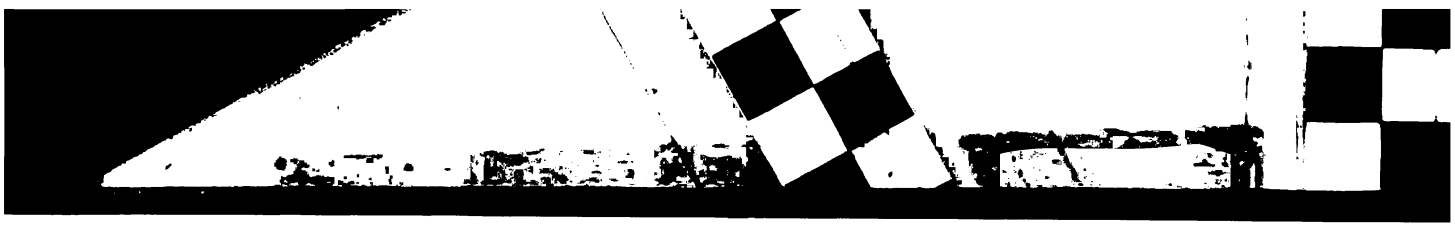
A HISTOCHEMICAL AND BIOCHEMICAL INVESTIGATION OF THE ESTERASES IN THE BRAIN AND LIVER TISSUES OF THE DEVELOPING CHICK

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY

Walter Edmond Page

1962



This is to certify that the

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A Histochemical and Biochemical Investigation of the Esterases in the Brain and Liver Tissues of the Developing Chick

presented by

Walter Edmond Page

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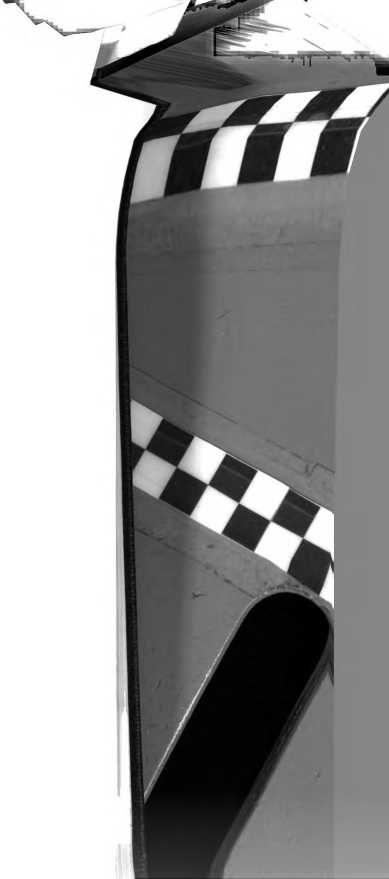
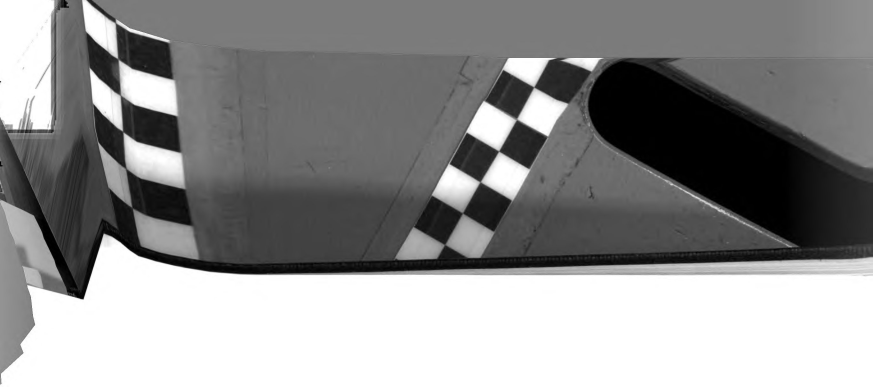




A HISTOCHEMICAL AND BIOCHEMICAL INVESTIGATION OF THE ESTERASES IN THE BRAIN AND LIVER TISSUES OF THE DEVELOPING CHICK

By

Walter Edmond Page

AN ABSTRACT

Submitted to the School of Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in Partial fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSPHY

Department of Zoology

1962

Approved Richard A. Fennell

ABSTRACT

A HISTOCHEMICAL AND BIOCHEMICAL INVESTIGATION
OF THE ESTERASES IN THE BRAIN AND
LIVER TISSUES OF THE
DEVELOPING CHICK

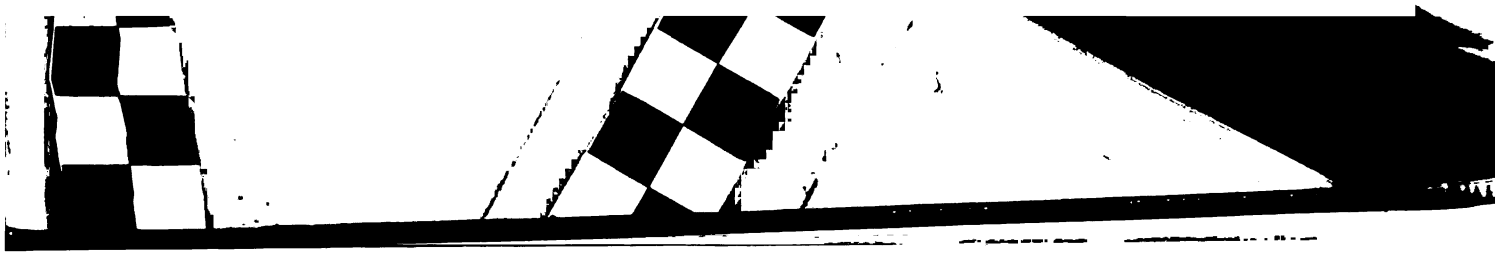
by Walter Edmond Page

A study was made of the esterase activity of the brain and liver tissue of developing chicks at three day intervals from the 9th through the 21st day of incubation. The amount of esterase activity was ascertained by use of the colorimetric procedure of Seligman and Nachlas (1950) using beta naphthyl acetate and beta naphthyl laurate substrates. Inhibitors used were sodium arsanilate, benzaldehyde, eserine sulfate, sodium fluoride, and sodium taurocholate.

Activity of both tissues with both substrates increased from the 9th to the 21st day of incubation. The activity of the liver tissue was greater than that of the brain tissue and the activity of both tissues was greater with beta naphthyl acetate than with beta naphthyl laurate.

The esterases were separated with the starch gel procedure of Smithies (1955) using both the starch and filter paper inserts. The bands were developed with alpha naphthyl acetate and Fast Garnet GBC. The inhibitors listed above were used for identification of the esterases.

Three esterases were identified in zymograms following separation of liver esterases by the starch gel electrophoresis procedure. Two esterases were identified in zymograms following the separation of the brain esterases when the homogenate was inserted into the gel strip suspended in granular starch, whereas 4 esterases were identified when the homogenate was inserted by suspending it on Whatman #3 filter paper.



The liver and brain tissues were sectioned on a cryostat-microtome for localization procedures. The tissue sections were incubated in 5-bromoindoxyl acetate substrate with eserine sulfate, sodium fluoride, silver nitrate and copper sulfate inhibitors. Sections were incubated in naphthol AS acetate substrate with eserine sulfate, sodium fluoride, and silver nitrate inhibitors. Sections were incubated in alpha naphthyl acetate substrate with eserine sulfate as the inhibitor.

Activity in the liver was very intense from the 9th to the 21st day of incubation. Activity of the brain was most intense in the medulla oblongata, medulla of the cerebellum, mesencephalon, diencephalon, choroid plexuses, brain nuclei and linings of the cavities.

The amount of protein nitrogen was ascertained by use of the Folin-Ciocalteu colorimetric procedure. The results were about 12% lower than those reported from an acid-digestion and iodine titration procedure.

Relating the results of this investigation with the concept of chromosomal differentiation, the evidence indicates the possibility that any chromosomal differentiation associated with the production of esterases in the brain of the developing chick has been completed by the 9th day of incubation. However, it appears that any chromosomal differentiation associated with the production of esterases in the liver of developing chick may not be completed by the 5th week of posthatching development.

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CURRICULUM VITAE

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Doctor of Philosophy

Final Examination: August 3, 1962

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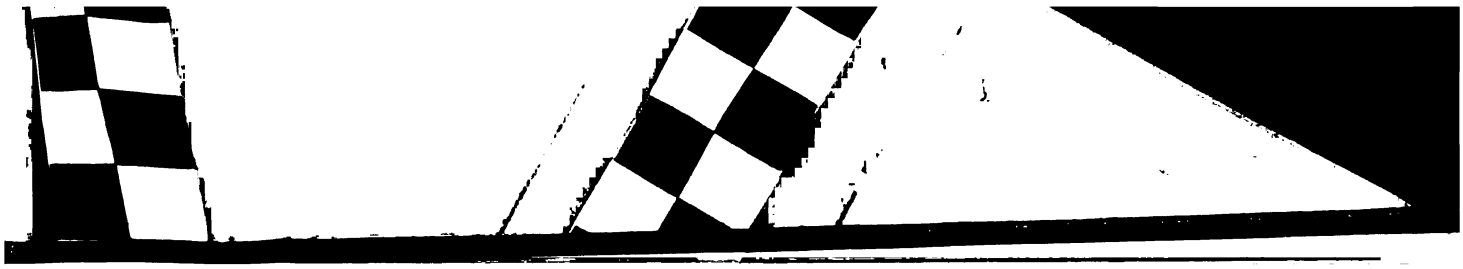

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INTRODUCTION

For many years the chick embryo has been used for the study of early embryonic development, for biochemical studies and for tissue and organ culture. However, a review of the literature reveals that the chick embryo has been used to a limited extent for histochemical investigations.

Tixier-Vidal (1954) studied the pituitary gland in relation to the production of the thyrotropic hormone. Histochemical results lead to the conclusion that the McManus positive cells (cells staining with periodic acid Schiff reagent) in the anterior extremity of the cephalic lobe are the cells which produce this hormone. Thommes (1960) was able to demonstrate insulin in beta granular cells of the pancreas which were sparsely distributed throughout relatively undifferentiated cellular components at 7 days of incubation. Grillo (1961a) found protein-bound sulfhydryl groups in beta cells of the islets of Langerhans of the pancreas on the 14th day of incubation. Bufio (1951) found the reaction for the sulfhydryl group very strong in the mesoblastic crescent, Hensen's node, anterior and posterior intestinal portals, neural plate, neural fold and neural tube in the period from the blastoderm to the 18 somite stage.

