A Biochemical Study on the Effect of Combination of Two Organophosphorus Compounds, EPN \([O\text{-}ethyl\ O\text{-}P\text{-}nitrophenyl\ Thionobenzene\ Phosphonate}\) and Malathion \([O,O\text{-}dimethyl\ S\text{-}(1,2\text{ dicarbethoxy})\ Ethyl\ Dithiophosphate}\) on Brain Cholinesterases

Olfat Awad
Loyola University Chicago

Recommended Citation
Awad, Olfat, "A Biochemical Study on the Effect of Combination of Two Organophosphorus Compounds, EPN \([O\text{-}ethyl\ O\text{-}P\text{-}nitrophenyl\ Thionobenzene\ Phosphonate]\) and Malathion \([O,O\text{-}dimethyl\ S\text{-}(1,2\text{ dicarbethoxy})\ Ethyl\ Dithiophosphate]\) on Brain Cholinesterases" (1961). Dissertations. 564.
https://ecommons.luc.edu/luc_diss/564

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.
Creative Commons License
This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 1961 Olfat Awad
A BIOCHEMICAL STUDY ON THE EFFECT OF COMBINATION OF TWO ORGANOPHOSPHORUS COMPOUNDS, EPN [O-ETHYL O-P-NITRO PHENYL THIONOBENZENE PHOSPHONATE] AND MALATHION [O,O-DIMETHYL S-(1,2 DICARBETHOXYETHYL) DITHIOPHOSPHATE] ON BRAIN CHOLINESTERASES.

by

Olfat Awad

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

February 1961
LIFE

Olfat Awad was born in Alexandria, Egypt in 1933. She attended University of Alexandria, Faculty of Science, between 1951 and 1955 and graduated in 1955 with a B.S. degree in chemistry.

She was a demonstrator in the department of Chemistry University of Alexandria, 1955-1956.

Between 1956 and 1957 she was a graduate student in the department of Biochemistry, University of Illinois, as University of Illinois Fellow. She graduated from Illinois with a M.S. degree (1957) upon completion of a thesis on development of new method and apparatus for micromasurement of tissue content of oxygen.

Since 1958 she became a graduate student, department of Pharmacology and Therapeutics, Loyola University Graduate School. While at Loyola she was a graduate Assistant and Standard Oil Fellow, 1959-1960.

She is author of the following publications:


ACKNOWLEDGMENTS

I am indebted in many ways to Dr. Alexander G. Karczmar, chairman of Department of Pharmacology, Loyola University, who as my adviser has given generously of his time and effort in directing the course of this investigation. Also, I wish to extend my sincere appreciation to my adviser for his guidance during the preparation of this thesis.

I wish to thank my mother and my father for their encouragement throughout the course of this investigation.
ABBREVIATIONS

Ach, acetylcholine
Proh, propionylcholine
Bucht, butyrylcholine
Bzhch, benzoylcholine
AcChE, acetylcholinesterase
ChE, cholinesterase
BuChE, pseudocholinesterase
C.N.S., central nervous system
N.M.J., neuromyal junction
RBC, erythrocyes
# TABLE OF CONTENTS

## Chapter I. INTRODUCTION:

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Historical note on cholinesterase</td>
<td>1</td>
</tr>
<tr>
<td>II. Distinction between the two types of cholinesterase</td>
<td>1</td>
</tr>
<tr>
<td>III. Physiological significance of AcChE</td>
<td>3</td>
</tr>
<tr>
<td>1. Neuromyial junction</td>
<td>5</td>
</tr>
<tr>
<td>2. Central nervous system</td>
<td>6</td>
</tr>
<tr>
<td>a. Brain ChE and central transmission</td>
<td>7</td>
</tr>
<tr>
<td>b. Spinal cord</td>
<td>7</td>
</tr>
<tr>
<td>c. Other sites in the C.N.S.</td>
<td>8</td>
</tr>
<tr>
<td>d. Physiological significance of other ChEs.</td>
<td>8</td>
</tr>
<tr>
<td>IV. Determination of ChE activity</td>
<td>9</td>
</tr>
<tr>
<td>1. Methods of determination</td>
<td>9</td>
</tr>
<tr>
<td>2. Units used in expressing ChE activity</td>
<td>10</td>
</tr>
<tr>
<td>3. Factors affecting the determination of ChE activity</td>
<td>11</td>
</tr>
<tr>
<td>V. Warburg method</td>
<td>11</td>
</tr>
<tr>
<td>1. The Warburg apparatus</td>
<td>11</td>
</tr>
<tr>
<td>2. The theory of the manometric technique</td>
<td>12</td>
</tr>
<tr>
<td>VI. Inhibitors of ChE</td>
<td>13</td>
</tr>
<tr>
<td>1. The organophosphorus anticholinesterases</td>
<td>13</td>
</tr>
<tr>
<td>2. Stereospecificity of organophosphorus anticholinesterases</td>
<td>14</td>
</tr>
<tr>
<td>3. Inhibition of ChE by organophosphorus compounds</td>
<td>15</td>
</tr>
<tr>
<td>4. Toxicity of organophosphorus anticholinesterases</td>
<td>19</td>
</tr>
<tr>
<td>5. Brain barrier and organophosphorus anticholinesterases</td>
<td>22</td>
</tr>
<tr>
<td>6. Metabolism of organophosphorus anticholinesterases</td>
<td>23</td>
</tr>
<tr>
<td>7. Biochemical and pharmacological action of EPN and Malathion</td>
<td>24</td>
</tr>
<tr>
<td>8. Potentiation by combination of organophosphorus anticholinesterases</td>
<td>27</td>
</tr>
<tr>
<td>VII. The purpose of the present study</td>
<td>28</td>
</tr>
</tbody>
</table>

## Chapter II. PROCEDURES:

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Technical approach to the present study</td>
<td>31</td>
</tr>
<tr>
<td>II. Preparation of the animal (general)</td>
<td>32</td>
</tr>
</tbody>
</table>
III. Preparation of the "upper animal" ............................................. 32
IV. Preparation for the liver incubation ........................................... 33
V. Dilution technique ............................................................... 34
VI. Dialysis technique ............................................................. 34
VII. Preparation for manometric analysis ......................................... 35
1. Procedure ................................................................. 35
2. Numerical example .......................................................... 36

III. EXPERIMENTAL RESULTS:

I. In vitro studies ............................................................... 40
1. Enzymatic inhibition by non-incubated and by liver-incubated EPN .......... 40
2. Irreversibility of ChE inhibition by EPN .................................. 40
3. Enzymatic inhibition by non-incubated and by liver-incubated Malathion ...... 43
4. Malathion inactivation by liver homogenate ................................. 43
5. Malathion-ChE interaction .................................................. 45
6. In vitro anticholinesterase effect of combined addition of EPN and Malathion .... 45
   a. Combination of non-incubated compounds ................................ 46
   b. Combination of liver-incubated compounds ............................. 50

II. In vivo studies ............................................................... 52
A. Single administration ........................................................ 52
1. Single I.V. administration .................................................. 52
   a. I.V. administration of EPN ............................................ 52
   b. I.V. administration of Malathion ................................... 54
2. In vivo interaction of EPN and Malathion with brain ChE .................... 58
3. Effects of EPN and Malathion on true and pseudo ChE of the blood .......... 58
4. Single administration in the "upper animal" ................................ 61
   a. Intrajugular administration .......................................... 61
      i. Non-incubated and liver-incubated EPN .......................... 61
      ii. Non-incubated and liver-incubated Malathion ............... 64
   b. Intraventricular administration .................................... 66
      i. Non-incubated and liver-incubated EPN ....................... 66
      ii. Non-incubated and liver-incubated Malathion .............. 69
5. Time-effect relationship of small doses of EPN and of Malathion, administered I.V. ..... 70
   a. Time-effect relationship of EPN .................................... 72
   b. Time-effect relationship of Malathion ............................. 72
   c. Effect of EPN and of Malathion on brain pseudo ChE .............. 72
B. Combined administration ................................................ 75
1. I.V. route ................................................................. 75
2. The effect of various sequences of combined I.V. administration ......................................................... 77
3. Combined administration to the "upper animal" .............................................................................. 83
   a. Intrajugular .............................................................. 83
      i. Non-incubated compounds ........................................ 83
      ii. Liver-incubated compounds ...................................... 83
   b. Intraventricular administration .................................. 87
      i. Non-incubated compounds ........................................ 87
      ii. Liver-incubated compounds ...................................... 87

IV. DISCUSSION:

I. Introduction ........................................................................................................................................ 89
II. Survey of the in vitro studies of EPN and Malathion .................................................................. 90
   1. Single addition .............................................................................................................................. 90
   2. Combined addition ....................................................................................................................... 91
   3. Type of interaction ......................................................................................................................... 91
III. Survey of the in vivo studies of the two compounds .................................................................... 92
   1. Single administration .................................................................................................................. 92
      a. Effects (biochemical and toxic) of single administration and relationship to toxicity ............... 92
      b. Role of metabolism .................................................................................................................... 95
      c. Differential affinities .................................................................................................................. 95
      d. Dual action of EPN .................................................................................................................... 98
      e. Effect on brain pseudo ChE ....................................................................................................... 98
      f. Role of C.N.S. barrier .................................................................................................................. 99
   2. Combined administration ............................................................................................................... 100
      a. Effects due to EPN given 30 minutes prior to Malathion ........................................................... 100
         i. Role of metabolism .................................................................................................................. 101
         ii. Role of C.N.S. barrier .............................................................................................................. 101
         iii. Relationship to toxicity ........................................................................................................ 102
      b. Effects arising from varying the sequence of EPN and Malathion administration .................. 103
IV. Mechanism of potentiation .................................................................................................................. 106
   1. Description of Cook's postulate .................................................................................................... 106
   2. Cook's hypothesis and the present results .................................................................................... 107
      a. Facts supporting Cook's hypothesis ........................................................................................... 109
      b. Facts which do not support Cook's hypothesis .......................................................................... 110
   3. New postulate .................................................................................................................................. 113
      a. The basis of the new hypothesis ............................................................................................... 113
      b. Speculative explanation of potentiation on the basis of the new postulate ............................... 115

V. SUMMARY ......................................................................................................................................... 118
BIBLIOGRAPHY .................................................................................................................................... 124
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. STASTICAL ANALYSIS OF BRAIN CHE ACTIVITY DETERMINED IN NORMAL ANESTHETIZED ANIMALS</td>
<td>39</td>
</tr>
<tr>
<td>II. MALATHION INACTIVATION BY LIVER HOMOGENATE IN VITRO</td>
<td>46</td>
</tr>
<tr>
<td>III. IN VITRO INHIBITION OF BRAIN CHE BY EPN AND MALATHION AND THEIR COMBINATION</td>
<td>48</td>
</tr>
<tr>
<td>IV. LETHALITY AND BRAIN CHE INHIBITION BY EPN AND MALATHION ON I.V. ADMINISTRATION</td>
<td>55</td>
</tr>
<tr>
<td>V. DETERMINATION OF HOMOGENIZATION EFFECT -IF ANY- ON PERCENT INHIBITION OF CHE</td>
<td>56</td>
</tr>
<tr>
<td>VI. EFFECT OF DIALYSIS ON THE IN VIVO EPN-BRAIN CHE INHIBITION</td>
<td>59</td>
</tr>
<tr>
<td>VII. EFFECT OF DIALYSIS ON THE IN VIVO MALATHION-BRAIN CHE INHIBITION</td>
<td>60</td>
</tr>
<tr>
<td>VIII. EFFECTS OF EPN AND MALATHION ON TRUE AND PSEUDO CHE OF THE BLOOD IN VIVO</td>
<td>62</td>
</tr>
<tr>
<td>IX. EFFECTS OF EPN GIVEN VIA. FEMORAL AND JUGULAR VEIN TO NORMAL AND &quot;UPPER&quot; ANESTHETIZED ANIMALS RESPECTIVELY</td>
<td>63</td>
</tr>
<tr>
<td>X. STATISTICAL ANALYSIS OF MEDULLARY CHE ACTIVATION BY INCUBATED EPN, 1 MG/KG ADMINISTERED INTRAJUGULARLY.</td>
<td>65</td>
</tr>
<tr>
<td>XI. EFFECTS OF MALATHION GIVEN VIA. FEMORAL AND JUGULAR VEIN TO NORMAL AND &quot;UPPER&quot; ANESTHETIZED ANIMALS RESPECTIVELY</td>
<td>67</td>
</tr>
<tr>
<td>XII. EFFECTS ON BRAIN CHE BY EPN ADMINISTERED BY THREE DIFFERENT ROUTES</td>
<td>68</td>
</tr>
<tr>
<td>XIII. EFFECTS ON BRAIN CHE BY MALATHION ADMINISTERED BY THREE DIFFERENT ROUTES</td>
<td>71</td>
</tr>
</tbody>
</table>
XIV. TIME-EFFECT RELATIONSHIP ON BRAIN CHE OF EPN, 2 MG/KG GIVEN I.V. TO NORMAL ANESTHETIZED ANIMAL ...... 73

XV. TIME-EFFECT RELATIONSHIP ON BRAIN CHE OF MALATHION 100 MG/KG GIVEN I.V. TO NORMAL ANESTHETIZED ANIMAL. 74

XVI. IN VIVO INHIBITION OF BRAIN CHE UPON COMBINED ADMINISTRATION OF DIFFERENT SEQUENCES AND OF DIFFERENT DOSAGE RATIOS OF EPN AND MALATHION ..... 76

XVII. MEASURED AND CALCULATED AED50 IN MG/KG OF COMBINED EPN AND MALATHION 79

XVIII. EFFECT OF INTRAJUGULAR COMBINED ADMINISTRATION OF EPN GIVEN HALF HOUR PRIOR TO MALATHION INTO THE "UPPER ANIMAL" 85

XIX. INTRAJUGULAR EFFECT OF COMBINATION OF EPN INCUBATED AND GIVEN TO THE "UPPER ANIMAL" PRIOR TO NON-INCUBATED MALATHION 86

XX. EFFECT OF INTRAVENTRICULAR ADMINISTRATION TO THE "UPPER ANIMAL" OF EPN GIVEN PRIOR TO MALATHION ... 88
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. a.</td>
<td>General formula for organophosphorus compounds</td>
<td>17</td>
</tr>
<tr>
<td>1. b.</td>
<td>Reactivation of CH, inhibited by organophosphorus compounds, by oxamides</td>
<td>17</td>
</tr>
<tr>
<td>2.</td>
<td>Structural formula for EPN and malathion</td>
<td>25</td>
</tr>
<tr>
<td>3.</td>
<td>Hydrolysis of ACh by brain CHE</td>
<td>37</td>
</tr>
<tr>
<td>4.</td>
<td>In vitro inhibition of brain CHE by liver-incubated and non-incubated EPN</td>
<td>41</td>
</tr>
<tr>
<td>5.</td>
<td>Irreversibility tests for EPN-CH reaction</td>
<td>42</td>
</tr>
<tr>
<td>6.</td>
<td>In vitro inhibition of brain CHE by liver-incubated and non-incubated malathion</td>
<td>44</td>
</tr>
<tr>
<td>7.</td>
<td>Irreversibility tests for malathion-CH reaction</td>
<td>47</td>
</tr>
<tr>
<td>8.</td>
<td>In vitro effects on brain CHE, of combination of non-incubated compounds</td>
<td>49</td>
</tr>
<tr>
<td>9.</td>
<td>In vitro effects on brain CHE, of combination of different concentrations of incubated and non-incubated compounds</td>
<td>51</td>
</tr>
<tr>
<td>10.</td>
<td>In vivo dose-effect relationship of EPN on brain CHE</td>
<td>53</td>
</tr>
<tr>
<td>11.</td>
<td>In vivo dose-effect relationship of malathion on brain CHE</td>
<td>57</td>
</tr>
<tr>
<td>12.</td>
<td>Effect on brain CH by combination of compounds given i.v. to normal anesthetized animal (EPN, 2 mg/kg given prior to malathion, 10 mg/kg)</td>
<td>78</td>
</tr>
<tr>
<td>13.</td>
<td>Effect of three different sequences of combined administrations of EPN, 2 mg/kg and malathion, 10 mg/kg</td>
<td>81</td>
</tr>
</tbody>
</table>
14. EFFECT ON BRAIN CHE OF COMBINATION OF NON-INCUBATED COMPOUNDS GIVEN INTRAJUGULARLY TO "UPPER ANIMAL" AT 60 AND 90 MINUTES AFTER THEIR ADMINISTRATION (EPN, 2 MG/KG GIVEN 30 MINUTES PRIOR TO MALATHION, 10 MG/KG) ........................................... 84

15. SCHEMATIC REPRESENTATION OF VENTRICLES IN THE BRAIN . 97
Chapter I

Introduction

I. Historical note on cholinesterase:

Dale (21) as early as 1914 suggested that an enzyme present in the blood brought about the destruction of Ach. Loewi (57) demonstrated in 1926 the existence of an enzyme in extracts prepared from heart muscle which inactivated a choline ester identified later as Ach. Engelhart and Loewi (29) in 1930 explained the destructive action of the blood Ach by suggesting the existence of an esterase which was completely inhibited by minute amounts of physostigmine. About the same time, the conditions for the inactivation of Ach were found to be consistent with the action of an esterase. Plattner and Huttner (71) were the first to show in 1930 that the enzyme is present in nearly all animal tissues. Stedman (76) proposed in 1932 the name cholinesterase for the enzyme that hydrolyzes choline esters.

II. Distinction between the two types of cholinesterase (ChE):

Uncertainty prevailed as to whether or not the hydrolysis of Ach was catalyzed by a specific enzyme. Stedman (76) assumed that many sera contains two enzymes, at least one being a ChE the other an ordinary esterase. Gilman and Goodman (37) first spoke about specific and non-specific ChE. In a review by
Augustinsson (5), an enzyme which hydrolyzed only choline esters is defined as a specific ChE. Such an enzyme has been proved to exist in animal organisms. The physiological substrate of this specific enzyme is Ach.

