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THE ACTIVITY OF ACETYLCHOLINESTERASE IN
THE BRAIN OF THE DEVELOPING CHICK EMBRYO
AND ITS MODIFICATION IN THE EARLY STAGES
WITH EXOGENOUS RNA

BY

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ABSTRACT

This work was carried out to investigate the acetylcholinesterase and pseudo-cholinesterase activities in the brain of developing chick embryos.

Inhibitors were used and activity units were calculated using Hestrin's colorimetric method.

Five distinct stages of varying acetylcholinesterase activity levels are reported in the chick brain embryos (2 to 21 day-old). These stages are correlated to the functional and developmental changes of the embryos.

Preliminary work on the effect of exogenous RNA on the enzyme synthesis and activity showed negative results.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
ps-ChE	pseudocholinesterase
ChE	acetylcholinesterase and pseudocholinesterase
DFP	di-isopropylfluorophosphate

CHAPTER I

INTRODUCTION

The powerful pharmacological action of acetylcholine (ACh) was first discovered by Hunt and Taveau in 1906. This led workers in the field to postulate that ACh is "a neurohumoral transmitter." Nachmansohn and coworkers (Nachmansohn, 1948; Welsh, 1946) proved that the action of the ester is essential for the production of bioelectric currents and thus the propagation of impulses along nerves. It is thought that these currents are produced by changing the permeability of sodium ions through the concentration gradient between the inside of the fiber and the environment. This process is regulated by acetylcholinesterase (AChE) which hydrolyzes the ester and restores the fiber to its initial state (Nachmansohn, 1948).

Cholinesterases:

In most animal tissues two cholinesterases can hydrolyze choline esters of fatty acids. These enzymes differ in function, distribution and substrate specificity. The enzyme AChE, which is found mostly in conductive tissues and erythrocytes, is responsible for the hydrolysis of ACh at myoneural junction and is vital for impulse transmission. True or specific cholinesterases are names often applied to this enzyme. The second called usually pseudo-cholinesterase (ps-ChE) or cholinesterase (ChE), has no specific function and is equally distributed in the body

and serum. This could be inhibited without ill effects (Nachmansohn, 1948; Augustinsson, 1949; Myers, 1953; Wilson and Alexander, 1962).

The most highly active AChE is found in the electric eel, Electrophorus electricus. It hydrolyzes 18 to 20 millimoles of acetylcholine per gram fresh weight per hour and has a Michaelis constant, K_m , of 4.6×10^{-4} M. Each enzyme molecule can hydrolyze about 300 molecules of ACh per millisecond, and has a molecular weight of 3×10^6 (Wilson and Harrison, 1961).

AChE is a stable enzyme; it can be stored at -20°C without loss of activity for years (Lowry et al., 1954). Both of the cholinesterases hydrolyze many esters of choline and others. While AChE hydrolyzes acetyl, propionyl, and butyryl, in decreasing order, the ps-ChE exhibits the opposite order. Substrate specificities vary with the species (Chessick, 1954).

Substrate Inhibition of Acetylcholinesterase

Hydrolysis of ACh by AChE is inhibited by excess substrate (Fig. 1). If the activity of AChE is plotted against the negative logarithm of ACh concentration, pS , the resulting curve is bell-shaped (Augustinsson, 1949; Wilson and Alexander, 1962). This is due to the fact that AChE may form a mono-substrate-enzyme complex as well as di-substrate-enzyme complex. The former results in hydrolysis, while the latter is completely inactive. Maximal activity of the enzyme towards ACh is exhibited at a concentration of 4 to 7 micromoles per milliliter. However, ps-ChE does not form a di-substrate-enzyme complex, and thus, does not show the characteristic bell-shaped curve mentioned above.

Active Sites

Inhibition studies (Bergmann, Wilson and Nachmansohn, 1950) revealed that the active site of the enzyme ChE is made of two components (Fig. 2). Anionic site, which binds the positively charged quaternary ammonium group of the substrate, ACh, and an esteratic site which brings about the actual hydrolysis of CO-O-R group. The sites are separated by a distance of 7 ⁰ Å. The esteratic site consists of an acidic and a basic group (Wilson and Bergmann, 1950). The basic group is concerned in binding the substrate although both are involved in the overall reaction. The former is consistent with an imidazole group of amino acid histidine, while the latter with a phenol of serine. Unlike AChE is ps-ChE, the anionic site does not exist. Nevertheless, like all other esterases, the esteratic site has similar amino acid sequence, glutamic acid and serine (O'Brien, 1963). The above theory explains why the two enzyme activities are pH dependent.

The enzyme is acetylated during the course of the reaction and the acetyl enzyme finally breaks to give enzyme, acetic acid and choline (Laedler, 1958). Figure 3 is a schematic representation of the course of hydrolysis of ACh by AChE.

Inhibitors

Since there is no specific substrate for AChE, inhibitors must be used to mask ps-ChE and other hydrolytic enzymes. The two inhibitors most widely used are di-isopropylfluorophosphate (DFP) and eserine. Eserine (Appendix A) contains an ester linkage and a nitrogen atom which is almost the same distance away as ACh. It inhibits ChE's

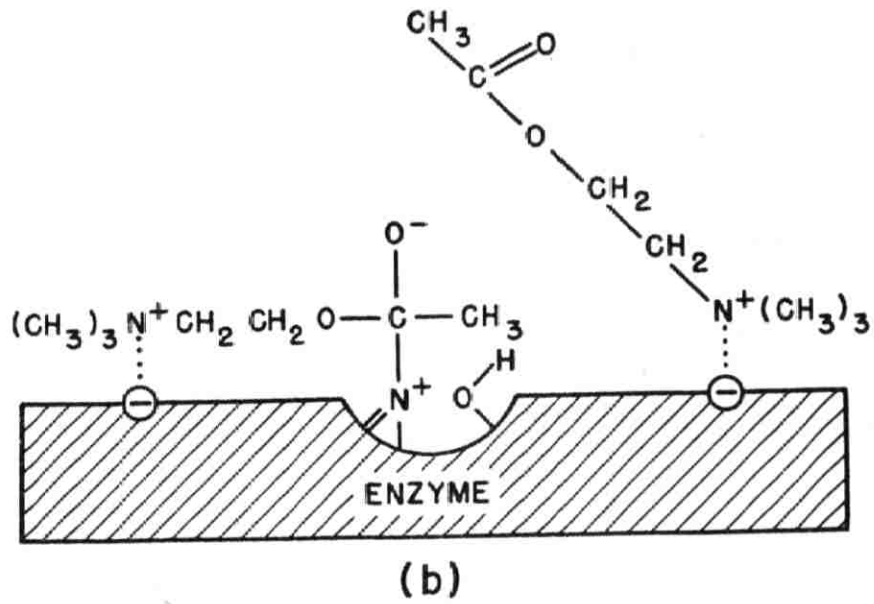


Fig. 1.- Acetylcholinesterase, showing the attachment of two substrate molecules. (From Laedler, 1958, p. 276).

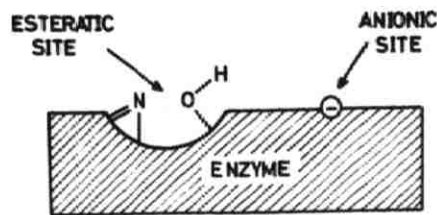


Fig. 2.- Schematic representation of the active centres of cholinesterase. (From Laedler, 1958, p. 264)

competetively (Laedler, 1958); while DFP (Appendix B) inhibits the enzymes by phosphorylating the serine residue of the active site (O'Brien, 1963). The inactive phosphorylated enzyme is very stable. In both cases, inhibitors must be preincubated with the enzyme in order to prevent substrate competition for the active site (Poulson and Alldridge, 1964).