De Gannaro (1959) studied the glycogen body (located on the dorsal side of the spinal cord at the level of the sciatic plexus) finding that it made its first appearance at 7-8 days of incubation. Luppa (1959) found that no glycogen was stored in the epithelium of the muscular and

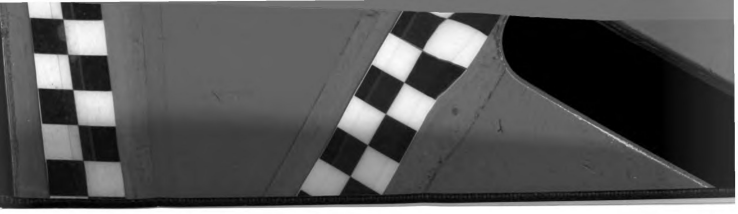
glandular stomachs during the ingestion of the albumen from the 13th to the 18th day of incubation. Van Alten and Fennell (1957) included some histochemical studies in their work on the digestive tract of the developing chick where they found mucopolysaccharides in the differentiated tissues. Reactions in the undifferentiated muscular, epithelial and connective tissues was due, in part, to glycogen. Metachromatic granules of unknown composition were found in the cardiac jelly of the truncus arteriosus, and ventricle of the chick embryo from the 2nd to the 3rd day of incubation (Barry, 1951).

Hancox (1954) found a strong alkaline phosphatase activity in the free border of the epithelial layer of the duodenal loop of the chick at 12 days of incubation. Moog (1944) found that alkaline phosphatase activity is usually quite high during rapid cell division and differentiation of tissues. Between 4 and 8 days of incubation, alkaline phosphatase of the liver parenchyma decreased while that of the liver endothelium increased. Moog and Richardson (1955) were able to induce precocious phosphatase activity and accelerate differentiation of duodenal epithelial cells by injecting corticoid hormones into the chorio-allantoic vesicle. Glycogen deposition in the epithelial cells increased prior to the differentiation of the digestive tube and then disappeared. Moog (1943) found alkaline and acid phosphatase in the neural tube on the 1st day of incubation. The alkaline phosphatase became localized in the white matter throughout the tube and in the gray matter and ependymal cells of the ventral half of the cord with the exception of the motor neurons. The acid phosphatase became restricted to the ventral half of the cord especially in the motor groups. The large cells of the spinal ganglia contained large amounts of both phosphatases. Hinsch and Knovacs

(1960) found alkaline phosphatase highly active in the mesenchyme surrounding the tracheal epithelium; but subsequent to the development of the cartilage enzyme activity was markedly lowered and was found only in the epithelial layer. The walls of the developing esophagus exhibited intense alkaline phosphatase activity until this organ differentiated and then it disappeared. Koning and Hamilton (1954) demonstrated that alkaline phosphatase, RNA, polysaccharides, cytochrome oxidase, succinic dehydrogenase, and tyrosinase were associated with the developing down feather. Junqueria (1952) found that the alkaline phosphatase of the mesonephros increased from the 4th to the 16th day of incubation then decreased, whereas in the metanephros it increased from the 11th to the 16th day, then decreased to a minimum on the 20th day of incubation.

Guha and Wegmann (1961) concluded from their work that the liver of the developing chick contained an active form of phosphorylase only. Grillo (1961b) demonstrated phosphorylase in cardiac muscle, liver and skeletal muscle on the 3rd, 7th and 13th days of incubation respectively. Also, glycogen was localized in the liver, cardiac muscle, and skeletal muscle on the 6th, 2nd and 3rd days of incubation respectively. Bot et al. (1960) found phosphorylase and phosphoglucomutase to be formed in large amounts in skeletal muscle from the 17th to the 21st days of incubation, whereas phosphorylase was present in the liver and cardiac muscle in large amounts even in the early stages of development. Billett and Mulherkar (1958) were able to demonstrate beta glucuronidase as early as the headfold stage at which time it was widely diffuse in the dorsal side of the embryo and adjacent to the area opaca. Later, it was found in the region of the somites and neural tube.

Kato (1959) showed that phosphomonoesterases of the duodenum,



liver, mesonephros and metanephros which were induced by injections of phenylphosphate into the chorioallantoic vesicle were localized in essentially the same places as that of the naturally occurring enzyme.

Buño and Mariño (1952) found that subsequent to the 3rd day of incubation lipase was identified in the liver and gut, whereas in the pancreas, it appeared on the 4th day of incubation. Other sites of lipase activity were mesenchyme of the notochordal sheath, endocardial cushion, precartilagenous blastema of the body and limbs, adipose tissue, mesonephric tubules, metanephros, genital ridge and white matter of the neural tube. George and Iype (1959) demonstrated lipase in the cardiac muscle of the chick embryo and suggested that there was a correlation between lipase activity and the heart rate.

Bonichon and Gerebtzoff (1958) found that nerve fibers which remained amyelinated retained part of the acetylcholinesterase, whereas the formation of a myelin sheath was preceded by an increase in the concentration of this enzyme at the synapse. Bonichon (1958) identified acetylcholinesterase in the neuroblasts of the optic lobe and medulla oblongata subsequent to the 4th day of incubation prior to morphological differentiation. When the enzyme was first identified, it was uniformly distributed throughout the cytoplasm; then it became concentrated at the periphery and ultimately migrated the length of the dendrite to the synapses.

Richardson, Berkowitz and Moog (1955) found esterase activity in the duodenal epithelium to be uniformly distributed on the 10th day of incubation; from the 14th to the 19th days of incubation intense activity was seen in the epithelium adjacent to the lumen of the duodenum. The underlying connective tissue showed some activity at the 10th day and

decreased until none was observed at 19 days of incubation. Zacks (1954) studied the esterase activity in the chick embryo through 96 hours of incubation. There was no esterase activity found in the unfertilized and unincubated eggs. He found activity in the anterior crescent, Hensen's node, primitive streak, neural folds, proamnion, extraembryonic areas, neural tube, somites, amnion, yolk sac entoderm, foregut, hindgut, retina, lens, heart and notochord.

The purpose of the present investigation was to study the esterases in the brain and liver tissue of the developing chick from the 9th to the 21st day of incubation. A quantitative study of esterase activity was made by the colorimetric procedure of Seligman and Nachlas (1950). A separation of the esterases was made by the starch gel electrophoresis procedure of Smithies (1955). A localization in the tissues was made by using the 5-bromoindoxyl acetate procedure of Holt and Withers (1952); the naphthol AS acetate procedure of Shnitka and Seligman (1961); and the alpha naphthyl acetate procedure after Gomori and Chessick (1953). A classification of the enzymes by substrate specificity and reaction to inhibitors was based on the procedures of Gomori (1952).

MATERIALS AND METHODS

Quantitative determination of esterase activity. The colorimetric procedure for the quantitative determination of esterase activity was essentially the same as that used by Seligman and Nachlas (1950). A 0.1M phosphate buffer with a pH of 7.4 was used instead of the Veronal buffer because the latter began to precipitate soon after it was mixed.

The homogenates for each test were prepared from the liver and brain tissue of five embryos in order to minimize differences due to individual variations. The brain and liver were removed from the embryos, weighed and homogenized in a glass tissue grinder with enough phosphate buffer to make a 1:10 dilution. Aliquots of the 1:10 dilutions were diluted with more buffer to give a final dilution of 1:1200 for the liver and 1:300 for the brain. These dilutions were necessary to bring the intensity of the colored solution within the range of the colorimeter.

For each test which was replicated the following conditions were used: tube 1 had 1.5 ml of distilled water; tubes 2-7 each had 0.2 ml of tissue homogenate; tube 2 had 1 ml of distilled water; 3 had 1 ml sodium arsanilate (40 mg/ml); 4 had 1 ml benzaldehyde (1 ml/350 ml H_2O); 5 had 1 ml eserine sulfate (5 mg/ml); 6 had 1 ml sodium fluoride (30 mg/ml) and 7 had 1 ml sodium taurocholate (8.9 mg/ml). The homogenate was exposed to the inhibitors at room temperature for 30 minutes prior to addition of the substrate. Five ml of substrate prepared according to Seligman and Nachlas (1950) was added to each tube. When beta naphthyl acetate was used as the substrate, the mixture was allowed to incubate for 30 minutes at room temperature. When beta naphthyl laurate was used, the mixture was allowed to incubate for 4 hours at 37° C.

After incubation was completed, the tubes were placed in an ice

water bath. One ml of a coupler solution composed of Naphthanil Diazo Blue B dissolved in ice water (4 mg/ml) was added to each tube and mixed by vigorous shaking. Two minutes after addition of the coupler, 1 ml of 40% trichloroacetic acid was added to each tube and mixed. The azo dye, produced by coupling beta naphthol (released from beta naphthyl acetate by esterase activity of the homogenate) with the diorthoanisidine in the Naphthanil Diazo Blue B, was extracted by the addition of 8 ml of ethyl acetate to each of the tubes. The ethyl acetate was measured with a 50 ml burette equipped with a Teflon valve. The tubes were again shaken vigorously and centrifuged to separate the azo dye-ethyl acetate solution from the aqueous solution. The colored extract was drawn off with a pipette and transferred to separate test tubes.

The intensity of the color was ascertained with a Bausch and Lomb Spectronic "20" colorimeter at a wavelength of 450 millimicra using the solution from tube 1 to adjust the colorimeter to 100% transmittance.

When it was discovered that the color of the azo dye faded on exposure to light, the tubes were kept in a rack provided with a hinged-top aluminum cover and tests following incubation were performed in a room with the light intensity reduced.

In order to convert the colorimeter readings to mg of beta naphthol released, a calibration curve was made by using pure beta naphthol of graded concentrations ranging from 0.02 to 0.18 mg at intervals of 0.02 mg. The volumes of the reagents used with each increment of beta naphthol were essentially the same as those used in the experiment except that distilled water was substituted for the tissue homogenate. Additional calibration curves were made following the addition of inhibitors to the substrate solution. Concentrations of beta naphthol used with the

inhibitors ranged from 0.01 to 0.09 mg at intervals of 0.02 mg.

The colorimeter readings obtained following incubation of tissue homogenate were converted to mg of beta naphthol released by the use of the calibration curves, and these figures in turn were converted to millimoles (mM) of beta naphthol released per mg of tissue (wet weight) per hour by the use of the following formula (Nachlas and Seligman, 1949a):

$$\frac{\text{mg beta naphthol} \times 1000}{\text{mg tissue (wet weight)} \times \text{time (hours)} \times \text{mol. wt. beta naphthol}}$$

Separation of the esterases with starch gel. Separation of the esterases was accomplished with the starch gel procedure of Smithies (1955). The starch gel was prepared from hydrolyzed potato starch and concentrated borate buffer obtained from Connaught Laboratories, Toronto, Canada. The buffer was made by diluting 8.8 ml of concentrated borate buffer to 200 ml with distilled water. Twenty-four and eight-tenths grams of starch were added to the buffer and immediately shaken so that no sticky lumps would be formed since they do not always dissolve on heating.

The starch-buffer mixture, in a 500 ml Pyrex suction flask, was heated over a Bunsen flame with constant shaking until it gelated and then solated. The latter occurred in about 3 minutes. The flask was removed from the flame, stoppered and evacuated by use of an aspirator to remove all air bubbles.

Enough liquified gel was poured into 4 plastic trays with inside dimensions of 0.6 x 2.3 x 23 cm so that each tray had an excess. The plastic covers were applied by lowering one end into contact with the gel and carefully lowering the other end so that no air bubbles were trapped. The excess gel was forced out by holding one end of the cover

in place and running the thumb and forefinger of the other hand firmly along the edges of the plastic cover. The cooled strips were refrigerated overnight. By so doing the gels were thoroughly cooled and the electrophoresis could be started early in the succeeding day.