The non-specific ChE is today defined as the enzyme which can hydrolyze other esters in addition to choline esters, the latter being split at a higher rate. Proof has been offered that at least such non-specific ChE occurs in the animal organism. However, the physiological function of this type of ChE is obscure and the natural substrate is not well known.

Investigation of sensitivity to different inhibitors of enzymes from various sources capable of splitting Ach led Mendel and Rudney (60) to propose a general classification of enzymes of this type. The term "true" or "specific" ChE was reserved for the enzymes of erythrocytes and nervous tissues, which acted only on choline esters, including Ach, acetyl β-methylcholine (Mecholyl) but not benzoylecholine, and which were inhibited by high concentration of Ach. This enzyme hydrolyzes choline esters at a rate inversely proportional to the length of the carbon chain. Certain common esters are split at rates which can be arranged in the following order: butyryl < propionyl < acetylcholine. Hence the name acetylcholinesterase (AcChE) was also given to the "true" ChE.

On the other hand, the term "pseudo" or non-specific ChE included the enzymes from serum, pancreas and other tissues which
hydrolyzed Ach, benzoylcholine and several non-choline esters such as tributyrin, tripropionin and methylbutyrate but not Mecholyl. This enzyme exhibited maximum activity in the presence of high concentration of Ach. The rate of hydrolysis by serum ChE increases with the length of the acyl chain in the following order: acetyl < propionyl < butyrylcholine, i.e. in reverse order compared to the sequence of rates exhibited by AcChE. Hence the name butyrylcholinesterase (BuChE) was also given to this enzyme.

III. Physiological significance of AcChE:

AcChE and its substrate Ach hold an important position in the physiology of the nervous system, while the importance of BuChE is still obscure. The properties of Ach examined by Dale in 1914, who made the assumption that Ach is a synaptic neurohumor. In 1921, Loewi was the first to prove the existence of a humoral transmitter for the parasympathetic nervous system and later postulated the hypothesis that parasympathetic nerves act by liberating from their terminals a chemical substance, assumed to be identical with Ach, and in this way transmit the nerve impulse to their effector organs. On the basis of the existence in the cells of conducting tissues of an enzyme with a relatively high affinity to Ach Nachmanson and his coworkers (1939-1947) hypothesized that Ach has a physiological role in C.N.S. Kinetics of the activity of AcChE satisfies the postulate that the electrical activity of the nervous system is initiated by the chemical
action of Ach at the synapses.

Altogether Nachmannson as recently as in 1960 held to a "unitarian" hypothesis according to which ChE is a prerequisite of axonal conduction as well as synaptic and neurohumoral transmission. Generally however the synaptic and neurohumoral role of ChE is more acceptable to most investigators in this field. Synaptic role of ChE at the neuromyial junction and in the C.N.S. is particularly pertinent to the present research and will be therefore described at some length.

1. Neuromyial junction:

Brown and Feldberg (12), Fatt and Katz (30) and others presented complete evidence for the chemical transmission at the end-plate region. ChE system is said to be an integral part of the mechanism responsible for the development and propagation of action potential. The localization of the hydrolyzing enzyme has been demonstrated by histochemical means and its local influence on the transmission of the impulse has been elucidated by the use of specific ChE inhibitors. The enzyme action at the neuromyial junction (N.M.J.) does not occur at other points of the nerve-muscle system.

The role of Ach as a synaptic transmitter and of ChE as the other component of the cholinergic transmission system has been most studied at the N.M.J. It was suggested (o.c.) on the basis of certain properties of this synapse that its transmission
system may have similar importance at other sites such as the C.N.S. As will be seen from the next section, evidence is today available indicating that such may be the case.

2. Central nervous system:
   a. Brain ChE and central transmission:

   The presence of such transmission system in the C.N.S. is possible if the components of this system are present in the brain. The components are the chemical transmitter (Ach), an enzyme to synthesize the transmitter (choline acetylase) and an enzyme to hydrolyze the transmitter (AcChE).

   i. Ach has been found in the brain in a concentration which could be correlated with the activity of the C.N.S. Generally no other choline esters have been shown to be present in the brain.

   ii. Choline acetylase (31) has been found in the brain and its presence could be correlated to an extent with the presence of Ach in the brain. Ach and choline acetylase have been found in high concentration in the cerebellum and in the reticular formation.

   iii. Both AcChE and BuChE have been found in the brain. AcChE predominates in the gray matter. It has now been established that the enzyme in all nerve tissues is either exclusively or predominantly specific for Ach. The localization of AcChE is correlated with that of Ach in the brain; both are found in high
concentration in the cerebellum and the reticular formation. The ChE activity is correlated with neurogenesis and ontogenetic development of brain function, including ontogenetic emergence of electrical activity of the cortex (32). Similar correlation was made with regard to choline acetylase.

b. Spinal cord:

Evidence exists which indicates that at least some of the important synapses in the spinal cord such as the synapse between the collateral and the Renshaw cell are cholinergic. The Renshaw cells (72) are located in the ventral root of the spinal cord. Recurrent collaterals of the motoneuron impinge on the Renshaw cells. It has been proposed by Eccles et al (28) that the Renshaw cell is an interneuron which can be fired by antidromic stimulation of the motor nerves and which is capable of evoking a wave of hyperpolarization in the motoneuron. The pharmacological characterization of the synapse between the recurrent collaterals of the motoneuron and the Renshaw cell indicates that this is a cholinergic synapse (27, 28). Longo et al (58) investigated the effects of physostigmine and dihydro β-erythroidine on the frequency of spike activity of the Renshaw cell. They found that dihydro-β-erythroidine decreased the frequency of the burst of the cell during the first five msec. Dihydro-β-erythroidine also attenuated the duration of the Renshaw burst (Eccles, 26). In agreement with Eccles (27, 28) Longo found that physostigmine prolonged the
duration of the discharge of the Renshaw cell burst. However measurements of the relationship between the time course of the Renshaw cell burst and stimulus strength yielded data inconsistent with the hypothesis that the duration of the burst is determined solely by the rate of the hydrolysis of Ach. The effect of physostigmine in prolonging the Renshaw cell burst can be explained on the basis that, in the presence of a reduced concentration of effective esterase, the rate of hydrolysis of Ach would proceed at a slower rate and the concentration of Ach available for combinations with effector receptors would be increased. Ach would cause then membrane change persisting beyond the time of its physical presence.

c. Other sites in the Central nervous system:

Other sites in the C.N.S. may be cholinergic as implied by certain pharmacological investigations. Karczmar and Long (49) have studied antagonism of the toxicity of an anticholinesterase (TEPP) by atropine substitutes, and suggested that both TEPP toxicity and protection against TEPP are central in nature. They have found that the structural changes in the atropine substitutes that increase the peripheral cholinolytic potency also increase the anti-TEPP action supplying therefore evidence for the hypothesis that cholinergic transmission is involved in the central action of TEPP and in its antagonism by atropine-like compounds.

Other pharmacological evidence for cholinergic transmiss-
ion in the C.N.S. was also obtained. It was shown for instance that "hemicholiniums" which are thought to block the formation of Ach by blocking the transfer of choline to the site of the reaction, possess a highly toxic action against respiration via a central mechanism, probably within the relay centers for respiration of the spinal cord (75). Other evidence for cholinergic transmission in the cortex has shown that when Ach is given I.A. or added locally to the cortex it increased the size of the spontaneous bursts of EEG, produced tremors and even convulsions. This indicates facilitation of cholinergic transmission at cortical synapse (for references cf. 53); Koppanyi (53) suggested therefore that Ach affects cholinergic electrical activity of the cortex. Hypothalamic and reticular transmission may be also cholinergic. D-tubocurarine, a cholinergic blocking agent at the N.M.J., blocks upon intra-ventricular injection the effects of hypothalamic stimulation on cardiac contractility and acceleration (Peiss, 70). Bradley (10) has shown that cholinergic compounds such as DFP, can alert the mid brain and reticular formation, probably at the thalamic level.

d. **Physiological significance of other ChEs:**

While no other esters of choline except Ach were generally found in the C.N.S., this is not necessarily true elsewhere. Homologues of Ach such as Proh have been demonstrated in ox spleen (9). BuChE has recently been demonstrated to be present in the
motor end-plate region (44). The physiological role of these esters as well as of BuChE or related ChE is not known. It was suggested by Whittaker (78) and Karczmar et al. (50) that the physiological role of BuChE might be to hydrolyze particularly in blood choline esters other than Ach, which may appear there as undesirable metabolites. BuChE may be also involved in intestinal motility and contractility (52). The physiological role of PCh is still obscure.

IV. Determination of ChE activity:

1. Methods of determination:

More than one method has been used to determine ChE activity.

a. Biological methods, in which Ach concentration was bioassayed on smooth muscle or on cat blood pressure before and after exposing the Ach solution to samples of biological material containing ChE.

b. Chemical methods, in which the acetic acid liberated from hydrolysis of Ach is determined:

A. By titrating with an alkaline solution of known concentration using an indicator or by electrometric titration.

B. Manometric methods:

i. Warburg method is most convenient for following the time course of hydrolysis of Ach, it is also most suitable for use in comparative and serial experiments. Ammon (3) was
first to use this method in assaying ChE activity. In this method CO₂ is liberated from buffered bicarbonate system by acetic acid formed during Ach hydrolysis by ChE of the sample; CO₂ is then measured manometrically.

ii. Van Slyke method is based on the same principle as that of Warburg and employs different apparatus.

iii. Microchemical gasometric method has been successfully applied to the study of the ChE activity by Linderstrom-Lang et al. (56). The principle of the Cartesian diver is applied in this method.

C. A photometric method is based on the ferric chloride reaction with acetic acid liberated by ChE from the substrate.

D. A histochemical method was first used by Koelle (51) and modified by Holmstedt (45) and others. The method depends upon complex formation by Ach thio-derivatives and cupric ion. By treatment with ammonium sulfide this complex changes to a black precipitate of CuS which localizes the enzyme microscopically. In this method thio-derivatives of Ach and Buch are used as substrates for determination of true and pseudo ChE respectively; differential inhibitors are used additionally.

2. Units used in expressing ChE activity:

a. Nachmansohn's Q and A values are defined as follows:

Q, mg Ach hydrolyzed in 60 minutes by 100 mg tissue at 20°C
A, mg Ach hydrolyzed in 60 minutes by 1 mg protein

b. Some investigators express enzyme activity in μl of
CO₂ evolved in 15 or 60 minutes by the sample. Figures for evolution of CO₂ can be used to calculate the amount of Ach hydrolyzed: 

\[ 1 \mu l \text{CO}_2 = 8.1 \mu g \text{Ach or} \ 1 \text{mg Ach} = 123.5 \mu l \text{CO}_2 \]

c. Augustinsson's Cf and b₃₀ values are defined as follows: 
\[ \text{Cf} = \frac{\text{Ach \mu mole destroyed}}{60 \text{ minutes/mg dry substance}}. \]
\[ b₃₀ = \frac{\text{CO}_2 \mu l}{30 \text{ minutes}}. \]

3. Factors affecting the determination of ChE activity:

a. Effect of H ions concentration; at pH 2 the non-specific ChE is completely destroyed; it starts to decline in the alkaline range. Its optimum pH is 8 to 8.5. Optimum pH for specific ChE is 7.5 to 8 (4). At pH 4.5 it is completely destroyed. The stability in alkaline reactions of AcChE also seems to be less than that of BuChE.

b. Optimum temperature for both AcChE and BuChE is 37° to 40° C (38).

c. Activators are said to be Ca⁺⁺, Mg⁺⁺, Mn⁺⁺ and some organic compounds (67).

d. Inhibitors are said to be Cu⁺⁺ and Co⁺⁺ (2, 67).

V. Warburg method:

1. The Warburg apparatus:

Conical flasks (15-20 ml volume) are used. Warburg manometers are filled up with Brodie solution (23 gm NaCl + 5 gm Na choleate in 500 ml water). Evan's blue dye is used in a solution having a density of 1.034, so that a 10,000 mm level difference of Brodie solution is 760 mm Hg. Contents of flasks
are mixed by means of shaking motors. The flasks are connected to the manometers and are placed in a water bath the temperature of which is constant and thermoregulated.

2. **The theory of the manometric technique:**

The conditions of the assay must be such that in a reaction medium a certain gas is either evolved from or absorbed by the reaction mixture. At constant volume the gas pressure can be recorded from the changes in the level of manometer fluid. In the case of ChE, the substrates such as Ach or Bzch are hydrolyzed by the enzymes. The products of hydrolysis are choline + acetic or benzoic acid. The acid is then reacted with the bicarbonate and the resulting CO\(_2\) gas is evolved. Its pressure is a measure of the activity of the ChE present in the reacting medium. The chemical reactions which occur are as follows:

i. \(\text{AcChE} + \text{Cl}(\text{CH}_3)_3\text{N-CH}_2\text{-CH}_2\text{-O-CO-CH}_3 \rightarrow \text{AcChE} + \text{choline} + \text{acetic acid}\)

ii. \(\text{CH}_3\text{-COOH} + \text{NaHCO}_3 \rightarrow \text{CH}_3\text{-COONa} + \text{H}_2\text{O} + \text{CO}_2\)

A thermobarometer flask which contains 3 ml of water and a control flask containing bicarbonate solution and substrate solution of the same concentrations as that added to the brain homogenates, are included in all experiments. Corrections for thermobarometer changes (due to alterations in temperature and pressure) and for the non-enzymic hydrolysis of the substrate are made.

The absolute volume of CO\(_2\) liberated by the enzymic hydrolysis of the substrate / unit time / mg brain tissue, can be
calculated. In the case of analyzing brain tissue treated with inhibitors, the percent of remaining ChE activity and of ChE inhibition can be calculated provided control values are known.

VI. **Inhibitors of ChE:**

1. **The organophosphorus anticholinesterases:**

   The anticholinesterase agents constitute one of the few classes of drugs for which a mechanism of action has been defined in terms of inhibition of a specific enzyme.

   Prior to War I, Dixon and Ransom and Fraser described pharmacological actions of physostigmine and demonstrated physostigmine miosis and slowing up of the heart as early as before the turn of the century. At that time these investigators naturally did not know that this substance is an anticholinesterase or, in fact, that this mechanism of action exists and they described actions of physostigmine as muscarinic or stimulant to the parasympathetic system.

   The mechanism of action of physostigmine was studied after the role of ChE was elucidated as already described. Straus and Goldstein (1943) showed that physostigmine and Ach enter into a competition for the active sites on the surface of the enzyme. Displacement of Ach by physostigmine nullifies the capacity of the enzyme to hydrolyse Ach.

   Another important development was the demonstration of anti-curare action of physostigmine (Walker). The clinical
similarity between the loss of skeletal muscle tone in myasthenia gravis and in curare poisoning led Mary Walker to conceive the idea that physostigmine which evoked a decurarizing action might be useful in the treatment of the disease. In 1934 she treated her first patient suffering from myasthenia with physostigmine salicylate and observed dramatic improvement.

Prior to War II, ChE was not of wide interest; the advent of the organophosphorus anti-cholinesterase agents at about the time of War II which was due to Schrader's synthesis of some of these compounds brought new aspects to this area and ChE determination have become of practical importance in industrial health, crop protection and study of insecticides, and in work on chemical warfare. The new agents served as antamyasthenics (TEPP, OMPA), or as war gases which were first synthesized in Germany and in England where Saunders prepared alkylphosphoryl fluoride (DPP), or finally as insecticides (Parathion, EPN and Malathion). The last two compounds are the compounds used in the present study and they will be described later in some detail with respect to their chemistry, biochemistry and pharmacology.

2. Stereospecificity of organophosphorus anticholinesterases

The ChE inhibitors of organophosphorus group can be given the general formula proposed as early as in 1937 by Schrader, for derivatives of phosphorus acid with insecticidal properties (Fig. 1a). Biologically active compounds are obtained when in addition
to oxygen or sulfur, two similar or dissimilar substituents are bound to the phosphorus atom and an organic or inorganic acid residue is also present. \(R_1\) and \(R_2\) are capable of almost infinite variation.

The stereochemistry of the anticholinesterases of the phosphorus group has been very little investigated although it is well recognized that enzymes exhibit stereospecificity. Such specificity is likely to be the consequence of the asymmetric structure of the enzyme surface. Recently the d- and l- enantiomorphs of an asymmetric organic phosphorus inhibitor of ChE were synthesized (1). It was found that the l- isomer inactivated irreversibly four ChE preparations from 10 to 20 times faster than the d- form (1).

3. Inhibition of ChE by organophosphorus compounds:

Wilson (77) has suggested a basic similarity between the interaction of Ach on one hand and of organophosphorus compounds on the other with ChE, despite the fact that the carboxyl esters such as Ach are usually substrates and the phosphoryl derivatives are inhibitors of ChE. The forces between Ach and the enzyme are thought to be of three kinds at least (66):

a. Ionic bond: The active surface of the enzyme protein contains a negatively charged site, the "anionic site" (77), which reacts by coulombic forces with the positive electric charge of Ach (the cationic nitrogen), and contributes hereby to the
attraction, fixation and orientation of the substrate upon the enzyme surface.

b. Covalent bond: There is also another group at the enzyme surface which reacts with the ester group of Ach by a covalent bond; this grouping has been referred to as the esteratic site.

c. Van der Waals attraction between the substrate Ach and the enzyme.

