity of each isoenzyme was determined. Percent inhibition of various microsomal isoenzymes of DFP-treated rats were determined from the relative specific activity of microsomal isoenzymes of control rats. Figure 13A shows the 4 isoenzymes of control microsomes and figure 14A shows the 4 isoenzymes of control nerve endings. In microsomes, the isoenzymes 2 and 4 were prominent and denser, whereas in nerve endings isoenzymes 4, 3 and 1 were prominent and denser.

B, C, D and E in Figure 13 represent the microsomal isoenzyme pattern of DFP-treated rats on day 1, 3, 5 and 15 following the injection. On the first day after DFP injection, the inhibition of microsomal isoenzyme 4, 3, 2 and 1 were 81%, 25%, 49% and 0% respectively (Table VIII). Fifteen days following DFP, the inhibition of isoenzyme 4 was 36% and of isoenzyme 2 - 25%. In the same period, inhibited isoenzyme 3 was completely replaced by newly synthesized AChE. During the period of 15 days, 45, 25 and 24 percent of inhibited isoenzyme 4, 3 and 2 was replaced by newly synthesized enzyme.
Figure 13: Treatment of microsomes with Triton were done according to the conditions described in Figure 11. 40-50 µg of Triton treated supernatant was applied to each gel. Separation was achieved at 4°C in Tris-glycine buffer (pH 8.4) at 3mA per tube for 3 hours as described in Methods. Incubation and staining of the gel were performed according to the procedure described in Methods. Gel A: Triton supernatant of microsomes from control rats. Gel B:C:D:E: Triton supernatant of microsomes on day 1, 3,15 following intracerebral injection of DFP at a dose of 3.5 mg/kg in 20-day old rats.
ACETYL CHOLINESTERASE ACTIVITY (%) OF 1% TRITON X-100 EXTRACTED MICROSONAL ISOENZYME OF DFP (3.5 mg/kg) TREATED 20 DAY OLD RAT DETERMINED BY DENSITOMETRIC SCAN AS COMPARED TO TRITON X-100 TREATED CONTROL MICROSONE

<table>
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<td>Day 15</td>
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<td>75</td>
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Table VIII: Triton treatment of microsomes and electrophoresis were performed according to the conditions described in Methods. Relative Specific Activity of each band was determined from the specific activity of enzyme applied to each gel.

Percent inhibition of various isoenzymes of the DFP-treated group were determined from the Relative Specific Activity of various isoenzymes of the control group.
These values indicate that the microsomal isoenzymes not only show differential rate of inhibition but also different order of synthesis. This might indicate a different turnover rate for these microsomal isoenzymes.

The inhibition and recovery of nerve ending isoenzymes were also studied in a manner similar to those of the microsomal isoenzymes. Figure 14 shows the 4 isoenzymes of control nerve endings. Isoenzyme 4, 3 and 2 were prominent and dense. Gel B, C, D and E represents the nerve ending isoenzyme pattern on day 1, 3, 5 and 15 following treatment with DFP. From the values of control rats, percent inhibition of the nerve ending isoenzymes were determined on the day of injection; the isoenzymes 4, 3, 2 and 1 were inhibited 83%, 30%, 4% and 82% respectively (Table IX). Fifteen days following DFP injection, the inhibition of the isoenzyme 4 and 1 was 16 and 25 percent, respectively. Inhibited isoenzyme 3 and 2 were completely replaced by newly synthesized enzyme. The inhibited isoenzymes 4, 3, 2 and 1 were replaced to the extent of 67, 30, 4 and 57 percent, respectively, during a period of 15 days. Here again the isoenzymes of nerve endings demonstrated different order of sensitivity to DFP and of the recovery rate indicating a different turnover rate.
Figure 14: Conditions as described in Figure 13.

Gel A: Triton supernatant of nerve endings fractions obtained from control rats.