Both the starch suspension and filter paper insert methods were used. For the starch suspension method a slot 1.5 mm wide was cut in the gel 6.0 cm from the cathode end with two single edged razor blades soldered together. The homogenate was prepared by homogenizing the tissue with enough distilled water to make a 1:11 dilution for the brain tissue and a 1:101 dilution for the liver tissue. Iodimetry starch was used to make a mixture just fluid enough to flow through a glass pipette with a 1.5 mm bore at the tip.

For the filter paper insert method a 1:11 dilution homogenate was used for both tissues. Whatman #1 paper was used for the liver homogenate and Whatman #3 paper was used for the brain homogenate. A slot 6.0 cm from the cathode end was cut with a single razor blade and widened by pushing the gel toward the cathode end. The filter paper, saturated with homogenate, was inserted into the slot, the razor blade was removed and then the cut surfaces of the gel were pressed firmly against the paper. The slots were sealed with melted vaseline in both methods.

Filter paper wicks for electrical contact between the gel strips and bridge solution were made from 3 thicknesses of Whatman #1 filter paper. After the wicks were in place, the trays were wrapped in Saran Wrap to prevent evaporation.

Two Pyrex baking dishes 1" x 6" x 10" were used for the bridge solution composed of 10.384 g of boric acid, 1.344 g sodium hydroxide and 800 ml distilled water.

The electrical current was supplied by a Heath Kit variable-voltage-regulated power supply model PS-3. Platinum electrodes were dipped into the bridge solutions. A current with a potential of 140 volts (6v/cm) was applied for 6 hours.

At the end of the migration period the strips were sliced once horizontally and once vertically so that four strips 3 x 11 mm were obtained from each tray. Notches cut in the strips provided an identifying code. Test tubes 25 x 200 mm were used as containers for developing the strips. One slice of starch gel with liver esterases and one with brain esterases were placed in 60 ml of 0.2 M phosphate buffer having a pH of 7.4. Also, one slice of gel for each tissue was placed in an inhibitor solution. The inhibitor solutions were made by dissolving the inhibitor in 60 ml of buffer. The following final concentrations of inhibitors were used: eserine sulfate 1×10^{-3} M; sodium arsanilate 1×10^{-1} M; sodium fluoride 7.14×10^{-2} M; sodium taurocholate 1.6×10^{-2} M; and benzaldehyde 2.67×10^{-2} M. After the gel strips were in the inhibitors at room temperature for 30 minutes, 2.5 ml of alpha naphthyl acetate substrate (4 mg alpha naphthyl acetate per ml acetone) were added to each tube and mixed by inverting the corked tubes. The tubes were incubated for 30 minutes at room temperature.

The gel strips were then rinsed in buffer and placed in a coupling solution composed of 400 mg of Fast Garnet GBC Salt (o-amino-azo-toluene) dissolved in 500 ml of phosphate buffer. After 30 minutes the gel strips were washed in tap water, put on a clear plastic platform and photographed with transmitted light using a green filter and Kodak Panatomic X film. The film was developed in Kodak D-11 developer and the prints were made with Kodabromide F-3 paper developed in Kodak D-72 developer.

Disc Electrophoresis. The buffer reservoirs were two 6-inch polyethylene refrigerator trays cut down to a 2.5 inch depth. Twelve 3/8th inch holes were drilled in the bottom of one reservoir so that they formed a 5-inch circle and the spaces between adjacent holes were equal. The holes were then lined with rubber grommets having 1/4 inch holes. Both reservoirs were equipped with a carbon electrode mounted in the center of the reservoir.

The tubes for holding the gel were glass tubing 63 mm long with an inside diameter of 5 mm. While the tubes were being prepared with the gel, a rubber serum vial cap was placed on one end of each tube. A capillary pipette was used to fill the tubes and a capillary pipette with a piece of string protruding through the bore was used to overlay the gel layers with distilled water. The string made possible a gentle stream of water which would minimize mixing the water with the monomer solutions. A 1-ml tuberculin syringe provided with a 20-gauge needle and a short length of polyethylene tubing was used to measure the large pore solution-tissue homogenate mixture into the tubes.

The tubes were filled up to 15 mm from the top with the small pore solution, overlayed with distilled water, and allowed to polymerize at room temperature for 30 minutes and at 37° C for 15 minutes. The water was poured out, the empty space was rinsed with large pore solution; a 3 mm layer of large pore solution was added and overlayed with distilled water. This layer of gel was polymerized by exposing it to a fluorescent light at a distance of 2 inches for 15 minutes. The water was again poured off and the remaining space was rinsed with large pore solution.

While the first layer of large pore solution (spacer layer) was

polymerizing, the large pore-homogenate mixture was prepared. The tissue was homogenized with an equal amount of distilled water and 0.02 ml of homogenate was mixed with 1 ml of large pore solution. A .15 ml aliquot of this mixture was added to each tube, overlayed with distilled water and polymerized by exposure to fluorescent light for 20 minutes.

After this last gel layer had polymerized, the tubes were removed from the rubber caps and the sample insert ends inserted into the rubber grommets of the buffer reservoir. This reservoir was set on a ring stand over the second reservoir. Each reservoir was filled with 400 ml of buffer composed of 0.6 g of Tris and 2.88 g of glycine per liter of solution. One ml of Bromphenol blue (0.001%) added to the upper reservoir provided a visible means of following the progress of migration. The upper reservoir was lowered until the tubes were immersed in the lower buffer solution to a depth of about 5 mm. The electric current was provided by the same power supply used for the starch gels. A current of 2 milliamperes per tube was applied for 45 minutes.

After migration was accomplished, the gels were removed by immersing the tubes into distilled water and loosening the gel at both ends by sliding a dissecting needle around between the gel and the glass. The esterases were demonstrated by developing the bands in a substrate solution composed of 50 ml of 0.2 M phosphate buffer with a pH of 7.3, 2 ml of alpha naphthyl acetate (4 mg per ml of acetone) and 80 mg of Fast Garnet GBC. The gels for the liver homogenate were developed for 15 minutes and those of the brain, for 30 minutes at room temperature. They were then placed in 7% acetic acid for fixing.

Localization of esterases. Localization of the esterases was accomplished by cutting frozen sections on a cryostat-microtome, fixing

in calcium-formol solution and using three different developing procedures.

Excess tissue from previously-used embryonic tissue was used to make an elevated base on the tissue carrier for the tissue to be cut. The brain was cut in half along the mid-sagittal line and the cut surface laid against the base tissue so that a sagittal section could be obtained. A piece of liver tissue from the same embryo was laid along the dorsal surface of the brain so that both liver and brain tissue could be obtained in the same section.

The tissue was quickly frozen by almost completely submerging it in isopentane cooled with solid CO_2 and acetone. If the tissue was completely submerged, the optic lobe would rupture. After the tissue was frozen, it was wrapped in Saran Wrap, placed in a tightly covered jar and stored in a deep freeze at -20° C until time for cutting.

The tissue was cut on an International Equipment Co. cryostat-microme with a chamber temperature of -10 to -15° C. The sections, 8 micra thick, were taken from near the mid-sagittal plane of the brain.

The tissue sections were picked up on 22 mm square glass cover slips which were kept at room temperature. The tissue was allowed to air dry so that it would adhere to the coverslip during the developing procedure. When the tissue was dry, it was fixed in cold calcium-formol fixative (1% calcium chloride in 4% formaldehyde).

5-Bromoindoxyl acetate procedure. The procedure of Holt and Withers (1952) was used, but with the ferro-ferricyanide redox buffer system reduced to 5×10^{-4} M, as recommended by Shnitka and Seligman (1961) for use following formalin fixative. The following formula was used:

5-bromoindoxyl acetate	1.3 mg
Ethanol	0.1 ml
0.2 M Tris (hydroxymethyl) amino methane/ HCl buffer (pH 7.2)	2.0 ml
0.05 M-potassium ferricyanide	0.1 ml
0.05 M-potassium ferrocyanide	0.1 ml
0.1 M-calcium chloride	1.0 ml
Distilled water to make 10 ml	

With the first series using eserine sulfate 1 x 10^{-3} M and sodium fluoride 7.14 x 10^{-2} M along with the control, the inhibitors were incorporated into the substrate medium without first exposing the tissue to inhibitor solutions alone. With the second series using silver nitrate 1 x 10^{-2} M and copper sulfate 1 x 10^{-3} M as well as a control, the control section was placed in distilled water and the other sections in their respective inhibitors for 30 minutes at room temperature. Then they were placed in the substrate for 30 minutes at 37° C. Inhibitors at the same final concentrations as above were incorporated into the substrate medium.

The tissue was rinsed in distilled water, counterstained in Mayer's Carmalum for 2 minutes, rinsed quickly in distilled water, and mounted on a slide with glycerine jelly.

Solutions were contained in plexiglass trays made to accomodate 6 coverslips.

Alpha naphthyl acetate procedure. The alpha naphthyl acetate procedure was modified after Gomori and Chessick (1953). The fixed tissue sections were developed in a medium composed of 10 ml of 0.1 M phosphate buffer having a pH of 8.1, 0.2 ml of 1% alpha naphthyl acetate in 50% acetone, 10 mg Naphthanil Diazo Blue B. This medium was mixed immediately prior to use and filtered quickly by suction with a Buchner funnel. Eserine sulfate with a final concentration of 1 x 10^{-5} M was incorporated into the medium for the inhibited section. Uninhibited

controls were also made. The sections were developed for 30 minutes at room temperature, rinsed in tap water, and mounted without counterstaining in glycerine jelly.

Naphthol AS acetate procedure. The naphthol-AS acetate procedure was that of Shnitka and Seligman (1961) using Fast Violet LB Salt (diazotized-5-benzamido-4-chloro-2-toluidine) as the coupling agent. Here again, the substrate medium was mixed immediately prior to use and quickly filtered. The inhibitors used were eserine sulfate, 1×10^{-4} M, sodium fluoride, 7.14×10^{-2} M; and silver nitrate, 1×10^{-2} M. The inhibited sections were exposed to the inhibitors in distilled water as well as having the inhibitors incorporated into the substrate medium. They were developed for 15 minutes at room temperature. After a tap water rinse, they were counterstained for 30 seconds with Harris' Hematoxylin diluted 1:4 with distilled water and no acetic acid. These sections were also mounted in glycerine jelly.

Determination of protein nitrogen. The amount of protein nitrogen in the brain and liver tissue was ascertained by the Folin-Ciocalteu colorimetric procedure (Litwack, 1960). The tissue was homogenized with enough distilled water to make an initial dilution of 1:10. Aliquots of the 1:10 dilutions were further diluted to 1:500 of which 0.5 ml samples were used in the tests. Since the number of tests and number of embryos varied with the age groups, these figures are given in table 14. A turbidity control was made by diluting 0.5 ml samples of the homogenate with 5.5 ml of distilled water (the same volume as that of reagents used in the tests) and reading the amount of absorbance at the same wavelength as for the tests.