The suggested similarity between the affinity of organophosphorus compounds and of Ach to the enzyme (13) can be demonstrated as follows:

\[
\begin{align*}
1. \quad H-G + CH_3-COOR & \leftrightarrow R-O-C-O \rightarrow C-O + ROH \\
(enzyme) & \quad (Ach) \quad CH_3 \quad CH_3 \\
2. \quad H-G + (RO)_2P=F & \rightarrow P-O + HF \\
& \quad RO \quad OR \quad (dialkyl phosphoryl enzyme)
\end{align*}
\]

The bonding between Ach and the enzyme is easily broken down by hydrolysis. As far as the interaction between the enzyme and the inhibitor is concerned, labeling of the organophosphorus inhibitors with \( P^{32} \) has shown that the bond between the enzyme and the inhibitor is so strong that it is not broken down by dialysis or by recrystallization of the enzyme (16). In the inhibitor-enzyme complex the phosphorus atom represents a strong
A. GENERAL FORMULA FOR ORGANOPHOSPHORUS COMPOUNDS

B. REACTIVATION OF CHE, INHIBITED BY ORGANOPHOSPHORUS COMPOUND, BY AN OXIME AGENT.
electrophilic center. This is a positive center which is attracted to a negatively charged center at the enzyme surface and therefore does not react with water to regenerate the active enzyme as readily as Ach-enzyme complex.

It is possible however to regenerate the active enzyme by using nucleophilic reagents which are attracted to a positively charged center i.e. P atom of the inhibitor. A large number of reactivators were studied, such as hydroxylamine, pyridine and hydroxamic acid methiodide (17). Reactivation occurs when because of coulombic attraction the reactivator can attack the phosphoryl group of the inhibited enzyme (Fig. 1b). Hydrolysis of the phosphoryl group is based on the same principle as Ach hydrolysis in the case of Ach-enzyme complex.

\[
\begin{align*}
\text{i.} & \quad \text{H}^+ + \text{G}^- \overset{\text{H-O + C-O}}{\overset{\text{HO-C-O}}{\overset{\text{H-G + CH}_3\text{-COOH}}{}}} \\
\text{ii.} & \quad \text{H}^+ + \text{P}^- \overset{\text{H-O + P-O}}{\overset{\text{P-O + OH}}{\overset{\text{H-G + RO OR}}{}}}
\end{align*}
\]

The organophosphorus anticholinesterases that form complexes with the enzyme which are not easily hydrolyzed such as DFP, are called "irreversible" inhibitors. When given in vivo they produce
inhibition such that the enzyme activity cannot be restored until new enzyme is synthesized. Other ChE inhibitors form easily hydrolyzable complexes with the enzyme and therefore are called "reversible" inhibitors, such as physostigmine. Toxicity caused by a reversible ChE inhibitor is temporary and normal activity of the enzyme is rapidly restored by its liberation from its complex with the inhibitor.

4. **Toxicity of organophosphorus anticholinesterases:**

DFP as an example of this group of agents produces the following general effects (64): Motor: unrest, muscle twitching, tremors, ataxia, weakness, respiratory stimulation, salivation, miosis, bradycardia, defecation, diarrhea, and finally convulsions which lead to respiratory depression and death.

These symptoms fall into two groups: a) peripheral effects due to muscarinic stimulation of respiratory and gastrointestinal tracts, and to nicotinic action on skeletal, particularly respiratory muscles, and, b) central effects.

The mechanism inducing this toxic picture undoubtedly involves ChE inhibition. This in turn leads to Ach accumulation which again probably occurs both centrally and at the periphery.

A direct toxic effect on the C.N.S. is considered of primary importance in ChE poisoning (23) and may be demonstrated by the following data:

a. Michaelis et al. (63) demonstrated that a phosphonate
anticholinesterase causes central accumulation of Ach.

b. Karczmar and Long (48) showed that the mechanism involved in the protective action against TEPP toxicity of atropine and its congeners is both central and cholinergic in nature, since their protection against central toxicity parallels that against peripheral toxicity.

c. Analysis of AcChE activity of pons and medulla following TEPP administration indicated that following marked initial potentiation of respiratory reflex which was maximal at 10 to 12 percent inhibition of AcChE there was a gradual but progressive decline in the potentiated respiratory reflex in parallel with the fall of AcChE activity of regions vitally concerned with respiration until respiratory failure was evident at 89 to 92 percent inhibition levels. The experiments suggested that the cholinergic transmitter Ach, may be a factor in respiratory control (62)

d. As far as the electrical activity of the cortex is concerned the effects of organophosphorus compounds comprise EEG desynchronization, convulsions and finally quiescence. The convulsions may ultimately block respiratory activity which will have already been reduced by muscarinic actions on the respiratory tract and by the depressant effect of anticholinesterase compounds on the respiratory centers and muscles. The desynchronization of EEG is the first sign of action of the anti-
cholinesterase compounds on the ascending reticular activating system of Moruzzi and Mangun. Recent studies by Bradley (11) and Himwich (14) indicate that cholinergic drugs do not appear to act only on a mechanism controlling EEG pattern but also on another mechanism which is not concerned with behavioural changes such as wakefulness and sleep.

e. It has been mentioned earlier that recurrent collaterals of the motoneuron and the Renshaw cell constitutes a cholinergic synapse.

On the other hand, there are some data which indicate that toxicity may not always depend either on general or central inhibition of ChE. Frawley et al. (33) have shown that DFP exhibited minimum symptoms when brain ChE was inhibited by 92 percent; on the other hand, Parathion and TEPP reduced the activity to 20 percent and 51 percent of normal respectively, concomitantly with severe symptoms. They concluded that while brain ChE inhibition may contribute to the symptoms observed, it is not always the main cause of death in the case of phosphonate toxicity. These data suggest that at convulsive doses central ChE inhibition is not equal with these two compounds; thus, either convulsions are central but not due to central ChE inhibition, or peripheral and localized at N.M.J.
An important consideration is which part of the brain was analyzed for the ChE activity. Small inhibition at one particular site does not necessarily mean that equally small inhibition occurred at all sites. The data of Frawley et al are also in disagreement with the results of Metz (62) showing that respiratory failure with TEPP was evident at 90 percent inhibition of medullary AcChE and with the results of Karczmar and Long (48) which were already described.

5. Brain Barrier and organophosphorus anticholinesterases:

It has been known long that some compounds penetrate more easily than other blood brain barrier. This is true for example of tertiaries which can cross the barrier more readily than the quaternary amines. This has been related to differences in lipid solubility. It has also been known long that certain substances cause central effects and at the same time damage blood brain barrier to such an extent that the damage can be visualized with vital staining by trypan blue. Tertiary ChE inhibitors produce central effects without destroying to any extent the blood brain barrier (68). The penetrability of sulfanilamide, which is measured by calculating the ratio of this substance that penetrate into the brain to that in plasma, into the brain was found to increase with increasing permeability of blood brain barrier. Paulet and Andre (69) used this test to investigate the relationship between the anticholinesterasic effect and the change in
permeability of blood brain barrier of some organophosphonate compounds. They found that some anticholinesterases such as Parathion and OMPA increased the permeability of blood brain barrier, and that this property appeared to be independent from the anticholinesterasic activity of those substances.

6. Metabolism of the organophosphorus anticholinesterases:

In 1950, Dubois (24) and Gardiner (36) reported metabolic conversion of organophosphorus compounds to anticholinesterase agents. Casida (16) and Cage (15) have shown that many organophosphate esters are toxic in vivo and yet inefficient as ChE inhibitors in vitro; thus their metabolic conversion to more active anticholinesterases can be suspected. Metcalf and March (61) used the Warburg technique to determine the activity of EPN and Malathion incubated with liver slices. House fly head enzyme was sensitive to those compounds after incubation. Para-thion metabolism has been shown to result in an isomerization between the thiono-sulfur and the ethoxy-oxygen, rendering it a more potent anticholinesterase. Thus, metabolism in vivo, catalyzed frequently by liver enzymes may lead to formation of more toxic compounds. The opposite can occur also, and is a major consideration in the evaluation of modern insecticides. Malathion, an insecticide of extreme importance at present, has the advantage of being much less toxic to mammals than to insects because it is broken down in vivo to inactive compounds as shown
in the hen, mouse and roach (59). In vitro studies by Cook et al (18) showed that Malathion is altered very quickly by rat liver when it is hydrolyzed at the diethyl succinate group to a compound which is ineffective and more water soluble. This breakdown of Malathion may be inhibited by certain organophosphorus compounds. It has been shown by Murphy and Dubois (65) and by Cook (19) that EPN as well as some - but not all - other organophosphorus compounds inhibit Malathion-d detoxifying liver enzyme. The recovery of EPN inhibited liver enzyme occurs in a matter of a few days. This point of great pertinence to the present research will be discussed in greater detail subsequently.

7. **Biochemical and pharmacological actions of EPN and Malathion:**

EPN was synthesized during the war and shown to be an insecticide belonging to the general class of compounds referred to as organophosphorus insecticides. Chemically it is O-ethyl-O-P-nitrophenyl thionobenzene phosphonate (Fig. 2). The technical EPN is a dark amber liquid, soluble in most of the common organic solvents and stable at ordinary temperature.

EPN produced marked inhibition of human erythrocytes ChE slowly increasing with time (42); decrease of ChE activity to 53 percent of initial activity occurred in 200 to 300 minutes. ChE inhibition by EPN is irreversible (42). EPN lacks high ChE inhibitory action in vitro. In vivo, the intoxication is cholin-
FIGURE 2

STRUCTURAL FORMULAE FOR EPN AND MALATHION
ergic in nature and exhibits C.N.S. manifestations. In general, intoxication symptoms are those characteristic of anticholinesterase agents. EPN is considered as one of the potent toxic organophosphorus compounds and its LD$_{50}$ in dogs is 20 to 30 mg/Kg orally.

Malathion is chemically O,O-dimethyl S-(1,2-dicarbethoxy) ethyl dithiophosphonate (Fig. 2). It was synthesized by Casaday (14). Malathion is a yellowish oily liquid, having a strong smell of garlic, slightly soluble in water, miscible in most commonly employed organic solvents and stable at ordinary temperature and pressure.

The pharmacological action of Malathion seems mediated by the inhibition of tissue ChE; the signs of toxicity after a large single dose in experimental animals are almost exclusively characteristic of cholinergic intoxication. Among those are excessive salivation, depression, tremors, convulsions, coma and death. Its inhibition of ChE is irreversible (40). It has a relatively low mammalian toxicity with an exceptionally wide range of activity against insects. It is one of the few organic phosphonate insecticides that is considered safe enough to justify availability in commerce for general insect control use. It is weak anticholinesterase when compared with Parathion and EPN. It was stated that in dogs, Malathion given I.V. inhibited plasma ChE more than RBC and brain ChE (40), while Parathion and
EPN inhibited more RBC and brain ChE. It was also said that an effective dose of Malathion increased blood pressure (40) followed by respiratory failure; Metrazol and artificial respiration failed to revive the animals.

8. Potentiation by combination of organophosphorus anti-cholinesterases:

The possibility exists that a worker may be exposed to two or more insecticides on the same or different days, and that intoxication may accidentally occur by ingesting several different food products, each containing a different insecticide. This prompted Frawley et al (34,35) in 1957, to carry out a series of experiments on the additive action of EPN and Malathion. The data showed potentiation of acute toxicity of EPN and Malathion when they were administered simultaneously to dogs. Potentiation was less marked in rats. Potentiation of inhibition of RBC ChE was noticed also. Frawley et al. suggested that the mechanism of potentiation is not a chemical reaction between the two phosphonates, but that the potentiation of ChE inhibition in RBC is a results of a prior reaction of one or both of the compounds with another biological system. Since it was demonstrated that phosphonates are enzymatically detoxified and that this can be blocked by certain other phosphonates, Cook et al. (19) suggested that this is the basis of the potentiation of Malathion by EPN. Rosenberg (73) confirmed that EPN potentiats toxicity
of Malathion when the two compounds are given orally simultaneously to rats. He showed also that combinations of Malathion with OMPA, Parathion, DFP or with physostigmine show minimal or no potentiation. Combinations of EPN-Parathion, EPN-OMP and OMPA-Parathion exhibited no potentiation of toxicity (73, 74). Besides potentiation of ChE inhibition, pharmacological potentiation at specific neuroeffectors, such as vagal, ganglionic, neuromyal and at the C.N.S. was also demonstrated (39).

However both potentiation of enzymic inhibition and of neuroeffector toxicity need not be entirely due to the mechanism suggested by Cook et al. (19) since it was shown earlier that even quaternary compounds which should not be hydrolyzed by the enzymes described by Cook (19) also potentiate each other (Lands, Hoppe, Karczmar and Arnold) 55). This is one of the points that led to the present research and will be discussed more in detail in the next section.

VII. The purpose of the present study:

A series of synthetic organophosphorus compounds, besides being employed as pharmacological tools, are used as cholinergic agents in therapy and as insecticides. Their application in agriculture is continuously expanding. Many questions regarding their pharmacological action must be answered, for the protection of the manufacturer as well as the consumer of the treated food. ChE investigations were designed to evaluate the relationship of
inhibition to symptomatology. A recent demonstration from our laboratories and from Federal Food and Drug Administration that certain anticholinesterase war gases and insecticides potentiate the toxicity of each other rather than act merely additively is to be considered in view of the possibility of simultaneous exposure to two or more of these compounds. This phenomenon has profound theoretical and practical implications and points out the need for caution in the use of drug combinations in the field of therapeutics and for additional investigations in this frequently overlooked phase of pharmacology and toxicology, particularly since organophosphorus compounds of particular interest in the present study, EPN and Malathion, are among those which are in greatest use in agriculture.

The potentiation was however inadequately studied. There was no previous systematic investigation of these two compounds as to the dose-effect relationship of their anticholinesterase action, the type of their interaction with the enzyme or to the relationship between the extent of inhibition particularly at strategic effector sites, and toxicity. Potentiation of ChE inhibition was generally demonstrated with reference to blood enzymes, and not enough data are available as to ChE response of other tissues, particularly of the C.N.S. Per se potentiation of inhibition of the blood enzyme cannot account for the rapid death observed, although it is generally thought
that the inhibition of this enzyme is responsible for the action of the individual compounds and also for the potentiation of the toxicity arising from the administration of their combinations.

Finally, inhibition of detoxification of one compound by the other was the explanation offered for the potentiation of the enzymic inhibition and of toxicity arising from combined administration of these compounds. This explanation naturally does away with the difficulty of understanding the mechanism whereby chemically similar compounds which should react with identical radicals of the enzyme act more than additively. However, indications that another mechanism was involved were already mentioned.

The above questions necessitate a detailed study of interactions between EPN, Malathion and the enzyme in vivo and in vitro with regard to potentiation of inhibition of C.N.S. enzyme, dependence of inhibition on EPN and Malathion metabolism, and type of EPN and Malathion interaction with the ChE. It was also necessary to reexamine the hypothesis of Cook, and to attempt if necessary to suggest alternative mechanisms for the potentiation. Finally, the present research is not conceived to elucidate present uncertainty of the mechanism of central transmission but may yield additional clues for the understanding of the function of ChE in the C.N.S.
CHAPTER II
PROCEDURES

I. Technical approach to the present study:

The two compounds in question, EPN and Malathion, were given singly and in combination and studied in vitro as well as in vivo; Warburg manometric technique was used for the measurement of ChE activity. In vivo studies were carried out by injecting the compounds into the femoral vein of anesthetized animals and intrajugularly and intraventricularly to the "upper animal". The "upper animal" (cf. infra) technique was used to supply information as to whether or not the metabolism of these compounds is necessary for their actions.

The in vitro studies were carried out by adding compounds to brain homogenates. In other instances they were incubated with liver, then added to brain homogenate. The in vitro studies had as their purpose first, to investigate the conditions in which the potentiation may or may not occur, particularly with regard to the necessity of liver incubation for potentiation. Secondly, it was ascertained whether or not the enzymic inhibition by compounds in question was irreversible as suggested by Hazleton et al. (40) and this was tested by dialysis and dilution techniques. Besides theoretical implications of this the
irreversibility of their action renders the measurements of inhibition occurring in vivo more reliable, since it precludes reversibility of inhibition during measurements.

II. Preparation of the animal (general):

Dogs were used for the present study. They were anesthetized with an average dose of 30 mg/Kg of Na pentobarbital, given I.P. The average weight of the animals was 8 Kg. The femoral vein was exposed for I.V. injection of the compounds in question. At a certain time after the injection, the animals were sacrificed by dividing the spinal cord at a level below the medulla. The skull was opened and the brain was taken out for biochemical analysis. In some instances, the brain samples were kept in the refrigerator and were analyzed the following day.

III. Preparation of the "upper animal":

This special animal preparation was done for the purpose of studying the effect of the compounds in question without compounds being circulated through the liver or any other visceral organs. The dogs were anesthetized as mentioned above, the trachea was exposed and a tracheal cannula was inserted for artificial respiration. The aorta was ligated above the level of the hepatic artery. This was done by making a small incision in the diaphragmatic membrane through which a ligature was clamped on the aorta. To reach the diaphragm, an incision was made in the abdomen, then the mesenteric visceral membrane was
separated from the abdominal wall. In this way, hemorrhage and blood loss was avoided to a great extent. Subsequently, the animal was kept under artificial respiration during the remainder of the experiment. At the end of most experiments the state of aortic ligature was checked to ascertain the success of this form of hepatectomy. Additional indication of the success of the procedure was that the blood vessels of the liver were always noticed to be collapsed.

The drugs were administered to the "upper animal" either intrajugularly in which case the external jugular vein was exposed for that purpose, or intraventricularly. In the latter case a small hole (1x\frac{1}{2} cm^2) was drilled out in the skull and 26 gauge needle was inserted into the brain ventricle. When injecting the drug, spinal fluid of a volume equal to that to be injected was withdrawn.