Gel B, C, D, E: Triton supernatant of nerve ending fraction isolated on days 1, 3, 5, 15 following intracerebral injection of DFP at a dose of 3.00 mg/kg in 20-day old rat.
ACETYL CHOLINESTERASE ACTIVITY (%) OF 1% TRITON-X-100 EXTRACTED SYNAPTOSOMAL ISOENZYME OF DFP (3.5mg/kg) TREATED 20 DAY OLD RAT DETERMINED BY DENSITOMETRIC SCAN AS COMPARED TO TRITON X-100 TREATED CONTROL

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Table IX: Triton treatment of nerve endings and electrophoresis were performed according to the conditions described in Methods. Relative Specific Activity of each band was determined from the specific activity of enzyme applied to each gel. Percent inhibition of various isoenzymes of the DFP-treated group were determined from the relative Specific Activity of various isoenzymes of the control group.
DISCUSSION: EFFECT OF DFP ON RAT BRAIN AChE

After attempting various methods of isolation of synaptosomes and analyzing the fractions by electron microscopy, we chose the method of Autilio and Appel (1968) as the fractions obtained by this method were relatively intact. Synaptosomes are difficult to obtain as intact preparations due to various contaminating membranous elements (from various organelles in nerve cells). As can be seen from the electron micrographs (Fig. 1, 2) we were able to obtain relatively intact preparations. Indeed, even the functional contacts between the pre- and post-synaptic terminal can be seen, as are the intraterminal mitochondria.

Synaptosomes are heterogeneous with respect to chemical composition. Synaptosomes derived from the whole brain or from cerebral hemisphere contain various transmitters and enzymes responsible for their synthesis and degradation. Synaptosomes contain relatively large number of enzymes; enzymes of the intra-terminal mitochondria, enzymes of the soluble cytoplasm and enzymes of the extra-synaptic membrane. It is difficult to assess for contamination as there are no specific markers for synaptosomes. Since our purpose was to study the protein synthesis in synaptosomes, we were concerned only with components which would complicate interpretation of the origin of protein
synthesis. The only contaminating fraction with which we were concerned were microsomes. Assaying for NADPH-dependent cytochrome C reductase in the synaptosomal and microsomal fraction through various isolation procedures is indicative of the degree of microsomal contamination. The contamination was found to be less than 15.0%. By using rotenone in the assay medium, we eliminated the results arising from the mitochondrial enzyme. (Table X).

We noted a low recovery of radioactivity in earlier experiments using (C\textsubscript{14})DFP. The radioactivity of the homogenate following injection with (C\textsubscript{14})DFP increased with increasing amounts of (C\textsubscript{14})DFP but the recovery was still low. The original compound (C\textsubscript{14})DFP was in propylene glycol. Since propylene glycol is an inert solvent, various media were tested to improve the binding of (C\textsubscript{14})DFP to the brain. As can be seen (Table 2), there was no improvement in the recovery of the counts when various media were employed. The low recovery of counts in various subcellular fractions indicated loss of radioactive counts following the injection.

Jandorf and McNamara (1950) injecting(\textsuperscript{32}P) labelled DFP intravenously into rabbits found that radio phosphorus was retained in relatively large amounts by kidney, liver and lung, while brain and other organs retained very insignificant amount of label. The brain, while exhibiting high levels AChE had
TABLE X

NADPH CYTOCHROME C REDUCTASE ACTIVITY OF MICROSOMAL AND SYNAPTOSOMAL FRACTIONS. SPECIFIC ACTIVITY IS EXPRESSED AS

\[ \text{Abs 550/minute per mg of protein} \]

<table>
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<tr>
<th>Fraction</th>
<th>0.255</th>
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<tr>
<td>Microsomal Fraction</td>
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<tr>
<td>Synaptosomal Fraction</td>
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</table>
the least amount of radioactivity. Cohen and Warringa (1954) studying the fate of DFP in human body found that its metabolism involved both protein binding and detoxification, about 80% of (32P) was excreted in urine as diisopropyl phosphoric acid and about 20% remained bound to the tissue. DFP bound nonspecifically to tissue proteins show little dealkylation compared to DFP which was found to brain enzymes. DFP bound nonspecifically to tissue proteins was hydrolyzed after a few hours.

During injections with (C14)DFP, in one set of experiments, we found large amount of radioactive counts in liver (Table 1). The liver radioactive counts may represent DFP lost in detoxification and in the course of nonspecific binding. Normally aging phenomenon following DFP occurs by the loss of one isopropyl group. In a uniformly labelled (C14)DFP, the aging phenomenon would result in a loss of 50% of the injected label. There may be further loss of label due to nonspecific binding of (C14)DFP to tissue proteins. The actual counts obtained by us might represent the label bound to acetyl cholinesterase. These may be the reasons for the low recovery of counts obtained. The low recovery of counts did not permit the determination of the binding of inhibitor to individual isoenzymes.
In our later experiments, using unlabelled DFP, a minimum of 2.0 mg/kg of DFP was required by intracerebral injections to inhibit 92% rat brain AChE (in 16 day old rats). Studying the inhibition with rat brain homogenate in vitro, $10^{-5}M$ DFP caused 92% inhibition of AChE. In terms of concentration at least seven times the in vitro concentration of DFP was required in vivo to inhibit the enzyme to the same extent.

