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CHAPTER I

INTRODUCTION

The physiological role of acetylcholine as a chemical transmitter of the nerve impulse in the peripheral nervous system has been known for many years. However, it has only been a few years that acetylcholine has been generally considered to serve the same function in the central nervous system. Since many vital functions of the body are regulated by the nervous system, and since cholinesterase has an important role in the metabolism of acetylcholine, elevated or lowered cholinesterase activity may have a profound influence in producing an alteration in these functions.

Enzymology has contributed a great deal to our present understanding of intra- and extracellular processes. This was made possible primarily by correlating enzymatic activity with the function of its substrate. The effect of drugs on enzyme systems has also emerged as a worthwhile field of pharmacological investigation. Some examples are those systems including the enzymes catalase, hexokinase, carbonic

anhydrase, succinic dehydrogenase and cholinesterase.

The inhibition of enzyme systems is known to occur and decreased cholinesterase activity has been associated with some toxicological and pathological processes. The toxic symptoms resulting from absorption of the organic phosphorus insecticides has been related to cholinesterase inhibition in the central as well as in the peripheral nervous system. Most of these toxic symptoms may be explained on the basis of an accumulation of acetylcholine resulting from a decrease in cholinesterase activity. Decreased cholinesterase activity has also been indicted in disorders of the nervous system such as epilepsy and catatonic stupor. Increased cholinesterase activity has been reported in chronic psychotic patients and in mentally ill patients showing autonomic overactivity.

Since biopsies of brain cannot ordinarily be taken it would be desirable to know if a correlation exists between the cholinesterase activity of brain, blood, and cerebrospinal fluid. Koelle and Gilman (25), Freedman, Willis and Himwich (20), Hagan and Frawley (21), and Frawley et al.(18) have conducted investigations to determine if a correlation exists between brain and blood cholinesterase activity and symptoms of toxicity following di-isopropyl fluorophosphate (DFP). Hagan and Frawley concluded that there was no correlation. The other three groups found no correlation

between serum and brain cholinesterase activity and symptomology. Freedman et al. (20) believed there was a slight correlation between the cholinesterase activity of brain and erythrocytes and symptomology following acute toxicity. Frawley et al. (18) believed that following a suspected acute exposure to a cholinesterase inhibitor, measurements of erythrocyte cholinesterase activity would be of little help in determining the degree of exposure and severity of symptoms. Further studies, particularly those of a correlative nature, are needed. Therefore, based on the above information, it was decided to study whether or not there was a correlation between the cholinesterase activity of parts of the brain, the cerebrospinal fluid, the erythrocytes, and blood plasma. This study was conducted in normal animals; animals treated with electroshock; and with drugs, particularly an irreversible cholinesterase inhibitor, di-isopropyl fluorophosphate (DFP).

CHAPTER II

HISTORY

Elliott (15), in 1905, introduced the revolutionary idea of the chemical transmission of the nerve impulse. He suggested that adrenaline might be the chemical mediator of the nerve impulse from sympathetic nerve endings to their effector cells. Subsequently, Loewi (29), in 1921, published experimental data showing the release of a substance which he called "Vagusstoff" into the perfusion fluid in a frog heart when the vagus nerve was stimulated. When the perfusate from this heart was perfused into the heart of another frog, responses occurred which were similar to vagal stimulation. Hunt and Taveau (24) had previously demonstrated the pharmacological action of acetylcholine. Loewi and Navatril (30) extended the work of Loewi (27) and it led them to assume that "Vagusstoff" is acetylcholine.

These early ideas and investigations comprise the actual beginning and basis of the concept of neurohumoral transmission of the nerve impulse. The hypothesis was that a chemical substance was liberated from nerve endings which served as a mediator for the initiation of the nerve impulse.

Dale and Dudley (11) isolated acetylcholine from the spleen and showed it to be a natural constituent of the animal body. Dale and Feldberg (12) showed that acetylcholine was liberated into the blood stream following stimulation of the gastric vagus of the eserinized dog.

It is now known that the liberation of acetylcholine is not confined to vagal nerve endings but is also concerned with nerve impulse transmission at all ganglionic synapses. Acetylcholine is also the mediator for nerve impulses to skeletal muscles, sweat glands, chromaffin cells of the adrenal medulla, piloerector muscles, and at some central nervous system synapses.

The possibility of a chemical mediator at the central synapse was considered by Dale (9) as early as 1934. In his Harvey Lectures (8) in 1936, he discussed the fact that Eccles considered the sympathetic ganglion to be an accessible model of a synapse in the gray matter of the central nervous system. He later remarked that Sherrington had told him in those early years that he considered the myoneural junction to afford a model of the process occurring at the central synapse (10). Feldberg (16), in his review in 1945, gave a rather complete picture of the mode of action of acetylcholine in the central nervous system. At that time he stated, "The present position of the theory of acetylcholine as central transmitter is all but settled". Almost ten years

later (17), when participating in the Symposium on Neurohumoral Transmission, he considered the central synaptic and peripheral synaptic transmission processes to be fundamentally identical.

When it was demonstrated that acetylcholine played an essential physiological role in the nervous system, it is not surprising that many investigators soon turned their thoughts to a study of it s metabolism. In 1914, Dale (7) suggested the existence of an enzyme in blood which terminated the pharmacological response to injected acetylcholine. He believed this to be broght about by hydrolysis of the compound. It was in 1930 that Matthes (32) showed the enzymatic nature of this hydrolysis. In the same year, Plattner and Hinter (47) found the enzyme to be present in nearly all animal tissues. Using horse serum, Stedman et al. (60) found that butyrylcholine was hydrolyzed at a higher rate than acetylcholine, and that following partial purification, non-choline esters were not hydrolyzed. They believed the enzyme to be specific for choline esters and called it cholinesterase. Subsequent investigations did not support the contention that this enzyme was specific for choline esters. In fact, Vahlquist (64) showed that human serum esterase was not specific for choline esters, although they were hydrolyzed at a higher rate than non-choline esters. About this time, Stedman and Stedman (59) pointed out that blood cholinesterase of various species did not seem to be

the same. They behaved quite differently with respect to rates of hydrolysis of different substrates. Then in 1940, Alles and Hawes (1) accomplished the first major step toward differentiating blood cholinesterase into its two components, serum and erythrocyte cholinesterase. They showed that erythrocyte cholinesterase is inhibited by an excess of substrate. This was in sharp contrast to serum cholinesterase which has no optimum substrate concentration. Further, they showed that only erythrocyte cholinesterase hydrolyzed acetyl-beta-methylcholine. Mendel and Rudney (34) extended this work and again utilizing substrate specificity as a means of differentiating the two enzymes, named the erythrocyte and serum enzymes, true- and pseudo-cholinesterase respectively. They also showed that the cholinesterase of conducting tissue is predominately of the true-cholinesterase type. Nachmansohn and Rothenberg (40,41) used three esters of choline differing only in the length of the acyl chain. They found that the rate of hydrolysis of these esters by the enzyme of conducting tissue and erythrocytes increased as the acyl chain was decreased from butyryl to acetylcholine. The reverse was true for the serum esterase, i.e., the rate of hydrolysis being greatest for butyrylcholine. They suggested the name acetylcholinesterase for the esterase of erythrocytes and conducting tissue and cholinesterase for the serum esterase. Augustinsson (2,3) showed that at high

acetylcholine concentrations, where the erythrocyte and nervous tissue cholinesterase activity was inhibited, triacetin was hydrolyzed, in some cases at a higher rate than acetylcholine.

It can be seen from the standpoint of chemical specificity that there is no sharp distinction between the true or specific and the pseudo or nonspecific types. Neither is there a clear cut distribution of these two enzymes as might be expected at first glance. Ord and Thompson (45) have determined the distribution of these two types in various tissues using differential substrate specificity methods.