The readings for the tests and controls were made in percent

transmittance and converted to optical density by a conversion table. The optical density of the turbidity control was subtracted from that of the tests for the liver. The revised figures were converted to micrograms of protein nitrogen by use of a standard curve. Multiplying the number of micrograms by the dilution factor and dividing by 1,000 gave the number of milligrams of protein nitrogen per gram of wet weight of tissue. Since the amount of absorbance by the turbidity control of the brain tissue was insignificant, no corrections were made.

The standard curve was made by the use of bovine albumen obtained from Armour Pharmaceutical Co. The assay value was 10.2 mg of protein nitrogen per ml. A graded series of dilutions from 10.2 to 81.6 µg protein nitrogen per ml of solution was used in duplicate tubes and processed in the same manner as the tests. The average values for the two tubes were plotted on linear-linear graph paper.

RESULTS

Quantitative determination of esterase activity. The results of the quantitative determination of esterase activity are recorded in tables 1-8 inclusive and figures 4 7 inclusive. The standard curves for converting percent transmittance to mg beta naphthol released are recorded in figures 1-3.

With beta naphthyl acetate as the substrate, the activity of the uninhibited liver tissue increased sharply from the 9th day to the 18th day, then increased only slightly on the 21st day (Table 2). Benzaldehyde produced only a slight amount of inhibition with the activity paralleling very closely that of the control to the 18th day. Inhibition was somewhat more marked at the 21st day. Sodium taurocholate reduced activity to 30-45% of that of the control; sodium arsanilate to 20-30%; eserine sulfate, to 17-27% and sodium fluoride, to 9-13% of the control.

With beta naphthyl laurate as the substrate, the activity of the uninhibited liver tissue increased gradually from the 9th to the 21st day (Table 4). Benzaldehyde and sodium taurocholate produced complete inhibition in homogenates at 9 days and allowed activity to increase to a maximum of 60% of that of the control on the 21st day. Sodium arsanilate produced complete inhibition on the 9th, 18th, and 21st days, and allowed a maximum activity of 17% on the 12th day. Eserine allowed a maximum activity of 33% at 15 days and reduced it to 4% on the 21st day. Activity of the liver tissue with beta naphthyl laurate was about 1% of that of liver tissue with beta naphthyl acetate as the substrate.

The activity of the uninhibited brain tissue homogenate increased gradually from the 9th to the 15th days and then more sharply to the

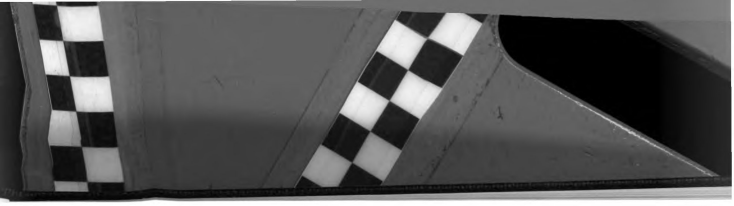
21st day (Table 6). Sodium arsanilate reduced activity to 85-90% of that of the control; benzaldehyde, to 20-63%, eserine sulfate, to 13-22%; sodium taurocholate, to 5-17%, and sodium fluoride produced almost complete inhibition.

Activity of brain tissue with beta naphthyl laurate as a substrate increased only very slightly from the 9th to the 21st day of incubation (Table 8). The total activity was very low. Activity with sodium arsanilate was 5-8% of that of the control, with eserine sulfate, 50-95%; with sodium fluoride, 53-67%. Sodium taurocholate produced complete inhibition from the 9th to the 21st day. Benzaldehyde produced complete inhibition from the 9th to the 15th day and allowed the activity to increase to 13% on the 21st day.

Activity of the brain tissue with beta naphthyl laurate as the substrate was about 5% of that with beta naphthyl acetate. Also, it was about 15% of that of the liver tissue with the laurate substrate. The brain tissue with beta naphthyl acetate substrate had an activity of 5% of that of the liver under comparable conditions.

Starch gel electrophoresis. The results of the starch gel separations are recorded in tables 9 and 10 and pictured in plates 1-10 inclusive. Using liver tissue and the starch suspension insert method, band A was present from the 9th day to the 21st day. This band was not affected by eserine sulfate. It was slightly inhibited by benzaldehyde on the 12th day and by taurocholate on the 9th day. It was completely inhibited by sodium fluoride and sodium arsanilate.

Band B first became visible at 15 days and increased to its maximum on the 18th day. This band was not affected by eserine sulfate or sodium taurocholate. It was completely inhibited by sodium fluoride and sodium arsanilate from the 9th to the 21st day.



Band C was first present on the 12th day and increased to its maximum intensity on the 18th day, then decreased slightly on the 21st day. This band was not affected by eserine sulfate or sodium taurocholate. It was inhibited by sodium fluoride and sodium arsanilate from the 9th to the 21st day.

When the filter paper method was used with the liver tissue, band A reached its maximum at 15 days. The rest of the results were the same as for those of the starch insert method.

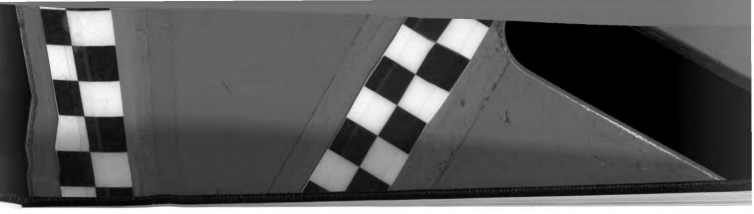
Band B was faintly discerible from the 15th through the 21st day. Its reactions to the inhibitors were the same as for those of the starch insert method. Band C exhibited its maximum intensity from the 12th through the 21st day. It was completely inhibited by sodium fluoride and sodium arsanilate, but was not affected by eserine sulfate, sodium taurocholate or benzaldehyde.

With brain tissue and the starch insert method, band A was faintly discernible from the 9th to the 21st day. It was not affected by eserine or benzaldehyde. It was completely inhibited from the 9th to the 21st day by sodium fluoride. Sodium arsanilate completely inhibited this band from the 15th to the 21st day. Sodium taurocholate inhibited this band on the 12th day.

Band B was faintly discernible from the 9th to the 21st day. It was not affected by eserine sulfate, sodium taurocholate, or benzaldehyde. It was completely inhibited by sodium fluoride from the 9th to the 21st day and by sodium arsanilate from the 15th to the 21st day.

Bands C and D did not appear as distinctly discernible bands with the starch insert method.

When the filter paper insert method was used with brain tissue,



bands A and B were present on the 9th day, only very weakly discernible on the 12th day and present as indistinct shadows on the 15th to the 21st days of incubation. They were completely inhibited by sodium fluoride but not by sodium taurocholate, eserine sulfate, sodium arsanilate or benzaldehyde.

Band C was present from the 9th to the 21st day. It was completely inhibited by eserine sulfate and sodium fluoride but not by sodium taurocholate, sodium arsanilate or benzaldehyde.

Band D was present from the 9th day and attained its maximum intensity at 21 days. It was completely inhibited by eserine sulfate and sodium fluoride. It was not affected by sodium taurocholate, sodium arsanilate or benzaldehyde.

At 5-weeks posthatching, no new bands were obtained with brain tissue and band A was no longer visible. With liver tissue a new band D was present and was inhibited only by eserine sulfate. Bands A and C were still present but band B was not.

Disc electrophoresis. The results of the disc electrophoresis are diagramed in figure 8. With liver tissue, band A was present from the 9th to the 21st days. Band C was present on the 15th and 18th days. Band D was present on the 21st day. The band comparable to band B of the starch gels was not observed. At 5-weeks posthatching, bands A and D were still present.

The brain produced bands A, B, C and D. Bands C and D increased in intensity from the 9th to the 18th day. Bands A and B exhibited maximum intensity throughout the test period. At 5-weeks posthatching, all four bands were present at the same intensity as at 21 days.

Localization observations. The intensity of the reactions was

estimated visually and tabulated using numbers from 0 through 8 where 0 indicates no visible reaction and 8 indicates the most intense reaction found in liver tissue. The estimations for the brain indicate the amount of reaction for the brain divisions as a whole.

5-Bromoindoxyl acetate. The results obtained with liver and brain tissue sections when 5-bromoindoxyl acetate was used as a substrate are recorded in table 11.

It is evident from table 11 that all controls of liver tissue exhibited maximum activity at each stage of the developing chick tested. Eserine and copper sulfate produced no detectable inhibition. Sodium fluoride reduced activity to about 75% of that of the control. Silver nitrate reaction was erratic.

The brain showed a general increase in activity up to 18 days of incubation and then decreased somewhat at 21 days in the sections which were developed immediately following fixation. When the sections were left in distilled water for 30 minutes between fixation and development, the reaction was somewhat reduced, giving an increase in reaction through 21 days. The strongest reaction was in the medulla oblongata, medulla of the cerebellum and mesencephalon. The next strongest was in the optic lobe, diencephalon and cortex of the cerebellum. The cerebrum showed the least amount of reaction.

Eserine sulfate produced complete inhibition in all divisions of the brain at 9 and 12 days of incubation and in the medulla oblongata and medulla of the cerebellum at 21 days. There was only a slight amount of reaction in all divisions during the remaining intervals.

Sodium fluoride produced complete inhibition in all divisions throughout the test period. Silver nitrate and copper sulfate reduced

activity to about 50-75% of that of the control sections left in water for 30 minutes.

The greatest concentration of reaction products was found in the cytoplasm and not in cell nuclei, nor was there a high concentration extra cellularly. The cytoplasm of the Purkinje cells of the cerebellum was darkly stained from the 12th day of incubation through the 21st day. The brain nuclei were darkly stained throughout the test period.

Copper sulfate produced no special areas of inhibition or lack of inhibition.

The walls of the blood vessels in the liver had an activity of about 25-50% of that of the liver tissue and were inhibited to about the same relative amount as the liver tissue.

Naphthol AS acetate. The results of employing naphthol AS acetate are recorded in table 12.

The liver control showed maximum reaction throughout the test period. Sodium fluoride produced no observable inhibition. Eserine sulfate reduced activity to about 75% of that of the control from 9 to 18 days of incubation and to about 88% on the 21st day. Silver nitrate reduced activity to about 12% at 9 and 12 days; to about 25% at 15 and 21 days and to about 50% on the 18th day.

Esterase activity was moderately strong even at the 9th day of incubation in the brain. The medulla oblongata gave a stronger reaction than the other brain divisions at 9 days and gave its strongest reaction at 15-21 days. The esterase activity of the medulla of the cerebellum, optic lobe and mesencephalon reached its maximum reaction on the 21st day. These last divisions (e.g. medulla of cerebellum, etc.) attained the same degree of intensity of esterase activity. The cerebrum and cortex of the cerebellum showed about the same intensity up to the

21st day at which time it increased somewhat.

Eserine sulfate reduced activity to about 50-75% of the control from 9 to 18 days and to 70-80% at 21 days. Silver nitrate reduced activity to about 10% at 9 and 15 days, to 0% at 12 days, to 50-80% at 18 days and 40% at 21 days. Sodium fluoride reduced activity to about 50-75% of the control.