In a homogenate, the structures of the organelle may be ruptured resulting in a better reaction with the inhibitor. On the other hand, in an in vivo system, (intact system), higher amount of inhibitor will be required to reach the enzyme bound to the membranes (synaptic junction). Once the inhibitor binds the enzyme, the binding becomes irreversible. Before it reaches the enzyme, it might bind nonspecifically to tissue proteins and proteolipids or might be carried in the blood stream away from the injected organ for the purpose of detoxification.

Another explanation for the requirement of high dose in vivo is related to the hydrolysis of the DFP by certain enzymes present in the system. Mazur (1946) observed that $10^{-2}M$ DFP in the presence of serum produced a considerable CO$_2$ evolution from a bicarbonate medium and that similar results could be obtained with tissue extracts and found this is due to DFP hydrolyzing enzyme. Aldridge also reported the hydrolysis of paraoxan (E-600) by serum esterase. The high
amount of nonspecific esterase, especially aryl esterase present in the brain might hydrolyze injected DFP.

PROTEIN SYNTHESIS AND AXOPLASMIC TRANSPORT:

There are a number of indications from our studies and that of others that nerve endings can synthesize their own protein.

The classic studies of Weiss (1943, 1947, 1959, 1967) have shown that a mass of neuroplasm material migrates down the axon at a rate of approximately 1 mm per day. Weiss proposed the concept of axonal flow as a mechanism for the transport of specialized cell products from the central site of manufacture to peripheral destination. Lasek (1968) using autoradiographic and scintillation counting technique studied the distribution of $^3$H-leucine injected into the cat dorsal root ganglion and ventral horn region of the rat spinal cord. Rates of axoplasmic transport up to 100 mm per day were found in the case of the motor neuron and of the dorsal root ganglion cells of the cat, respectively. Lasek proposed that two categories of axoplasmic transport may be distinguished, in one case, the materials mostly protein is transported from the cell body at a few millimeters per day, as observed by Weiss and his co-workers; in the other, the protein is transported with a wide range of only a few millimeters up to 100 to 500 mm per day. Various theories have been proposed regarding the
mechanism of axoplasmic flow. None of them is firmly supported by experimental data. Weiss (1967) and Ochs (1963) proposed that axoplasmic transport occurs by peristaltic movement and energy is obtained from periaxonal elements. Recent tissue culture suggests the presence of a protein which by change in configuration provide the necessary energy for transport. Borisky and Taylor (1967) found a protein associated with microtubules and filaments in mammalian brain and squid axoplasm which had a high affinity for colchicine. The role of this protein in transport is being considered. A survey of concentration of this protein in a variety of cell types showed that protein extracts and specifically the extracts of axoplasm were the richest source of colchicine binding protein which is presumed to be subunit of microtubules. (Shelanski and Taylor, 1970). It is a major soluble protein and may also exist in particulate form. Half life of this protein was shown to be about 4 days. Therefore microtubules are influx rather than structural elements. It has been shown that this protein migrates with a slow component of axoplasmic transport. Although nerve endings do not characteristically contain any identifiable microtubules, substantial proportion of soluble component of isolated nerve ending fraction is microtubular protein.
Studying the axonal transport of cholinergic enzymes, (Fonnum et al, 1973) the estimated rate for AChE was 430 mm/24 hours for vagus and 73 mm/24 hours for the hypoglossal nerves. These rates correspond to the fast or intermediate rate of axonal flow. They also found that only small proportion of the enzymes (5-20%) were mobile and concluded that only a part of AChE in the vagus and hypoglossal nerve migrated. A retrograde transport of the enzyme was also demonstrated. The amount of enzyme transported this way, represented only a small portion of the total transport of cholinergic enzymes.