IV. Preparation for the liver incubation:

Dubois technique (24) for liver incubation of the organophosphorus compounds was followed. The incubation medium for in vitro studies contained the following components in the concentrations indicated: Dog liver homogenate 2.5 percent; diphosphopyridine nucleotide (DPN) 0.1 percent; nicotinamide 1x10^{-3} M; and phosphate buffer at pH 7.2. In certain experiments DPN was excluded from the medium. The incubation medium for the in vivo study contained the following components: EPN, 1 mg/gm liver
homogenate and Malathion, 100 mg/gm liver homogenate; DPN 0.8 percent; nicotinamide $8 \times 10^{-3}$ M; and phosphate buffer at pH 7.2. Usually, EPN and Malathion were incubated for 25 to 30 minutes at $38^\circ$ C then added to brain homogenate in vitro or injected into the "upper animal".

The concentration of EPN (1mg/gm) was selected so as to inhibit only the liver enzyme in its incubation medium and not to protect Malathion detoxification by its own medium when EPN and Malathion were used in combination following incubation, in vitro or in vivo. In fact, the concentration used inhibited about 90 percent of the liver detoxifying enzymes (65). In some instances, different concentrations of EPN and Malathion were also used.

V. Dilution technique:

The inhibited brain homogenate was diluted four or more times. Solutions of various dilutions were analyzed to determine their ChE activity and percentual inhibition of ChE obtained at various dilutions was compared.

VI. Dialysis technique:

Microdialysis technique as described by Craig (20) was used. Phosphonate-inhibited brain homogenate was placed into a dialysis bag. Inside this bag a glass rod smaller in diameter than the dialysis bag was placed so that the layer of the dialyzed solution (inner medium) would be of very small thickness.
Dialysis bag and its contents were placed in a container having bicarbonate buffer (outer medium) identical with that used for homogenizing the brain. This allowed better exchange of ions between the outer and the inner media, and eliminated the necessity of stirring (20). Dialysis was carried out for a period of 12 to 72 hours and the brain tissue was analyzed to determine its ChE activity before and after dialysis.

VII. Preparation for manometric analysis:

1. Procedure:

Calibration was made for each Warburg flask and its manometer, and the flask constant "K" was calculated. Homogenates were diluted in bicarbonate buffer containing 0.025 M Na bicarbonate and 0.005 M MgCl₂. Bicarbonate was gased from a cylinder containing a mixture of 5 percent CO₂ and 95 percent N₂. 1×10⁻³ M Ach and 1×10⁻² M Bzch were the substrates used to determine AcChE and BuChE activities respectively (cf. Augustinsson, 1957). The brain tissues analyzed were gray and white matter of the cortex and of the medulla. Forty five to 50 mg brain tissue homogenates, in 2.75 ml of the bicarbonate buffer were placed in the main compartment of the Warburg flask. The final volume of solution in each flask was 3 ml. A flask containing only bicarbonate buffer and the substrate and another flask serving as thermobarometer and containing 3 ml of water were included in all experimental series. All the flasks were
then connected to their manometers and placed in water bath at 38°C. Equilibration was carried out for 15 minutes, the CO₂ and the N₂ gas mixture was bubbled through for 5 minutes, the air trapped inside the manometers was then expelled and all air vents of the flasks closed. Brodie solution was adjusted in the right arms of the manometers at the 150 ml mark and zero readings in the left arms of the manometers taken. The substrate was then tipped into the main compartments of the flasks and the shaking motor of the machine turned on. Manometers were read every 5 to 10 minutes for a total of 60 minutes; solution was adjusted in the right arm at the 150 mark and the left arm level measured.

2. Numerical example of the analytical results:

Fig. 3 shows the evolution of CO₂ during 60 minutes period in the case of 100 mg homogenates of cortical gray matter and of medulla, Ach substrate. The calculation of the flasks constant "K_{CO₂}" and of the CO₂ evolved were carried out as follows:

a. The calculation of the flask constant for CO₂:

\[
K_{CO₂} = \left(\text{flask volume in } \mu l - \text{fluid volume in } \mu l\right) \times \frac{t}{T+T^0} + \left(\text{fluid volume in } \mu l \times \alpha_{CO₂}\right)
\]

\[
p^0 = \frac{273}{\text{gas volume in } \mu l \cdot \frac{T}{T} + \text{fluid volume in } \mu l \cdot \alpha_{CO₂}}
\]
FIGURE 3

HYDROLYSIS OF ACHE BY BRAIN CHE
\[ K_{\text{CO}_2} = \text{flask constant for CO}_2 \text{ gas} \]
\[ \alpha_{\text{CO}_2} = 0.567 \text{ at } 37^\circ C \]
\[ T = T_0 + t = 273 + t \]
\[ P_0 = 10,000 \]

Fluid volume = 3000 μl

b. The calculation of CO₂ evolved in μl/time/dry weight of tissue (Ach substrate):

<table>
<thead>
<tr>
<th>Flask #1</th>
<th>Flask #2</th>
<th>Flask #3</th>
<th>Flask #4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermo-</td>
<td>Bicarbon-</td>
<td>Cortical</td>
<td>Medullary</td>
</tr>
<tr>
<td>barometer</td>
<td>tissue</td>
<td>tissue</td>
<td></td>
</tr>
<tr>
<td>(T.B.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>1.8</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Manometric difference/40 minutes 7 12 43 99

T.B. subtraction 5 36 92

Multiplication by \( K_{\text{CO}_2} \) 9 72 184

Correction for non-enzymic hydrolysis of Ach 63 175

μl CO₂/100 mg dry tissue/40 minutes 29 67.5

To measure the variability of biochemical data, a statistical analysis was carried out with regard to cortical and medullary ChE activity (Table 1). Fifteen dogs were studied in this series of experiments over a period of 1 year. In the case of each dog 3 to 6 determinations of the medullary and cortical enzyme were made with Ach as substrate and the results averaged. The variability of 6 determinations from the same
animal was \( \pm 0.06 \mu l \text{ CO}_2 \); in the case of all 15 dogs the values for cortical and medullary ChE activity were \( 27.6 \pm 0.46 \) and \( 64 \pm 0.71 \mu l \text{ CO}_2 \). Thus the variability of the manometric data for 15 animals over a period of one year was less than 2.0 percent indicating high reliability of the method.

**TABLE I**

**STATISTICAL ANALYSIS OF BRAIN CHE ACTIVITY DETERMINED IN NORMAL ANESTHETIZED ANIMALS. ACH SUBSTRATE**

**15 DOGS. IN THIS EXPERIMENT AND IN ALL THE SUCCESSING ONES EACH SAMPLE WAS DUPLICATED.**

<table>
<thead>
<tr>
<th>( \mu l \text{ of CO}_2/100 \text{ mg dry wt. of cortical tissue/40 minutes} )</th>
<th>( \mu l \text{ of CO}_2 \text{ evolved/100 mg dry wt. of medullary tissue/40 minutes} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>28.5</td>
<td>62.4</td>
</tr>
<tr>
<td>31.5</td>
<td>65.4</td>
</tr>
<tr>
<td>26.5</td>
<td>65.4</td>
</tr>
<tr>
<td>26.0</td>
<td>61.1</td>
</tr>
<tr>
<td>26.5</td>
<td>60.3</td>
</tr>
<tr>
<td>26.0</td>
<td>67.5</td>
</tr>
<tr>
<td>26.0</td>
<td>66.8</td>
</tr>
<tr>
<td>28.0</td>
<td>61.5</td>
</tr>
<tr>
<td>28.0</td>
<td>66.6</td>
</tr>
<tr>
<td>26.0</td>
<td>62.4</td>
</tr>
<tr>
<td>27.0</td>
<td>61.5</td>
</tr>
<tr>
<td>28.0</td>
<td>67.5</td>
</tr>
<tr>
<td>29.0</td>
<td>60.5</td>
</tr>
<tr>
<td>26.0</td>
<td>64.5</td>
</tr>
<tr>
<td>31.0</td>
<td>67.4</td>
</tr>
</tbody>
</table>

The mean 27.6 64.0

Standard deviation \( \pm 0.46 \) \( \pm 0.71 \)
CHAPTER III
EXPERIMENTAL RESULTS

I. In vitro study:

1. Enzymatic inhibition by non-incubated and liver-incubated EPN:

Different concentrations of non-incubated EPN were added in vitro to brain homogenate. Also EPN incubated with liver homogenate with DPN added, as well as EPN incubated with liver homogenate alone were added to brain homogenate. As indicated in Fig. 4, non-incubated EPN has weak inhibitory action on brain ChE in vitro, while percentual inhibition of ChE was much higher when EPN was incubated with liver and DPN. On the other hand, there was no definite increase in anticholinesterase activity of EPN when this compound was incubated without DPN.

2. Irreversibility of the ChE inhibited by EPN:

The character of the interaction between EPN and the enzyme ChE was studied by testing the irreversibility of their bonding complex. Physical rather than chemical means were used for this purpose, namely:

a. Dilution technique:

The results of this study are shown in Fig. 5. They indicate that dilution has no effect on the bonding between EPN.
FIGURE 4

IN VITRO INHIBITION OF BRAIN CHE BY LIVER-INCUBATED AND NON-INCUBATED EPN.
EACH POINT REPRESENTS AN AVERAGE VALUE OF FOUR DETERMINATIONS FROM FOUR DOGS.
ACH SUBSTRATE.
FIGURE 5

Irreversibility tests for EPN-CHE interaction. Each point represents an average value of three determinations from three dogs. ACH substrate.
and ChE irrespective of EPN concentration.

b. **Dialysis technique:**

The attachment between EPN and ChE was not destroyed by dialysis of from 24 to 72 hours (Fig. 5), suggesting again that EPN inhibited brain ChE irreversibly.

The results listed under (a) and (b) indicate the reliability of the biochemical determinations carried out in vivo.

3. **Enzymatic inhibition by non-incubated and by liver-incubated Malathion:**

Malathion alone, as well as Malathion incubated with liver homogenate was added in different concentrations to brain homogenates. The effects on brain ChE are shown in Fig. 6. Malathion inhibited brain ChE at the \( I_{50} \) of \( 0.8 \times 10^{-5} \) M; inhibition of ChE was higher when Malathion was not incubated than when it was incubated with liver homogenate. The results indicate also (Fig. 6) that Malathion lost its activity to the same extent whether it was incubated with liver homogenate or with liver homogenate and DPN.

4. **Malathion inactivation by liver homogenate:**

To study the rate of Malathion inactivation by the liver Malathion was incubated with dog liver homogenate in a concentration of 50 mg/gm liver. Incubation was carried out for 0, 15 and 30 minutes. After each period, the incubated Malathion was added to brain homogenate and brain ChE activity determined. At
FIGURE 6

IN VITRO INHIBITION OF BRAIN CHE BY LIVER-INCUBATED AND NON-INCUBATED MALATHION. EACH POINT REPRESENTS AN AVERAGE VALUE OF FOUR DETERMINATIONS FROM FOUR DOGS. ACH SUBSTRATE.
zero time, Malathion inhibited 80 percent of the brain ChE, while after 30 minutes of incubation with liver homogenate it inhibited only 34 percent of brain ChE (Table 2). Thus, when Malathion-liver ratio is about 1:20 Malathion "half life" is about half hour. This suggests a relatively rapid destruction of Malathion by the liver enzyme.

5. Malathion-ChE interaction:

As was done with EPN, dialysis and dilution techniques were used to study the type of interaction of Malathion with brain ChE in vitro.

a. Dilution technique:

The results of this study shown in Fig. 7 indicate that the binding between the inhibitor and ChE was not destroyed by dilution. This suggested an irreversible type of interaction.

b. Dialysis technique:

The results shown in Fig. 7, confirmed the observation obtained by the dilution technique, of the irreversibility of Malathion inhibition of Brain ChE. Again this shows the reliability of the method used for measuring in vivo inhibition by Malathion. Moreover, these data in conjunction with similar results obtained with EPN, indicate at least a certain degree of similarity between EPN and Malathion with regard to the type of their interaction with ChE.

6. In vitro anticholinesterase effect of combined addition
TABLE II

MALATHION INACTIVATION BY LIVER HOMOGENATE IN VITRO. EACH VALUE REPRESENTS AN AVERAGE VALUE OF FOUR ANIMALS.

<table>
<thead>
<tr>
<th>Time of incubation in minutes</th>
<th>Percent ChE inhibition in vitro (Ach substrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>70</td>
</tr>
<tr>
<td>15</td>
<td>56</td>
</tr>
<tr>
<td>30</td>
<td>34</td>
</tr>
</tbody>
</table>

of EPN and Malathion:

a. Combination of the non-incubated compounds:

Non-incubated EPN and Malathion were added in vitro to brain homogenate, singly as well as in combination. After 30 to 60 minutes the activity of brain ChE was determined using Ach substrate in each case, from which extent of inhibition was calculated. Table 3 and Fig. 8 show percentual inhibition of brain ChE resulting from combined effect of different concentration of EPN and Malathion. It appeared that the effect of combination of non-incubated compounds in vitro was merely additive. In fact, in certain cases ChE inhibition produced was somewhat less than additive (Table 3). It seemed that when high concentration of Malathion was added in combination with EPN to brain homogenate, the inhibition appeared to be equal to ChE
**Figure 7**

Irreversibility tests for Malathion–Che interaction. Each point represents an average value of three determinations from three dogs. ACh substrate.
IN VITRO INHIBITION OF BRAIN CHE BY EPN AND MALATHION AND THEIR COMBINATION. EACH VALUE REPRESENTS AN AVERAGE VALUE OF FOUR DOGS. EACH SUBSTRATE.

<table>
<thead>
<tr>
<th>The compound</th>
<th>Concentration</th>
<th>Percent ChE inhibition by: EPN or Malathion singly</th>
<th>EPN and Malathion combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malathion</td>
<td>3.2 $10^{-5}M$</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>EPN</td>
<td>4.0 $10^{-5}M$</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>Malathion</td>
<td>2.2 $10^{-5}M$</td>
<td>80</td>
<td>95</td>
</tr>
<tr>
<td>EPN</td>
<td>2.0 $10^{-4}M$</td>
<td>50</td>
<td>95</td>
</tr>
<tr>
<td>Malathion</td>
<td>2.0 $10^{-5}M$</td>
<td>78</td>
<td>85</td>
</tr>
<tr>
<td>EPN</td>
<td>7.5 $10^{-5}M$</td>
<td>25</td>
<td>85</td>
</tr>
<tr>
<td>Malathion</td>
<td>0.2 $10^{-5}M$</td>
<td>42</td>
<td>68</td>
</tr>
<tr>
<td>EPN</td>
<td>8.5 $10^{-5}M$</td>
<td>28</td>
<td>68</td>
</tr>
</tbody>
</table>
FIGURE 8

IN VITRO EFFECTS, ON BRAIN CHE, OF COMBINATION OF NON-INCUBATED COMPOUNDS, ACH SUBSTRATE. EACH VALUE REPRESENTS AN AVERAGE OF FOUR DETERMINATIONS FROM FOUR DOGS.
inhibition produced by Malathion alone. This may be due to Malathion having higher affinity to brain ChE than EPN, and preventing the action of EPN at certain sites of ChE molecule. It may be also that the ChE inhibition by EPN is a slow process allowing therefore Malathion effect to predominate at the time (30 to 60 minutes) that the reading was taken.

b. Combination of liver-incubated compounds:

EPN incubated with liver and DPN and liver-incubated Malathion were added to brain homogenate singly and in combination. The activity of brain ChE was then determined and its inhibition calculated. The results obtained (Fig. 9) indicate that combination of EPN incubated with liver and DPN and liver-incubated Malathion resulted in a potentiation in vitro of the brain ChE inhibition. This might be explained by Cook's theory if we were to assume that EPN inhibits detoxification of Malathion by the liver homogenate added in vitro in these experiments.

However the question arises as to whether or not this is the only mechanism that functions here. Potentiation occurred also when incubated Malathion was added to brain homogenate after the addition of EPN. The concentration of liver incubated with Malathion was selected so that most Malathion should have been destroyed. The concentration of EPN (0.2 to 0.8 mg/gm liver) was selected so as to inhibit the liver enzyme in its own incubation medium and not to protect detoxification of Malathion.
FIGURE 9

IN VITRO EFFECTS, ON BRAIN CHE, OF COMBINATION OF DIFFERENT CONCENTRATIONS OF LIVER-INCUBATED AND NON-INCUBATED COMPOUNDS. ACH SUBSTRATE. EACH VALUE REPRESENTS AN AVERAGE OF FOUR DETERMINATIONS FROM FOUR DOGS.
by its own medium after combination with EPN sample; indeed, at these concentration of EPN inhibition of the "Malathionase" of its own homogenate was incomplete and no EPN was left over to protect Malathion from detoxification (cf. P. 33)

The pertinent results were as follows:
15 percent brain ChE inhibition by EPN, 0.8 mg/gm liver
12 percent brain ChE inhibition by Malathion, 20 mg/gm liver
65 percent brain ChE inhibition by EPN-Malathion combination.

When concentration of Malathion was kept constant and EPN concentration was lowered, potentiation still occurred:
4 percent brain ChE inhibition by EPN, 0.2 mg/gm liver
12 percent brain ChE inhibition by Malathion, 20 mg/gm liver
40 percent brain ChE inhibition by EPN-Malathion combination.

II. In vivo studies:
   A. Single administration:
      1. Single I.V. administration:
         a. I.V. administration of EPN:
            A systematic study of the in vivo effects of EPN and of its dose-effect relationship was carried out. Non effective and lethal doses were also used. Fig. 10 indicates that EPN exerts in vivo anticholinesterase effect on brain enzyme, and that this action follows a clear cut dose-effect relationship. EPN appeared as a rather in vivo potent inhibitor and its anticholinesterase effect first appeared at a dose of 2 to 3 mg/Kg
FIGURE 10

IN VIVO, DOSE-EFFECT RELATIONSHIP OF EPN ON BRAIN CHE. EACH POINT REPRESENTS AN AVERAGE VALUE FROM SIX DOGS. ACH SUBSTRATE.
(Ninety to 100 percent inhibition was obtained with 10 to 12 mg/Kg of EPN.