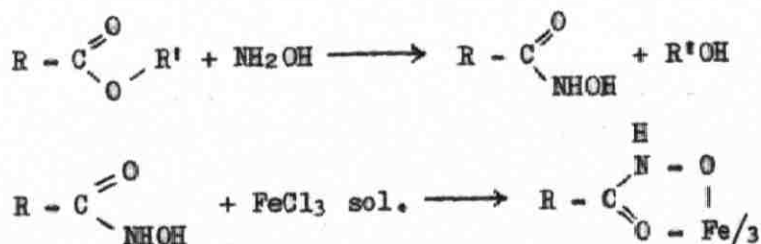
Review of Assay Methods

Various methods have been used to determine AChE activity of which some will be mentioned.

1. Titrimetric method: The acetic acid released on the hydrolysis of ACh is titrated with an alkali and the activity of the enzyme is calculated (Stedman, et al., 1933).

2. Manometric method: The enzyme and the ester are incubated in a bicarbonate buffer. The acid produced releases carbon dioxide and the change of pressure in the Warburg tube is measured, thus making the enzymatic activity accessible (Bergmayer, 1965).

3. Colorimetric method: The method makes use of the property of hydroxylamine reacting with the excess substrate, ACh, left after incubation with the enzyme. This yields hydroxamic acid quantitatively which when reacted with ferric chloride forms a brown-violet complex, the absorption maximum of which is at 540 mμ (Hestrin, 1949).



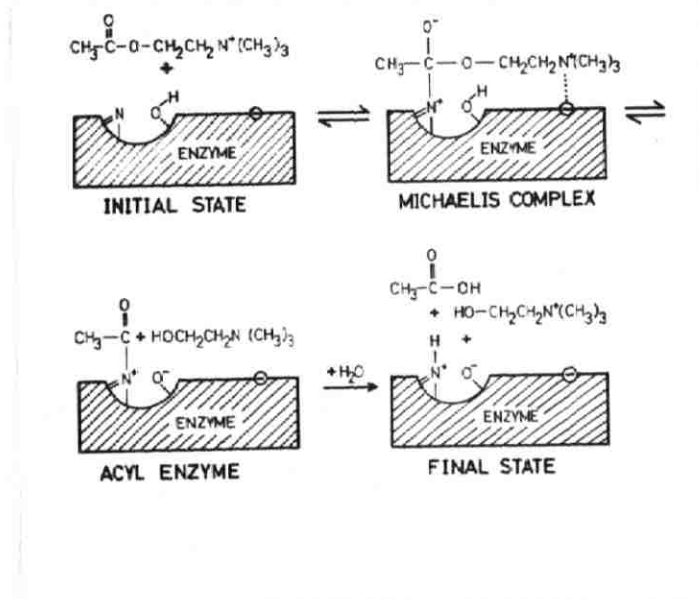


Fig. 3.- Schematic representation of the course of hydrolysis of acetylcholine by acetylcholinesterase. (From Laedler, 1958, p. 264)

4. Histochemical method: The method described by Koelle (1950) consists of incubating the tissue preparation in a medium of acetylthiocholine and copper glycinate for 60 minutes. Then the tissue sections are rinsed with copper thiocholine solution. White precipitates are formed which when immersed in ammonium sulfide are converted to dark brown deposits of copper sulfide. Histochemical methods give precise information on the location of enzymes; however, it suffers two drawbacks: difficulty of obtaining quantitative estimations and applicability to large amounts. The biochemical method does not suffer such limitations.

Distribution of Cholinesterases in Vertebrates

Recent advances in molecular biology show that the levels of different biomolecules bear a direct relationship to the morphological development of species.

Youngstorm (1938) was the first to correlate cholinesterase level to functional activity. He showed that ChE was present in three species of Amphibia before the appearance of the nervous system and its activity increased steadily during the period of developing motility.

The ChE activity in the central nervous system of the human fetus and of Amblystoma punctatum was studied histochemically by Youngstorm (1941) and Manometrically by Boell and Shen (1950). Their results suggested a correlation between the increase in enzymatic activity and functional differentiation. Metzler and Humm (1951) measured ChE activity in the rat whole brain; Palladin (1955) measured it in the rabbit and Himwich and Aprison (1955) in the guinea pig and sheep. The

enzymatic activities in quinea pig and sheep brain showed higher values in fetal and early postnatal life while the contrary applied for the rat and the rabbit. It is believed that this is due to the higher anatomical and physiological maturity of the latter compared to that of the rabbit and rat at the time of birth.

Activity of Cholinesterases in Chick Tissues

Nachmansehn (1938) was first to suggest the relationship of enzyme activity to morphological changes and function in chick brain.

Morphological differentiation has been studied by several investigators in certain parts of the chick brain; Reddick (1951) for the medulla, Shen et al. (1956) for the retina, Kahlenbeck (1937, 1938) for the diencephalon and telencephalon, Rogers (1960) for the whole brain.

Kuo (1932) has studied the behaviour of chick embryo throughout the incubation period. Lindeman (1947) tested pupillary reflex experimentally in the late embryo. Reflex activity was detected by Windle and Orr (1934) on the fifth or sixth day of incubation. Peters and coworkers studied electrical functions of the cortex (1956), in the eye and optic lobes (1958) and cerebellum (1960) of the chick embryo. The conclusion of these studies was that the nervous system becomes functional in the following chronological sequence: medulla, spinal cord, midbrain, diencephalon, cerebral lobes and optic system. The results of Elkes and Todrick (1955) on the rat were consistent with those of the chick.

Ammon and Shutte (1934) studied the development of lipase, esterase

and ChE in whole chick embryos. ChE activity was not studied in embryos younger than six days of incubation. Nachmansohn (1939), using the manometric method, determined the ChE activity of chick brain in embryos fifteen and sixteen days of incubation, newly hatched chicks, and young chickens. He found that the enzymatic activity increases sharply up to the eighth day after hatching and then falls slightly.

Rogers et al. (1960) studied ChE activity in different parts of brain homogenates of the chick embryo from six days to six to nine weeks posthatching. Data showed that the maximum for all parts occur between sixteen to nineteen days of incubation. Their results are consistent with electrical studies of Peters et al. (1958). Except for the cerebral hemispheres, all other brain tissues show a decreasing ChE activity on nearing hatching time.

Rogers et al. (1960) studied ChE activity in chick retina and found a sharp and continuing rise after the seventeenth day of incubation until eight weeks posthatching.

Zacks (1954) using the histochemical method, demonstrated the existence of AChE in early chick embryo of 0 - 96 hours. AChE activity was first detected at the fifteenth hour in the region of Hensen's node and in the primitive streak. As the embryo developed, the enzymatic activity was localized on the surface of neural folds and then at the twenty-sixth hour it appeared on the surface of the prosencephalon. In older stages (30 to 96 hours) little enzymatic activity was demonstrated in the brain and interior portion of neural tube except the retina and metencephalon. The decrease in enzyme activity in older embryos was attributed to an increase in brain mass without equal increase in AChE

content. Concurrently, the decrease in ChE in the nervous system in sixty days old Amblystoma embryo was attributed to this dilution effect by Sawyer (1943).

Moog (1944) noted the decrease in acid and alkaline phosphate activity in later stages of development when enzymes became more specifically localized. This could be the case for ChE in the chick since according to Lillie (1929) neuroblasts appear at the end of the third day of incubation. Kuo (1939) studied the development of acetylcholine in chick embryos. He noted a great increase in ACh levels in four-day old embryos and this level fluctuates until the twelfth day. He also noted the absence of synapses in the nervous system; these appeared on the third day. Thus the enzyme AChE appears long before the appearance of synapses or the substrate do.

Recently, Maynard (1966) succeeded in separating three anodically moving proteins having the properties of cholinesterases, from homogenates of whole chick brains (3 to 19 day-old) using acrylamide-gel-electrophoresis. Embryos of 7 to 19 days old showed an increase in activity. Furthermore, the preference of these ChE's to ACh was shown, thus suggesting that they are similar to AChE rather than ps-ChE. The three electrophoretically obtained AChE's could be due to either protein or molecules binding to the enzyme or to AChE in different polymerization state.