A high concentration of reaction products in the borders of the cerebral ventricle and cavity of the optic lobe persisted throughout the test period. A high concentration of reaction products in the cytoplasm of the Purkinje cells and in the area immediately surrounding them appeared at 12 days and persisted through 21 days of incubation. There was a strong reaction in the brain nuclei and choroid plexuses throughout the test period. Eserine sulfate and sodium fluoride produced a general decrease in reaction and prevented the nuclei and Purkinje cells from staining so darkly as they did in the control sections.

For reasons given in the discussion, no attempt is made to indicate areas of special concentration of reaction for silver nitrate.

The reaction products appeared to be located more extracellularly than intracellularly.

The blood vessels of the liver had an intensity of about 50% of that of the liver tissue and were not affected by eserine sulfate or sodium fluoride, but were inhibited by silver nitrate.

Alpha naphthyl acetate. The results with alpha naphthyl acetate are recorded in table 13.

The liver gave its strongest reaction at 9 days of incubation and a constant but less intense reaction for the remainder of the test period. Eserine sulfate had no observable effect on the reaction in the liver. The blood vessels were indistinguishable from the liver tissue.

All brain divisions showed a progressive increase throughout the test period with the strongest reaction in the medulla oblongata and the medullary portion of the cerebellum. The optic lobe and mesencephalon had the next strongest reaction, and the cortex of the cerebellum, diencephalon and cerebrum had the weakest reaction.

Eserine sulfate had very little effect until the 21st day of incubation when it reduced activity to about 50-75% of that of the control.

In the brain, the choroid plexuses, linings of the cerebral ventricle and cavity of the optic lobe gave a strong reaction from the 9th to the 21st day. The other regions showed a progressive increase in intensity of reaction. The Purkinje layer of the cerebellum first became prominent at 12 days and became markedly more intense as development progressed. The reaction products are primarily within the cytoplasm of the cells.

Eserine sulfate produced a general reduction in the intensity of the reaction in the various brain regions except for the choroid plexuses and the linings of the cavities which were not affected.

Protein nitrogen. The results of the protein nitrogen determinations are recorded in figure 9 and table 14. The amount of protein nitrogen in the liver tissue increased from 19.5 mg per gram of wet weight of tissue at 9 days to 22.0 mg from 15 to 18 days, then decreased to 20.5 mg at 21 days of incubation. In the brain tissue there were 10.3 mg at 9 days, decreasing to 9.5 mg at 12 days, then increasing to 13.8 mg at 21 days of incubation.

DISCUSSION

Classification of esterases. Gomori (1952) defines esterases as enzymes which hydrolyze carboxylic acid esters of alcohols and phenols. Also, he distinguishes between lipase and esterases on the basis of substrate specificity and reactions with inhibitors and activators. Lipase shows a preference for esters of glycerol whose aliphatic components have carbon chains longer than 12 carbons. It is activated by bile acids such as taurocholic acid, is not affected by arsanilic acid and only slightly inhibited by sodium fluoride. Aliesterases show a preference for esters which are formed by aromatic and short-chained fatty acids with monohydric alcohols. These esterases are inhibited by arsanilic acid, sodium fluoride and bile salts. The cholinesterases are distinguished from the aliesterases in that they show a preference for esters of choline.

Augustinsson and Nachmansohn (1949) classify the cholinesterases into the true or acetylcholinesterases and pseudocholinesterases. Acetylcholinesterase is highly reactive with low concentrations of acetylcholine, whereas, the pseudocholinesterases are highly reactive with acetylcholine only when the substrate concentration is relatively high. Mendel and Rudney (1943a) made the same statement.

Pearse (1960) subdivides the non-specific esterases into B esterases which are sensitive to organophosphates, A esterases which are resistant to organophosphates and inhibited by para-chloromercuribenzoate, and C esterases which are resistant to organophosphates and activated by para-chloromercuribenzoate. He also separates the cholinesterases into acetyl- and pseudocholinesterases.

Hawkins and Gunter (1946) considered that the use of the term

pseudocholinesterase was appropriate since the selective inhibition of pseudocholinesterases in live rats by the use of NU-683 (2-hydroxy-5-phenylbenzyl)trimethyl-ammonium bromide, did not produce any signs of acetylcholine poisoning. Hawkins and Mendel (1949) selectively inhibited the true cholinesterases in live rats by using NU-1250, N-p-chlorophenyl-N-methyl carbamate of m-hydroxyphenyltrimethyl-ammonium bromide, and found that symptoms of acetylcholine accumulation appeared. These two experiments indicate that the pseudocholinesterases do not hydrolyze a significant amount of acetylcholine in vivo.

Unfortunately the classification of esterases has its complications. Chessick (1954) suggests the use of the term eserine-sensitive for those sensitive to eserine of 10^{-5} M concentration and eserine-resistant for those resistant to eserine of 10^{-3} M concentration. Then he states that among the eserine resistant esterases there exists a whole spectrum of enzymes, the properties of which vary so unpredictably among species, organs, and tissues, that they defy any kind of classification. Gomori (1952) made the statement that the differences between the aliesterases and the cholinesterases are not so marked as formerly believed since both groups hydrolyze choline and non-choline esters. Whittaker (1951) observed that the true and pseudocholinesterases react with non-choline esters. They both react with esters having a configuration similar to the choline esters and at rates comparable to those obtained when choline esters were used as substrates. Mendel and Rudney (1944) concluded that a classification of cholinesterases on the basis of activity-substrate concentration relationship alone is invalid. Factors such as the addition of colloids, which change the electrical charge on the enzyme molecules, change this relationship. They state that the best distinction is made on the basis of substrate specificity.

Mendel and Meyers (1955) modified the preceding observation by stating that the distinction of true and pseudocholinesterases cannot be made on the basis of substrate specificity alone because there is too much overlapping. Instead, they recommend a combination of substrates and inhibitors.. Meyers (1953) states that true cholinesterase from the brain of the chicken hydrolyzes propionyl choline more rapidly than it does acetylcholine and that acetyl-beta-methylcholine cannot be used as a specific substrate for true cholinesterase in the chicken. Mendel and Rudney (1943b) used acetyl-beta-methylcholine as a specific substrate for true cholinesterase in studies on the brains of chicken, mouse, rat, guinea pig, rabbit, dog, cat, cow and pig.

It will be noted in this investigation that the results do not adhere to the requirements for a precise classification. There are enough variations so that a classification must be made and accepted with reservations.

In view of the foregoing observations, one can see that it is difficult to be precise in classifying the esterases.

Quantitative determination of esterase activity. During the process of attempting to establish a calibration curve for beta naphthol, it was found that readings with the same concentration of beta naphthol were frequently widely divergent. When duplicate tubes were prepared and read at 15-minute intervals, the percent transmittance continued to rise, indicating that the color was fading. When one tube of each concentration tested was placed in the dark, the color of these tubes remained the same while those left in the light continued to fade. For this reason precautions mentioned under materials and methods were taken to prevent the color from fading. This discovery contradicts the claim

by Seligman et al. (1951) that the naphthol-azo dye complex in ethyl acetate did not fade. They said the only problem was a concentration of color due to the evaporation of the ethyl acetate.

Beta naphthyl acetate and beta naphthyl laurate have both been used as substrates for the colorimetric determination of esterase activity (Nachlas and Seligman, 1949a). Esterases will hydrolyze both substrates but the acetate ester is more readily hydrolyzed than the laurate ester. These authors believe that the effectiveness of the enzyme depends more on the length of the acid chain than on the type of alcohol to which it is attached. They found that the liver tissue of the mouse, rat, guinea pig, rabbit, dog and man hydrolyzed both substrates. Also, inhibition was produced by eserine, sodium taurocholate, and sodium fluoride. Gomori (1955) obtained inhibition of esterases with sodium fluoride in the human liver. These results are similar to those from the developing chick liver in this investigation.

Seligman, Nachlas and Mollomo (1949), using beta naphthyl acetate, beta naphthyl laurate and beta naphthyl palmitate-stearate substrates with sodium fluoride and sodium taurocholate inhibitors, concluded that there was no lipase in the liver of the dog and the human. Buño and Mariño (1952) found strong lipase activity in the chick embryo liver at 5 days. In this investigation, the evidence supports the conclusion that there is lipase in the chick liver from the 9th to the 21st days of incubation. Benzaldehyde, a lipase inhibitor (West and Todd, 1957), reduced activity only a small amount with naphthyl acetate which is more readily hydrolyzed by esterases but reduced activity more strongly when naphthyl laurate was used. Naphthyl laurate is more readily hydrolyzed by lipase.

Nachlas and Seligman (1949a) found that sodium taurocholate not

only inhibited liver activity [esterase activity] but accelerated pancreas activity [lipase activation]. . Examination of figure 4 reveals that taurocholate produced an activity intermediate to that of the uninhibited control and to that obtained by eserine, arsanilate and sodium fluoride. This fact could be the result of inhibiting the esterases but activating the lipase. The strong inhibition by arsanilate, sodium fluoride, and eserine indicates that esterases are the most responsible for the activity of the liver.

Mendel and Rudney (1943b) using acetyl-beta-methyl-choline as a substrate concluded that only true cholin esterase was present in the brain of chickens and 11 other animals which they tested. Gomori (1955) found human nerve cell bodies to have acetyl-esterase enzymes but not cholinesterase, whereas dog nerve cells which were eserine sensitive, were accordingly thought to contain cholinesterase.

The reactions obtained when the brain homogenate reacted with beta naphthyl acetate indicate that much of the activity could be due to cholinesterase. There is a strong inhibition with eserine and sodium fluoride. The results of starch gel analysis indicate sodium fluoride may produce an inhibitory effect on cholinesterases. The very small amount of activity with beta naphthyl laurate and no taurocholate activation indicate that there is no lipase activity even though benzaldehyde has produced a small amount of inhibition with both substrates. The activity with the laurate substrate would be that of aliesterases since the strongest inhibitions are produced by taurocholate, sodium fluoride and arsanilate.

Perhaps it should be emphasized here that the ordinates in figures 5 and 6 showing the activity of liver homogenate with the laurate substrate and of brain homogenate with the acetate are magnified ten fold

as compared to that of figure 4. The ordinates of figure 7 showing the activity of brain homogenate on the laurate substrate are magnified 100 fold as compared to that of figure 4.

Rogers et al. (1960) studying acetylcholinesterase in the separate divisions of the brain found that activity of the optic lobe, cerebrum and cerebellum continued to increase to the 21st day of incubation. The medulla reached its peak at 14 days; the diencephalon, at 18 days and the midbrain, at 19 days of incubation. A visual estimation of the average of these separate curves looks as though it would closely resemble the curve for the activity of the developing chick brains studied in this investigation. Rogers et al. (ibid.) found that there was a close correlation between the time of a sharp increase in alkaline phosphate activity and morphological differentiation in the brain of developing chicks between 6 and 21 days of incubation. This sharp increase in the phosphatase activity immediately precedes a corresponding increase in cholinesterase activity. It appears that the esterase activity of the brain increases as the parts of the brain differentiate to their functional condition.