Table 4 shows the anticholinesterase effect of EPN with respect to the death frequency in anesthetized dogs. The death caused by toxic doses seemed to parallel fairly close the level of inhibition of brain AcChE. Death did not occur until the inhibition reached about 90 percent of the normal activity of the enzyme. Cholinergic symptoms such as defecation salivation, muscle twitches, convulsions and respiratory embarrassment were observed with lethal doses of EPN, milder cholinergic symptoms also were observed with lower effective doses of EPN (Awad and Karczmar, 8).

Brain samples from animals given EPN I.V. added to brain sample from untreated animal; the two samples then homogenized together and ChE activity measured in this mixed sample. In this case the enzyme of the untreated brain sample was not inhibited as shown in Table 5. This indicates that no free phosphonate compounds were present in the brain tissue, of animal given EPN in vivo.

b. I.V. administration of Malathion:

In a similar way experiments were carried out with Malathion. Fig. 11 indicates that Malathion has an anticholinesterase effect on brain enzyme in vivo which follows a clear cut dose-effect relationship. Malathion is a weak inhibit-
<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose in mg/Kg</th>
<th>Percent ChE inhibition (average values)</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>Medulla</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>50</td>
<td>0/5</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>74</td>
<td>0/6</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>90</td>
<td>2/5</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>100</td>
<td>2/2</td>
</tr>
<tr>
<td>Malathion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0/2</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>25</td>
<td>18</td>
<td>0/4</td>
</tr>
<tr>
<td>200</td>
<td>50</td>
<td>38</td>
<td>0/6</td>
</tr>
<tr>
<td>300</td>
<td>60</td>
<td>51</td>
<td>1/4</td>
</tr>
<tr>
<td>400</td>
<td>72</td>
<td>65</td>
<td>3/3</td>
</tr>
</tbody>
</table>
TABLE V

DETERMINATION OF HOMOGENIZATION EFFECT -IF ANY- ON PERCENT INHIBITION OF CHE EACH VALUE REPRESENTS AN AVERAGE VALUE OF THREE DOGS. ACH SUBSTRATE.

<table>
<thead>
<tr>
<th>Brain tissue</th>
<th>Percent inhibition of brain ChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain tissue treated with EPN (12 mg/Kg)</td>
<td>100</td>
</tr>
<tr>
<td>I:1 of brain tissue treated with EPN : brain tissue untreated homogenized together</td>
<td>47</td>
</tr>
</tbody>
</table>
FIGURE 11

IN VIVO, DOSE-EFFECT RELATIONSHIP OF MALATHION ON BRAIN CHE. EACH POINT REPRESENTS AN AVERAGE VALUE FROM SIX DOGS. ACH SUBSTRATE.
or when compared to EPN; its anticholinesterase effect first appeared at a dose level of about 50 to 60 mg/Kg. Maximum inhibition of 65 to 70 percent was noticed with doses of 400 mg /Kg.

Cholinergic symptoms were also observed with lethal as well as with effective doses of Malathion. The severity depended on how large a dose was given. Table 4 shows the relationship between brain ChE inhibition and lethality; it appeared that death did not occur until the dose of about 400 mg /Kg of Malathion in the anesthetized animals. As stated, at that time residual activity of ChE was still relatively high.

2. In vivo interaction of EPN and Malathion with brain ChE:

To determine the degree of irreversibility of the ChE inhibition produced by EPN and by Malathion in vivo, the dialysis technique was used. Table 6 shows that the in vivo inhibition by EPN was not reversed by dialysis, irrespective of dose. Similar type of irreversible interaction occurs in vivo between Malathion and ChE (Table 7). These results as well as the data on irreversibility of interaction between EPN and Malathion in vitro indicate that the measurement of the in vivo inhibition is a faithful indication of the actual inhibition in the animal which was not reversed or modified by the procedure.

3. Effects of EPN and Malathion on true and pseudo ChE
TABLE VI

EFFECT OF DIALYSIS ON THE IN VIVO EPN-BRAIN CHE INHIBITION. ACH SUBSTRATE
EACH VALUE REPRESENTS AN AVERAGE VALUE
OF THREE DOGS.

<table>
<thead>
<tr>
<th>Dose of EPN mg/Kg</th>
<th>Brain Tissue</th>
<th>Percent ChE inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before dialysis</td>
<td>After 24 hours</td>
</tr>
<tr>
<td>5</td>
<td>Cortex</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>70</td>
</tr>
<tr>
<td>8</td>
<td>Cortex</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>80</td>
</tr>
</tbody>
</table>
### TABLE VII

**EFFECT OF DIALYSIS ON THE IN VIVO MALATHION BRAIN CHE INHIBITION. ACH SUBSTRATE.**

**EACH VALUE REPRESENTS AN AVERAGE VALUE OF THREE DOGS.**

<table>
<thead>
<tr>
<th>Dose of Malathion mg/Kg</th>
<th>Brain tissue</th>
<th>Percent ChE inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before dialysis</td>
<td>After 24 hours dialysis</td>
</tr>
<tr>
<td>50</td>
<td>Cortex 11</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>Cortex 28</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Medulla 17</td>
<td>15</td>
</tr>
</tbody>
</table>
of the blood:

Three mg/Kg of EPN was given I.V. to the whole anesthetized animal. An hour later, a blood sample was withdrawn and analyzed for its plasma BuChE and RBC AcChE activities, using Bzch and AcCh substrates. Sixty mg/Kg of Malathion was injected similarly and blood activities of ChE's were determined. The results shown in table 8 indicate that EPN inhibited RBC enzyme more than plasma ChE, while Malathion inhibited plasma enzyme somewhat more than RBC enzyme.

4. Single administration in the "upper animal":

The purpose of the study of the "upper animal" was to determine whether or not metabolism is necessary for the activity of EPN and Malathion as well as for their potentiative effect to occur in vivo.

a. Intrajugular administration:

1. Non-incubated and liver-incubated EPN:

Various doses of EPN were administered into the jugular vein in the "upper animal". Brain tissue was then analyzed to determine its ChE activity. The effects of EPN on brain enzyme when given into the jugular vein to the "upper animal" can be compared (Table 9) with its action when EPN was given into the femoral vein to the normal animal. It appeared that at a dose of 1.5 mg/Kg EPN produced some brain ChE inhibition when injected into the normal anesthetized animal and had no
TABLE VIII

EFFECTS OF EPN AND MALATHION ON TRUE AND PSEUDO CHE OF THE BLOOD IN VIVO. EACH VALUE REPRESENT AN AVERAGE VALUE OF THREE ANIMALS.

<table>
<thead>
<tr>
<th>Dose of compound in mg/Kg</th>
<th>Percent of ChE inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBC</td>
</tr>
<tr>
<td></td>
<td>(Ach substrate)</td>
</tr>
<tr>
<td>EPN, 3 mg/Kg</td>
<td>63</td>
</tr>
<tr>
<td>Malathion, 60 mg/Kg</td>
<td>32</td>
</tr>
</tbody>
</table>
### TABLE IX

EFFECTS OF EPN GIVEN I.V. AND INTRA-JUGULARLY TO NORMAL AND "UPPER" ANESTHETIZED ANIMALS RESPECTIVELY. EACH VALUE REPRESENTS AN AVERAGE VALUE OF FIVE ANIMALS. ACH SUBSTRATE.

<table>
<thead>
<tr>
<th>Dose in mg/Kg</th>
<th>Brain ChE activity</th>
<th>Control = 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Given I.V. to normal anesthetized animal</td>
<td>Given intrajugularly to &quot;upper animal&quot;</td>
</tr>
<tr>
<td></td>
<td>Non-incubated</td>
<td>Incubated</td>
</tr>
<tr>
<td>1.5</td>
<td>90</td>
<td>116</td>
</tr>
<tr>
<td>10.0</td>
<td>30</td>
<td>10</td>
</tr>
</tbody>
</table>
effect when injected into the "upper animal"; in the latter case EPN did not circulate through the animal's lower part and its visceral organs. At a dose of 10 mg/Kg EPN produced about 70 to 90 percent brain ChE inhibition when injected into the normal animal, and had again no effect when given to the "upper animal". From this comparison it seems again that EPN had to be metabolized, presumably in the liver, in order that it can exert its anticholinesterase effect in vivo.

To find out more about this phenomenon other series of experiments were carried out with various doses of liver-incubated EPN given via the jugular vein to the "upper animal". Five experiments were carried out with a dose of EPN of 1.5 mg/Kg and 10 mg/Kg. Table 9 shows that the incubated EPN in a dose of 1.5 mg/Kg instead of inhibiting, increased brain ChE activity, particularly that of the medulla. A statistical study has been done to ascertain the occurrence of this phenomenon. The EPN dose used in this case was 1.0 mg/Kg. Table 10 shows the results of this analysis. The increase in brain ChE activity found in this series of 7 experiments was statistically significant.

11. Non-incubated and liver-incubated Malathion:

Experiments similar to the above were carried out with Malathion in the "upper animal". Non-incubated and liver-incubated Malathion was administered into the jugular vein.
TABLE X

STATISTICAL ANALYSIS OF MEDULLARY CHE ACTIVATION BY INCUBATED EPN, IMG/KG ADMINISTERED INTRAJUGULARLY. EACH VALUE REPRESENTS AN AVERAGE OF TWO DETERMINATIONS. ACH SUBSTRATE.

<table>
<thead>
<tr>
<th>µl of CO₂ evolved/40 minutes/50 mg brain tissue (untreated)</th>
<th>µl of CO₂ evolved/40 minutes/50 mg brain tissue (EPN treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32.25</td>
<td>43.20</td>
</tr>
<tr>
<td>31.50</td>
<td>42.70</td>
</tr>
<tr>
<td>32.25</td>
<td>47.50</td>
</tr>
<tr>
<td>28.25</td>
<td>47.50</td>
</tr>
<tr>
<td>32.50</td>
<td>42.50</td>
</tr>
<tr>
<td>34.00</td>
<td>45.50</td>
</tr>
<tr>
<td>34.00</td>
<td>46.50</td>
</tr>
</tbody>
</table>

The mean

| The mean  | 32.10 | 45.50 |
| Standard deviation | 0.60 | 0.80 |
| 95% CL. | 32.1 ± 1.4 | 45.5 ± 1.9 |
| Brain ChE activity | 100% | 141.5% |

"t" IS SIGNIFICANT AT THE 0.01% LEVEL
Brain tissue was analyzed in each case to determine its ChE activity. The results obtained (Table 11) indicate that Malathion, 50 to 100 mg/Kg, had anticholinesterase actions when injected I.V. to the normal anesthetized animal. This effect was more pronounced when non-incubated Malathion was given intrajugularly to the "upper animal" at the same dose level in contradistinction to the results obtained with EPN. Even at lower dose levels (Table 11) Malathion inhibited brain ChE when given intrajugularly to the "upper animal". Incubation with liver homogenate reduced anticholinesterase activity of Malathion, again contrary to the results obtained by EPN. Thus, liver metabolism of Malathion seems instrumental in the weak potency of Malathion as anticholinesterase agent when given I.V. to the normal anesthetized animal. Finally, unlike EPN, at dose level as low as 10 mg/Kg and as high as 100 mg/Kg Malathion did not produce activation of ChE; only inhibition was observed with minimal effective doses.

b. Intraventricular administrations:

i. Non-incubated and liver-incubated EPN:

The purpose of using this route of administration was to determine whether or not blood-brain barrier has any effect on EPN action. EPN incubated with liver and DPN as well as non-incubated EPN were injected into the brain ventricle of the "upper animal". Results obtained (Table 12) indicate that
TABLE XI

EFFECTS OF MALATHION GIVEN I.V. AND INTRAJUGULARLY TO NORMAL AND "UPPER" ANESTHETIZED ANIMALS RESPECTIVELY. EACH VALUE REPRESENTS AN AVERAGE VALUE OF FIVE ANIMALS. ACHE SUBSTRATE.

<table>
<thead>
<tr>
<th>Dose in mg/Kg</th>
<th>Percent brain ChE inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Given I.V. to normal anesthetized animal</td>
</tr>
<tr>
<td></td>
<td>Cortex</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
<td>22</td>
</tr>
</tbody>
</table>
TABLE XII

EFFECTS ON BRAIN CHE BY EPN ADMINISTERED BY THREE DIFFERENT ROUTES. ACH SUBSTRATE. EACH VALUE REPRESENTS AN AVERAGE OF THREE ANIMALS.

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Dose in mg/Kg</th>
<th>Brain Che activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cortex</td>
<td>Medulla</td>
</tr>
<tr>
<td>Brain ventricle (U.A.)</td>
<td>0.5</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>100</td>
<td>69</td>
</tr>
<tr>
<td>Jugular vein (U.A.)</td>
<td>1.5</td>
<td>100</td>
<td>92</td>
</tr>
<tr>
<td>Femoral vein (N.A.)</td>
<td>1.5</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

U.A. INDICATES "UPPER ANIMAL"
N.A. INDICATES NORMAL ANIMAL
non-incubated EPN at a dose of 10 mg/Kg produced no inhibition of brain ChE when administered intraventricularly. It should be born in mind that this dose produced 70 to 90 percent inhibition of brain ChE when administered I.V. to normal anesthetized animal. Liver-incubated EPN in a dose of 0.5 mg/Kg given intraventricularly produced greater increase of ChE activity (Table 12) than that produced by intrajugular administration of a dose of 0.5 mg/Kg. Larger doses of EPN produced brain ChE inhibition (Table 12), provided liver-incubated EPN was employed.

These data as well as the results obtained upon I.V. administration of EPN to the "upper animal" (cf. above, section 4) suggest that EPN metabolism is necessary for its activation as an anticholinesterase rather than to improve its capacity to penetrate blood-brain barrier. Whether given intraventricularly or I.V. in the "upper animal" EPN inhibits more after incubation; particularly in the former case blood-brain barrier was by-passed and yet the compound inhibited depending on its activation in the liver.

11. Non-incubated and liver-incubated Malathion:

Liver-incubated Malathion and non-incubated Malathion were administered into the brain ventricle of the "upper animal". Even half of the dose of non-incubated Malathion which was ineffective when administered I.V. to the whole animal inhibited brain ChE when administered intraventricularly into
the "upper animal" (Table 13). Here also as in the in vitro experiments, liver incubation reduced the anticholinesterase activity of Malathion. ChE inhibition by non-incubated Malathion given to the "upper animal" was almost equal quantitatively by both intrajugular and intraventricular routes; Malathion at 25 mg/Kg produced 16 and 14 percent inhibition of cortical ChE when given into the ventricle or via the jugular route respectively. These data suggest that blood-brain barrier has no control over Malathion action. Also the effects were generally similar upon intrajugular and intraventricular administrations of liver-incubated Malathion. However, the choice of the route seemed to influence the distribution of Malathion. When given intraventricularly, Malathion inhibited medullary enzyme more than the cortical enzyme while when administered I.V. into either normal or "upper animal" it inhibited cortical more than the medullary enzyme. This dependence on the route of selectivity of action of Malathion toward ChE of different parts of the brain is not understood (cf. Discussion).

5. Time-effect relationship of small doses of EPN and of Malathion, administered I.V.:

The indication obtained above that EPN given via intrajugular and intraventricular routes causes at low dose level increase in ChE activity urged more careful study of EPN effect given by the I.V. route in normal anesthetized animal. Similar
TABLE XIII

EFFECTS ON BRAIN CHES BY MALATHION
ADMINISTERED BY THREE DIFFERENT ROUTES
EACH VALUE REPRESENTS AN AVERAGE VALUE
OF THREE ANIMALS. ACH SUBSTRATE.

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Dose in mg/Kg</th>
<th>Percent ChE inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain ventricle (U.A.)</td>
<td>25</td>
<td>16 10 28 18</td>
</tr>
<tr>
<td>Jugular vein (U.A.)</td>
<td>25</td>
<td>14 7 9 0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>25 12 15 0</td>
</tr>
<tr>
<td>Femoral vein (N.A.)</td>
<td>50</td>
<td>8 0</td>
</tr>
</tbody>
</table>

U.A. INDICATES "UPPER ANIMAL"
N.A. INDICATES NORMAL ANIMAL
study was also carried out in the case of Malathion.

a. **Time-effect relationship of EPN:**

EPN in a dose of 2 mg/Kg was given I.V. to the normal anesthetized animal. At different lengths of time after the injection the animal was sacrificed and the brain was analyzed for its ChE activity. It appeared (Table 14) that EPN in small doses increased the activity of brain ChE. The increase reached its peak at 40 minutes; return to control or slight inhibition followed. These results were in agreement with those obtained with small doses of EPN administered into the "upper animal".

b. **Time-effect relationship of Malathion:**

Malathion in a dose of 10 to 100 mg/Kg was given I.V. to normal anesthetized dogs. Animals were sacrificed after different time intervals, and brain ChE activity was determined. With small doses of Malathion (10 to 25 mg/Kg) there was no increase in ChE activity in the brain; slight inhibition occurred with larger dose (Table 15). It could be calculated from the dose-effect relationship of EPN and Malathion that 1 mg/Kg dose of the former which as seen activated the enzyme, was equi-effective with 45 mg/Kg dose of the latter which was ineffective or somewhat inhibitory. Whenever Malathion caused inhibition it was nearly constant for more than two hours.

c. **Effects of EPN and of Malathion on brain pseudo ChE:**
### TABLE XIV

**TIME-EFFECT RELATIONSHIP ON BRAIN CHE OF 2. MG/KG OF EPN GIVEN I.V. TO NORMAL ANESTHETIZED ANIMAL. ACH AND BZCH SUBSTRATE.**

**Each value represents an average value of three animals.**

<table>
<thead>
<tr>
<th>Time at which brain samples were taken after the injection</th>
<th>Cortical activity Control = 100</th>
<th></th>
<th>Medullary activity Control = 100</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AcChE</td>
<td>BuChE</td>
<td>AcChE</td>
<td>BuChE</td>
</tr>
<tr>
<td>20 minutes</td>
<td>95</td>
<td>85</td>
<td>123</td>
<td>125</td>
</tr>
<tr>
<td>40 minutes</td>
<td>75</td>
<td>85</td>
<td>131</td>
<td>140</td>
</tr>
<tr>
<td>75 minutes</td>
<td>80</td>
<td>70</td>
<td>112</td>
<td>106</td>
</tr>
<tr>
<td>150 minutes</td>
<td>75</td>
<td>65</td>
<td>84</td>
<td>90</td>
</tr>
</tbody>
</table>
TABLE XV

TIME-EFFECT RELATIONSHIP ON BRAIN CHE OF 100 MG/KG OF MALATHION GIVEN I.V. TO NORMAL ANESTHETIZED ANIMAL. EACH VALUE IS AN AVERAGE VALUE OF THREE ANIMALS. ACH AND BZCH SUBSTRATES.