If it was assumed that AChE was transported by a fast transport, there must exist a mechanism by which it is transported to the presynaptic membrane. Tubular protein which was considered to be involved in transport was found to migrate only with slow component of axoplasmic transport. Furthermore, nerve endings do not characteristically contain any identifiable microtubules. Fonnum et al (1973) found only a small percent of the enzyme being transported and retrograde transport was much lower than this. So there must exist a system for breaking down the enzyme at nerve endings. Lyso- somes have not been shown to be present in the nerve ending fraction. A small amount of proteinase activity with a PH range of 5.3 to 6.9 was found at NE (Palladin and Belik, 1970). Whether the proteinase is responsible for the breakdown of
AChE in the nerve ending is not known. As the main function of AChE is at nerve ending, a higher percent of the enzyme should be expected to be transported if it was dependent on the cell body for its synthesis. Large amount of enzyme was found to be stationary. Axoplasmic transport of AChE still remains an open question.

On the other hand, there is evidence for local synthesis of protein in the nerve axon as shown in vivo and in vitro studies. The recent extensive work with the unicellular organism Acetabularia (Bracket, 1967) constituted an important stimulus to consider the possibility that protein may be synthesized at sites which are very distant from cell nucleus. There is evidence that axon can survive for a considerable period of time after its transection from the cell body and can synthesize ribonucleic acid and protein particularly in chloroblasts.

Protein synthesis in isolated synaptosomes have been shown by various workers as discussed earlier (cf. Introduction; Autilio and Appel, 1968; Austin and Morgan, 1967 and Goldberg, 1971). The lack of visible ribosomes in both the axon and its endings does not rule out the possibility of protein synthesis in such tissues, since RNA with base ratios consistent with ribosomal RNA has been found in Mauthner axons of fish
(Edstrom et al., 1962) and in cranial nerve roots in cat (Koenig, 1965). RNA is present in synaptosomes in particular in the non-mitochondrial membrane deprived from these synaptic organelles (Austin and Morgan, 1967). Studying protein synthesis in vitro synaptosomal preparation, Austin and Morgan (1967) showed that uptake of radioactive amino acid was linear for an hour. The synaptosomal fraction was broken down to its components by osmotic shock. Membranes, soluble protein and mitochondria derived from osmotically shocked synaptosomes were all labelled with amino acid showing the most rapid rate of labelling. Their results strongly suggested that synaptosomes can synthesize protein and since not only mitochondria but also synaptosomal membranes and soluble protein were labelled, it seemed possible that a protein synthetic machinery exists either in the membrane or the cytoplasm of nerve ending. Various workers used protein synthesis inhibitors to differentiate the biosynthesis of proteins in the synaptosomes and in particular to determine whether the synthesis is of synaptosomal or mitochondrial type. It could be argued that the amino acid incorporation is not into synaptosome at all, but is merely the result of an uptake by some contaminants such as bacteria, mitochondria or ribosomes. Autilio et al. (1968) showed that bacteria isolated from the incubation mixture incorporated leucine linearly for at least
5 hours, whereas the rate of incorporation into synaptosomal protein fell off rapidly after 20-30 minutes. These bacteria were very sensitive to chloramphenicol inhibition, whereas the synaptosomes are not. Mitochondria from brain synthesize protein (Campbell et al., 1966, Bachelard, 1966) but despite the inclusion of mitochondria within the nerve ending, all of the protein synthesis cannot be due to mitochondria. The sensitivity of mitochondrial protein synthesis to chloramphenicol has been demonstrated for mitochondria from a wide range of tissue including brain (Kroon, 1963, Clark-Walker and Linnane, 1967) and yet synaptosomal protein synthesis was inhibited by only 25%, a figure which suggests contribution made to the system by both synaptosomal and contaminating mitochondria (Austin and Morgan, 1967). Mitochondria derived from synaptosome showed less than 2% of the incorporated isotope in a soluble fraction isolated after sonication (Autilio et al., 1968). The soluble protein isolated from synaptosome after incubation with leucine had about 20% of the total incorporated label.

Contamination by free ribosomes released from other structures is not a problem in this system. Brain ribosomal system is inhibited almost completely by incubation with ribonuclease, but the intact synaptosomal system is unaffected (Campbell et al., 1966). When labelled microsomes added to a brain homogenate did not contaminate the synaptosomal fraction which was
subsequently isolated from the homogenate (Austin and Morgan, 1967).

The origin of axonal RNA is unknown. It could be transported from the cell body or from Schwann cells or glia along the axon. It does seem however that the nerve receives the RNA or RNA precursor in some way by transport along the nerve rather by direct uptake from the blood.