Starch gel electrophoresis. Smithies (1959) stated briefly some of the principles involved in electrophoresis. The force applied to an ion in an electrical field is directly proportional to the charge on the ion; the charge carried by protein molecules in a buffer solution is a function of the protein and the pH of the solution. The composition of the buffer affects the charge since the protein binds buffer ions, providing a different charge on the protein molecule without changing the pH. The mobility of the molecule through the solution is determined by the balance of electrical force and physical retardation. Serum proteins are

separated with greater success in starch gel than with other methods because the frictional retardation is more nearly proportional to the molecular size. When the pore size is large as in free solutions, filter paper, or plain starch grains, the larger molecules possessing a greater charge experience a greater propelling force than the smaller molecules. Without a selective frictional retardation, separation is not so sharp.

Smithies (ibid.) also made the observation that the use of the vertical starch gel procedure, which eliminates the necessity of a suspending medium for the sample insert, made possible a better entry of the proteins into the gel without any interfering adsorption. In this investigation there were reaction products at the insertion end but these were probably adsorbed to or contained in particles since the homogenate was not centrifuged before preparing the insert. Hunter and Burstone (1960) postulated that such esterases which did not enter the gel are the same as those which did migrate. When they used filter paper instead of starch as a suspending medium for the insert, no activity was observed on the paper after migration had taken place. Koehler (1960) working with Tetrhymena pyriformis W obtained 5 bands using starch insert but only 4 when he used the filter paper.

In this investigation no difference was noted between the two methods of insert when working with liver homogenate. The brain homogenate suspended in starch produced only 2 bands, whereas, it produced 4 bands when it was suspended on filter paper. The 2 bands which did not appear with the starch insert may have been adsorbed to the starch or the amount of the sample may have been too small for them to show up under the conditions of the test. No attempt was made to ascertain which, if either, of these possibilities might have been involved. If one can assume

that the 4 bands obtained with the disc electrophoresis were the same 4 and in the same order as those obtained with the starch gels, the 2 bands obtained with the starch insert were the weakest of the 4 obtained with the disc electrophoresis. This evidence indicates that concentration may not be the explanation.

That the different bands represent different, distinct enzymes and not artifacts due to homogenizing procedures or some other reason, is supported by the fact that the patterns of different organs and their reactions to substrates and inhibitors are reproducible. (Hunter and Markert, 1957). Paul and Fottrell (1961) have stated that the esterase bands are not artifacts due to adsorption to proteins since a comparison of the esterase bands with those of protein bands obtained from the same source do not show any correlation. They also found that esterase patterns varied from one organ to another within the species and varied in the same organ from one species to another. For example, the liver of the guinea pig produced 12 esterase bands; the human liver, 7 bands; rat liver, 13 bands; and the liver of a chicken, 5 bands. Reed (1962) found that the number of bands in mice increased from 5 in the embryo to 10 in the adult.

In this investigation, the liver of the developing chick produced 3 bands with starch gel during the incubation period. A check with a chick 5-weeks posthatching resulted in a 4th band not present before hatching. A band comparable to the latter in position was observed at the 21st day of incubation with the disc electrophoresis. The fact that a more highly concentrated homogenate was used may account for the appearance of this band. It appeared with both methods at 5-weeks posthatching. Since it was inhibited by eserine, it was probably cholinesterase.

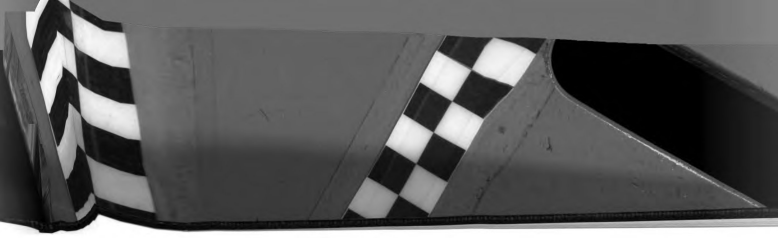
There are at least 2 possible explanations why 5 bands were not found in this investigation. It is possible that the 5th was not present at all or not in high enough concentration to show up. A study of chicks after hatching could clarify this point. The 2nd possibility is the difference in buffers. Paul and Fottrell (ibid.) used a Tris buffer instead of a phosphate buffer.

Hunter and Burstone (1960) studied the differences in reactivity of 30 different substrates for developing starch gels and found that the primary difference was in the rate of reaction and not in specificity. By using different couplers, such as Fast Garnet GBC, Fast Violet BB and Fast Blue RR, they found only a difference in intensity. These facts show that a worker has a wide selection of materials from which to choose.

All 3 bands produced by the liver homogenate were probably aliesterases since they produced a strong reaction with a short-chained fatty acid ester; there was no obvious inhibition by benzaldehyde or eserine and there was inhibition by sodium fluoride and arsanilate. Why taurocholate did not inhibit these bands is not known. No attempt was made to note any possible differences in the rates at which the different bands appeared. This information might provide helpful information.

A comparison of the quantitative results and the starch gels shows an agreement of the controls, benzaldehyde, fluoride and arsanilate reactions. Eserine and taurocholate show more inhibition in the quantitative results than in the starch gels. It is not known whether there may be some enzymes which did not migrate or were not concentrated enough to show up in the starch gels.

Of the 4 bands which were produced by the brain homogenate, bands A and B conform partly to the expected results for aliesterases in that



they were not inhibited by eserine and benzaldehyde and were inhibited by fluoride and taurocholate. However, they were not inhibited by arsanilate. Bands C and D, having been inhibited by eserine and fluoride, are probably cholinesterases. Melnick and Lawrence (1961) working with human blood serum and alpha naphthyl acetate were able to demonstrate a band which they had classified as a cholinesterase band by using 6-bromo-2-naphthyl carbonaphthoxy choline and its reaction to eserine.

A comparison of the quantitative results and the starch gels shows a fair correlation with bands C and D. Taurocholate shows more inhibition in the quantitative results than in bands C and D but bands A and B may make the difference.

Disc electrophoresis. The disc electrophoresis was used primarily on an experimental basis for a comparison with the starch gel procedure. There are certain advantages with the disc electrophoresis. Higher concentrations of sample may be used without obtaining a "smearing" effect often obtained with the starch gels. Smaller samples may be used with the disc method. The migration time is only about 45 minutes with the disc method as compared to 6 hours with starch gels.

The number of bands obtained with the disc method using brain homogenate was the same as for the starch gels, but the liver homogenate with the disc method did not produce band B.

Localization. Localization was accomplished using frozen sections cut on an International Equipment Co. Cryostat-microtome. This procedure has a decided advantage over other procedures for enzyme work. The enzymes are not exposed to fixatives, dehydrating agents or heat for prolonged periods of time. Nor is there a long waiting period as is necessary for the freeze-drying procedure. Tissues frozen with a CO_2 jet can be cut immediately if ice crystal artifacts do not impair the value of the results.

These artifacts can be eliminated, or reduced, by using solid CO_2 and acetone with a temperature of about -70° C or an isopentane bath cooled with liquid air having a working temperature of about -160° C. The brain and liver tissue used in this investigation showed no detectable difference when frozen by these 2 latter methods.

Tissue frozen at extremely low temperatures could not be cut immediately since it would crumble. Wrapping the tissue in Saran Wrap and storing it in an air-tight jar at -20° C overnight or for 2-3 days, proved to be very satisfactory for preserving esterase activity. Pearse (1960) stated that some tissues can be stored satisfactorily under these conditions for 1-2 weeks, however, brain and liver tissue suffer distortion in less time.

The chamber temperature of the cryostat had to be maintained at -10 to -15° C. If the temperature became lower, the tissue would crumble; and if it became warmer, the tissue would stick to the anti-roll guide or compress on the knife edge. Maintaining the knife temperature alone is not the complete answer to temperature control because temperature fluctuations too rapid to permit the knife temperature to change would affect the cutting.

Barrnett (1953) found that brief fixation in cold acetone reduced the activity of aliesterases, lipase and nearly abolished cholinesterase activity. Doyle and Liebelt (1954) found that rabbit appendix fixed in cold formalin for 1 hour had only 5-8% of the activity compared to that of freeze-dried tissue. However, none of the activity was diffusible. Seligman, Chauncey and Nachlas (1951) reported that thin slices of tissue fixed in 10% neutralized formalin at 4° C for 24 hours still had much activity left and was sufficiently firm to be cut on a

freezing microtome. Taxi (1952) reported that formalin fixation at 4° C had less inactivating effect than it had at higher temperatures. The tissue slices in this investigation were fixed for 10 minutes in a calcium-formal fixative (1% calcium chloride in a 10% solution of commercial formaldehyde) which was kept in an ice water bath. Control sections left in distilled water 30 minutes before developing with 5-bromoindoxyl acetate showed slightly less activity than those developed immediately, apparently there was some loss of enzyme possibly due to insufficient fixation.

The results of the silver nitrate inhibition are not considered reliable since the silver nitrate produced much precipitation. Since it is not known what was precipitated, it is not possible to evaluate the reactions. It will be noticed in tables 11 and 12 that the results do not look consistent.

Pearson and Grose (1959) listed several advantages for the use of 5-bromoindoxyl acetate as a substrate. It is a soluble, colorless substrate which becomes an insoluble pigment as a result of enzyme action. The end product is a fine uniform particle with little tendency to form crystals. The tissue sections can be dehydrated in alcohol and cleared in xylene if desired. There are certain disadvantages, however. It is not a specific substrate since it is hydrolyzed by esterases, lipase, specific and nonspecific cholinesterases. Positive reactions are produced by chymotrypsin, imidazol and histamine. Underhay (1957) stated that indoxyl esters show structural analogies to acetylcholine esters and aromatic esters such as phenyl and naphthol esters. Therefore, reactions with the indoxyl esters probably demonstrate the combined activity of several esterases.

Alpha naphthyl acetate is more desirable for localization work than the beta form since there is less diffusion of the reaction products

obtained with the alpha form (Gomori, 1950 and Chessick, 1953).

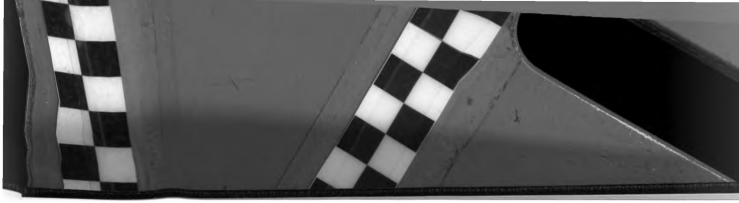
Naphthol AS acetate produced some crystals immediately in this investigation and crystallization increased after several weeks.

The reaction of liver tissue with 5-bromoindoxyl acetate and alpha naphthyl acetate, recorded in tables 11 and 13 respectively, support the conclusion that the esterases are primarily aliesterases as stated earlier. Eserine did not produce any inhibition of liver tissue reacting with 5-bromoindoxyl acetate or alpha naphthyl acetate substrates. Sodium fluoride produced only partial inhibition with 5-bromoindoxyl acetate. Shnitka and Seligman (1961) found fluoride sensitive and fluoride resistant esterases in liver tissue. Eserine produced partial inhibition when naphthol AS acetate was used, suggesting the presence of cholinesterase in harmony with the quantitative results.