<table>
<thead>
<tr>
<th>Time at which brain samples were taken after the injection</th>
<th>Cortical inhibition</th>
<th>Medullary inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AcChE</td>
<td>BuChE</td>
</tr>
<tr>
<td>20 minutes</td>
<td>22</td>
<td>40</td>
</tr>
<tr>
<td>40 minutes</td>
<td>24</td>
<td>39</td>
</tr>
<tr>
<td>90 minutes</td>
<td>22</td>
<td>37</td>
</tr>
</tbody>
</table>
This was studied in the course of the investigations on EPN and Malathion time-effect relationship. Table 14 indicates that EPN at small doses activates and at large doses inactivates medullary brain pseudo ChE in parallel with its action upon brain AcChE. In the case of Malathion there was only inhibition of pseudo ChE (BuChE) as well as of AcChE (Table 15). In other words effects of EPN and Malathion on BuChE were always analagical to those on AcChE, whether in case of inhibition as with EPN and Malathion or activation, as in the case of EPN alone.

B. Combined administration:

1. I.V. route:

This study was carried out to analyze the effects of combination of EPN and Malathion on brain ChE. EPN was administered I.V. to animals in different doses ranging from 1 to 10 mg /Kg. One half hour later, Malathion was administered in doses ranging from 10 to 50 mg/Kg. Brain tissue was analyzed for ChE activity. Table 16 shows percent brain ChE inhibition and the death frequency with different combined doses of EPN and Malathion. To describe more specifically the phenomenon occurring with one dose combination, the toxicological and enzymic effects of administration of 2 mg/Kg of EPN and 10 mg/Kg of Malathion will be described. Death occurred within 10 minutes after the injection of the second compound (Malathion) in about 85 percent of the treated animals; it was preceded by cholinergic symptoms
<table>
<thead>
<tr>
<th>Dose in mg/Kg</th>
<th>Sequence of administration</th>
<th>Percent ChE inhibition (Average values)</th>
<th>Death ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPN 1 mg</td>
<td>EPN prior to Malathion</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>Malathion 50 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPN 2 mg</td>
<td>EPN prior to Malathion</td>
<td>85</td>
<td>65</td>
</tr>
<tr>
<td>Malathion 10 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPN 2 mg</td>
<td>EPN and Malathion simultaneously</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Malathion 10 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPN 2 mg</td>
<td>Malathion prior to EPN</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Malathion 10 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPN 10 mg</td>
<td>Malathion prior to EPN</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>Malathion 50 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPN 5 mg</td>
<td>Malathion prior to EPN</td>
<td>69</td>
<td>68</td>
</tr>
<tr>
<td>Malathion 50 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPN 3 mg</td>
<td>Malathion prior to EPN</td>
<td>62</td>
<td>68</td>
</tr>
<tr>
<td>Malathion 60 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
already described. Brain ChE inhibition was about 85 percent in the medulla and about 65 percent in the cortex (Fig. 12). In all those cases the blood ChE inhibition was always complete while brain enzyme inhibition was not.

A simple algebraic equation was used (Table 17) to ascertain whether this extent of inhibition was due to additive action or to potentiation. The equation allowed the calculation of the combined AED$_{50}$ for both compounds (AED$_{50}$ is the average effective dose producing 50 percent inhibition of ChE) as well as for their combinations. It appears that a potentiative inhibition occurred since the combined AED$_{50}$ was much higher when calculated (91 mg/Kg) than when measured (51 mg/Kg, Table 17).

2. The effect of various sequences on combined I.V. administration:

Since potentiation occurred when EPN was administered prior to Malathion the question arises as to whether or not potentiation would also occur if the sequence of the combined administration is reversed. To answer this question four experimental series were carried out.

a. EPN, 2 mg/Kg, was administered I.V. 30 minutes after 10 mg/Kg of Malathion

b. EPN, 2 mg/Kg, was administered simultaneously with 10 mg/Kg of Malathion.

c. EPN, 10 mg/Kg was administered I.V. 50 minutes after Malathion, 50 mg/Kg
FIGURE 12

EFFECT ON BRAIN CHE BY COMBINATION OF COMPOUNDS GIVEN I.V. TO THE NORMAL ANESTHETIZED ANIMAL (EPN, 2 MG/KG GIVEN PRIOR TO MALATHION, 10 MG/KG) EACH VALUE REPRESENTS AN AVERAGE OF FIVE DOGS. ACH SUBSTRATE.
TABLE XVII

MEASURED AND CALCULATED AED$_{50}$ IN MG/KG OF COMBINED EPN AND MALATHION. EACH VALUE REPRESENTS AN AVERAGE VALUE OF SIX ANIMALS. EACH SUBSTRATE.

<table>
<thead>
<tr>
<th></th>
<th>Measured</th>
<th>Calculated from the equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>12</td>
<td>24.3</td>
</tr>
<tr>
<td>Medulla</td>
<td>51</td>
<td>91.8</td>
</tr>
</tbody>
</table>

THE EQUATION:

\[
\frac{I}{AED_{50} \text{ of combination}} = \frac{\text{PERCENT, RATIO EPN in combination}}{AED_{50} \text{ EPN}} + \frac{\text{PERCENT RATIO, MALATHION in combination}}{AED_{50} \text{ MALATHION}}
\]
d. EPN, 3mg/Kg was administered 50 to 120 minutes I.V., after Malathion, 60 mg/Kg.

The results of those experiments are shown in Fig. 13 and Table 16 and can be summarized as follows:

i. Cholinergic symptoms developed in all series of experiments.

ii. Potentiation of brain ChE inhibition occurred in (c) and in (d) but not in series (a) and (b), i.e., when EPN, 2 mg/Kg was given simultaneously with or after Malathion, 10 mg/Kg.

iii. Lethality occurred again in series (c) and (d) but not in series (a) and (b). The fact that potentiation of toxicity and of brain ChE inhibition did not occur in series (a) and (b) does not necessarily signify that the dose of Malathion in question (10 mg/Kg) was destroyed in the liver without having the chance of being protected by EPN since Malathion at this particular dose given intrajugularly to the "upper animal" and thus not destroyed by the liver, did not produce a significant brain ChE inhibition.

iv. When EPN, 10 mg/Kg was given after Malathion (series c) lethality may be due to toxic effect of EPN since at this dose it sometimes produces death. However, this does not seem to be the only cause of death since potentiation of both lethality and the enzyme inhibition occurred also when minimal effective dose of EPN (3 mg/Kg) was given prior to minimal
FIGURE 13

EFFECT OF THREE DIFFERENT SEQUENCES OF COMBINED ADMINISTRATION OF EPN, 2 MG/KG AND MALATHION, 10 MG/KG. EACH VALUE REPRESENTS AN AVERAGE OF THREE DETERMINATIONS FROM THREE DOGS. ACH SUBSTRATE.
effective dose\(^{(*)}\) (60 mg/Kg) of Malathion.

v. In series (d) in spite of the fact that death and potentiation of brain ChE inhibition occurred the latter was not as extensive as when EPN was given prior to Malathion; potentiation of inhibition amounted to 20 to 30 percent above values expected on the basis of pure addition.

vi. In series (d) in which Malathion was given prior to EPN death occurred within 60 minutes of the administration of the second compound (EPN) while when EPN was given prior to Malathion death occurred at only 7 to 10 minutes after the administration of the second compound (Malathion).

The biochemical data obtained in this series of experiments agree fairly well with the pharmacological data obtained in the same laboratory, since potentiation of pharmacodynamic effects was noticed in a study of actions of EPN and Malathion at specific neuroeffectors. For instance, Malathion 28 mg/Kg given 45 minutes prior to EPN, 2.5 mg/Kg, produced increase of muscle response to indirect stimulation while Malathion and EPN given singly, produced increased muscle twitches at doses of more than 250 and 12 mg/Kg respectively (Kaczmar, Awad and Blachut, unpublished).

\(^{(*)}\) The minimum effective dose is the smallest dose capable of producing significant (\(\approx\) 15 percent) inhibition.
3. Combined administration to the "upper animal":

a. Intrajugular:

i. Non-incubated compounds:

In the "upper animal" EPN, 0.5 to 2.0 mg/Kg was administered into the jugular vein 30 minutes prior to Malathion, 10 to 25 mg/Kg. The results obtained (Fig. 14) indicate that non-incubated combined EPN and Malathion did not produce potentiation at 30 minutes in the "upper animal" after the administration of the second compound. However at 60 minutes after their administration potentiation of brain ChE inhibition developed, particularly in the cortex.

ii. Liver-incubated compounds:

EPN was incubated with liver and DPN and administered via the jugular vein to the "upper animal" 30 minutes prior to administration of incubated Malathion. The results obtained (Table 18) indicate that potentiation of ChE inhibition was produced 30 minutes after the administration of the second compound.

Potentiation occurred also from combination of incubated EPN and non-incubated Malathion (Table 19). In this experiment conditions for destruction of Malathion were not present, since Malathion was administered without liver and since EPN concentration was selected so as to destroy "Malathionase" of its own liver medium. Thus, potentiation in this
FIGURE 14

EFFECT ON BRAIN CHE OF COMBINATION OF NON-INCUBATED COMPOUNDS GIVEN INTRAJUGULARLY TO THE "UPPER ANIMAL" AT 60 AND 90 MINUTES AFTER THEIR ADMINISTRATION (EPN, 2 MG/KG GIVEN 30 MINUTES PRIOR TO MALATHION, 10 MG/KG). EACH VALUE REPRESENTS AN AVERAGE OF FIVE DETERMINATIONS FROM FIVE DOGS. ACH SUBSTRATE.
TABLE XVIII

**EFFECT OF INTRAJUGULAR COMBINED ADMINISTRATION OF EPN GIVEN HALF HOUR PRIOR TO MALATHION INTO THE "UPPER ANIMAL". ACH SUBSTRATE. EACH VALUE REPRESENTS AN AVERAGE VALUE OF THREE ANIMALS.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose in mg/Kg</th>
<th>Incubated</th>
<th>Non-incubated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Brain ChE activity control = 100</td>
<td>Brain ChE activity control = 100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortex</td>
<td>Medulla</td>
</tr>
<tr>
<td>EPN</td>
<td>0.5</td>
<td>97</td>
<td>121</td>
</tr>
<tr>
<td>Malathion</td>
<td>25.0</td>
<td>94</td>
<td>100</td>
</tr>
<tr>
<td>EPN prior to Malathion</td>
<td>0.5</td>
<td>55</td>
<td>60</td>
</tr>
</tbody>
</table>
TABLE XIX

INTRAJUGULAR EFFECT OF COMBINATION OF EPN GIVEN TO THE "UPPER ANIMAL" PRIOR TO NON-INCUBATED MALATHION. EACH VALUE IS AN AVERAGE VALUE OF THREE ANIMALS. EACH SUBSTRATE.

<table>
<thead>
<tr>
<th>Compound in mg/Kg</th>
<th>Brain ChE activity control = 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
</tr>
<tr>
<td>EPN, 0.5</td>
<td>97</td>
</tr>
<tr>
<td>Malathion, 25</td>
<td>86</td>
</tr>
<tr>
<td>EPN, 0.5</td>
<td>41</td>
</tr>
<tr>
<td>Malathion, 25</td>
<td></td>
</tr>
</tbody>
</table>
case could not depend on protection afforded by EPN to Malathion against detoxification.

b. Intraventricular administration:

i. Non-incubated compounds:

In the "upper animal" EPN, 0.05 mg/Kg, was injected into the brain ventricle 30 minutes prior to administration of Malathion, 5 mg/Kg. The results (Table 20) showed that at 30 minutes after the injection of the second compound (EPN prior to Malathion) brain enzyme inhibition was somewhat greater than that expected on the basis of mere additive effects. This again suggests that potentiation does not necessarily depend on protection of Malathion by EPN.

ii. Liver-incubated compounds:

Concentrations of EPN in the liver homogenate were selected with the precaution mentioned earlier (cf. p. 33). EPN was incubated with liver in presence of DPN and given intraventricularly to the "upper animal", 30 minutes prior to liver-incubated Malathion. The results obtained (Table 20) indicate that at 30 minutes after the administration of the second compound (Malathion) potentiation of brain ChE inhibition occurred. Potentiation was much more pronounced than that occurred at 60 minutes after combined intraventricular administration of the non-incubated compounds.
### TABLE XX

**EFFECT OF INTRAVENTRICULAR ADMINISTRATION TO THE "UPPER ANIMAL" OF EPN GIVEN PRIOR TO MALATHION. EACH VALUE IS AN AVERAGE VALUE OF THREE ANIMALS. ACHE SUBSTRATE.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose in mg/Kg</th>
<th>Incubated</th>
<th>Non-incubated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Brain Che activity control = 100</td>
<td>Brain Che activity control = 100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortex</td>
<td>Medulla</td>
</tr>
<tr>
<td>EPN</td>
<td>0.05</td>
<td>96</td>
<td>135</td>
</tr>
<tr>
<td>Malathion</td>
<td>5.00</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>EPN prior to Malathion</td>
<td>0.05</td>
<td>60</td>
<td>70</td>
</tr>
</tbody>
</table>

88
CHAPTER IV

DISCUSSION

I. Introduction:

Frawley in 1957 has shown that potentiation of the acute toxicity arises from simultaneous administration of EPN and Malathion; inhibition of blood ChE was also found to be more than additive. No data were obtained with reference to the effect of this combination on C.N.S. ChE. One of the purposes of the present study was to establish whether or not inhibition of brain ChE is also potentiated.

Up to the present time it was suggested that the potentiation phenomenon of ChE inhibition produced by combined administration of organophosphorus anticholinesterase compounds is due to the inhibition by one compound of the detoxification of the other (Cook's postulate). With regard to the two compounds studied at present, EPN and Malathion, Cook (18) has shown that in vitro EPN inhibits Malathion detoxification by the liver.

The present systematic study shows in detail how brain ChE is affected by the combination of those two compounds, how their enzymic effect is influenced by their liver metabolism, and whether or not the effect upon brain enzyme can be correlated with the toxicity of the combination. These data will be now summarized and certain points stressed to show that while some
of the results supports Cook's postulate others do not; accordingly, an alternative hypothesis is presented at the end of this discussion.

ii. Survey of the in vitro studies of EPN and Malathion:

1. Single addition:

In vitro studies showed that EPN has weak anticholinesterase action and that incubation with liver homogenate and DPN leads to the conversion of EPN into a potent inhibitor of ChE. This suggests that in vivo EPN has to be metabolized, probably in the liver, to be effective as an anticholinesterase agent, and confirms earlier results on activation of EPN by its incubation with the liver (61). Incubation of EPN with liver homogenate in absence of DPN has shown no definite increase in its anticholinesterase action.

In vitro studies of Malathion showed that a relatively rapid decrease rather than increase of anticholinesterase effect, followed its incubation with the liver homogenate. This agrees with the data obtained by Cook (19) showing that in vitro Malathion is inactivated within half hour of incubation. Parathion, EPN and many other organophosphorus compounds on the other hand are not inactivated by the liver. Malathion seems to be inactivated similarly whether or not DPN was added to the incubation medium. This indicates that destruction of Malathion and activation of EPN are carried out by two different enzyme
systems of the liver.

2. **Combined addition:**

Combined non-incubated EPN and Malathion produced in vitro inhibition of brain ChE equivalent to the inhibition predictable on the basis of an additive action. On the other hand combination of EPN and Malathion incubated with liver homogenate produced potentiation of their anticholinesterase effects. This agrees with the data of Rosenberg (73) indicating that in vitro EPN and Malathion combination potentiate the inhibition of rat brain ChE only after these compounds were incubated with liver.

3. **Type of interaction:**

In vitro studies have shown also that the interaction between EPN and Malathion with ChE are of an irreversible type. Moreover, when brain samples from animals treated in vivo with EPN were added to and homogenized together with, brain samples from untreated animals, the enzyme of the latter was not inhibited. This indicates that no free phosphonate compounds were present in the brain of EPN treated animals and presumably not in the extra cellular spaces of the tissues. However, final proof of this latter point depends on the use of different other techniques.

This set of data shows that the manometric method can be reliably used to measure the inhibition in vivo since the latter
could not be reversed in the course of the preparation for analysis nor could the actual inhibition be increased by the release of the free compounds during homogenization. They also show that there is at least a certain degree of similarity between EPN and Malathion both being irreversible inhibitors of ChE.

III. Survey of the in vivo studies of the two compounds:

Systematic investigations have been carried out with regard to EPN and Malathion administered by various routes singly or in combination to anesthetized animals.

1. Single administration:

a. Biochemical and toxic effects of single administration and relationship to toxicity. On I.V. administration EPN was found to be a fairly potent anticholinesterase agent; significant brain enzyme inhibition begins with a dose of about 2 to 3 mg/Kg. Cholinergic symptoms developed and its severity was parallel to the dosage given. Death did not occur until about 90 percent inhibition of normal activity of brain AcChE was recorded.