Since acetylcholine is concerned with vital functions of the nervous system, inhibitors of cholinesterase are of great concern. The physiological function of this enzyme-substrate system has been better ascertained through the use of these agents. One of the most familiar inhibitors is the alkaloid physostigmine, which forms a reversible complex with the enzyme. During World War II a second group of powerful cholinesterase inhibitors were synthesized which produced an irreversible inhibitor-enzyme complex (31,33). They were obtained from dialkylphosphoric acid esters. Since they are potential warfare agents, many investigations have been conducted to study their mode of action.

Koelle and Gilman (27) pointed out that although many

drugs inhibit cholinesterase, this may be of minor importance in their pharmacological activity. Furthermore, specific inhibitors may also act directly upon the effector cells. However, most studies substantiate the fact that the major pharmacological effects of DFP are those resulting from cholinesterase inhibition. The results of Mazur and Bodansky (33); Koelle and Gilman (25); and Freedman, Willis and Himwich (20) indicated that the symptomology following DFP is closely related to a low brain cholinesterase activity. Koelle and Gilman (25) studied the regeneration rates of blood and brain cholinesterase activity following DFP. They believed it was futile to attempt to relate symptomology to serum cholinesterase inhibition. Frawley et al. (18) have presented the best evidence to date that some of the symptoms of toxicity resulting from DFP poisoning may be due to factors aside from cholinesterase inhibition.

Much of the work related to correlation between brain and blood cholinesterase activity following DFP has been done using rats. Groups of about six animals were used to determine the mean enzymatic activity. To this writer's knowledge, no attempt has heretofore been made to establish a correlation between brain and blood cholinesterase activity in individual animals, particularly the dog.

In 1937, McGeorge (35) examined a group of hospitalized, depressive patients and could find no relationship between

the mental state of the patient and serum cholinesterase activity. In the same year, Tod and Jones (62) determined serum cholinesterase activity in patients who exhibited anxiety and depressive states. They found elevated serum cholinesterase activity in cases showing autonomic over-activity. In the more acute cases, enzymatic activity was even higher. In another publication, Tod and Jones (63) found a significant difference between patients exhibiting anxiety states, catatonic stupor and epilepsy. Those with anxiety showed higher than normal serum cholinesterase activity while those with catatonic stupor and epilepsy showed lower than normal activity. Platania and Pappalardo (46) made a study of 100 mentally ill patients. Those showing schizophrenia with intense psychomotor activity seemed to have higher values of serum cholinesterase than the quiet ones. In the epileptic group, the value increased with the number of convulsions. They believed the nervous system regulated the amount of cholinesterase in blood serum. Early et al. (14) found an elevated serum cholin-esterase activity in over 50 per cent of their chronic psychotic patients. On the other hand, in some patients they reported no serum cholinesterase activity at all. They believed this to be due to lack of production of cholines-terase. Ravin and Altschule (51) reported that the mean values of serum cholinesterase activity for a group of

mental patients were slightly higher than the mean of a control group. Following electroshock no significant change occurred although some patients improved while others did not. They reported a transient rise in serum cholinesterase activity following electroshock but believed this was probable due to hemoconcentration which has been shown to occur. DeCaro and Altea (13) could find no significant change in cholinesterase activity in the blood of 53 patients and in the cerebral tissue and blood of rabbits following electroshock. Lagnara and Preziosa (28) reported that following electroshock in 30 patients and after convulsions in 10 epileptics, serum cholinesterase activity was greatly increased associated with an increase in acetylcholine. Takagi (61) reported that electroshock had no effect on cholinesterase. Rowntree et al. (54) found the erythrocyte cholinesterase activity to be in the normal range in schizophrenia and in manic depressive psychosis. However, they found that schizophrenics had more tolerance to DFP. This they called autonomic hyporeactivity. Further depression of the manic depressive state was produced by DFP. The administration of DFP to the schizophrenic produced activation of the psychosis. Blood cholinesterase activities were the same in each group despite the different clinical effects produced by DFP. They also found those in the manic depressive state exhibited a rise in blood

pressure when given DFP. In 1952, Sherwood injected purified human erythrocyte cholinesterase into the lateral ventricles of a patient in deep catatonic stupor (55). Marked improvement resulted but following cessation of therapy the patient returned to the former catatonic state. Later, in another publication, Sherwood et al. (56) also demonstrated that anticholinesterases aggravated the signs and symptoms of schizophrenia. An interesting experiment was carried out by this group whereby they produced a syndrome in cats which they believed to be indistinguishable from that of human catatonia. The cats were made catatonic by producing lesions in the brain with a stereotaxic instrument. When cholinesterase was injected intraventricularly, recovery occurred within 2 days. When acetylcholine was injected via the same route, symptoms were again aggravated. Randall (49) administered large daily doses of insulin to schizophrenics and found an elevated serum cholinesterase activity in the 4th week of treatment. Randall and Jellinek (50) found an elevated serum cholinesterase activity in acute schizophrenics one week following insulin shock whether or not improvement occurred.

CHAPTER III

METHODS

Animals

Mongrel dogs ranging in weight from 9 to 17 Kg. were used. The animals were killed with air emboli. The air, which was injected into the cephalic vein through a hypodermic needle, was supplied by a small pump. Rubber tubing was used to connect the hypodermic needle to the pump. A T-tube was inserted in the tubing between the pump and the needle to provide a by-pass for the air to escape until the needle was inserted. When the needle was in the vein, the air was forced to enter by closing the side arm of the T. Following cessation of respiration, the animals were bled by severing the juglar vein.

Tissues

(1) Brain. The brain was exposed in the following manner: An incision was made through the skin just below a point midway between the eyes which was extended just posterior to the occipital protuberence. The temporal muscles were cut away and an opening was made through the skull by means of a trephine. Bone forceps were used to

remove the calavarium and expose the brain. The dura was reflected, the midbrain sectioned transversely, and the supratentorial portion of the brain removed. The brain specimen was then sectioned in half by cutting through the corpus callosum, third ventricle and hypothalamus in the midsagittal plane. The caudate nucleus is made accessible by removing the septum pellucidum. The right and left caudate nuclei were removed. When cerebral cortex was used, the arachnoid-pia layer was removed from the sulci and gyri approximating Brodmann Areas 4 and 6. The gray matter was freed as carefully as possible from the white matter. The tissue to be analyzed was weighed and ground in a ground-glass homogenizer. In experiments using metrazol, insulin, and atropine, a 1:8 homogenate of caudate nucleus in distilled water was made and centrfuged at about 800 G. for 15 minutes. The supernatant liquid was used to make a 1:10 dilution. One ml. of this dilution was used to determine the cholinesterase activity. Later it was found that the greater part of brain cholinesterase activity remains with the precipitate. Therefore, in all subsequent experiments, the whole homogenate was used. A 1:25 dilution was made using 1 ml. of the 1:8 homogenate. One ml. of this dilution was then used to determine the cholinesterase activity. In those experiments using cerebral cortex, a 1:8 homogenate in distilled water was made and 1 ml. of this dilution was used to determine the enzyme activity.

(2) Blood. Heparin was used to prevent clotting and the blood was centrifuged at about 800 G. for 15 minutes. The plasma was withdrawn and the erythrocytes were washed with 0.9% NaCl solution and again centrifuged for 15 minutes. Following a second washing with 0.9% NaCl solution, the erythrocytes were centrifuged for 20 minutes and then suspended in 0.9% NaCl solution as described by Michel (38).

(a) Plasma. The plasma was diluted with distilled water so that each ml. of solution contained 0.007 ml. of plasma. The buffer capacity of one ml. of this solution when mixed with one ml. of buffer (Buffer II under Determination of Cholinesterase Activity) averaged 7 per cent of the total as determined by titration between pH 8 and 7.