Pearson and Grose (1959) using 5-bromoindoxyl acetate found the activity to be quite uniform throughout the lobule of the rat liver. The blood vessels of the rat liver did not show any activity. The chick liver in this investigation showed slightly greater activity adjacent to the central veins and some activity in the blood vessel walls.

Nachlas and Seligman (1949b) studying the livers of rats, rabbits, guinea pigs, dogs and humans found them to stain intensely with beta naphthyl acetate. The blood vessels usually were positive for esterases. These results are the same as those obtained with the developing chick liver following incubation in this substrate.

Chessick (1953) in his study of human, cat, rat, and mouse tissue found intense activity in the liver using alpha naphthyl acetate and naphol AS acetate substrates. However, he did not find any activity in the blood vessel walls as was found in the developing chick liver tissue in this investigation.



Eserine strongly inhibited the activity of the brain tissue reacting with 5-bromoindoxyl acetate and naphthol AS acetate indicating the presence of cholinesterases. However, eserine (10^{-5} M) did not inhibit activity with alpha naphthyl acetate until the 21st day of incubation, indicating that low concentrations of acetylcholinesterase are present until late in development.

Since sodium fluoride completely inhibited activity with 5-bromoindoxyl acetate, it is obvious that it inhibits cholinesterases as well as aliesterases. This point was noted in the starch gels, also.

The evidence obtained with the localization procedures show that there are differences in esterase intensities in the different brain divisions but the inhibitor reactions indicate that the same enzymes were present in the various divisions. This conclusion was substantiated by a starch gel test where the brain tissue of chicks at 21 days of incubation were divided into the prosencephalon, mesencephalon and rhombencephalon divisions. These divisions were tested separately and found to have the same 4 bands.

Chessick (1953) found that human, cat, rat, rabbit, and mouse neuron cell bodies stained poorly with naphthol AS acetate but more strongly with alpha naphthyl acetate. The neuron cell bodies of the developing chick brain in this investigation stained well with naphthol AS acetate and more rapidly than with alpha naphthyl acetate.

Nachlas and Seligman (1949b) using beta naphthyl acetate reported that esterases are not present in the cerebrum and cerebellum of the rat, rabbit, guinea pig and man. These same divisions of the developing chick brains in this investigation very definitely possessed esterases.

Peters, Vonderahe and Powers (1956) making electrical studies on

the brain of developing chicks between 6 and 21 days of incubation concluded that the brain first becomes functional along a posterior-anterior gradient, e.g., rhombencephalon, mesencephalon, prosencephalon. The medulla oblongata becomes functional first followed by the midbrain, diencephalon and then the cerebral hemispheres. These findings agree with alkaline phosphatase and acetylcholinesterase activity (Rogers et al., 1960). In this investigation the cholinesterase activity as shown by the reactions with 5-bromoindoxyl acetate correlates with the above findings as much as can be expected with a test of this type. There were no obvious contradictions.

Protein determination. Fruton and Simmonds (1959) mention briefly several methods used for the quantitative determination of proteins. The most accurate is the Kjeldahl method in which the bound nitrogen is converted to ammonia by digesting the protein with sulfuric acid in the presence of an appropriate catalyst. The mixture is then made alkaline and the ammonia is steam distilled into an excess of acid. The amount of excess acid is ascertained by titration. From this information, the amount of nitrogen can be calculated and converted to the amount of protein by multiplying by 6.25. This factor is based on nitrogen as 16% of the protein which is the average of a 12-19% range for the nitrogen content of proteins in general.

Another procedure is the Biuret reaction where a purple color is produced when an alkaline copper sulfate solution reacts with peptide bonds. The intensity of this color is proportional to the number of peptide bonds and can be ascertained colorimetrically. A calibration curve can be established with some protein such as albumen.

The Folin-Ciocalteu procedure is based on the formation of a

blue color produced by phosphomolybdotungstic acid solution reacting with tyrosine. For greatest accuracy the calibration curve should be made by using the specific protein which one is studying. Where one is testing a mixture of proteins a standard such as bovine albumen can be used and the results interpreted accordingly.

In this investigation the liver homogenate produced a turbidity which affected the colorimeter readings (Table 14). Attempts to clarify the homogenate by centrifugation, extracting with ethyl acetate and freeze-thawing reduced the intensity of the color so much that it could be detected visually. Kingsbury, Alexanderson, and Kornstein (1956) reported that the amount of lipids in the liver increased during development. At least some of the turbidity was caused by lipids because there was a layer of material floating on the homogenate following centrifugation. The use of a turbidity control was chosen to correct the colorimeter readings.

The protein content of the developing chick brains in this investigation was 6.4% at 9 days, decreased to 6% at 12 days and then increased to 8.4% at 21 days of incubation. The liver protein content increased from 12% at 9 days to 14% at 18 days and then decreased to 13% at 21 days of incubation. Dumm and Levy (1949) reported the protein content of the developing chick liver increased from 14% at 5 days to 16% from 9 through 21 days of incubation. They used an acid digestion and iodometric procedure. It is obvious that the Folin-Ciocalteu is not a satisfactory procedure to use with tissue homogenates. The amount of heavy sediment obtained with centrifugation indicates that the tissue may not have been thoroughly homogenized to release all protein for reaction. This possibility could account for the difference in results between the two procedures.

The full significance of the results of this investigation cannot be completely assessed but there are some speculations which can be made associating them and some of the accepted opinions concerning the relationships between enzyme formation and embryonic development.

The esterase activity of the liver tissue increased strongly from the 9th to the 18th day of incubation. Moog (1959) gives the three following interpretations for the increase of enzyme activity: (1) a dissociable activator has appeared or an inhibitor has disappeared; (2) the enzyme itself has undergone differentiation; (3) there has been an actual synthesis of a specific protein that is identified by its enzymatic activity. Possibility number one is ruled out as a result of work done with mixing a homogenate having a low activity with one having a high activity. The activity of the mixture was an average for the two homogenates. If an inhibitor were responsible for reducing the activity, the activity of the mixture should be less than an average of the two homogenates. If an activator were involved, the activity of the mixture should have been greater than an average of the two homogenates. The second possibility is ruled out for lack of supporting experimental evidence at present. The results of the electrophoretic analysis of the developing chick liver clearly supports the third possibility. Based on the results of the two electrophoretic procedures used, the liver esterase bands B and D attained a concentration which made it possible to demonstrate them as new bands appearing during the course of the 9th to 21st days of incubation. The intensity of the bands also roughly indicated an increase in the activity of the esterases forming the bands.

Hunter and Markert (1957) made the statement that each tissue exhibits its own repertory of esterases and that during embryonic development and early postnatal development the esterases appear one after another until

the adult number is attained. The developing chick liver shows this same trend. As indicated earlier, the 5th band, reported by Paul and Fottrell (1961), did not appear by the 5th-week posthatching. Further work on older chickens may demonstrate when this last band does appear.

It is still unknown what initiates the production of enzymes. Moog (1956) states the concept, which appears to be accepted by some other authors, that enzyme production occurs as a result of an inducing substance which may be the substrate upon which the enzyme will react. Although most of the present evidence for enzyme induction has been obtained from work with microorganisms, there are some examples from vertebrates. Burkhalter, Jones, and Featherstone (1957) were able to induce an increase of acetylcholinesterase in chick embryo lung tissue grown in vitro by the addition of acetylcholine. The addition of acetate or choline alone or both of them together had no effect on the production of acetylcholinesterase. Non-specific esterases were not affected by the addition of the acetylcholine. Stearns and Kostellow (1958) reported the work of Knox and Mehler who concluded that the induction of the enzyme trytophan peroxidase in the liver of adult R. pipiens followed injection of tryptophan. The in vivo function and substrate are known for acetylcholinesterase but not for the other esterases. One might speculate that the production of esterases of the developing chick liver may be induced by metabolic products of fat metabolism and may be involved in the metabolism of these products. Baldwin (1959) stated that 90% of the material oxidized by the developing chick is fat. This possible involvement in fat metabolism could account for the presence of aliesterases in the brain. It is well known that the liver is an important organ for the interconversion of fats, carbohydrates and proteins. This last fact may account for the high esterase activity in the liver.

The esterase activity of the brain tissue in this investigation showed a small increase as compared to that of the liver and there was no change in the number of esterase bands obtained by electrophoresis. Since the nervous system begins morphological differentiation so early, it is possible that its chemical differentiation (specifically esterase enzymes in this study) is attained before the earliest age of this investigation. If acetylcholine is the inducing substance for the production of acetylcholinesterase *in vivo*, the mechanism for the production of acetylcholine must precede the production of acetylcholinesterase.

Markert (1958) referred to the works of Pavan and of Beerman with chromosomes of larval tissue of certain insects. They have observed localized thickenings called Balbiani rings on the chromosomes. The occurrence and distribution of these rings along the chromosomes are specific for the tissue and the stage of development. These findings indicate a differentiation of the chromosomes. Markert (*ibid.*) refers also to the work of King and Briggs with the transplantation of nuclei from the various stages of developing frog embryos to enucleated frog eggs. These workers found that the nuclei from older embryos did not support normal development after transplantation, indicating that there must be some kind of chromosomal differentiation as development progressed.

Cautiously relating the findings cited in the above paragraph to the results in this investigation, it appears that chromosomal differentiation (if such a phenomenon occurs in the developing chick) associated with esterase production in the liver was not completed even by the 5th-week posthatching. However, any possible chromosomal differentiation in the brain related to esterase production may have been completed before the 9th day of incubation. Only a study of adult brain tissue will show whether any more esterases are produced after 5-weeks posthatching.

SUMMARY

1. A study of the esterase activity was made on the brain and liver tissue of developing chicks at 3-day intervals from 9 days through 21 days of incubation.

2. The amount of activity was ascertained by use of the colorimetric procedure of Seligman and Nachlas (1950), using beta naphthyl acetate and beta naphthyl laurate substrates. Inhibitors used were sodium arsanilate (40 mg/ml), benzaldehyde (1 ml/350 ml H_2O), eserine sulfate (5 mg/ml), sodium fluoride (30 mg/ml), and sodium taurocholate (8.9 mg/ml).

3. Activity of both tissues with both substrates increased through the 9-21 day period. The activity of the liver was greater with both substrates than was that of the brain tissue. Activity of both tissues was greater with beta naphthyl acetate than with beta naphthyl laurate.

4. Reactions with the inhibitors indicate that the activity of the liver was primarily that of aliesterases with a small amount due to lipase. Activity of the brain tissue was primarily that of cholinesterases with a small amount due to aliesterases.

5. A separation of the esterases was made by use of the starch gel procedure of Smithies (1955). Using both the starch and the filter paper inserts, the bands were developed with alpha naphthyl acetate and Fast Garnet GBC. Inhibitors used were eserine sulfate (1×10^{-3} M), sodium fluoride (7.14×10^{-2} M), sodium taurocholate (1.6 x 10-2 M), sodium arsanilate (1×10^{-1} M), and benzaldehyde (2.67×10^{-2} M).