Our study was undertaken to study the mechanism by which AChE is formed at the external membrane of the pre- and post-synaptic terminal and the junctional complex. The question was whether it is formed locally or was transported from the cell body. AChE is associated chiefly with membranous structures in rat brain (Toschi, 1959). At the synaptic area, it has been shown to be localized on the external membrane of the presynaptic terminal and in the junctional complex. The hypothesis on the functional significance of AChE is based in part on their known localization. The membrane-bound enzyme has implications for function which are different than those for the function at the cytoplasmic-localized enzyme and the hypothesis of the functional AChE and the related hypothesis of Koelle (1961) are based on the identification of functional AChE at the synaptic membranes.
We studied the synthesis of AChE in the 20-day old rat following DFP injections (3.5 mg/kg). For a period of 15 days nerve endings synthesized or replaced 47% of the inhibited AChE, while microsomes replaced 42% of the inhibited enzyme. Since this period represents a period of rapid growth in the animal, attempts were made to differentiate the enzyme arising due to normal growth and those arising due to enzyme replacement after DFP inhibition. After the appropriate corrections, it was found that AChE synthesized to replace the DFP inhibited enzyme amounted to 43% at the nerve endings and to 16% at the microsomes.

The ability of synaptosomes to incorporate ($^{14}$C) leucine into their proteins was found to decrease with the age of the rat (Austin and Morgan, 1967). However our data indicate the presence of a AChE synthesizing machinery at the nerve endings of older (58 days older) as well as of younger animals (20 days old). In 58 day old rats following DFP injections (2.0 mg/kg), their nerve endings synthesized and replaced 64% of the inhibited AChE, while microsomes replaced 45% of the inhibited enzyme.

According to some investigators (Weiss and Hiscoe, 1948) proteins are presumed to be transported to nerve endings from the cell body following its synthesis at microsomes. If nerve ending AChE had its origin in the cell body and was transported from the microsomes at the cell body, a faster rate of micro-
somal AChE synthesis compared to its appearance at nerve endings could be expected following DFP administration. There would be a time lag, if replacement is dependent on microsomal synthesis for the enzyme and its subsequent transport from microsomes to nerve endings. Altogether it would take time for AChE to be transported from the cell body to the nerve endings. Since AChE is a protein of high molecular weight, it would probably be transported by a slow process. We never found a time lag in nerve endings either in 20 day or 58 day old rats, in fact recovery data indicate an earlier onset of AChE synthesis. This evidence indicates that the synthesis of AChE may occur in the nerve endings. The higher rate of synthesis of AChE in nerve endings as compared to the microsome of older animal following DFP might be due to higher initial inhibition of AChE which occurred in these animals. Our results throw a light on the paradox of coexistence of free cytoplasmic acetyl choline and acetylcholinesterase in the same cell which is concomitant with the concept of the axoplasmic transport. As the nerve ending fraction synthesized their own AChE at the membrane, there will not be a need to supply the enzyme from the cell body and there would be no cytoplasmic enzyme capable of AChE hydrolysis.

PROPERTIES OF THE MEMBRANE BOUND AChE:

Several enzymes have been extracted from the membranes and their properties were found to be different from those of
the naturally-bound enzymes. This phenomenon has been termed allotopy (Racker, 1967). The first example of allotopy to be described was that of mitochondrial ATPase (F1) (Racker, 1967). In its membrane-bound form, the enzyme was sensitive to oligomycin and N,N-dicyclohexyl carbodiimide; in the soluble form this sensitivity was lost but the enzyme became cold labile. Solubilizing the succinic dehydrogenase from the inner membrane of mitochondria, various workers found modifications of properties of the enzyme. The catalytic properties of the membrane-bound enzyme was found to be different from the soluble dehydrogenase (Cerletti et al., 1967). The iron chelating agent TTA (2 Thionyl Trifluoreacetone, 4,4,4,4 Trifluoro-1-(2-thionyl) 1-3 butane dione) was found to inhibit only the membrane-bound enzyme at low concentrations (Redfearn, 1965). The membrane-bound dehydrogenase was found to be more stable than the solubilized one (King, 1963). Other enzymes have also been shown to exhibit this phenomenon of allotopy.