Maynard's results are supported by the electron microscopy studies of Terack and Barnett (1962) who have shown that cholinesterase activity at different sites in the neuron can be associated with the cell membrane, cytoplasmic vesicles or with the membrane of the endo-

plasmic reticulum. Therefore different molecular forms of the enzyme can be attributed to association with different tissues.

Choice of Inhibitors

Hestrin's method does not allow the distinction between the activities of the different esterases, and therefore inhibitors were used.

Until 1943 the term cholinesterase used in papers referred to the enzyme which hydrolyzed ACh. Thus, it may be either AChE of the nervous tissues or ChE found in various tissues. One of the generally used inhibitors of ps-ChE is DFP. Cholinesterase activity is selectively inhibited by low concentration of eserine (Richter and Croft, 1942).

Zacks (1954) showed histochemically, that in the embryonic axis of chick embryos of 30 to 96 hours of incubation, the only esterase is AChE, because the specific chromogenic substrate for ps-ChE, carbonaph-toxycholine iodide was not hydrolyzed by the enzyme of the embryonic axis.

The choice of DFP and eserine was made through the use of the property that within one species the ChE differs from tissue to tissue and the enzyme of a given tissue differs from species to species (Chessick, 1954). Thus, chick retina (Shen, 1956) which contains AChE exclusively, the plasma, ps-ChE (Blaber and Cuthbert, 1962; Maynard, 1966), the heart, both AChE and ps-ChE (Sippil, 1955), and the brain, AChE and a small percentage of ps-ChE (Myer, 1953; Blaber et al., 1962) were used to determine specific concentration of an inhibitor. For the brain, a suggested concentration of 5.0×10^{-9} M DFP is given

to produce inhibition of ps-ChE.

The present work was mainly undertaken to examine in a systematic way the changing pattern of AChE activity during development of the chick brain and its relation to differentiation and onset of function.

Effect of Exogenous RNA on Protein Synthesis and Differentiation

Growth and differentiation are the essence of all developmental processes. Both aspects are a result of synthesis of new molecules and for the increase in the level of already existing ones. The newly synthesized species must be oriented to do a specific biological function. Enzymes are the best examples of biosynthesis brought about by the appearance of new messenger RNA species to code for new protein.

Niu (1956) found that in cell-free inductors RNA could induce protein synthesis, thus specific cell structure appears.

This led many investigators to study the effect of exogenous RNA (extracted from adult tissue) on developmental potency. The exact effects of RNA created conflicting ideas among the investigators. Niu (1958), Hillman and Niu (1963), Sanyal and Niu (1966) stated that RNA induced the target cells to form structures which are specific to the organ source of RNA. Whereas, others as Butros (1963, 1965), Finnegan and Biggin (1966), Eisenberg and Pierson (1964), Hillman and Hillman (1967) reported that exogenous RNA does not induce the synthesis of proteins specific to the source of RNA.

According to Butros (1963), the RNA added to the culture system was hydrolyzed forming oligonucleotides, nonspecific protein synthesis.

On the other hand Niu (1963) believes that RNA macromolecules penetrate the host cells and attach themselves to the chromosomes, thereby inducing the production of RNA (of its kind) and subsequently, proteins specific to the inducer.

Very recently Hillman and Hillman (1967) reported that ectoderm responds to both heart and brain RNA, but the same response is found when exposed to boiled brain RNA. This led them to assume that the induction was nonspecific.

Morphological differentiation on tissue level (such as the development of neural tissue by the induction of exogenous brain RNA) has been used as a criterion for new protein synthesis (Niu, 1958; Butros, 1963, 1965; Hillman and Niu, 1963; Sanyal and Niu, 1966; Finnegan and Biggin, 1966). Also the biosynthesis of new enzyme could be taken as another criterion. Weisberger (1962) using sickle-cell RNA induced the production of an abnormal hemoglobin; and Niu (1963) showed that kidney RNA induced the biosynthesis of L-Amino acid oxidase, whereas liver-RNA induced glucose-6-phosphatase and tryptophan pyrolase in mouse ascite cells.

Preliminary work on the effect of brain-RNA on the AChE level in chick embryo is reported in this work, following the main studies on the enzyme pattern. The purpose of this study is to check on the large number of conflicting suggestions of different workers on whether exogenous RNA has or does not have specific effect on the level of the enzyme, AChE (in 3 day-old embryos).

CHAPTER II

MATERIALS AND METHODS

Reagents

All chemicals used were of analytical grade.

1. Acetylcholine Solution: 10.0 ml of 0.5 M of Acetylcholine-chloride solution (Nutritional Biochemicals Corp.) was prepared in distilled water weekly. Fresh preparations were necessary since Acetylcholine is known to hydrolyze when kept for a long period of time. It was kept at 0°C.

2. Barbital Buffer (0.1 M): 2.06 g sodium diethylbarbiturate (May and Baker Ltd.) and 2.12 g anhydrous Na₂CO₃ were dissolved in 180 ml of distilled water, approximately 1.9 ml of concentrated hydrochloric acid was added. This was followed by 2.18 g MgCl₂·6H₂O and 0.04 g KCl and the volume was brought to the 200 ml mark with distilled water. The pH of the solution was checked and found to be (8.0 - 8.2). It is known to be stable indefinitely if stored at 4°C. This buffer was chosen because of the small drop in pH (0.2 units) occurring during the assay.

3. Acetylcholine Buffer Solution (0.05 M): Just before use nine volumes of the buffer were mixed with one volume of Acetylcholine solution.

4. Hydroxylamine 14% (2 M): 14.0 g Hydroxylamine hydrochloride ($\text{NH}_2\text{OH}\cdot\text{HCl}$, May and Baker Ltd.) were dissolved in distilled water, final volume 100 ml. The solution is known to be stable indefinitely at 4°C .

5. Alkaline Hydroxylamine: Equal volumes of 14% Hydroxylamine and 14% NaOH were mixed immediately before use.

6. Ferric Chloride Reagent (0.37 M): Ferric Chloride ($\text{FeCl}_3\cdot 6\text{H}_2\text{O}$, Hopkins and Williams Ltd.) 0.37 M was prepared in 0.1 N Hydrochloric acid.

7. Eserine Solution (1.0×10^{-4} M): Solution of di-isopropyl-fluorophosphate (Merck, Darmstadt) was prepared weekly and kept at 4°C . The solution was then diluted ten times in the buffer to make it 5.3×10^{-8} M.

9. Acid: Concentrated Hydrochloric acid, sp. gr. 1.18 diluted with two parts by volume of water.

10. Holin-Ciocalteu Reagent: (Fisher Scientific Co.).

Procedure

Eggs from white Leghorns obtained from the University Farm were stored at 16°C until used (not longer than a week). Eggs incubated at 37.5°C for 2 to 21 days were used. The tissues examined were hole brain from 7 day-old embryos and older; the head minus the eyes from 5 and 4 day-old embryos; the whole head from 2 and 3 day-old embryos (2 heads per experiment). The embryos were transferred to ice-cold 0.9% NaCl solution. The brains were removed and placed in fresh ice-cold 0.9% NaCl. The optic lobes with the retina and external membranes and their blood vessels were removed. The tissues which were blotted to remove

excess fluid were weighed quickly and then homogenized immediately in a cold, hand-operated glass tissue-homogenizer. The homogenate, diluted in thirty to forty volumes of buffer of pH 8.0, was centrifuged for 20 minutes at 4°C at about 12,000 x g (I E C International Model HR - 1 Refrigerated Centrifuge, Boston 35, Mass. U. S. A.). The resulting supernatant was used as the enzyme preparation. The solution was kept at 0°C until used.

Because of high surface to volume ratio of brain tissue, evaporation is fairly rapid, thus the determination of fresh weight can not be accurate. Therefore, the weight provided only a rough estimate of the mass of the sample and was not used for expression of enzyme activity. However, AChE activity was expressed per mg of protein of the sample.

Protein Determination with Folin-Ciocalteu Reagent

The method used is that of Lowry et al. (see Colowick et al., 1955). The violet blue colour obtained due to the addition of alkaline copper solution and Folin reagent is thought to be produced by: (1) biuret reaction of protein with copper in alkaline, (2) the reduction of phosphomolybdic-phosphotungstic reagent (Folin-Ciocalteu) by the tyrosine and tryptophan present in the treated protein.