(b) Erythrocytes. One ml. of washed and suspended erythrocytes was hemolyzed in 4.8 ml. of 0.01 per cent saponin solution. The buffer capacity of one ml. of the erythrocyte hemolysate when mixed with one ml. of Buffer I averaged 12 per cent of the total as determined by titration between pH 8 and 7.

(3) Cerebrospinal Fluid. Cerebrospinal fluid was withdrawn from the cisterna magna immediately following death. It was diluted with distilled water so that 1 ml. of this solution when mixed with one ml. of Buffer I averaged 12 per cent of the total as determined by titration between pH 8 and 7. However, some experiments were conducted using 1 ml. of undiluted cerebrospinal fluid.

Determination of Cholinesterase Activity

Cholinesterase activity was determined using a modification of the electrometric method of Michel (36). The principle of this method consists in measuring the change in pH which occurs as a result of the hydrolytic action of cholinesterase on acetylcholine. The products of the reaction are choline and acetic acid. The acid formed is measured in a buffered solution and enzyme activity is expressed as Δ pH, the change in pH in one hour. All determinations were made in duplicate at 25-27° C. using a Beckman Model G pH meter. Corrections for non-enzymatic hydrolysis were made using the table of corrections published by Michel (38). The corrections for non-enzymatic hydrolysis given by him for erythrocytes were used for brain cholinesterase activity determinations. This table was determined for human erythrocytes and plasma and probably is not strictly accurate for dog brain and blood. However, this was a study to compare rates of activity and the table is acceptable and should not affect the results appreciably. The table for non-enzymatic hydrolysis of human serum cholinesterase activity was also used for the determinations of dog serum cholinesterase activity.

Michel states that "The rate of pH change will be a satisfactory measure of enzyme activity if an approximately

linear relationship between these two quantities can be established". This was confirmed in the present study by doubling the enzyme concentration which resulted in about a two-fold increase in velocity. The kinetics are those of a first order reaction during the reaction period used.

Cholinesterase activity decreases with a decrease in pH (4). The pH range covered by this method is from 7.9 to 6.0. The buffering capacity of the added enzyme source necessarily affects the total buffering capacity and must be taken into account. This has been minimized by sufficiently diluting the tissues so that their buffering capacity is small compared to that of the buffer.

After mixing of either erythrocyte hemolysate or brain homogenate with the buffer, the mixture does not give a pH of 8.00 as Michel found with human erythrocytes. The pH of the mixture in these experiments was always between 7.5 and 7.9 when using erythrocytes, 7.9 and 8.05 when using brain and 7.89 and 7.95 when using plasma. These values are very near the optimal pH range of 7.50 to 8.00 for true-cholinesterase and 8.00 to 8.50 for pseudo-cholinesterase (4). Since the initial pH was always very near the same value from sample to sample, these differences are of little or no significance.

(1) Buffers.

(a) Buffer I (for erythrocytes, cerebrospinal

fluid, and brain): 0.02M sodium barbital (4.1236 Gm.); 0.004M KH_2PO_4 (0.5446 Gm.); 0.60M KCl (44.730 Gm.). For 1 liter of buffer the reagents are dissolved in 900 ml. of water; 28.0 ml. of o.1N HCl are added while shaking the solution, and the volume is then made to the mark. The pH of Buffer I should be 8.10 at 25° C.

(b) Buffer II. (for plasma): 0.006M sodium barbital (1.2371 Gm.); 0.001M KH_2PO_4 (0.1361 Gm.); 0.30M NaCl (17.535 Gm.). For 1 liter of buffer the reagents are dissolved in about 900 ml. of distilled water and 11.6 ml. of 0.1N HCl are added before diluting to volume. The pH of Buffer II should be 8.00 at 25° C.

(2) Substrate. Ampules of acetylcholine chloride (Merck and Co., Inc.) containing 100 mg. were used. A small hole was drilled into each ampule through which 3.33 and 5.00 ml. of distilled water were added to obtain 0.165M and 0.11M solutions, respectively. A substrate concentration of 0.165 M was used for plasma and 0.11M for erythrocytes, brain, and cerebrospinal fluid.

Electroshock

An I.O.L.-Liberson Brief-Stimulus Therapy Apparatus Type 735 made by Offner Electronics Inc.[1] was used to

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[1] Donated to the Department of Pharmacology by Dr. Coyne Campbell of the Coyne Campbell Sanitarium, Oklahoma City, Oklahoma.

produce convulsions. Since the resistance through the head varies from individual to individual, this apparatus is so designed that the resistance is measured and thus permits adjustments to be made in order to administer a current of predetermined intensity. A pulse duration of 1 millisecond and a frequency of 120 cycles per second were used in all experiments. The current and duration of treatment were varied and are given in the section under experimental results.

The animals were anesthetized with pentobarbital sodium (Nembutal, Abbott)[2], 30 mg./Kg. I.V. The hair from an area on each side of the head, posterior and lateral to the eyes, was clipped and dovered with electrode jelly. Electrodes were placed over gauze squares which had been soaked in NaCl solution and which covered the shaved areas. Electrical current was then passed from bitemporal electrodes through the skull to produce convulsions.

Drugs

(1) Atropine dissolved in distilled water, 50 mg./ml.

(2) Metrazol ampules containing 100 mg./ml.

(3) Serpasil (Reserpine, Ciba) ampules containing 2.5 mg./ml.

(4) Iletin (Insulin, Lilly) containing 80 units/ml.

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[2] Abbott Laboratories, Veterinary Nembutal Sodium. Each ml. contains Nembutal Sodium 60 mg., alcohol 10% and propylene glycol 20%.

(5) Di-isopropyl fluorophosphate (Bios Laboratories) in propylene glycol, 100 mg./ml.

CHAPTER IV

EXPERIMENTAL RESULTS

Controls

Since each dog could not serve as its own control for determining brain cholinesterase activity, it was necessary to compute the mean value for a group of control dogs. Table 1 shows the results from six dogs. Determinations were made on the caudate nucleus, cerebral cortex, erythrocytes, plasma, and cerebrospinal fluid. Cholinesterase activity was either absent from the cerebrospinal fluid of the controls or was present in such small amounts that it was not detectable by this method. This was true even when 1 ml. of undiluted cerebrospinal fluid was used as the enzyme source. Thereafter, the cholinesterase activity of the cerebrospinal fluid from the experimental animals was not determined.

Nembutal

Nembutal was employed as the anesthetic in all experiments using electroshock. Therefore, it was necessary to determine if nembutal itself had any effect

TABLE 1

CHOLINESTERASE ACTIVITY* IN CONTROL ANIMALS

CAUDATE NUCLEUS	CEREBRAL CORTEX	RBC	PLASMA	SEX
1.04	0.98	.36	.32	M
1.04	0.84	.32	.23	M
1.07	1.70	.39	.19	M
0.87	0.92	.29	.28	M
0.86	0.89	.35	.28	M
0.90	0.82	.37	.22	F

* $\triangle$ pH/Hour

TABLE 2

CHOLINESTERASE ACTIVITY* AFTER NEMBUTAL**

CAUDATE NUCLEUS	CEREBRAL CORTEX	ERYTHROCYTES BEFORE	ERYTHROCYTES AFTER	PLASMA BEFORE	PLASMA AFTER	SEX
1.35	1.10	.51	.53	.25	.28	M
1.00	0.83	.40	.41	.17	.20	F
1.12	1.01	.44	.40	.27	.26	M
0.87	0.79	.36	.35	.17	.18	M
1.08	0.93	.38	.39	.15	.12	M

* $\triangle$pH/Hour
** 30 mg./Kg. I.V.

upon cholinesterase activity. The results of the experiments obtained with a dose of 30 mg./Kg. intravenously are shown in Table 2. The analysis of the results using the standard "t" test shows that in these experiments nembutal had no significant effect on cholinesterase activity.