THE PROPERTIES OF THE MEMBRANE-BOUND ENZYME are different due to the difference in their surroundings. The properties may be influenced by 1) the general chemical and physical nature of the membrane, 2) by specific interactions of the enzyme with various molecules in its immediate neighborhood and 3) by local effect of the enzyme and that of its neighbors.
We decided to solubilize the membrane-bound enzyme and study the difference in property between the soluble enzyme and the membrane-bound enzyme. Initially as a model system, we studied the effect of Triton X-100 on the human brain AChE.

We found considerable activation of the enzyme with 1% Triton X-100 (Table 6). Other workers also found activation of AChE with Triton X-100. Harwood and Hawthorne (1969) found considerable stimulation of AChE with Triton X-100 (cf. also Crone, 1971). Fiszer and De Robertis (1967) claimed only solubilization of the enzyme with Triton X-100 though their Triton X-100 treated mitochondrial fraction showed an increase in activity.

In the case of the human brain, we found an increase in AChE activity in the supernatant with Triton X-100, without concomitant change in the activity of the sediment. The analytical data indicate that the increase in acetylcholinesterase activity that is demonstrable with the use of Triton X-100 in the extractive media is not due to more complete extraction of the enzyme from the particulate to which it is bound but to changes in the properties of the catalytic protein. This is evidenced by change in supernatant activity without any commensurate changes in the activity of the residue. These changes could be due to the catalytic protein released from
association with membrane fragments; as originally associated with the membrane, AChE may have been not fully active. The nature of the binding of enzyme to membrane could be altered. This can lead to either the unmasking of hidden catalytic sites by release of an allosteric inhibitory mechanism or by conformational changes in the molecule due to rupture of lipophilic bonds. It appears that Triton X-100 alters the conformation of the enzyme resulting in an enhancement of catalytic activity.

The effect of Triton X-100 on the AChE activity of subcellular fractions of DFP (3.5 mg/kg)-treated 20-day old rats was also studied. The Triton X-100 treated microsomal and synaptosomal fraction displayed a lower percent of AChE inhibition following DFP as compared to buffer treated fractions. The difference in inhibition between the Triton X-100 treated enzyme and the soluble enzyme was around 10-12% in the case of microsomes and nerve endings. This fraction might represent a pool of reserve enzyme or the membrane-bound enzyme which is not accessible to DFP. It is also possible that Triton X-100 treatment opens active sites which are not normally available for interaction with substrates or it might represent the newly synthesized enzymes which are not available to substrates normally.
Effect of Triton X-100 on nerve ending microsomal AChE of control and DFP-treated rats at various intervals of time in 20 day old rats (age 20-35 days). In the case of microsomes (Fig. 11), the specific activity of the enzyme released by Triton X-100 decreased with the age of the animal, while the specific activity of the soluble enzyme increased with age. In the DFP-treated group, the soluble enzyme increased, while the enzyme released by Triton X-100 remained almost constant during aging. This situation was markedly different in the nerve ending fraction (Figure 12) as in this case the soluble as well as Triton-released enzyme increased both in control as well as DFP treated animals. The large increase in Triton-released enzyme found in nerve endings at various ages may represent a reserve or a pool enzyme. In microsomes only the soluble enzyme increased at various ages whereas the Triton released enzyme decreased during the various time intervals.

The main physiological function of AChE is at the nerve endings. It is possible to have a pool of enzyme acting as a reservoir at nerve endings. The increase in Triton releasable enzyme at nerve ending following DFP was not accompanied by a similar increase or decrease at the microsomal enzyme level. This further indicates that the nerve endings possess an independent protein synthesizing machinery of nerve endings. The ability of the nerve endings to synthesize the soluble as well
as the membrane-bound enzyme is clearly shown.

We excluded by appropriate tests the possibility that our results were related to microsomal contamination of the synaptosomes (cf. Table X). If the nerve endings were contaminated with microsomes, the enzyme recovery would follow a pattern similar to microsomes. The absence of such pattern diminishes the possibility of the results arising from microsomal contamination. As the percent of enzyme activity was calculated against the values from control rats, the minimal error which may arise from microsomal contamination is eliminated to a large extent.