This method is as accurate as Nesslerization and far more sensitive than measurement of optical density at 280 mu, or the biuret test, yet it is far less complicated than any of the above mentioned methods.

To 0.2 ml of enzyme preparation 1.0 ml. of alkaline copper solution (50 ml of 2% Na₂CO₃ in 0.1 N NaOH and 1.0 ml of 0.5% CuSO₄.5H₂O in 1% sodium-potassium tartrate) were added. The content of the tube was mixed

well and allowed to stand for more than 10 minutes at room temperature. The 0.10 ml of 10 times diluted Folin-Ciocalteu reagent was added, followed by vigorous mixing. After 30 minutes the absorbance of the sample was read at 750 m μ , (the samples were usually diluted 3 times with distilled water).

The protein concentration of the samples were calculated from a standard curve.

A protein standard curve was obtained using a standard commercial sample of bovine serum ("Known Specimen" Lab-Trob Dade Reagent. Inc.).

Determination of the Enzyme Activity

Incubating Systems: The method of Hestrin (1949) was used in this work.

In all the experiments the assay tube contained 0.2 ml of enzyme preparation; 0.2 ml enzyme preparation and 0.3 ml 5.3×10^{-8} M DFP (final concentration 5.3×10^{-9} M) or 1.0 ml of 1.0×10^{-4} M eserine solution (final concentration 1.0×10^{-5} M). Triplicate samples were used throughout this work. All volumes were brought to 2.7 ml with the barbital buffer. The tubes were stoppered to prevent contact with CO₂ and O₂, which might have an inhibiting effect on AChE, and to decrease evaporation (Bonting and Featherstone, 1956). The tubes were placed in a steel rack which was then immersed in a water bath at 37°C. After 10 minutes, 0.3 ml of substrate (ACh buffer mixture final concentration 5.0×10^{-3} M) was added with an automatic pipette to each tube. The tubes were shaken and immediately reincubated at 37°C for one hour. Since ACh hydrolyzes in solution, five control tubes containing buffer instead of enzyme were included. Two of these contained eserine of

similar concentration as above. It was found that eserine stabilizes to a certain extent the hydrolysis of the substrate (Hestrin, 1949).

Assay

After one hour of incubation, 2.0 ml of alkaline hydroxylamine reagent were added to each tube containing 1.0 ml of the incubated solutions. The tubes were shaken. The quantitative conversion to hydroxamic acid has been found to occur after one minute of the hydroxylamine addition at room temperature. The concentration of the former remained constant for at least five hours (Hestrin, 1949). It is known that the addition of alkaline hydroxylamine to the enzyme solution brings about the termination of the enzyme assay. It is reported that the formation of ferric-acethydroxamic acid complex is maximal in the pH range of 1.2 ± 0.2 . Consequently, before adding any ferricchloride reagent the pH of the solution was adjusted so as to yield the highest optical density. The purple brown solution when too dense to give any readings at 540 m μ on the spectrophotometer, was diluted with 0.074 M of ferric chloride in 0.1 N HCl. This was necessary since the iron ion complex would break at low concentration of ferric chloride. At 540 m μ ferric chloride shows no absorption while the ferric-acethydroxamic acid complex has a peak. Iron binding anions (phosphates, sulfates, fluorides and oxalates) were avoided because of their competitive effect towards the iron ion (Lipmann and Tuttle, 1945). Their effect is usually suppressed when excess iron ions are added. The absorbancy readings obtained were then used to evaluate the activity of the enzyme preparation. The procedure used consisted of evaluating the concentration of

substrate before and after incubation. To avoid the evaluation of gas bubbles in the cuvettes, the tubes were swirled vigorously after each addition of reagent. The colorimetric readings were made with a Beckman spectrophotometer Model DU.

Brain Tissue Culture with Exogenous RNA

Brain tissue slices were first treated with versene (1 mg per 1 ml of Mg-Ringer solution containing 7.0 g NaCl, 0.2 g KCl, 0.2 g MgCl₂.6H₂O and 2 g glucose) for 30 minutes. Then they were incubated for one hour in a solution of one volume of RNA (chick brain extract and half volume of Mg-Ringer solution (pH 7.2 and final concentration 0.75 mg per one ml)). The incubation period of one hour is believed to be sufficient to allow the exogenous RNA to penetrate the brain cells.

The RNA extract was prepared* according to the modified method of McCarthy (1966). The RNA was characterized by its absorption ratio at 230 and 260 mu; the concentration of the solution was then calculated from a standard using the 260 mu wavelength absorption (N.B.C. standard RNA solution containing 1 mg per ml has an O.D. of 20 at 260 mu).

The culture vessels were 65 mm watch glasses supported on a wet cotton ring in petri-dishes (Fell and Robison, 1929). Sterile techniques were used throughout. To the watch glass fitted with a stainless steel mesh, a solution of 8 drops of horse-serum and 12 drops of TC₁₉₉ (Defco) was added till it was in contact with the metallic mesh (Dodson, 1967). The already incubated brain tissues removed from the RNA were placed on

* By Prof. J. Butros and Dr. S. Deeb at the University where the experiment was carried.

the mesh and allowed to be incubated up to 20 hours at 37.5°C.

In one control group similar brain tissues were incubated in the nucleic acid solvent (0.15 M NaCl) and in another boiled RNA solution was used.

The tissues thus incubated were removed and the AChE activity was determined in the usual manner.

CHAPTER III

RESULTS

Preliminary Experiments to Determine the Optimal Conditions for the Assay

1. Effect of Substrate Concentration on the Rate of Reaction:

The effect of substrate concentration on the rate of hydrolysis at a constant enzyme concentration was determined over the range of ACh concentration from 3×10^{-3} M to 3×10^{-2} M (Fig. 4). It was found that optimal concentration was 1.05×10^{-2} M after which a rapid drop of rate was exhibited. In this work, final substrate concentration of 5.0×10^{-3} M was used for embryos more than five days old. For embryos less than five days old the optimal concentration was found to be 3.3×10^{-3} M.

2. Effect of Enzyme Concentration on the Rate of Acetylcholine Hydrolysis: The rate of ACh hydrolysis was studied varying enzyme concentrations at two different substrate concentrations (5.0×10^{-3} M and 3.3×10^{-3} M). The rate increases proportionately with increase of enzyme concentration up to the point where substrate concentration becomes limiting (Fig. 5).

All assays were done in the linear portion of the graph.

(See Appendix C for the kinetics of enzyme reactions).

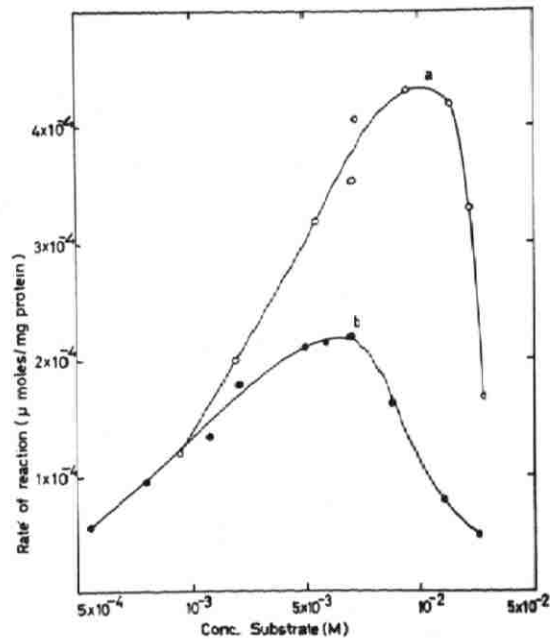


Fig. 4.- Effect of substrate (ACh) concentration on the rate of the reaction when a limited amount of enzyme (AChE) is present. (a) Effect of substrate concentration on the rate of hydrolysis by AChE prepared from chick brain homogenate of 10 day-old embryos, (protein concentration, 0.93 mg. per ml). (b) Effect of substrate concentration on the rate of the reaction when the enzyme (AChE) is prepared from chick brain homogenate of 5 day-old embryos, (protein concentration 0.24 mg. per ml).