On the other hand Malathion given I.V. to anesthetized dogs was found to be a rather weak brain anticholinesterase

It was noticed that EPN, 5 mg/Kg given I.V. to atropinized dogs did not produce brain ChE inhibition while it did so in non-atropinized dogs (five animals were used in each case). This might have a bearing on similarity between the enzyme and the central receptor sites surfaces.
agent; inhibition began with a dose of about 50 to 60 mg/Kg. The lethal dose of Malathion agreed fairly well with that which produced toxicity of non-anesthetized dogs, and was found to be about 400 mg/Kg. The severity of cholinergic symptoms were also parallel to the dosage given; however, brain AcChE inhibition at toxic level was about 60 to 70 percent, i.e. less than the inhibition produced by toxic doses of EPN. The level of inhibition of about 90 percent characteristic for the lethal doses of EPN is in close agreement with the results of Metz (62) who produced respiratory failure with TEPP at 90 percent inhibition levels of medullary AcChE. In case of TEPP as probably also in that of EPN death can be attributed mostly to inhibition of brain AcChE or more specifically to that of medullary AcChE which is higher than that of the cortical enzyme. On the other hand ChE inhibition by Malathion was lower at death than that which obtained not only with lethal doses of EPN but also with lethal doses of several other organophosphorus compounds. Malathion toxicity seems therefore to be due to a combination of brain enzyme inhibition and other factors. The factors that may be involved are as follows:

1. Peripheral ChE inhibition. At 70 percent inhibition of C.N.S. ChE the enzyme of the N.M.J. and of the ganglia may be completely inhibited; in fact, in the case of Malathion blood ChE is completely inhibited when C.N.S. enzyme is only 70
percent inhibited, while with EPN central and peripheral cholinesterase inhibition may run parallel. The importance of this factor cannot be ascertained, since the activity of ChE in the peripheral—other than in the blood—has not been determined in the present investigation.

11. Non-cholinesterasic factors such as direct neuronal toxicity central or peripheral or both. Actually, central toxicity of Malathion may depend on a condition of direct neuronal toxicity and of central ChE inhibition.

In fact, the data obtained with regard to Malathion toxicity might agree with Frawley data (33) indicating that in the case of certain organophosphonates convulsive doses produce relatively little central enzymic inhibition. The alerting effects of EPN and Malathion upon the cat EEG as well as their antagonism of atropine EEG, studied in this laboratory (7) are of interest in this context. The effective dose is 8 mg/Kg and 250 mg/Kg for EPN and Malathion respectively (7); actually alerting sometimes could not be obtained with any dose of Malathion. This is in agreement with the fact that the lethal dose of Malathion did not inhibit brain AcChE above 70 percent level. This may also suggest that of the two possibilities mentioned above of either central non-cholinesterasic factors or of peripheral probably cholinesterasic factors as contributing at Malathion toxicity the latter should be considered particularly.
b. Role of metabolism. Metabolism of EPN and Malathion affects their anticholinesterase activities. This was shown by the difference in potency of their in vitro and in vivo anticholinesterase actions. EPN was a weak inhibitor in vitro (\( I_{50} = 2 \times 10^{-4} \) M) but a potent inhibitor in vivo, while Malathion was fairly potent inhibitor in vitro (\( I_{50} = 0.8 \times 10^{-5} \) M) and rather weak inhibitor in vivo. The effect of metabolism is also indicated by the difference between anticholinesterase potency of the non-incubated and of liver-incubated compounds administered to the "upper animal" intrajugularly or intraventricularly. In this case EPN, 10 mg/Kg, did not affect brain ChE when administered via either route without previous incubation, while it caused significant inhibition when given by either route after liver-incubation; moreover when this dose of EPN (10 mg/Kg) is administered I.V. to the normal animal and thus metabolized (activated) in vivo 90 percent inhibition of brain AcChE results.

Non-incubated Malathion administered intrajugularly to the "upper animal" significantly inhibited brain ChE at the doses of 25 to 50 mg/Kg, and only by half as much after incubation with liver homogenate. The same doses when given I.V. to the normal anesthetized animal inhibited ChE very slightly.

c. Differential affinities. Interestingly enough, Malathion and EPN seemed to exhibit differential affinities to various areas of the brain. Independently of the route of admini-
stration EPN always inhibited the medullary more than the cortical enzyme; Malathion on the other hand showed more affinity to cortical than to medullary enzyme when administered to the normal animal or intr jugularly to the "upper animal". However, when Malathion was administered intraventricularly, it inhibited the medullary enzyme more than the cortical one.

Compounds administered I.V. are generally expected to have more access to medulla than to cortex. The explanation for this being as follows. The two choroid plexi in the brain enclose the forth ventricle close to medullary area and the lateral ventricles in the neighborhood of the cortex (Fig. 15). As interstitial fluids and compounds capable of crossing blood-brain barrier pass from the blood to brain ventricles through the choroid plexi and as the fluid in the lateral ventricles is conveyed down to the fourth ventricle, the medulla should have the chance of absorbing compounds both from the choroid plexus in its vicinity and from the fluid descending upon it from the lateral ventricles. On the other hand on intraventricular administration into the lateral ventricles the cortex would be the nearest to the depot of the drug and more likely to be affected by their actions. Obviously these topographical factors did not control the distribution of EPN and Malathion since the medulla was the site of EPN action upon intraventricular administration while cortex was mostly affected when Malathion was
FIGURE 15

SCHEMATIC REPRESENTATION OF VENTRICLES IN THE BRAIN
given I.V. Other factors must be involved.

d. Dual action of EPN. Another interesting observation
was that EPN had two actions upon brain ChE depending on its
dose. In doses as small as 1 to 2 mg/Kg given I.V. to the
normal anesthetized animal or given via the jugular or the
ventricular route to the "upper animal" EPN significantly in-
creased its activity. When EPN was given I.V. in these doses
to normal anesthetized animal the increase in activity of brain
ChE reached a maximum at 40 minutes and was followed by return to
control level or by slight inhibition. Malathion on the other
hand did not increase the activity of brain enzyme at any sub-
inhibitory dose level tried (10 to 100 mg/Kg).

e. Effect on brain pseudo ChE. The action of EPN and
Malathion towards brain pseudo ChE should be also mentioned. It
has been found that their action on pseudo ChE was parallel to
that on AcChE. In the case of EPN both activation and inhibition
occurred in parallel with regard to true and to pseudo brain ChE.
It is to be borne in mind that the amount of pseudo ChE present
in the brain is small and that therefore its inhibition might be
expected not to be of great importance. However Desmedt (22)
claimed that toxicity of organophosphorus compounds is due to
inhibition of pseudo ChE rather than to that of AcChE. This
conclusion can be criticized because most of the compounds used
by Desmedt could only poorly penetrate through blood-brain
f. Role of C.N.S. barrier. Non-incubated EPN, 10 mg/Kg did not produce any effect on brain AcChE when given via the ventricular route to the "upper animal". At 20 mg/Kg, non-incubated EPN produced some inhibition of the enzyme when given intrajugularly to the "upper animal". This indicates that blood-brain barrier did not prevent the non-incubated (not activated) EPN from reaching the brain, and that the inhibition obtained upon I.V. administration was that which could be expected on the basis of quantitative penetration of non-incubated EPN into the brain. It seems then that while metabolism of EPN increased its inhibitory potency since at a dose of level of 10 mg/Kg only the incubated compound inhibited brain ChE regardless of the route used, activation was not required for making it absorbable via the blood-brain barrier. In other words metabolism of EPN increases its capability of attacking the brain enzyme at the cell site.

Non-incubated Malathion inhibited brain ChE whether administered via the jugular vein or into the ventricle of the "upper animal". This shows also that Malathion penetration to the ventricle is not controlled by the blood-brain barrier. The reduction in inhibition was similar when liver-incubated Malathion was administered via either route. This indicates that metabolism does not alter Malathion penetrability through the
blood-brain barrier.

2. **Combined administration:**

a. **Effects due to EPN given 30 minutes prior to Malathion**

While inhibition of brain ChE after EPN-Malathion combination was independently of the doses of the two compounds always much higher than the inhibition which would result from additive effect of both compounds, certain other results of the combined administration to anesthetized dogs depend on the dosage of the two components of the combination. When the doses of EPN and of Malathion were 2 and 10 mg/Kg respectively 85 percent of animals died. Death occurred within 10 minutes and inhibition of brain ChE was greater in the medulla (85 percent) than in the cortex. On the other hand, when EPN and Malathion doses were 1 and 50 mg/Kg respectively death occurred within 40 minutes and cortical enzyme was inhibited more than the medullary enzyme. This indicates that larger the EPN fraction in the combined administration the faster death occurred and the more inhibited was medullary ChE. To the contrary, when EPN dose was lowered and Malathion dose increased death occurred slowly and the cortical enzyme was inhibited more than the medullary one. Thus as either Malathion or EPN content of the combination is increased, inhibition at the site characteristic for the pertinent compound is found to predominate. Thus in the case of 2 and 10 mg/Kg EPN-Malathion combination medullary enzyme was inhibited by 85
percent while in the case of 1 and 50 mg/Kg combination the inhibition was only 50 percent yet death occurred in both series. This again is another evidence indicating that Malathion toxicity is not entirely due to brain ChE inhibition. The data may also suggest that Malathion even when administered 30 minutes after EPN seems to prevent EPN from exerting its full action at the medullary site.

i. Role of metabolism. In the "upper animal" combined intrajugular or intraventricular administration of non-incubated EPN and Malathion produced: after a characteristic delay (60 minutes after the administration of the second compound) potentiation of the brain ChE inhibition. Potentiation of inhibition was particularly pronounced in the cortex. When these compounds were similarly administered after incubation with liver homogenate potentiation of brain ChE inhibition was produced faster (at 30 minutes after the administration of the second compound), to a greater extent, and it occurred in the cortex and in the medulla. Again, the inhibitory effect was potentiative rather than additive.

ii. Role of C.N.S. barrier. When EPN, Malathion or their combinations upon intraventricular administration do not inhibit at certain dose levels unless incubated, this naturally means that the metabolism is necessary for activation and increase of inhibitory action since blood-brain barrier does not
matter in the case of this route; on the other hand, lack of ChE inhibition upon intrajugular administration unless incubated of single compounds or of their combinations may mean that incubation is needed both for activation and penetration across the blood-brain barrier.

The present data obtained from combined administration of EPN and Malathion indicate that metabolism is necessary for faster and for more pronounced inhibitory potentiation. Combination of liver-incubated compounds produced potentiation of brain ChE inhibition in both cortex and medulla, at 30 minutes after the administration of the second compound, while with non-incubated compounds potentiation of the enzyme inhibition arises to a lesser extent and not until 60 minutes after Malathion administration; this latter potentiation occurred only in the cortex. Similar degree of potentiation occurred on intrajugular and intraventricular administration to the "upper animal". This indicates that blood-brain barrier does not affect the penetration of those compounds into the brain.

iii. Relationship to toxicity. Since metabolism (activation) of EPN increased the potentiative and anticholinesterase activity in the C.N.S., the potentiation of toxicity with regard to combined administration of EPN and Malathion could be at least partly related to brain AcChE inhibition. If high enough the latter could cause death directly or bring about secondary factors such as cardiovascular disturbance which in
turn may contribute to toxicity. However peripheral ChE inhibition at sites such as N.M.J. are very likely to contribute to the potentiation of toxicity arising on combined administration, since such factors seem to contribute to the toxicity of Malathion when given singly.

The biochemical data obtained in the present study agree with the pharmacological data: In this laboratory it has been shown that good antagonism of atropine EEG and marked alerting as well as convulsive EEG pattern resulted from combined administration of EPN, 2 to 4 mg/Kg and Malathion, 8 to 12 mg/Kg (unpublished). In this case the neuropharmacological data could be correlated with the inhibition of brain ChE. On the other hand, pharmacologically the potentiation was not confined to the C.N.S. and could be observed also at peripheral neuroeffectors such as neuromyal junction and at the ganglia (Karczmar, Awad and Blachut, unpublished). Also as mentioned earlier the present data showed that in the case of combinations with a relatively high content of Malathion the extent of central ChE inhibition at toxic doses is rather low. The above results suggest that both peripheral and central sites may be involved in biochemical and pharmacological potentiation and also in the potentiation of toxicity. Also, direct neuronal toxicity cannot be excluded.

b. Effects arising from varying the sequence of EPN and
Malathion administration. The potentiation of brain ChE inhibition described above was obtained when EPN was administered prior to Malathion. The question then arises whether or not potentiation of ChE inhibition would also occur if the sequence of administration of Malathion and EPN were reversed. As will be seen this has a bearing on the mechanism involved in potentiation.

Studies were carried out with Malathion, 10 mg/Kg administered prior to or simultaneously with EPN, 2 mg/Kg. While ChE inhibition occurred there was no death nor potentiation of inhibition, although distinct cholinergic symptoms and toxicity arose. The medullary ChE inhibition arising from the above combination seemed to be less than that produced by similar doses of EPN given singly, suggesting that Malathion prevents full anticholinesterase effect of EPN from being exerted. Similar interpretation can be applied to the fact that when Malathion was given 30 minutes after EPN medullary ChE reached 85 percent level with 10 mg/Kg dose of Malathion but was only 50 percent when Malathion dose in this combination was increased to 50 mg/Kg.

It should be remembered that the dose of Malathion of 10 mg/Kg did not produce per se inhibition of brain enzyme even when administered into the "upper animal" where it escapes inactivation by the liver. Thus it cannot be concluded on the
basis of this experiment that potentiation of inhibition cannot arise from this particular administration sequence; that this actually can be the case, appears from the scrutiny of the following experiments.

When Malathion, 50 mg/Kg, was given prior to EPN, 10 mg/Kg, death occurred within half hour and brain ChE inhibition reached 80 and 100 percent levels in the medulla and cortex respectively. Actually only 80 percent inhibition of the cortical enzyme could be expected on the basis of additive effects of Malathion and of EPN. Similarly when Malathion, 60 mg/Kg, was given 50 to 120 minutes prior to EPN, 3 mg/Kg, death occurred within 60 minutes and again some potentiation of ChE inhibition occurred amounting to an average of 23 to 30 percent increase in inhibition above the levels expected on the basis of additive effects. Those results are in agreement with data obtained in this laboratory indicating that potentiation of toxicity and of neuroeffector action develops on administering Malathion prior to EPN in non-anesthetized dogs. In the case of the N.M.J. potentiation of the muscle response to indirect stimulation developed in anesthetized cats when subeffective doses of Malathion were given 50 minutes prior to subeffective doses of EPN (Karczmar, Awad and Blachut, unpublished).

There was a relatively good correlation between toxicity and brain ChE inhibition when subeffective doses of EPN
was administered prior to subeffective doses of Malathion. On reversing this sequence of administration potentiation of the enzyme was not as high in the medulla and did not exceed 70 percent in either cortex or medulla, at the time of occurrence of death. In this case other non-cholinesterasic factors and/or peripheral cholinesterasic factors could have contributed to the toxicity. Thus, when EPN is administered prior to Malathion toxicity appears to be mainly due to potentiation of central enzymic inhibition, although particularly in the case of high content of Malathion in the combination peripheral factors may have contributed. When on the other hand Malathion was administered first, toxicity may be due to central potentiative effect and also to potentiation at peripheral sites. This can be supported by the fact that in combination there was always complete inhibition of blood ChE (a peripheral site) while this was not the case with brain ChE. We may assume in this context that Malathion concentrates and contributes to potentiation preferentially at peripheral sites. Certain pharmacological data support this suggestion (Karczmar, Awad and Blachut, unpublished). However, as indicated above, direct neuronal toxicity of combination may also exist.

IV. Mechanism of potentiation:

1. Discription of Cook's postulate (Malathionase system and its inhibition by EPN):
In 1957 Frawley showed that combined administration of EPN and Malathion produced potentiation of ChE inhibition of RBC as well as potentiation of toxicity in rats. Accordingly, Cook (19) hypothesized that the potentiation occurs due to the inhibition by one compound of detoxification of the other. He showed that in vitro Malathion is metabolized and inactivated very rapidly by liver homogenate. Cook et al. (19) showed also that detoxification was due to hydrolysis of one of the succinate ester groups of Malathion. They assumed therefore that the enzyme is an esterase and called it "Malathionase". None of many other organophosphorus compounds such as EPN, Chlorthion, Guthion, Parathion etc. was markedly altered by the liver. Cook et al. (18, 19) showed also that phosphonate compounds such as EPN have significant inhibitory effect on the degradation of Malathion when added to the liver homogenate either simultaneously with or prior to the addition of Malathion. This was confirmed by Murphy and Dubois (65) who showed that 50 percent of rat liver detoxification of the enzyme "Malathionase", occurred with a concentration of $2.8 \times 10^{-5}$ M of EPN in vitro. When 1.5 mg/Kg was administered to rats 1 hour prior to Malathion, this dose of EPN brought about a reduction in the LD$_{50}$ of Malathion from 1100 to 550 mg/Kg. (1)

2. **Cook's hypothesis and the present results:**

The results of the present study partially support and
(1) There is no evidence to prove that "Malathionase", the liver enzyme described by Cook is not a cholinesterase. Cook et al. argued as follows in favor of specific "Malathionase":

a. The very rapid alteration in vitro of Malathion by the liver differs from that shown for organophosphate pesticides generally. When cholinesterase and some aliesterases hydrolyze these phosphate esters, the enzymes are in turn inactivated or inhibited so that one mole of enzyme hydrolyzes only one mole of phosphate before it becomes inactive.

b. On the other hand, in the case of enzymes such as DFPase and Tabunase, one mole of enzyme hydrolyzes a large number of moles of the susceptible phosphate without the enzyme being inhibited during the process.