Reserpine

Plummer et al (48) found that the most obvious effect of reserpine was the production of a state of quietude and sedation. Sixty to ninety minutes following the injection of 0.25 to 0.30 mg./Kg. of reserpine intravenously, they found that a dog usually developed this quiescent state although miosis appeared much sooner. They also observed an augmentation of motor activity in the gastrointestinal tract. In the present study, a dosage of 0.4 mg./Kg. of reserpine[1] was injected intravenously and after two hours the animals were sacrificed. This dosage regularly produced miosis and defecation. The results of these experiments are shown in Table 3. It is evident that reserpine had no significant effect on erythrocyte or plasma cholinesterase activity. Analysis of the difference between the mean value of brain cholinesterase activity of these animals and of the control animals shows that there is no significant change in cholinesterase activity following reserpine.

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[1]Supplied by Ciba Pharmaceutical Products, Inc.

TABLE 3

CHOLINESTERASE ACTIVITY* AFTER RESERPINE**

CAUDATE NUCLEUS	ERYTHROCYTE		PLASMA		SEX
	BEFORE	AFTER	BEFORE	AFTER	
1.09	.36	.38	.24	.23	M
1.11	.34	.37	.22	.21	M
0.92	.38	.37	.27	.23	M
1.04	.37	.39	.20	.20	M
1.06	.41	.38	.15	.16	M

* $\triangle$ pH/Hour
** 0.4 mg./Kg. I.V.

TABLE 4

CHOLINESTERASE ACTIVITY* AFTER ELECTROSHOCK**

CAUDATE NUCLEUS	CEREBRAL CORTEX	ERYTHROCYTE BEFORE	ERYTHROCYTE AFTER	PLASMA BEFORE	PLASMA AFTER	SEX	HEMATOCRIT % BEFORE	HEMATOCRIT % AFTER
1.08	0.90	.30	.29	.09	.11	F	-	-
1.24	0.92	.28	.28	.23	.24	M	43	42
1.22	0.86	.44	.40	.40	.21	F	40	40
1.04	0.80	.32	.36	.14	.14	M	37	36
1.06	0.89	.34	.35	.24	.27	M	40	40

* $\triangle$ pH/Hour
** 80 milliamperes for 1.3 seconds

Electroshock

The animals in this group were anesthetized with Nembutal, 30 mg./Kg. intravenously. Table 4 summarizes the results obtained by passing a current of 80 milliamperes for 1.3 seconds (104 milliampere-seconds) through the intact skull from bitemporal electrodes. This is comparable to the range in milliampere-seconds used clinically in humans. Hemoconcentration is known to occur in patients following electroshock, which should increase the plasma cholinesterase activity. The dosage used in these experiments did not alter the hematocrit values and an analysis of the data shows that there was no significant change in cholinesterase activity of brain or blood.

Atropine, Metrazol and Insulin

Table 5 shows the results obtained with atropine, metrazol, and insulin. These experiments were conducted using the brain supernatant liquid and consequently little information is to be obtained from the measurements of brain cholinesterase activity. However, the data shows that none of the drugs in this group had a significant effect upon either erythrocyte or plasma cholinesterase activity. At the dosage levels indicated, convulsions were produced by each of these drugs.

TABLE 5

CHOLINESTERASE ACTIVITY* AFTER ATROPINE, METRAZOL, AND INSULIN

DRUG	DOSE	BRAIN**	ERYTHROCYTE BEFORE	ERYTHROCYTE AFTER	PLASMA BEFORE	PLASMA AFTER	SEX
Atropine	20 mg./Kg. I.V.	.45	.38	.34	.20	.21	M
Atropine	60 mg./Kg. I.V.	.80	.39	.39	.26	.27	M
Metrazol	20 mg./Kg. I.V.	.66	.30	.29	.17	.19	M
Metrazol	20 mg./Kg. I.V.	.63	.46	.43	.30	.31	M
Metrazol	20 mg./Kg. I.V.	.73	.46	.46	.16	.14	M
Insulin	400 units I.V.	.82	.34	.37	.23	.22	M
Insulin	200 units I.V.	.80	.38	.44	.22	.23	M

* $\triangle$pH/Hour

** The mean cholinesterase activity of 15 control dogs when using brain supernatant liquid was 0.78

DFP and DFP Followed by Electroshock

Table 6 summarizes the results obtained following DFP, 2 mg./Kg. I.V., and Table 7 the results of the same dose of DFP followed by electroshock. Nembutal, 30 mg./Kg. I.V., was given to the animals prior to electroshock. In these experiments using electroshock, the dosage was 80 milliamperes for 5 seconds and this was repeated 5 times within 5 minutes. This dosage regularly produced violent convulsions accompanied by urination, defecation, and profuse salivation. Hemoconcentration occurred in 4 of 6 experiments; however, there was no change in either the plasma or the erythrocyte cholinesterase activity. Analysis of the difference between the mean brain cholinesterase activity of these animals and the mean brain cholinesterase activity of those animals receiving only DFP shows there is no difference between the two groups.

Since no change in cholinesterase activity was observed following nembutal, reserpine, and electroshock with nembutal, the brain cholinesterase activity determinations of all these dogs were grouped together to serve as controls. This gives a total of 21 dogs whose mean brain cholinesterase activity is 1.05 $\pm$ .03 pH units. The standard deviation is 0.12 and the median value is 1.06. Blood cholinesterase determinations were made on 61 dogs. The control erythrocyte mean

TABLE 6

CHOLINESTERASE ACTIVITY* AFTER DFP**

CAUDATE NUCLEUS	ERYTHROCYTE BEFORE	ERYTHROCYTE AFTER	PLASMA BEFORE	PLASMA AFTER	SEX
.59	.55	.28	.22	.03	F
.31	.44	.22	.14	.02	F
.39	.40	.19	.21	.01	M
.51	.47	.31	.20	.02	F
.32	.50	.21	.26	.03	F

* $\triangle$ pH/Hour
** 2 mg./Kg. I.V.

TABLE 7

CHOLINESTERASE ACTIVITY* AFTER DFP** FOLLOWED BY ELECTROSHOCK***

CAUDATE NUCLEUS	ERYTHROCYTE		PLASMA		SEX	HEMATOCRIT %	
	BEFORE	AFTER	BEFORE	AFTER		BEFORE	AFTER
.26	.55	.16	.20	.03	M	45	56
.21	.27	.10	.37	.02	M	54	54
.52	.35	.20	.23	.05	M	40	40
.43	.24	.11	.15	.06	M	40	51
.51	.56	.34	.28	.04	F	42	49
.51	.30	.19	.19	.03	M	49	60

* $\triangle$ pH/Hour
** 2 mg./Kg. I.V.
*** 80 milliamperes for 5 seconds and repeated 5 times within 5 minutes

was 0.41±.02 pH units with a median value of 0.39. The control plasma mean was 0.23±.00 pH units with a median value of 0.23. The difference between the mean and the median was not statistically significant; one-third of all the observations were±1 standard deviation outside the mean; and 1.25 times the average deviation closely approximated the computed standard deviation of brain, erythrocyte, and plasma cholinesterase activity. This shows that the enzyme in each of the three tissues studied follows a normal distribution curve. Therefore, the mean value for brain cholinesterase activity which was computed from 21 observations can be used as the control value for each experimental animal with a reasonable degree of accuracy.

Figure 1 shows brain cholinesterase activity plotted against the erythrocyte cholinesterase activity for the control group. There is a considerable spread of the points around the line, and the coefficient of correlation (0.486) indicates that there is only a fair degree of correlation between the two variables.