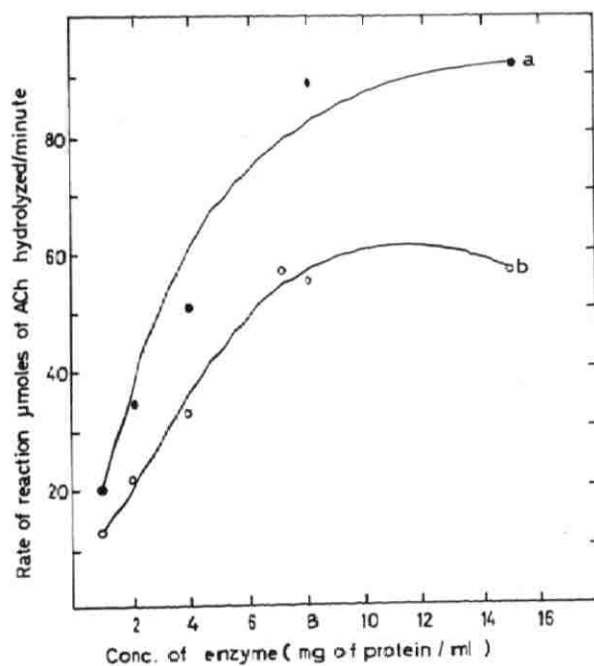


Fig. 5.- Variation of the rate of hydrolysis of the acetylcholine with enzyme concentration. (a) Represents the effect of the enzyme concentration (prepared from the chick brain homogenate of 10 day-old embryos) on the rate of hydrolysis of ACh substrate concentration 5.0×10^{-3} M. (b) Represents the effect of the enzyme concentration (prepared from the chick brain homogenate of 5 day-old embryos) on the rate of hydrolysis of ACh substrate concentration 3.3×10^{-3} M.

3. Inhibiting Effect of Eserine Concentration on the Rate of

Reaction: Eserine concentrations ranging from 2.0×10^{-7} M to 8.0×10^{-5} M were added to enzyme substrate mixture (Fig. 6). It was found that the inhibition over these two limits increased by fifteen fold and so concentrations below 8.0×10^{-5} M could be used without endangering the course of the reaction. Throughout the work a concentration of 1.0×10^{-5} M of eserine was used. Both ChE-s (AChE and ps-ChE) are inhibited at this concentration.

4. Inhibiting Effect of DFP Concentration on the Rate of Reaction:

The same experiment was repeated as above using DFP concentrations ranging from 5.0×10^{-4} M to 5.0×10^{-7} M (Fig. 7). Concentration 5.3×10^{-9} M was used throughout the work knowing that 5.0×10^{-9} M to 5.4×10^{-9} M DFP inhibited ps-ChE completely (Myers, 1953; Blaber, 1962).

5. Calibration Curves: As mentioned earlier (methods and materials), calibration curves were prepared for protein and substrate, where known concentrations of both were plotted against optical densities.

a) Protein Calibration Curve. The colours of Folin-Ciocalteu reagent with different concentrations of protein produced, were read with a spectrophotometer at a wavelength of 750 m μ and the optical density was plotted (Fig. 8). The graph shows an excellent agreement with Beer's Law. This curve was used to obtain protein concentration per milliliter.

b) Acetylcholine Calibration Curve. On the addition of ferric chloride, the different optical densities of solutions containing

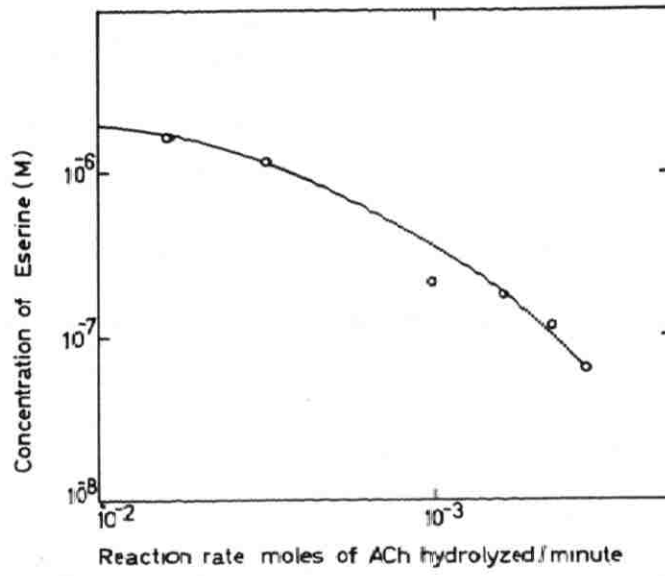


Fig. 6.- Inhibition of AChE by eserine. The reaction mixture contained of 0.96 mg. per ml protein and 5.0×10^{-3} M of acetylcholine.

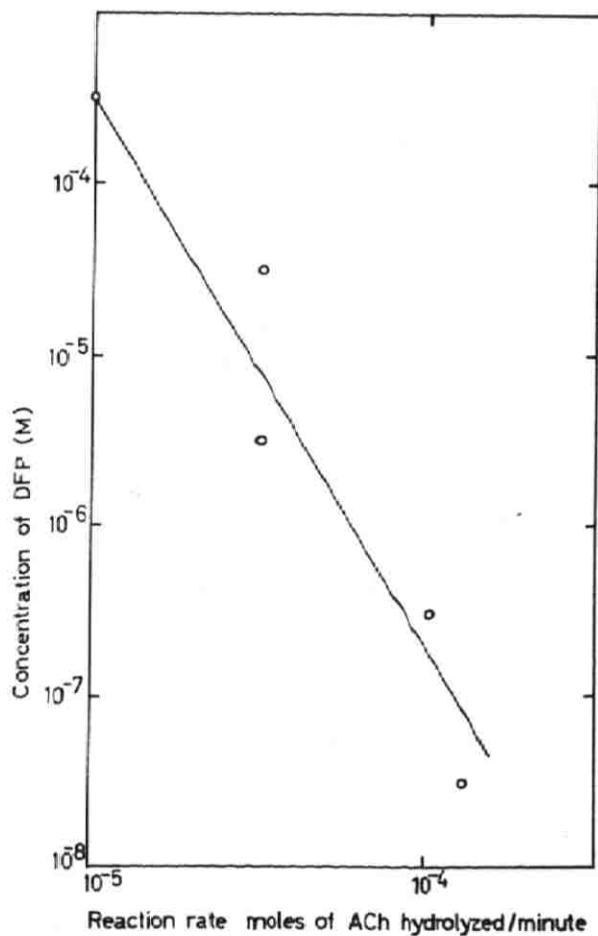


Fig. 7.- Inhibition of AChE by DFP. The reaction mixture contained 0.96 mg. per ml protein and 5.0×10^{-3} M of acetylcholine.

different amounts of ACh (quantitatively transformed to hydroxamic acid) were plotted (Fig. 9). Hydroxamic-acid-ferric complex follows Beer's Law.

Activity of AChE During Development of the Chick

Acetylcholine activity in chick embryos of different ages was determined, using a modified method of Hestrin as described earlier in this work. An example of the actual measurement of activity is included in Table 1. Enzyme unit was defined as that amount of the enzyme which will hydrolyze one millimicromole of substrate per minute under optimal conditions; specific activity was expressed as units per mg. of protein. Three eggs of each age group were used in triplicates. Averaged results obtained on AChE and ps-ChE are plotted (Fig. 10). The means (over 9 experiments) and their standard deviations are tabulated (see Table 2).