ISOENZYMES OF ACETYLCHOLINESTERASE

We have resolved several bands with AChE activity in rat brain tissue by polyacrylamide gel electrophoresis. Since the soluble extract gave a similar pattern in the case of the microsomes and of the nerve endings, we used a 1% Triton X-100 extract which showed differences in isoenzyme pattern of nerve ending and microsomes. As 1% Triton produced consistent results, it was used in this experiment. Five hours after DFP injection (3.5 mg/kg), microsomal isoenzymes extracted by 1% Triton X-100 showed differential inhibition patterns (Fig. 13). After analyzing the results by a densitometer, the order of inhibition of the 4 microsomal isoenzymes on the day of injec-
tion was 82, 49, 25 and 0% for isoenzymes 4, 2, 3 and 1 respectively. The rate at which these isoenzymes were replaced by newly synthesized enzyme was also different (Table 8).

The nerve ending isoenzymes underwent a different order of inhibition and recovery as compared to microsomal isoenzymes. On the day of injection, order of inhibition of the isoenzymes of nerve endings was as follows: Isoenzyme 4 (83%), Isoenzyme 1 (72%), Isoenzyme 3 (30%), Isoenzyme 2 (4%). In a period of 15 days, the inhibition of isoenzyme 4 was reduced to 16% and isoenzyme 1 to 25%. The other two isoenzymes were completely replaced by newly synthesized enzyme. Again, the recovery of nerve ending isoenzyme were different from that of microsomal isoenzymes. If the two subcellular fractions, namely microsomes and nerve endings exhibited similar pattern of inhibition and recovery, then the question of contamination of microsomes in nerve ending arises. Furthermore, comparing the differences between the inhibition and recovery of microsomal and nerve ending isoenzyme confirms the notion of independent synthesis of AChE in the nerve endings. On the day of injection, the inhibition on microsomal isoenzyme 1 was zero, but the nerve ending isoenzyme 1 was inhibited 82%. Isoenzyme 2 was on the way to recovery in microsomes, whereas at nerve endings it was completely replaced by newly synthesized enzyme for the same period. Isoenzyme 4 which seems to be a dominant one in both
subcellular fractions, was synthesized to 67% at the nerve endings and to 45% at the microsomes following inhibition with DFP. These data again indicate the independent protein synthesizing machinery at the nerve ending.

Microsomal and nerve ending isoenzyme pattern were reproduced following various freezing and thawing procedures. This indicates that they are separate units and are not formed by recombination of subunits. If the nerve ending isoenzymes were supplied by the microsomes by axoplasmic transport, then the order of inhibition with DFP and resynthesis would follow the same pattern. The two sets of isoenzymes (microsomal and nerve ending) follow entirely a different course following their inhibition with DFP. These results further support the presence of an independent protein synthesizing machinery at the nerve endings which is also responsible for the synthesis of isoenzymes. Davies and Agranoff (1968) in three different kinds of experiments have demonstrated that there is an isoenzyme of rat retina with an unusual half life of about 3 hours. Studies with several enzymes have indicated that these proteins are synthesized with a zero order reaction rate and degraded randomly with a first order rate (Rechagl and Weston, 1967).

In our preparations, isoenzyme 4 from nerve ending had a half life of 11 days while the microsomal isoenzyme 4 had a half life of 16 days. Rest of the isoenzymes at the micro-
somes and nerve ending exhibited half lives ranging from 11 to 50 days. The significance of this difference in the turnover rate of these isoenzymes is uncertain. The isoenzyme with a fast turnover rate may act as a precursor to other isoenzymes. These isoenzymes may have a different physiological role. In the case of nerve endings, the rapidly recovering isoenzyme might represent the functional enzyme responsible for the transmitter hydrolysis. The other three isoenzymes might have different roles. They may act as a precursor to the other enzymes. In microsomes, a different set of conditions may exist.

Wenthold et al., (1974) also confirmed the existence of multiple active forms of rat brain acetylcholinesterase and observed six forms by electrofocusing the enzyme. The six forms separated by electrofocusing were similar in several respects. They found them to be glyco-protein containing a subunit of same molecular weight with a distinct, reproducible isoelectric point. Specific isoenzymes were found to be concentrated in different subcellular locations. Similar to our results, they also noted the existence of enzyme in soluble and membrane-bound forms and the differences in the enzymes from these two different origin were also seen. On treating the isoenzymes with neuraminidase, they did not find any alteration in the isoelectric pattern.
Studying AChE isoenzymes in mouse brain, Kahayashi et al., 1973, found three different isoenzymes differing in PH optima, Km and inhibitory properties. The three isoenzymes were found to be distributed in different subcellular fractions. They proposed different physiological function for these isoenzymes.