Table 8 presents the data obtained from 20 of the 21 control dogs in another manner. It shows the quotients of brain cholinesterase activity/erythrocyte cholinesterase activity. The 2nd observation in Table 4 was not included in this series since the quotient is much larger than the others. The mean value obtained was 2.82 and the standard

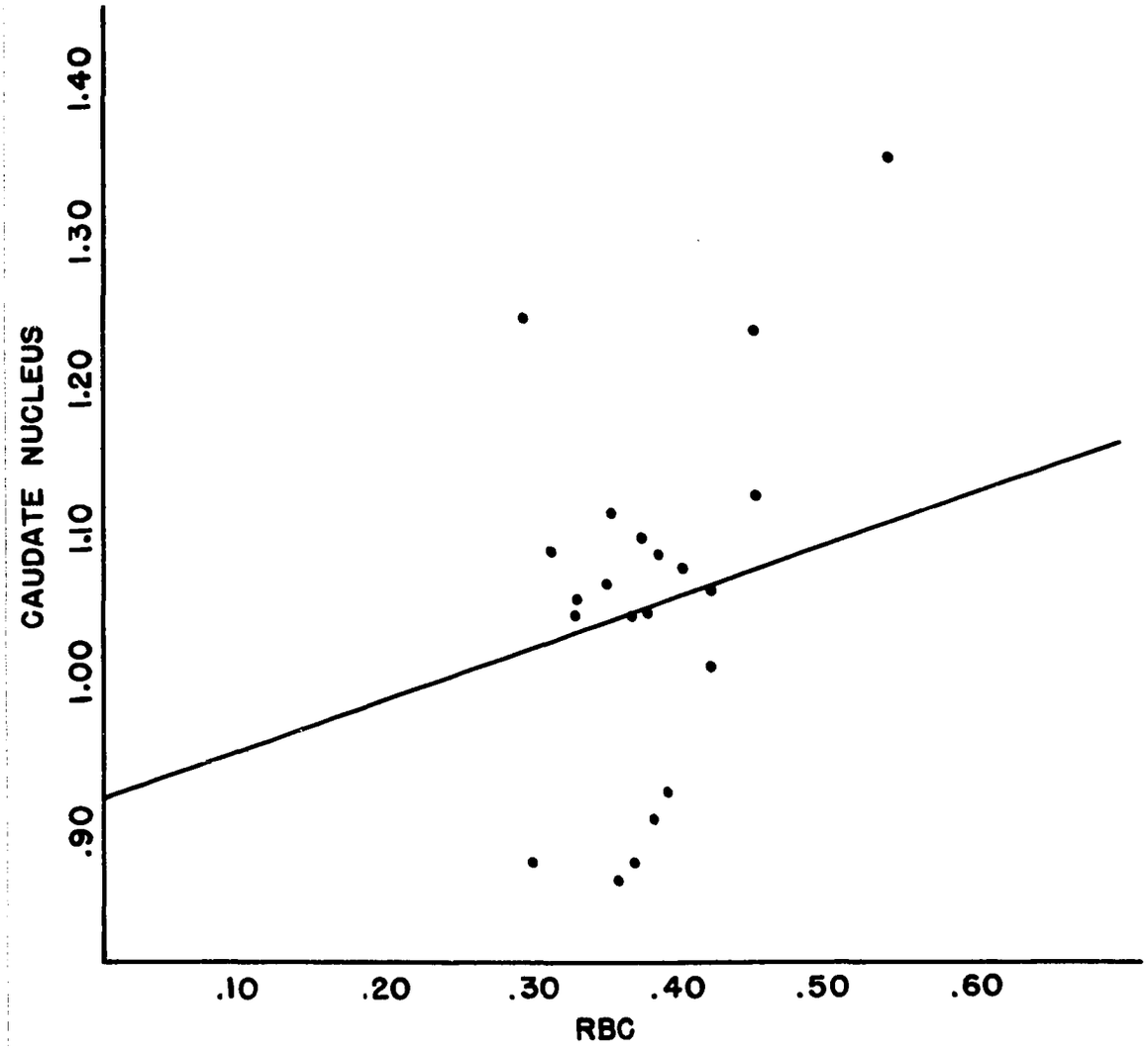

FIG. 1.—RELATIONSHIP BETWEEN CHOLINESTERASE ACTIVITY OF CAUDATE NUCLEUS AND RBC OF CONTROLS AS Δ pH/HOUR

TABLE 8

RELATIONSHIP BETWEEN BRAIN AND ERYTHROCYTE CHOLINESTERASE ACTIVITY* OF THE CONTROLS

CAUDATE NUCLEUS	RBC	QUOTIENT CAUDATE NUCLEUS RBC
1.04	.36	2.88
1.04	.32	3.25
1.07	.39	2.74
0.87	.29	3.00
0.86	.35	2.45
0.90	.37	2.43
1.35	.53	2.54
1.00	.40	2.50
1.12	.44	2.54
0.87	.36	2.41
1.08	.38	2.84
1.08	.30	3.60
1.22	.44	2.77
1.04	.32	3.25
1.06	.34	3.11
1.09	.36	3.02
1.11	.34	3.26
0.92	.38	2.42
1.04	.37	2.81
1.06	.41	2.58

* Δ pH/Hour

deviation was 0.34. The mean deviation of all the observations from 2.82 is only 0.28 or 10%. This demonstrates a better correlation between the brain cholinesterase activity and the erythrocyte cholinesterase activity of the control group than when the data is plotted graphically. A good correlation would be expected to exist between these two variables prior to administration of DFP if one was to exist following its administration.

DFP, 2 mg./Kg., was injected intravenously. A concentrated solution containing 100 mg./ml. was used so that the total volume injected ranged from about .18 to .35 ml. This dosage produced no overt signs of toxicity. After thirty minutes, a blood sample was taken and the animal sacrificed. Nachmansohn and Feld (42) reported that DFP which has not reacted with the enzyme, is retained in brain tissue. They showed this by determining the brain cholinesterase activity of a control animal and the brain cholinesterase activity of an experimental animal and then homogenizing together a piece of brain tissue from each animal. The observed difference averaged 33 per cent greater inhibition than the calculated difference. If this is true, it would appear to explain why a better correlation of blood to brain enzyme activity following inhibition by DFP has not been established. However, in the present study, this could not be confirmed by experimentation and it appears

that in the dosage employed, there is no DFP remaining in or upon the nerve cell membrane to react with free enzyme in the homogenate. Freedman and Himwich (19) found no evidence of retained DFP when using the whole rat brain following a dose of 2 mg./Kg. subcutaneously.

It can be seen from Tables 6 and 7 that plasma cholinesterase is most sensitive to inhibition by DFP, followed by brain and then erythrocyte cholinesterase. It has been shown by other investigators (25,44) that symptoms of toxicity are not manifested until the brain cholinesterase activity has been depressed to about 10-20% of control levels. In 11 of 12 experiments, the brain enzyme is inhibited to a greater extent than the erythrocyte enzyme. Figure 2 shows the relationship between brain and erythrocyte cholinesterase activity as the per cent of control activity remaining following DFP. Included in this group are those which were given DFP followed by electroshock. It can be seen that the level of activity of brain and erythrocyte cholinesterase approach zero simultaneously. Extrapolation of the computed line to intersect the axis which represents erythrocyte cholinesterase activity indicates that when the brain enzyme activity is at zero, the erythrocyte activity might still be about 2 to 3 per cent of control levels.