The curve shows five stages (see Fig. 10). A low activity of AChE is exhibited by embryos 2 to 5 days-old (3.10 x 4.68 units per mg. of protein). Then a sharp increase (4.68 to 14.35 units per mg. of protein) is noted between the fifth and sixth day-old embryos. This level rises gently and steadily till the fourteenth day (19,50 units per mg. of protein) after which a faster increase is observed and eventually in the eighteenth day-old embryo the AChE activity level rises steeply (30.8 to 70.2 units per mg. of protein). The increase of AChE activity between the 2 day-old and 21 day-old embryo is about twenty-fold. However, for ps-ChE the level remains low and constant until the end of the twentieth day when the level increases steeply.

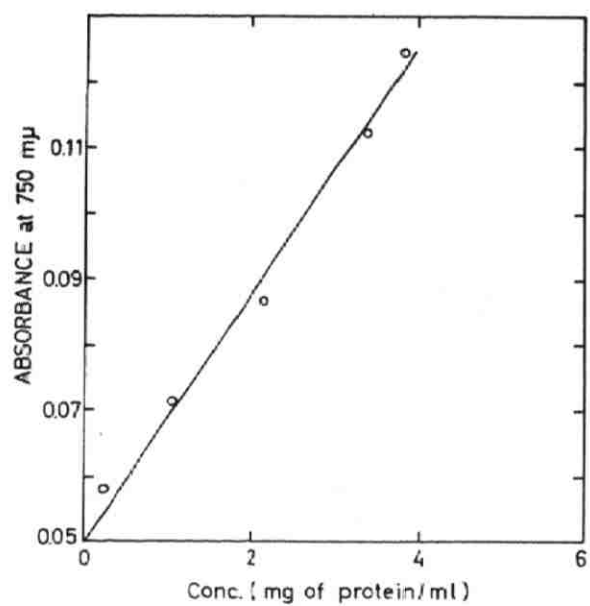


Fig. 8.- Protein calibration curve.

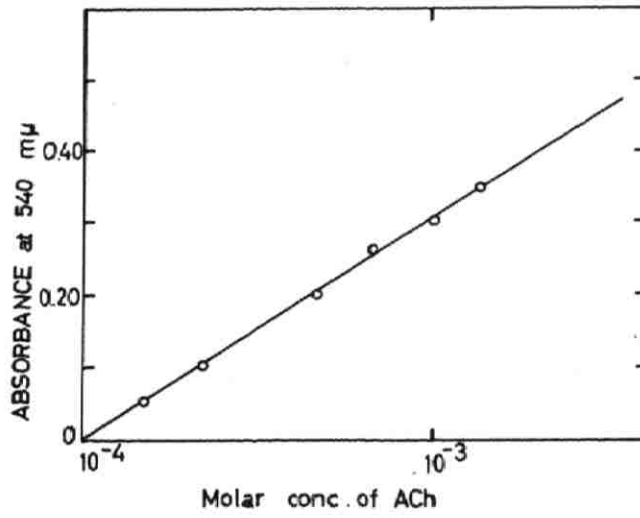


Fig. 9.- Substrate, acetylcholine calibration curve.

Effect of Exogenous RNA on the Biosynthesis of AChE

Chick brain pieces of 3 day-old embryos, treated with 0.75 mg. per ml of RNA showed no significant difference from those treated with the RNA-solvent and boiled-RNA (used as control). The specific activities were much lower than that observed earlier in the work.

TABLE I

TYPICAL ASSAY METHOD OF AChE AND ps-ChE

Incubating mixtures having a total vol. of 3 ml contained the following substrate 5.0×10^{-2} M Concentration of brain homogenate is 1.32 mg./ml of protein (5.3×10^{-9} M DFP and 1.0×10^{-5} M eserine).

SAMPLE	O.D. at 540 mu			Substrate (ACh) Conc. M 10^{-4}	Substrate (ACh) hydrolyzed umoles/hr.	Units of Activity hyd. mmoles /min.	Specific Activity units/mg. protein
	a	b	c				
S*	0.192	0.192	0.195	0.192	6.78		
S + Eserine	0.224	0.228	0.220	0.223	7.30		
S + E**	0.110	0.104	0.106	0.106	2.96	57.30	43.41
S + E + DFP	0.112	0.114	0.113	0.113	3.15	54.40	41.20
S + E + Eserine	0.200	0.224	0.191	0.205	5.32	29.70	22.50

S* - Substrate

S** - Enzyme

Specific Activity
(units/mg of protein)

43.41

2.20

18.70

AChE + ps-ChE + simple esterases

ps-ChE

AChE

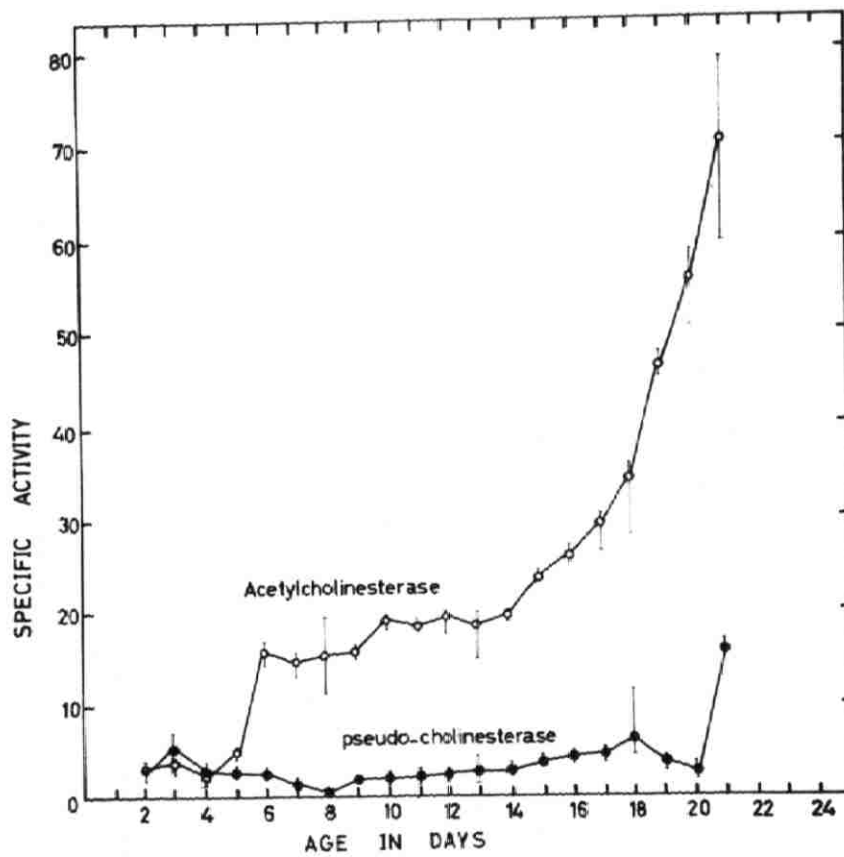


Fig. 10.- Developmental changes in the specific activity of AChE and ps-ChE in embryonic chick brain.

TABLE 2

SPECIFIC ACTIVITIES OF AChE AND ps-ChE (micromoles of ACh hydrolyzed per min. per mg. of protein) AT DIFFERENT DEVELOPMENTAL STAGES OF THE CHICK EMBRYO AND THEIR STANDARD DEVIATIONS

Age in Days	Specific Activity of AChE	Standard Deviation \pm	Specific Activity of ps-ChE	Standard Deviation \pm
* 2	3.10	0.020	3.03	0.850
* 3	3.73	0.370	5.07	0.420
** 4	2.28	0.370	3.74	0.830
5	4.68	0.054	2.77	0.660
6	15.57	1.090	3.53	0.300
7	14.35	0.960	1.13	0.380
8	15.25	3.300	0.27	0.081
9	15.45	0.640	1.83	0.054
10	18.94	0.280	2.15	0.450
11	18.26	0.260	2.07	0.630
12	19.20	0.370	2.10	0.280
13	18.45	1.500	2.99	1.200
14	19.50	0.330	2.90	0.490
15	23.50	0.490	3.93	0.450
16	25.96	0.430	4.22	1.340
17	29.20	1.670	4.10	0.400
18	30.80	3.470	6.12	1.700
19	46.17	0.820	3.50	0.560
20	55.52	4.200	2.73	0.700
21	70.20	8.010	15.80	0.220

* Each figure is the mean of triplicate estimations of three separate homogenates, and each homogenate represent pooled samples from 2 separate brains.