The heterogenity of the rat brain synaptosomal AChE has been shown electrophoretically by Glebov et al., (1970). Using a different extraction medium, they found differences in number of AChE bands in synaptosomes and in mitochondria (Glebov et al., 1971). The physiological function of the isoenzymes remains to be determined, but it seems likely that they correlated to the different subcellular localization of the enzyme. In the case of the choline acetyltransferase, Fonnum and Malthe Sorensen (1973) sought to correlate a higher isoelectric point and higher positive charge with enhanced tendency to bond to negatively charged residues in membranes. So far the charge distribution among the different forms of AChE does not correlate in any obvious way with electrostatic binding properties of the membranes. Probably when more is known about structure of neuronal membranes, light will be thrown regarding the localization of these AChE isoenzymes to the membrane surface. The isoenzymic forms though catalyzing the same reaction do generally have distinct catalytic properties which presumably enables each of them to function in accord with physiological requirements either at different sites within the cell or in
distinct metabolic sequences (Stadtman, 1968). The most important biological question posed by isoenzyme is simply their existence. They must clearly confer great advantages to an organism, else they would not be so common. But just what each isoenzyme does that distinguishes it from others is still largely unknown.
CONCLUSIONS AND SUMMARY

The protein requirement of synapses poses a unique problem as they are located at some distance from the cell body. The macromolecular requirement of synapses is a problem by itself in normal state as well as in pathological conditions. The question arises as to whether synapses synthesize all their proteins or do they depend on the cell body for proteins. As a model to study such a mechanism, we chose a system of isolated synaptosomes and microsomes from 20 and 58 day rat brain previously injected with DFP and studied specifically the synthesis of AChE. In 20-day old rats, following DFP, 47% of the inhibited AChE was synthesized at nerve endings, while for the same period 42% of the inhibited enzyme was synthesized at microsomes. Since the period represents a period of rapid growth in the animal, attempts were made to differentiate the enzyme arising due to normal growth and those arising due to enzyme replacement after DFP inhibition. After the appropriate corrections, it was found that AChE synthesized to replace the DFP inhibited enzyme amounted to 43% at the nerve endings and to 16% at the microsomes. In 58 day old rats, 64% of the inhibited nerve ending AChE was synthesized and for the same period microsomes synthesized 45% of the inhibited enzyme. This phenomenon was not an age dependent one as it was found in both age groups. AChE was found to
be synthesized at nerve endings without a concomittant increase or decrease of microsomal enzyme which suggested an independent synthesizing machinery of nerve endings. When Triton X-100 was used to solubilize AChE, the inhibition due to DFP was considerably less in nerve terminals and microsomes compared to DFP inhibition measured in buffer treated fractions. These results suggest that a reservoir of the enzyme is not available to substrate and to inhibitors such as DFP.

We also found differences in the rate of inhibition and recovery of isoenzymes in the microsomal and nerve ending fraction following DFP which further confirmed the independent synthesizing machinery of the nerve endings. On the day of injection, the order of inhibition of the 4 microsomal isoenzymes was 81, 49, 25 and 0% for isoenzymes 4, 3, 2, 1. Fifteen days following DFP, the inhibition of isoenzyme 4 was 36% and isoenzyme 2 was 25%. In the same period, inhibited isoenzyme 3 was completely replaced by newly synthesized AChE. The inhibition and recovery of nerve ending isoenzyme were also studied in a manner similar to those of the microsomal isoenzymes. On the day of injection, the isoenzymes 4, 3, 2 and 1 were inhibited 83, 30, 4 and 82% respectively. Fifteen days following DFP injection, the inhibition of isoenzyme 4 and 1 was 16 and 25 percent respectively. Inhibited isoenzyme
3 and 2 were completely replaced by newly synthesized enzyme. The isoenzymes of nerve ending demonstrated different order of sensitivity to DFP and of the recovery rate indicating a different turnover rate.

Certain hypothesis were made regarding the function of these isoenzymes. Microsomes might supply the enzyme around the cell body which prevents the proliferation of Ach into cell from other sources. Nerve endings might supply the enzyme at the junctional complex whose main role is to hydrolyze the transmitter following chemical transmission.
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The dissertation submitted by Rajammal Srinivasan has been read and approved by a committee from the faculty of the Graduate School, Loyola University of Chicago.

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.

Date
Signature of Advisor

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