Figure 2 shows graphically that there is a good re-

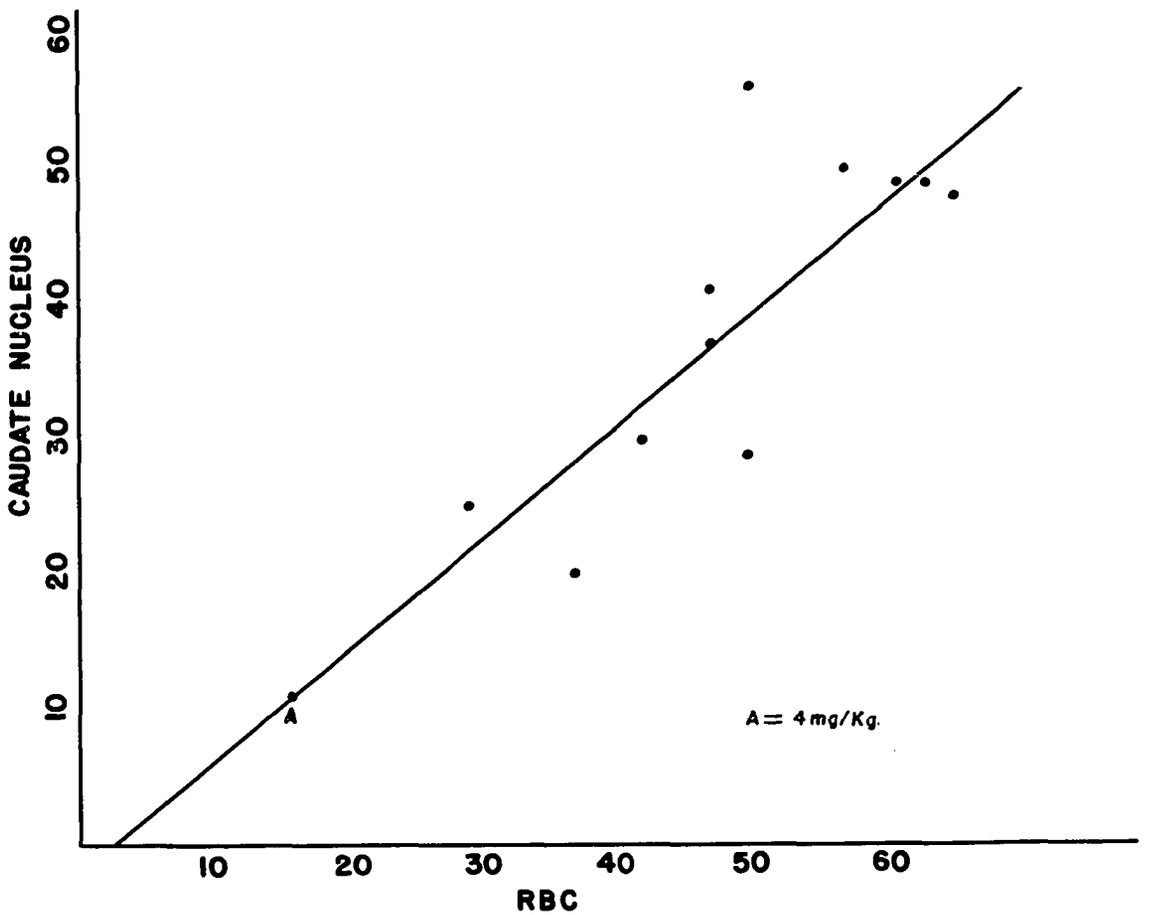

FIG. 2—RELATIONSHIP BETWEEN CHOLINESTERASE ACTIVITY OF CAUDATE NUCLEUS AND RBC AS PER CENT OF CONTROL ACTIVITY REMAINING AFTER DFP, 2 mg/Kg.

lationship between brain and erythrocyte cholinesterase activity following the administration of DFP. The line in this figure was fitted by the method of least squares. It shows a good degree of linearity between the two variables and by analysis it is found to be significant to the .01 level. The computed coefficient of correlation for these 12 observations is .87. It will be noted that this is considerably better than that found for the control group.

Figures 3 and 4 show the results when the data from Tables 6 and 7 are plotted separately. Further analysis shows that the coefficient of correlation of brain cholinesterase activity to erythrocyte cholinesterase activity for Table 6 is .860, and when the line for Figure 3 is fitted by the method of least squares, it is significant to the .02 level. Fitting the line in Figure 4 and analyzing for its significance shows it to be significant to the .01 level. The computed coefficient of correlation for Table 7 is very high at .960.

Plasma cholinesterase activity appears to have no correlation to erythrocyte or brain cholinesterase activity, either in the control or experimental group.

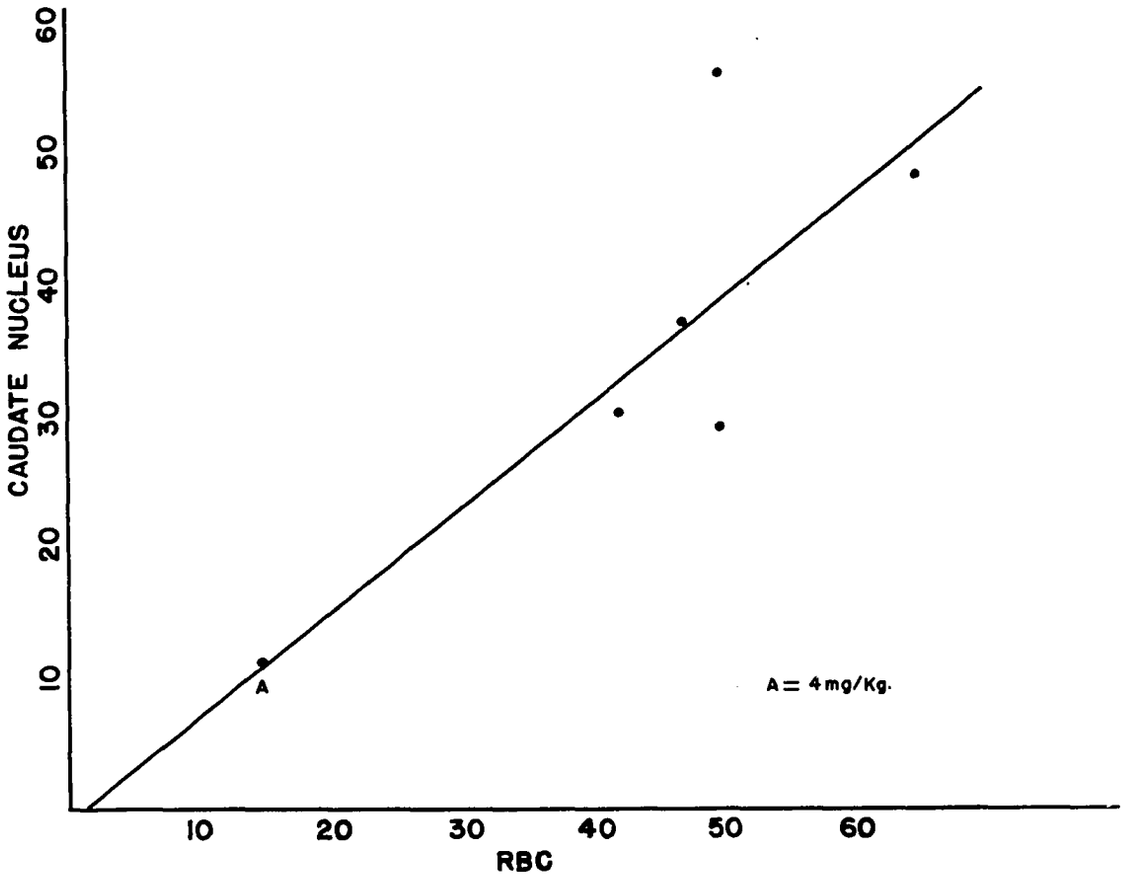

FIG. 3 — RELATIONSHIP BETWEEN CHOLINESTERASE ACTIVITY OF CAUDATE NUCLEUS AND RBC AS PER CENT OF CONTROL ACTIVITY REMAINING FOLLOWING DFP, 2mg/Kg.