** Each figure (4 day and older embryos) is the mean of triplicate estimations of three separate homogenates.

CHAPTER IV

DISCUSSION

Study of the pattern of enzyme activity during development could lead to the identification of critical changes in developing organs and tissues. These critical changes reveal acquisition of new functions in tissues.

This work was undertaken in order to establish in a systematic and chronological manner such a relationship, between AChE activity and brain development in the chick. No published work so far has treated this aspect fully.

Nachmansohn (1939) studied ChE activity in brain homogenates of the chick, for a few age groups such as 15 and 16 day old embryos, newly hatched chicks and young chickens. He noted that the enzymatic activity increases up to the eight' day after hatching and then falls slightly. The work of Rogers et al. (1960) covering a wider range of development than that of Nachmansohn's, (starting with 6 day-old chick embryo) shows distinctly critical changes in the total ChE activity in chick brain parts. Unfortunately they do not differentiate between the two ChE-s. The ChE activity in the cerebral hemispheres (Appendix D) continues to rise after hatching. Whereas the activity of ChE in the diencephalon, like all other brain parts, fall down slowly before hatching. The general pattern of the former resembles the curve obtained in this work.

As they comprise a big portion of the embryonic brain, the overall activity of the brain keeps rising, even after birth.

In this work AChE and ps-ChE were differentiated and their activities were determined over a wider range of incubation (3 to 21 day-old embryos). The colorimetric method of Hestrin (1949), used in this work, has several advantages:

1. Rapidity and simplicity of the determination of enzyme activity.
2. Applicability of the method to small volumes (50 micrograms of nervous tissue).
3. The pH of the incubation medium can be varied from 5.5 to 10.0.
4. The high precision of the method: the reproducibility of the determination of acetylcholine concentration in the range of 0.01 to 0.1 micromoles is ± 0.008 micromoles and that of the ChE assay is 0.0024 micromoles per hour, in the range of 0.01 to 0.1 micromoles per hour (Bonting and Featherstone, 1956).
5. It permits the use of inhibitors, thus allowing the differentiation between the esterases.
6. Protein content can be determined simultaneously by using Lowry's method based on spectrophotometry which allows protein determinations to within 0.1%.

The colorimetric method is so standard and accurate that most clinical biochemists use it in routine analysis (Bergmeyer, 1965).

Pattern of AChE Activity During Chick Development

In Fig. 10, five activity levels are distinctly observed. A very

low level of AChE activity (3.70 to 4.68 units per milligram of protein) for 2 to 5 day-old embryos is observed. A sharp increase follows, after which a steady rising slope (15.00 to 25.96 units per milligram of protein), covering ages 6 to 14 day-old embryos, is exhibited. Then two sharply increasing slopes, one from 14 to 18 day-old embryos, the second, from 18 day-old to the time of hatching are observed (30.8 to 70.2 units per milligram of protein).

The small drop of AChE activity at ages 3 to 4 day-old embryos could be explained by the localization of the enzyme, since neuroblasts and synapses appear at the end of the third day of incubation (Lillie, 1929; Kuo, 1939). The sharp increase between the fifth and sixth day of incubation is expected, since according to Windle and Orr (1934) and Kuo (1932), reflex activity starts to develop at this stage and the enzyme AChE is thus required.

The steady rise between the sixth and the fifteenth day of incubation in the enzymatic activity, could be accounted for. This is the period when most cytodifferentiation and morphological development of organs take place. The period between the fifteenth and seventeenth day of incubation is known to be the period when general biosynthesis of biomolecules occur. Consequently, the AChE activity level increases too and the brain becomes fully developed (as found histochemically by Rogers, 1960). The AChE activity increases to a level where it allows the newly formed chick to possess full function.

Pattern of ps-ChE Activity During Development

The curve for ps-ChE (Fig. 10) does not show such interesting variations. It shows a low and more or less constant level until prehatching time. This stands from the fact that ps-ChE is not involved in nerve-impulse transmission and its function in the brain is not known (Myers, 1953; Blaber et al., 1962).

Comparing the results of the two enzymes, AChE and ps-ChE, in respect to their pattern of development, shows that there is no correlation between the activity levels. No relationship has been found between the AChE and ps-ChE activities in the rat embryonic brain (Elkes and Todrick, 1955).

The great variability of AChE activities in embryonic brain deserves comment. Other workers have observed such variations (see Appendix D). The results indicate that embryonic tissues can tolerate wide differences in enzyme activities during their development which differ by only few hours at most.

Comparison With Results of Other Investigators and Other Enzyme

Patterns of Embryonic Chick Brains

Mandel et al. (1949) studied proteins, nucleic acids and phospholipids during development using entire chick brains. Ribonucleoproteins and protein levels rise at an accelerating rate in 10 to 19 day-old embryos; in contrast to DNA which rises at a lower and steadier rate. Galactolipids, although fairly high at an early age, keep on rising between the thirteenth to sixteenth day of incubation, whereas, sphingomyelin appears suddenly at this stage (Palladin, 1955).

Curves of biochemical studies of different enzyme levels such as glutamotransferase (Rudnick and Walsch, 1955), alkaline phosphatase (Rogers et al., 1960) show that the time of sharp rise corresponds to the time of morphological differentiation of the brain and coincides with the period of the most rapid synthesis of protein, RNA and phospholipids. The enzymatic level of the two enzymes declines after the embryonic peak. Enzymes such as ATPase, succinic dehydrogenase, cytochrome oxidase, carbonic anhydrase and glutamic acid decarboxylase show an increase in enzymatic activity at a common critical period. Their activities are all at a lower level in the chick embryo (15 to 19 day-old), as compared to that in the adults (Flexner, 1955). Progressive increase of alkaline phosphatase and creatin-kinase (found in muscle and nervous tissue) levels in chick brain coincide with that of AChE (Eppenberger et al., 1964).

In comparison to the time relations for the early growth of ChE activities by different species, it is revealed that there is a correlation with their degree of development at birth. Guinea pig, sheep as well as chick, born relatively more mature anatomically and physiologically than the rabbit or rat, exhibit higher values in fetus.

Histochemical Studies and the Role of AChE in very Early Stages

AChE activity in the very early life of chick embryo (0 - 40 hours) could not be determined biochemically, since very little enzyme is found. However, the level of AChE could be determined histochemically. Zacks (1954) detected traces of the enzyme at the fifteenth hour stages in the region of Hensen's node and primitive streak before the substrate

(ACh) could be detected. However, this work does not agree with his assumption that only AChE is present at this age, since traces of ps-ChE could be detected. The possible role of acetylcholinesterase at such an early stage of the embryo's nervous system is not yet known. However, it is believed that an adequate level of the enzyme must be present before function is possible (Boell et al., 1950; Sawyer, 1943). This must be the case in the chick embryo because as mentioned earlier, AChE activity is detected in the developing nervous system before morphological differentiation of neuroblasts and before its substrate acetylcholine is detected.

According to the observations of Kuo (1939) acetylcholine does not appear in the chick nervous system until two and one-half days of incubation.

It would be interesting to study the variation of activity of AChE with morphological changes of brain parts and other nervous centers. There is no doubt that a pure preparation of AChE extracted from the brain of various age groups can lead to a more exact determination of activity.

Maynard (1966) has reported three anodically migrating zones, similar to AChE activity, which probably are comparable to the three isoenzymes of Bernsohn et al. (1964). The present curve (Fig. 10) for AChE activity represents all these isoenzymes with an overall increasing activity.

The need for more precise measures of relative activities, substrate affinities and inhibition sensitivities of multiple forms, of

chick cholinesterase (during development) by kinetic studies are suggestive.

Preliminary Experiments on the Effect of Exogenous RNA

Contrary to Niu (1963) who has reported the biosynthesis of enzymes in cultures with exogenous RNA, the brain-RNA that was used in this work could not induce the biosynthesis of AChE.