Cook stated that "Malathionase" action does not appear to resemble the activity of either of these two enzymes. It is however not clear how does Cook know that "Malathionase" is not destroyed while hydrolysing Malathion and thus differs from the aliesterases; in fact, there was no quantitative study showing how many moles of Malathion are hydrolyzed by one mole of the enzyme. Neither is it proven that "Malathionase" behaves unlike DFPase.

c. The enzyme attacks Malathion at the diethyl succinate substituent rather than at a P=S or P=O linkage as is done by cholinesterase, DFPase, and Tabunase. However it is not known whether or not cholinesterase cannot attack this very linkage. Thus, it may be more in keeping with available data to state at present that the liver enzyme capable of destroying Malathion may be a cholinesterase. In fact Cook suggested that those phosphonate compounds which inhibited "Malathionase" become more potent upon oxidation which however increased also their anticholinesterase actions. Similar effect of oxidation on these two anti-enzymic activities of the organophosphorus compounds suggests that we deal here actually with one activity only, namely that of a cholinesterase. It is further possible that Malathion reacts with and is hydrolyzed by, this enzyme thus producing a reversible type of interaction, while EPN as well as other organophosphorus compounds react with but are not hydrolyzed by, this enzyme thus producing an irreversible type of interaction.
partially contradict Cook's hypothesis.

a. **Facts supporting Cook's hypothesis:**

i. Liver metabolism and Malathion. The present study has shown that Malathion in vitro is a fairly potent anticholinesterase agent. When incubated with liver homogenate its activity decreases. A dose of Malathion which does not inhibit brain ChE when given I.V. produces about 14 percent inhibition when given intrajugularly to the "upper animal". Incubated Malathion produces less inhibition in the "upper animal" than the non-incubated compound. Thus, also in vivo Malathion is metabolized, probably in the liver, to a less active compound. This agrees with Cook's data indicating that a liver esterase detoxifies Malathion.

ii. Effects of non-incubated compounds in vitro. Combined treatment of brain homogenate in vitro by non-incubated EPN and Malathion caused no potentiation but additive ChE inhibition. This again agrees with Cook's postulate since it indicates that in order for potentiation to occur EPN has to protect Malathion from destruction.

iii. Effects of non-incubated compounds in vivo. Combined administration to the "upper animal" of non-incubated EPN and Malathion given intrajugularly and intraventricularly did not produce potentiation of brain ChE inhibition at 30 minutes after the administration of the second compound (Malathion) which
is the time at which combination of the incubated compounds produced potentiation. This is an in vivo confirmation of Cook's suggestion that metabolism is necessary in order for potentiation to develop.

iv. Effects of changing the sequence of administration
When EPN was administered I.V. prior to Malathion in normal anesthetized animal potentiation of inhibition of brain ChE was produced by ineffective doses of both compounds. The reversal of the sequence of administration lessened the action. This agrees with Cook's postulate that EPN by being administered prior to Malathion can protect the latter from detoxification by the liver enzyme, thus causing potentiation of Malathion action.

b. Facts which do not support Cook's hypothesis:

i. Potentiation by incubated compounds. In vitro combination of liver-incubated EPN and Malathion produced potentiation of the inhibition of brain ChE. Also in vivo, administration to the "upper animal" of the liver-incubated combination of EPN and Malathion produced potentiation of brain ChE inhibition. It might appear at first glance that this favors Cook's statement since potentiation occurred only in the presence of liver and since EPN could prevent in these conditions destruction of Malathion. However this is not the necessary case because:

A. The concentration of EPN added was selected
so (cf. also p. 33) that EPN inhibited liver enzyme only and was not available to protect any further detoxification of Malathion.

B. In the case of non-incubated EPN and Malathion administered intrajugularly to the "upper animal" potentiation of inhibition of brain ChE did not occur at 30 minutes after administration of the second compound (Malathion) as already stated. However, at 60 minutes potentiation did appear, particularly in the cortex. Apparently then potentiation can occur in conditions in which Malathion cannot be metabolized; thus, the protection by EPN is not the only factor involved.

C. Finally incubated EPN given intraventricularly potentiates ChE inhibition by non-incubated Malathion. In this case again conditions for Malathion destruction are not present.

II. Further analysis of the effect of changing the sequence of administration. When Malathion is given 40 or even 120 minutes prior to EPN potentiation of brain ChE inhibition and toxicity did arise in the case of minimal effective doses of these two compounds. Thus EPN seems to potentiate Malathion even when given after it, thus not being in position to protect Malathion from destruction. It should be stated here that Cook's postulate becomes a working hypothesis only if we analogize Malathion to A11; in other words if we assume that Malathion is...
just about as rapidly metabolized as Ach. In this case it could be easily visualized how simultaneous administration of EPN and Malathion or particularly pretreatment with EPN may lead to potentiation of the effects of Malathion. To the contrary even if 60 minutes after administration of Malathion a certain amount of it remains intact and is protected by EPN from further destruction it is hard to visualize how this protection should render Malathion effective at that time.

iii. Finally two more facts not reported in this work but available from other investigations seem to contradict Cook's hypothesis:

A. Rosenberg has shown that Chlorthion and Malathion are both hydrolyzed by the liver and that Chlorthion is potentiated by EPN and also potentiates Malathion. On the other hand Cook (18) has reported that Chlorthion is not hydrolyzed by the liver and that both EPN and Chlorthion inhibit Malathion detoxification by the liver. If this is true then Chlorthion potentiation by EPN cannot be explained according to Cook's hypothesis. On the other hand, if we accept that Chlorthion is hydrolyzed by the liver it is difficult to visualize how it can potentiate Malathion by the mechanism suggested by Cook.

B. Potentiation cannot always depend on the interference of one compound with the detoxification of the other since similar potentiation was reported in the case of combination
of neostigmine and oxamide bis-quaternaries (55). Those compounds are not hydrolyzed by the liver.

3. New postulate:

Since several facts seem to argue against Cook's hypothesis and since particularly the question raised in b-ii (p. 111) seems to exclude EPN protection of Malathion as a major mechanism for potentiation, another postulate is proposed.

a. The basis for the new hypothesis:

Some in vitro as well as in vivo results indicate that Malathion when given after, prior to or simultaneously with EPN can prevent full effect of the latter to be exerted. This could be due to Malathion reacting faster with the enzyme than EPN. It may be that once Malathion combines with the enzyme it blocks EPN from the site of interaction with the enzyme. If the enzyme has only one cationic site (which can be phosphorylated) Malathion could compete for it with EPN. This does not seem to be the case since less than additive inhibition occurs even when Malathion produces only partial inhibition thus seemingly permitting EPN to exert additional inhibition. A similar difficulty in explaining interference by one ChE inhibitor with the action of another was recorded in the past. Koppanyi and Karczmar (54) have shown that small doses of physostigmine presumably capable of limited inhibition of ChE prevented pharmacological actions of DFP. While it can be readily understood (Koelle, 51) how
relatively large doses of physostigmine can protect ChE from DFP by reversibly binding the enzyme, it cannot be easily seen why ChE inhibited say by 10 percent only, should not be fully inhibited by suitable doses of DFP.

On the other hand, both potentiation and mutual competition could be expected if phosphonate and related compounds could act upon the enzyme in two different ways. Actually EPN and Malathion do not react identically with ChE though they both seem to be irreversible inhibitors. It is suggested accordingly that ChE has more than one cationic site of action with regard to EPN and Malathion. This postulate could explain also blockade in certain conditions of EPN by Malathion, since one site could be attacked by Malathion while the other could be attacked by EPN and blocked by Malathion. This postulate of multiple cationic sites on ChE molecule and of differential activities and affinities of various anticholinesterases at these sites may obviate the difficulty in explaining both potentiation and interference phenomena occurring with combinations of anticholinesterase agents. The postulate is strengthened by the fact that anticholinesterase actions of EPN and Malathion differ in several respects, since this again can be explained if ChE molecule had more than one cationic site:

1. Malathion cannot produce 100 percent inhibition of brain ChE while EPN can.
ii. In vivo inhibition by EPN is greater at medullary than at cortical levels. The opposite is true in the case of Malathion.

iii. EPN inhibits RBC AcChE more than it inhibits plasma ChE; again the opposite is true in the case of Malathion.

iv. Brain ChE inhibition by EPN correlates with the toxicity produced while Malathion seems to exhibit either peripheral or non-cholinesterasic components of toxicity.

v. An important difference is that EPN was shown to have a dual effect and can depending on the dose, activate or inactivate brain ChE.

b. Speculative explanation of potentiation on the basis of the new postulate:

i. Two types of EPN-ChE interactions.

EPN is metabolized presumably in the liver in two steps, first to a compound which increases the activity of brain ChE. This can occur either with regard to the number of free or active enzyme or with regard to the number of active sites on the enzymic molecule. The former may take place by competing with endogenous anticholinesterases (49); the latter may take place by exposing active sites on the enzyme surface which are normally hidden.

Second step of EPN metabolism brings about another compound which has anticholinesterase activity.
ii. Potentiation would then result when Malathion having access to more active sites of the enzyme after the effect of EPN took place, would inhibit ChE more efficiently. Given alone, it would have difficulty getting to all sites, which is evidenced by the fact that Malathion is unable to produce 100 percent inhibition of brain ChE. In fact, the actual inhibition by EPN given singly may be greater than the apparent one since the brain sample from an EPN-treated animals is compared to a control sample the ChE of which is not activated by EPN.

iii. Occurrence of toxicity. It is assumed that the bound enzyme is in equilibrium with the functional enzyme and acts as storage from which the latter can be replenished. In combination, EPN and Malathion will free and expose the bound enzyme and inhibit it when it is in the unbound form; thus, nothing of the stored enzyme would be left available to replace the functional enzyme when it gets inactivated. When both the stored and the functional enzymes are inhibited, Ach would accumulate and produces death, presumably via central effects such as convulsion and respiratory failure.

iv. Occurrence of toxicity after the change of sequence of administration. When Malathion is given prior to EPN, some of it gets attached to the brain enzyme and at the periphery. This fraction of Malathion is unable to inhibit until EPN activates the enzyme. At that time potentiation of
inhibition of ChE would be a result of more efficient effect of EPN and Malathion. In this particular sequence of administration toxicity occurs rather slowly; while Malathion can exert its action immediately, EPN metabolism and activation takes time, and the enzymic and pharmacologic effect is thus delayed. On the other hand, when the administration sequence is reversed, Malathion is administered at the time of activation of ChE by EPN; in this case toxicity occurs faster than in the former. All this does not necessarily imply that protection of Malathion by EPN from the liver detoxifying enzyme is not one of the mechanism contributing to potentiation; in fact it is felt that the mechanism postulated by Cook as well as that suggested at present may be both operative. Finally, peripheral cholinesterasic as well as peripheral and central non-cholinesterasic factors cannot be excluded at present.
CHAPTER V

SUMMARY

I. Purpose of this study:

In 1957 Frawley et al. have shown that combined administration of organophosphorus compounds, EPN and Malathion, produced potentiation of toxicity and of inhibition of RBC ChE in rats. Cook et al. (19) postulated that potentiation is due to inhibition of detoxification of one compound by the other. They demonstrated that in vitro Malathion is destroyed by the liver and that EPN protects Malathion from this destruction. This was confirmed by data of Murphy and Dubois (65) suggesting that EPN inhibits irreversibly the liver "Malathionase".

Since death could not be attributed to inhibition of ChE of RBCs and since no studies were carried out with regard to the effect of organophosphorus combinations upon the brain enzyme, detailed biochemical investigations of brain ChE and of its interaction with EPN and Malathion and with their combinations were undertaken. Suitable data were also collected for reexamination of Cook's postulate.

II. Procedures:

1. Anesthetized dogs as well as "upper animal" preparations were used in the present study. In the case of the latter the
agents could be administered so that they would not circulate in
the liver or any other visceral organs. Both liver-incubated
and non-incubated compounds were administered to the "upper
animal" so that the effect of metabolism on the compounds in
question could be studied in vivo in absence of the animal's own
"Malathionase".

2. Intraventricular administration of incubated and of non-
incubated compounds was used to study the role of their meta-
bolism as well as of the blood-brain barrier in action of the
compounds and of their combinations.

3. A systematic study of EPN and of Malathion actions on
brain ChE was carried out in vitro as well as in vivo. In vivo,
different parts of brain tissue were analyzed for their ChE
activity.

4. Warburg manometric technique was used exclusively for
measurement of AcChE and BuChE activities.

5. Interaction of EPN and of Malathion with ChE was studied
using dialysis and dilution techniques. The compounds inhibit
the enzyme irreversibly; also, no free compounds could be de-
monstrated in tissue samples from animals treated in vivo.
Thus, no free compound could be released during homogenization
and additionally inhibit the enzyme.

6. Irreversibility of inhibition and absence of free
compounds after their administration indicate reliability of
of measurements of ChE inhibition.

III. Results:

1. EPN was found to be a weak ChE inhibitor in vitro, but rather potent inhibitor in vivo. It has to be metabolized by the liver to become an effective anticholinesterase.

2. In vivo, EPN inhibited medullary enzyme more than cortical one.

3. At toxic dose of EPN inhibited nearly 100 percent of brain ChE activity.

4. Malathion was found to be fairly active anticholinesterase in vitro; in vivo it was weak inhibitor due to the fact that it is destroyed by liver enzyme.

5. Malathion inhibited cortical more than medullary enzyme. At toxic dose it inhibited maximally only 70 percent of brain ChE; this suggests peripheral or non-cholinesterasic components of toxicity.

6. EPN was found to have a dual effect; in small doses it increased brain ChE activity; large doses caused inhibition; on the other hand Malathion did not increase the enzyme activity at any dose studied.

7. EPN and Malathion inhibited in vivo brain pseudo ChE similarly to the true ChE.

8. Potentiation of enzymic inhibition in vitro occurred only from the combination of liver-incubated compounds.
9. Potentiation of brain ChE inhibition in vivo occurred when EPN was given prior to Malathion. When Malathion was given prior to EPN, potentiation occurred in the case of certain doses but it was less extensive than in the case of the former sequence.

10. With both sequences of administration death occurred. The extent of ChE inhibition of different parts of the brain and the speed with which toxicity occurred depended on the ratio of the two compounds in the combination.

11. In the "upper animal" rapid and extensive potentiation of brain ChE inhibition occurred from combination of EPN and Malathion when both compounds or EPN alone were incubated with liver homogenate. Some potentiation occurred however even in the case of non-incubated compounds after a prolonged delay.

12. In the case of intraventricular administration potentiation occurred upon administration of liver-incubated compounds.

13. Analysis and comparison of data obtained upon administration of EPN and Malathion and their combinations intrajugularly and intraventricularly in the "upper animal" or I.V. in the whole animal suggest that liver incubation of EPN activates the compound but is not necessary to enable it to cross the blood-brain barrier; liver incubation of Malathion inactivates it but again does not affect its capacity to penetrate the blood-brain barrier.

IV. Analysis of Cook's postulate:
Some of the results obtained support Cook's hypothesis; some other do not.

1. Potentiation of toxicity and brain ChE inhibition occurs not only by administering EPN prior to Malathion but also when this sequence is reversed. According to Cook's postulate when EPN is administered after Malathion little or no Malathion is left in the case of Malathion-EPN sequence by the time of EPN administration and the protection of Malathion residue by EPN could not result in potentiation.

2. Potentiation occurs when incubated EPN is administered with Malathion intraventricularly or intrajugularly in the "upper animal". It is shown that EPN in these conditions does not protect Malathion from hydrolysis in its incubation medium.

3. Other data as well as certain results of other investigators also do not agree with Cook's postulate.

4. A new postulate is based on the suggestion that provided EPN and Malathion do not react identically upon ChE, the potentiation may result from their interaction with the enzyme and does not have to depend on the protection afforded one compound from destruction by the other. Some of the differences between effects of EPN and Malathion are as follows:
   a. EPN but not Malathion has a dual action upon ChE.
   b. EPN inhibits preferentially RBC and Malathion plasma ChE
c. EPN inhibits more medullary than cortical ChE; the opposite is true for Malathion.

d. EPN but not Malathion can produce 100 percent inhibition of brain ChE at death.

5. The new postulate suggests that:

a. EPN is metabolized in two steps. Metabolite 1 activates ChE; Metabolite 2 inhibits it.

b. Activated ChE is more liable to inhibition than control ChE.

c. Malathion attaches itself to ChE but cannot inhibit it efficiently until the activating action of EPN takes place, hence potentiation.

d. Certain other aspects of the new postulate and their relation to data available in literature are analyzed further in the discussion.
BIBLIOGRAPHY

   I. Stereochemistry of asymmetric phosphorus compounds.
   II. Stereospecificity in the irreversibility of inactivation
   of cholinesterases by the enantiomorphs of organophosphorus

2) Ammon, V. R. und Pr. Konigsberg. Die Hemmungskorper der

3) Ammon, R. Manometric Methods for hydrolysis of Ach with

4) Augustinsson, K. B. Studies on blood cholinesterase.

5) Augustinsson, K. B. Cholinesterases. A study on compara-


7) Awad, O., S. Ridlon and A. G. Karczmar. Central anti-
   cholinesterase and EEG actions of EPN and Malathion.

8) Awad, O., and A. G. Karczmar. Anticholinesterase actions
   of thiophosphonate compounds, EPN and Malathion and their

9) Banister, J., The occurrence of homologues of Ach in ox

10) Bradley, P. B., C. Elkes and J. Elkes. Some effects of DFP
    on the electrical activity of the brain of the cat.

11) Bradley, P. B., The central action of certain drugs in
    relation to the reticular formation of the brain.
    Henry Ford Hospital International Symposium on Reticular

normal mammalian muscle to Ach and to eserine. J. Physiol. 87: 394, 1936.


APPROVAL SHEET

The dissertation submitted by Olfat Awad has been read and approved by five members of the faculty of the graduate school.

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 thesis 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.

December 19, 1960

Signature of Adviser