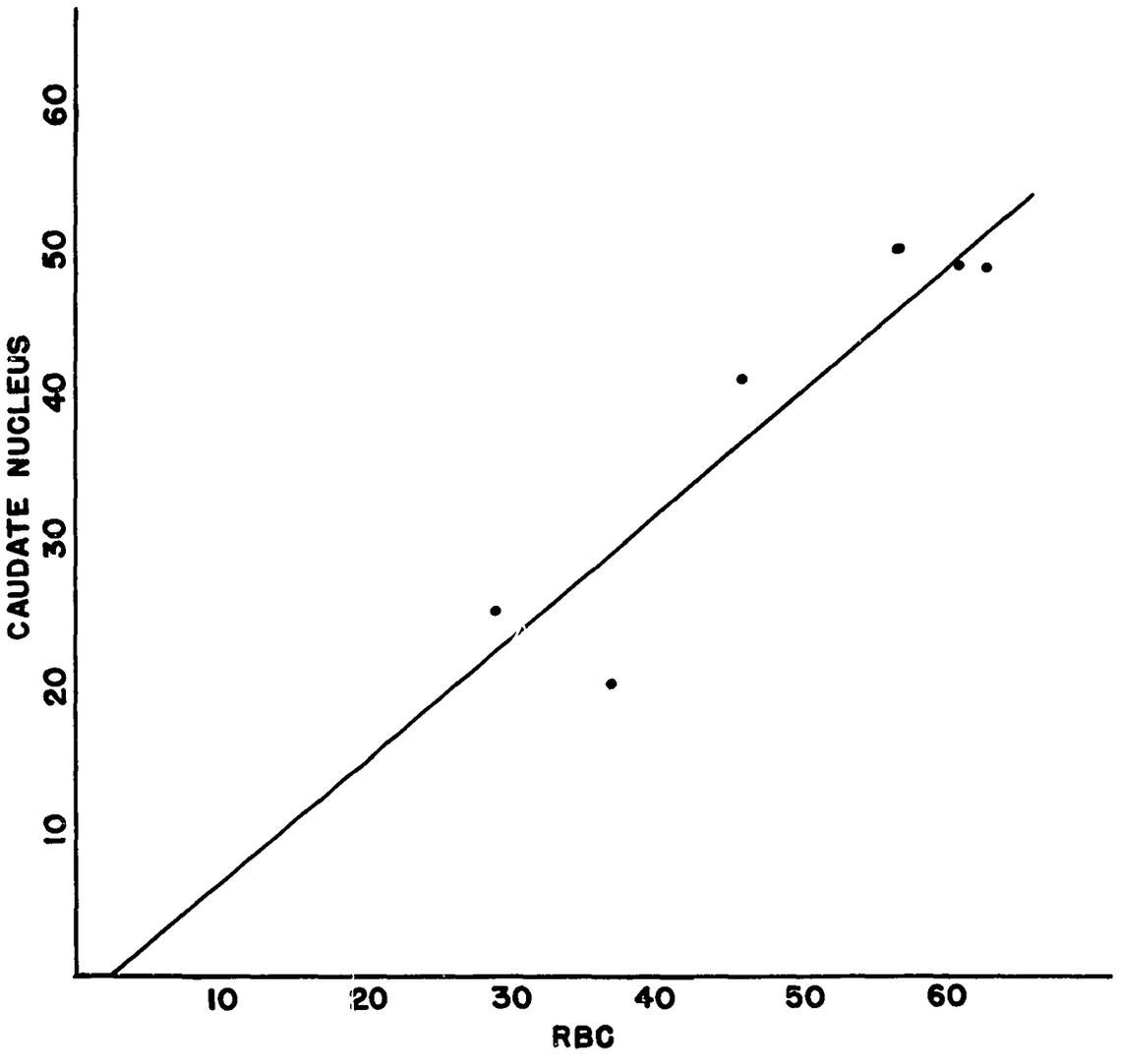

FIG. 4—RELATIONSHIP BETWEEN CHOLINESTERASE ACTIVITY OF CAUDATE NUCLEUS AND RBC AS PER CENT OF CONTROL ACTIVITY REMAINING FOLLOWING DFP, 2 mg/Kg., PLUS ELECTROSHOCK

CHAPTER V

DISCUSSION

Data obtained from measurements of the cholinesterase activity of tissues (caudate nucleus, cerebral cortex, erythrocytes, plasma and cerebrospinal fluid) of the dog have been presented. This was a study requiring statistical methods of analysis. Two approaches utilizing statistical methods were taken in the analysis: (1) The significance of the difference between the means of the control and the experimental group and (2) A mathematical derivation of the correlation between two biological variables.

As long as it is recalled that quantitative biological measurements include inherent variations; that a statistical analysis is primarily a function of probability; and that the experimental data must be of such a nature that the application of statistics is reasonable, then over-emphasis of the results obtained by such procedures is less likely to occur.

The data presented show that there is no effect of nembutal on cholinesterase activity of blood and brain. It is known that an accumulation of bound-acetylcholine occurs following nembutal. This probably is the result of decreased central activity (6). Several investigators have used the

supernatant liquid of homogenized brain tissue to determine the effect of drugs upon cholinesterase activity "in vivo". Using these methods, barbiturates have been reported to decrease cholinesterase activity approximately 5 per cent in chronic experiments (57,58). Nachmansohn and Feld (42) have shown that the major part of brain cholinesterase activity resides in the sediment. This was confirmed in the present study. Therefore, results obtained using the supernatant liquid are probably not accurate, especially when changes of small magnitude are observed.

The evidence for interference with the acetylcholine-cholinesterase system in certain mental disorders has been given. Convulsive therapy, utilizing either insulin, metrazol or electroshock is used in some types of mental illness. On the basis of Sherwood's results (56), an increase in cholinesterase activity following convulsive therapy might be expected. However, there are other possibilities which might bring about a temporary reduction of enzymatic activity following electroshock: (1) It is known that an increased hydrogen ion concentration will lower cholinesterase activity. Consciousness and other high levels of central nervous system activity depend upon the normal function of central synapses. Minute pathological changes occurring in the enzyme-substrate system at localized sites might be transientaly corrected by an increased concentration of hydrogen ions as a result of

electroshock. Since the structure of acetylcholine does not change with a change in pH, Wilson (66) believes changes in the rate of its hydrolysis may involve alterations in the protein structure of the enzyme. He postulated the following explanation for the change in cholinesterase activity with a change in pH,

$$EH_2 \overset{H^+}{\rightleftharpoons} EH \overset{OH^-}{\rightleftharpoons} E^- + H_2O$$

where EH, the active enzyme, is arbitrarily assigned a relative charge of zero. The forms EH_2 and E^- either cannot form complexes at all or they are not active. (2)The other possibility whereby electroshock might produce cholinesterase inhibition consists of enzymatic inhibition as a result of the substrate concentration in excess of the optimum. The bound-acetylcholine content of the brain is reduced following electrical stimulation (52). Although it is unlikely that large quantities of acetylcholine are released into the circulation following electrical stimulation, it is known that cholinesterase is located exclusively at the neuronal surface where bioelectrical phenomena are said to occur. A sudden release of large quantities of acetylcholine could, therefore, conceivably produce inhibition of the enzyme, because "in vitro" studies have shown that true-cholinesterase activity is inhibited by substrate concentrations which are greater than the optimum. It is

thought that the active surface of the enzyme possesses a negative and a positive combining site. The quaternary nitrogen atom in the acetylcholine molecule is electro-positive while the esteratic site has been shown to possess electro-negative properties. Therefore, the enzyme is believed to interact with the substrate at two sites to form an active enzyme-substrate complex. It has been shown that one molecule of acetylcholine probably reacts with one molecule of enzyme. Haldane (22) has postulated that two molecules of substrate may react with one molecule of enzyme and thereby form an inactive complex, resulting in enzyme inhibition. However, it appears from the results obtained in this study, that no change in cholinesterase activity occurred following insulin, metrazol, atropine, or electro-shock.