Hillman and Hillman (1967) reported that they could induce the synthesis of new proteins, when ectoderm was cultured with brain-RNA or boiled-brain-RNA. There was no response when the RNA solution was washed three times with ether. Their results suggested a Folin-positive material in RNA preparation, which was responsible for the nonspecific induction. Previous results were consistent, in that the inducer was RNA.

Many reasons could be attributed to the negative outcome of the results which could be accounted for by the short time of incubation in the RNA solution (one hour), and the low penetration of macromolecules into the cells.

Before much work is done, the RNA extraction and technique should be perfected. Experiments with radioactive material may suggest the extent and the fashion of penetration of the macromolecules (RNA) into the cultured cells.

CHAPTER V

CONCLUSION

The developmental changes in the specific activities of the enzymes, AChE and ps-ChE in embryonic chick brain have been determined colorimetrically from the second day of incubation to hatching.

Curve of AChE activity in the brain shows sharp increases at five levels.

The time of sharp rise of AChE activity approximately corresponds to the period of morphological differentiation and to the highest level of synthesis of RNA, proteins and phospholipids.

A low activity exhibited in embryos 2 to 5 day-old was attributed to the localization of the enzyme in neuroblasts and synapses which appear on the second and third day.

The sharp rise between the fifth and sixth day of incubation was attributed to the start of reflex activities.

The level of AChE activity rising steadily between 6 to 14 day-old embryos was accounted to the general biosynthesis of all biomolecules and to the cyto-differentiation of developing organs.

The very sharp increase at the fifteenth day of incubation to hatching time, was attributed to the increase in activity of the embryo in its preparation for hatching.

It is concluded that AChE, in the later stages in the development

of the brain, has some relationship to differentiation process in preparation for function. This is supported by the work of others on rats, rabbits, sheep and guinea pigs.

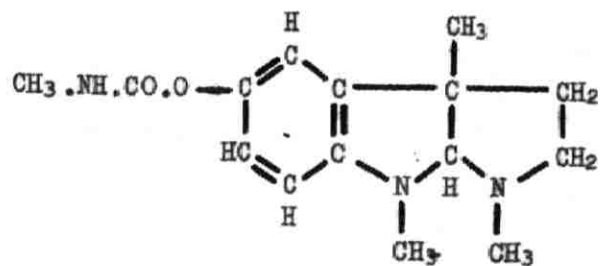
The level of activity for ps-ChE is low and shows a sharp rise only at the twentieth day, just before hatching. No relationship to AChE activity can be drawn.

Preliminary experiments on tissues cultures with exogenous RNA gave negative results. The RNA did not induce the biosynthesis of AChE in the brain of 3 day-old embryos.

CHAPTER VI

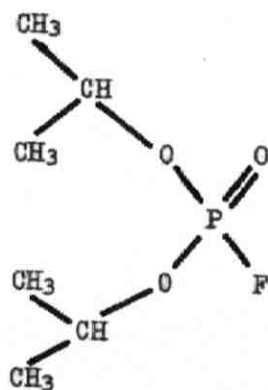
APPENDICES

APPENDIX A



Structure of Eserine

APPENDIX B



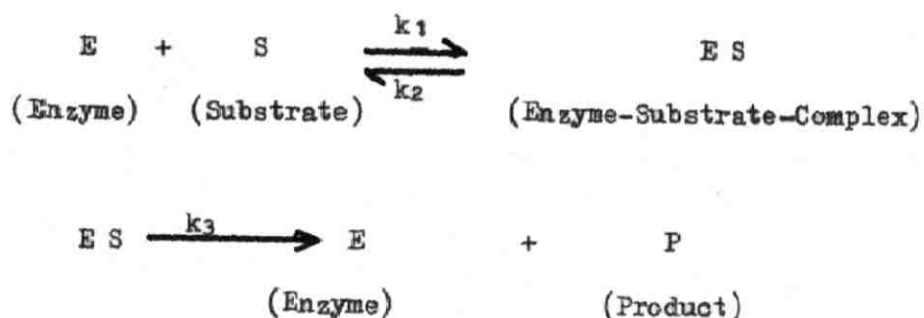
Structure of Di-isopropyl-fluorophosphate

(DFP)

APPENDIX C

Kinetics of Enzyme Reaction

As a first attempt to analyze results of enzymatic reaction Michaelis and Menten (see Laedler, 1958) postulated the following scheme.

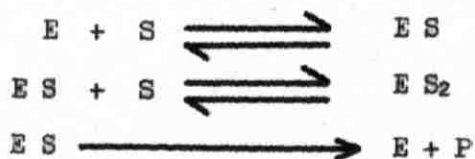


where k_1 , k_2 and k_3 are the respective rate constants of the three processes. The rate expression for these reactions is given by

$$V = \frac{k_3 E}{1 + \frac{K_s}{S}}$$

where K_s is the Michaelis constant and $K_s = \frac{k_1}{k_2 + k_3}$.

Later on and due to the pioneering work of Haldane and coworkers, other important features were discussed. It was Haldane who first noted and analyzed substrate inhibition (see Dixon and Webb, 1964).



APPENDIX C cont'd.

where $E S$ is enzyme mono-substrate and $E S_2$ is enzyme-di-substrate complexes.

$$V = \frac{k_3 [E]}{1 + \frac{K_s}{S} + \frac{K'_s}{S}}$$

where K_s and K'_s are the Michaelis constants for the enzyme-substrate and enzyme-di-substrate complex respectively.

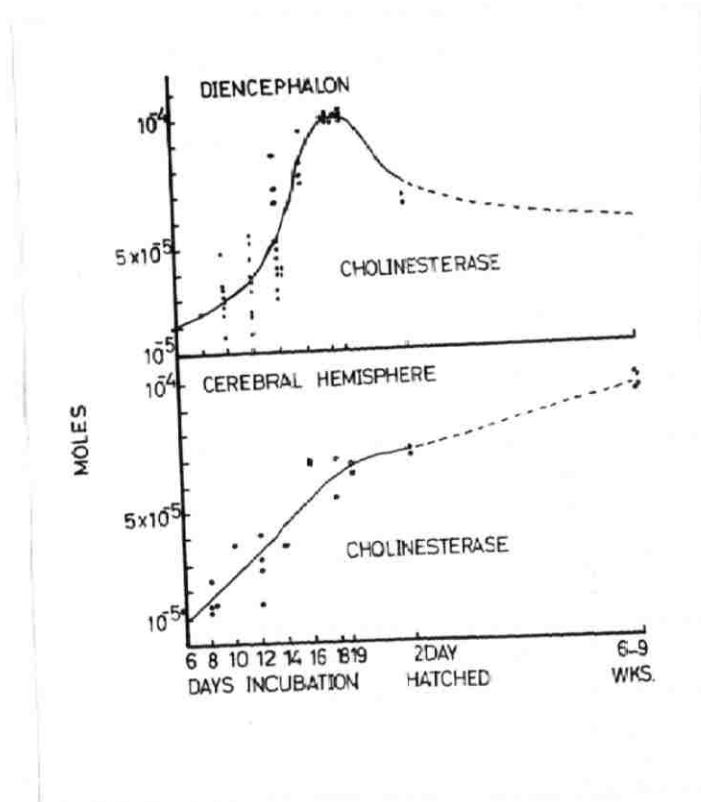
For a system of enzymes acting on one substrate, the total rate will be given by the sum of the individual rates. Thus, for the two ChE-s and other esterases acting on the same (S) the rate will be:

$$V = \frac{k_3^I [E_1]}{1 + \frac{K_1}{S}} + \frac{k_3^{II} [E_2]}{1 + \frac{K_2}{S}} + \frac{k_3^{III} [E_3]}{1 + \frac{K_3}{S}}$$

The use of inhibitors will allow the estimation of each individual rate separately.

APPENDIX D

Developmental Changes of ChE.



Moles of substrate split per hour for each milligram of tissue nitrogen plotted against developmental age of chick or chicken. (from Rogers et al., 1960, 164:96-97).

CHAPTER VII

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