The clinical and experimental evidence presented by Sherwood (55,56) suggested the possibility of decreased cholinesterase activity or of acetylcholine accumulation in schizophrenia. Therefore, it was decided to simulate this condition by reducing brain cholinesterase activity by about 50 per cent with DFP and then applying electroshock. It is generally believed that the release of bound, inactive acetylcholine to the free, active form occurs as a result of the nerve impulse. Experimental evidence indicates that either all or nearly all of the bound acetylcholine is released when the nerve stimulus is of threshold intensity.

The present experiment suggests several mechanisms whereby acetylcholine would be expected to accumulate in brain tissue for release by electroshock. Following DFP, the concentration of acetylcholine is approximately 102 per cent greater than before DFP (37). An increase in bound-acetylcholine was found to occur in brain under nembutal anesthesia (6). Experimental evidence indicated an increase synthesis of bound acetylcholine when free acetylcholine was added to the "in vitro" system (36). Furthermore, Augustinsson has pointed out that the optimal substrate concentration changes as the protein cholinesterase molecule changes in polarity. As the molecule increases in negativity, the optimal activity-substrate concentration decreases. Since cholinesterase is located at the neuronal surface, he believes that when depolarization occurs, the neuronal surface become negative and enzyme activity will be optimal at lower acetylcholine concentrations. In spite of these factors, no significant change in cholinesterase activity was found following electroshock. The hydrolysis of acetylcholine by cholinesterase occurs at an extremely high rate of speed. One molecule of enzyme may split one molecule of acetylcholine in about 3 or 4 microseconds (43). Nachmansohn states that, "No other chemical reaction known to be connected with nerve activity has a comparable speed". This may be a decisive factor in the failure of these experiments to detect a

change in cholinesterase activity following DFP plus electro-shock.

Reserpine has the following pharmacological properties: It has been reported to increase intestinal motility, to produce miosis, and bradycardia (48). Following excision of the ciliary ganglion, miosis does not occur which shows that pupillary constriction is not produced peripherally (5). Reserpine does not increase the movements of the isolated intestine (65,5). The site of action is thought to be in the hypothalamus (48). It is possible that reserpine could either selectively inhibit cholinesterase in the hypothalmus or could produce selective inhibition on the basis of a differential enzyme concentration in this region. In this event, the selection of tissue other than the hypothalamus probably would not show enzyme inhibition. It is unlikely that the above two conditions would exist without associated inhibition in the caudate nucleus. Sometimes only a slight change may result in a considerable functional difference. An example is the diuresis resulting from about one per cent change in reabsorption of water from the renal tubules. However, as previously mentioned, brain cholinesterase must be inhibited to a considerable extent before marked funtional changes occur. Nachmansohn has shown by "in vitro" studies that an increased concentration of DFP is required to produce a comparable inhibition as the dilution of

cholinesterase is increased. This would indicate that areas containing a lesser concentration of cholinesterase would require a higher concentration of DFP than would be required for an area of greater cholinesterase concentration in order to produce equal inhibition. Therefore, those areas possessing more cholinesterase should show enzyme inhibition earlier than those areas possessing less enzyme.

The data presented show that a relationship exists between brain and erythrocyte cholinesterase activity following DFP. Koelle and Gilman (25) showed the sensitivity of dog cholinesterase to inhibition by DFP when tested "in vitro". The sensitivity was in the increasing order of brain, erythrocyte, and serum cholinesterase. However, their chronic study using 4 dogs (26) shows the brain enzyme to be inhibited to the greatest extent at the end of 24 weeks following an initial injection of DFP. This was explained by the fact that since DFP produces irreversible inhibition, new enzyme must be formed in order to show a return of activity. It appears that the plasma and erythrocyte enzyme are formed at a faster rate than that of brain. This is a reasonable explanation and such has been shown to occur in rats (20,25). It is also known that DFP has a high lipid solubility and for this reason might be expected to inhibit brain cholinesterase to a greater extent than erythrocyte cholinesterase if measurements are made soon after a single dose. In the present study, brain cholin-

esterase was in fact inhibited to a greater extent than that of erythrocyte, and the solubility of DFP in lipids may be the reason why this data is in disagreement with the sensitivities reported by Koelle and Gilman.

According to the experiments of various investigators, it appears that the species, dosage, and time of measurement of enzyme activity following DFP all affect the correlation of brain to blood enzyme activity. For instance, "in vitro" studies have shown that rabbit, rat and human brain cholinesterase are more sensitive to inhibition by DFP than is their erythrocyte enzyme (25,33). The reverse is true in the dog (25). Freedman, Willis and Himwich (20) have shown that following the administration of DFP, 1 mg./Kg. subcutaneously, the rat brain cholinesterase activity is depressed to a greater extent than that of erythrocytes. This extends from a period immediately following it s administration throughout the remaining ten day experimental period. In another experiment, they found that following a larger dose, 2 mg./Kg., the erythrocyte enzyme was depressed more than that of the brain for about 4 days, after which time they believe erythrocyte cholinesterase regeneration rates overtook those of the brain, and consequently the brain enzyme was inhibited to a greater extent.

It is generally agreed that the symptoms following toxic doses of DFP are the result of an accumulation of acetylcholine which in turn results from cholinesterase

inhibition. Holmstedt (23) has shown the relationship between toxicity and cholinesterase inhibition. He plotted the logarithm of the $L.D._{50}$ dose of the inhibitor against the logarithm of the concentration producing 50 per cent inhibition. A straight line resulted. Freedman, Willis and Himwich (20) have done about the same thing in another way. They correlated signs of toxicity which cholinesterase inhibition and found a significant correlation.

Some evidence indicates that DFP produces symptoms of toxicity that cannot be accounted for by cholinesterase inhibition; however, to date no mechanisms have been proposed.

CHAPTER VI

CONCLUSIONS

1. The effect of physical and chemical agents upon cholinesterase activity was studied in the brain (caudate nucleus and cerebral cortex), plasma, erythrocytes, and cerebrospinal fluid.

2. The method of Michel was modified to measure cholinesterase activity of the above tissues of the dog.

3. The whole tissue homogenate of the brain was found to be necessary to determine quantitatively the effect of drugs upon cholinesterase activity "in vivo".

4. Nembutal was found to have no significant effect upon cholinesterase activity.

5. Dogs appeared to show no overt signs of toxicity when brain cholinesterase activity was depressed to within 30 to 50 per cent of control levels, and when erythrocyte cholinesterase activity was depressed to within 30 to 60 per cent of control levels.

6. Cerebrospinal fluid was found to contain no detectable cholinesterase activity.

7. It was found that erythrocyte cholinesterase activity measurements afford a relatively accurate means of

estimating brain cholinesterase activity when measurements are made soon after a single dose of DFP.

8. Contrary to expectations, depression of brain cholinesterase activity to within about 50 per cent of normal levels by DFP followed by electroshock produced no significant change in cholinesterase activity.

9. Metrazol, insulin, and atropine were found to have no effect upon cholinesterase activity of blood although convulsions were produced.

10. No retained di-isopropyl fluorophosphate (DFP) was found in brain tissue following a dose of 2 mg./Kg. I.V.

11. The sensitivity to inhibition by DFP "in vivo" was in the increasing order of erythrocytes, brain, and plasma cholinesterase activity.

CHAPTER VII

SUMMARY

Cholinesterase activity is inhibited by di-isopropyl fluorophosphate (DFP) and a correlation was found to exist between erythrocyte and brain cholinesterase activity. A better relationship appears to exist following DFP plus electroshock than with DFP alone. There was no significant change in cholinesterase activity following nembutal, reserpine, insulin, metrazol, atropine or electroshock. The cerebrospinal fluid either contains no cholinesterase or it was present in such small quantities that it was not detected by this method. Plasma cholinesterase activity appears to bear no relationship to erythrocyte or brain cholinesterase activity, either before or after the drugs used. Following an acute exposure to DFP, brain cholinesterase activity was inhibited to a greater degree than was erythrocyte cholinesterase.

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