6. The liver tissue produced three bands, two of which were present through the 9-21 day period and a 3rd which was present 12-21 days.

All three were completely inhibited by sodium fluoride and sodium arsanilate indicating that they were aliesterases.

7. The brain tissue produced four bands. Only the two fast moving bands, A and B, appeared with the starch insert. With the filter paper insert, all four bands appeared on the 9th day. From the 12th through the 21st day, only the slow moving bands, C and D, appeared.

8. Bands A and B were inhibited with sodium fluoride and sodium arsanilate indicating that they were probably aliesterases.

9. Bands C and D were inhibited with sodium fluoride and eserine sulfate indicating that they were probably cholinesterases.

10. Localization was accomplished with sections cut on a cryostat-microtome, fixed in cold calcium formol, and developed by three different procedures. The 5-bromoindoxyl acetate procedure was used with eserine sulfate (1×10^{-3} M), sodium fluoride (7.14×10^{-2} M), silver nitrate (1×10^{-2} M), and copper sulfate 1×10^{-3} M). Naphthol AS acetate was used with eserine sulfate (1×10^{-4} M), sodium fluoride (7.14×10^{-2} M), and silver nitrate (1×10^{-2} M). Alpha naphthyl acetate was used with eserine sulfate (1×10^{-5} M).

11. The liver tissue produced intense activity with all three substrates from the 9th to the 21st day of incubation. The walls of the blood vessels exhibited less activity than the liver tissue.

12. Activity in the brain with all three substrates was most intense in the medulla oblongata, medulla of the cerebellum, mesencephalon, diencephalon, choroid plexuses, brain nuclei and linings of the cavities.

13. Reactions with the inhibitors support the conclusion that the liver has primarily aliesterases and that the brain has primarily cholinesterases.

14. The amount of protein nitrogen was ascertained by the Folin-Ciocalteu colorimetric procedure. The results were about 12% lower than those reported where an acid-digestion and iodometric procedure was used. The protein content of tissue homogenates cannot be ascertained accurately with the Folin-Ciocalteu procedure.

15. Relating the results of this investigation with the concept of chromosomal differentiation, the evidence indicates the possibility that any chromosomal differentiation associated with the production of esterases in the brain of the developing chick has been completed by the 9th day of incubation. However, it appears that any chromosomal differentiation associated with the production of esterases in the liver of the developing chick may not be completed by the 5th week of posthatching development.

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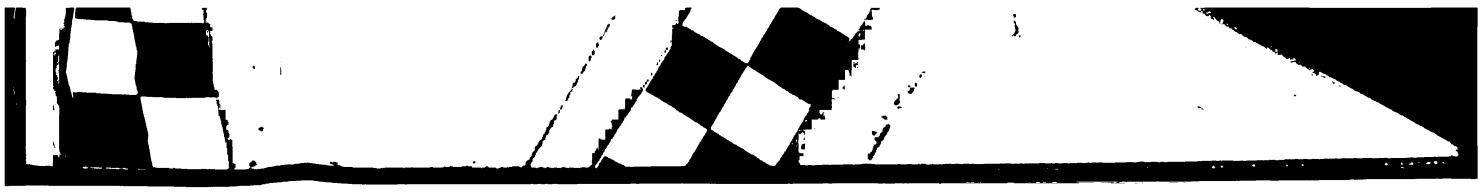

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FIGURE I

Calibration curve for the estimation of the amount of beta naphthol released. Known quantities of beta naphthol were used with the same reagents and following the same procedure as with the tests. The addition of eserine sulfate produced the same results as when no inhibitor was added. Ordinates, percent transmittance at a wavelength of 450 mμ; abscissae, mg beta naphthol.

FIGURE I

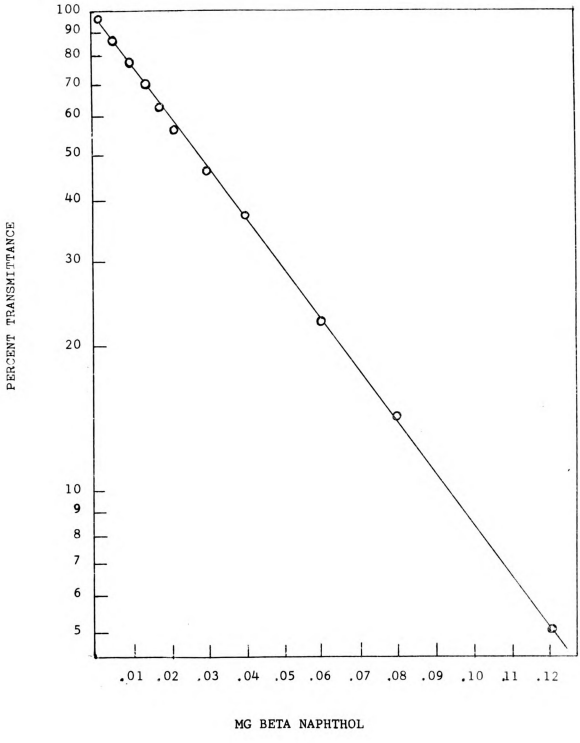

PERCENT TRANSMITTANCE
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
.01 .02 .03 .04 .05 .06 .07 .08 .09 .10 .11 .12
MG BETA NAPHTHOL

FIGURE 2

Calibration curve for the estimation of the amount of beta naphthol released. Known quantities of beta naphthol were used with the same reagents and following the same procedure as with the tests. Ordinates, percent transmittance at a wavelength of 450 mμ; abscissae, mg of beta naphthol.

Legend: □ Beta naphthol with sodium arsanilate

o Beta naphthol with benzaldehyde

FIGURE 2

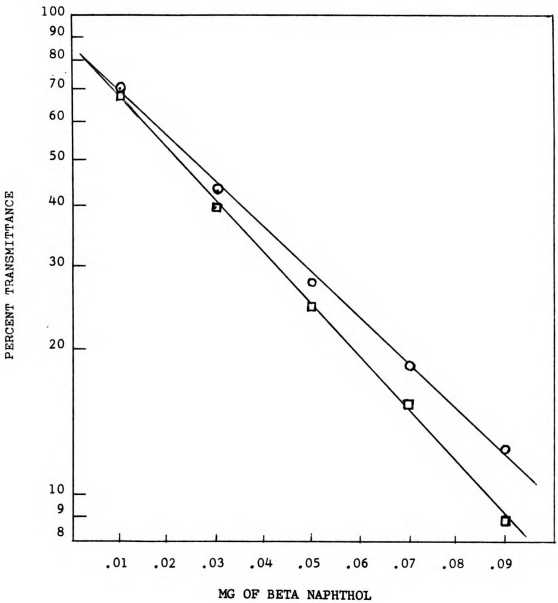

100
90
80
70
60
50
40
30
20
10
9
8
PERCENT TRANSMITTANCE
.01
.02
.03
.04
.05
.06
.07
.08
.09
MG OF BETA NAPHTHOL

FIGURE 3

Calibration curve for the estimation of the amount of beta naphthol released. Known quantities of beta naphthol were used with the same reagents plus inhibitors and following the same procedure as with the tests. Ordinates; percent transmittance at a wavelength of 450 mμ; abcissae, mg of beta naphthol.

Legend: O Sodium fluoride

□ Sodium taurocholate

FIGURE 3

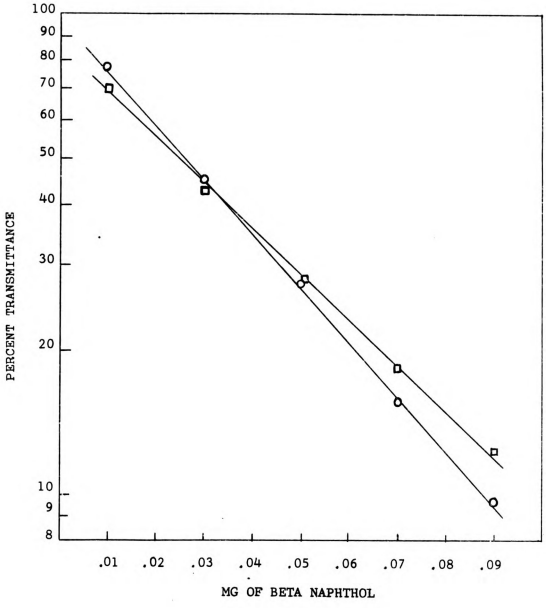

100
90
80
70
60
50
40
30
20
10
9
8
PERCENT TRANSMITTANCE
.01
.02
.03
.04
.05
.06
.07
.08
.09
MG OF BETA NAPHTHOL

TABLE I

Percent transmittance of the azo dye solution following the incubation of liver homogenate with beta naphthyl acetate and the addition of Diazo Blue B.

Inhibitor	Age in days of incubation				
	9	12	15	18	21
Control	39.4	19.6	10.8	5.8	5.2
Sodium arsanilate	66.0	53.4	44.2	38.7	44.8
Benzaldehyde	38.7	18.7	11.6	5.9	6.2
Eserine sulfate	80.1	65.6	54.8	52.4	57.7
Sodium fluoride	89.2	79.1	73.0	70.3	76.4
Sodium taurocholate	64.0	48.3	38.1	25.6	25.5

TABLE 2

Esterase activity in liver homogenate using beta naphthyl acetate. Activity is expressed in millimoles of beta naphthol released per milligram of tissue per hour.

Inhibitor	Age in days of incubation				
	9	12	15	18	21
Control	3.134	5.474	7.480	9.611	9.987
Sodium arsanilate	0.878	1.504	2.215	2.674	2.173
Benzaldehyde	2.674	5.098	6.686	8.984	8.817
Eserine sulfate	0.736	1.421	2.006	2.173	1.855
Sodium fluoride	0.318	0.694	0.961	1.086	0.836
Sodium taurocholate	1.086	2.173	3.092	4.638	4.638

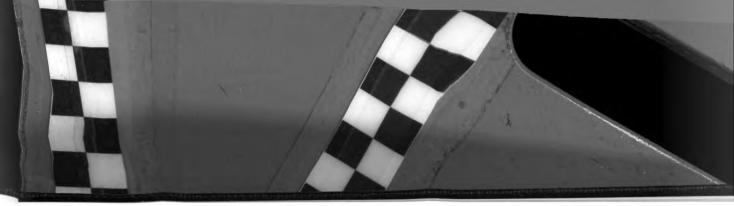


FIGURE 4

Esterase activity of liver homogenates of developing chicks at 3-day intervals from 9 days through 21 days of incubation using beta naphthyl acetate substrate expressed in millimoles of beta naphthol released per milligram of wet weight of tissue per hour of reaction time. Each point represents the average of 6 tests. Ordinates, millimoles of beta naphthol; abscissae, age in days of incubation.

Legend:

- O Uninhibited control
- ● Sodium arsanilate 8×10^{-2} M
- ⊘ Benzaldehyde 1.6×10^{-3} M
- ◑ Eserine sulfate 8×10^{-3} M
- ■ Sodium fluoride 1×10^{-1} M
- ◪ Sodium taurocholate 2×10^{-2} M

FIGURE 4

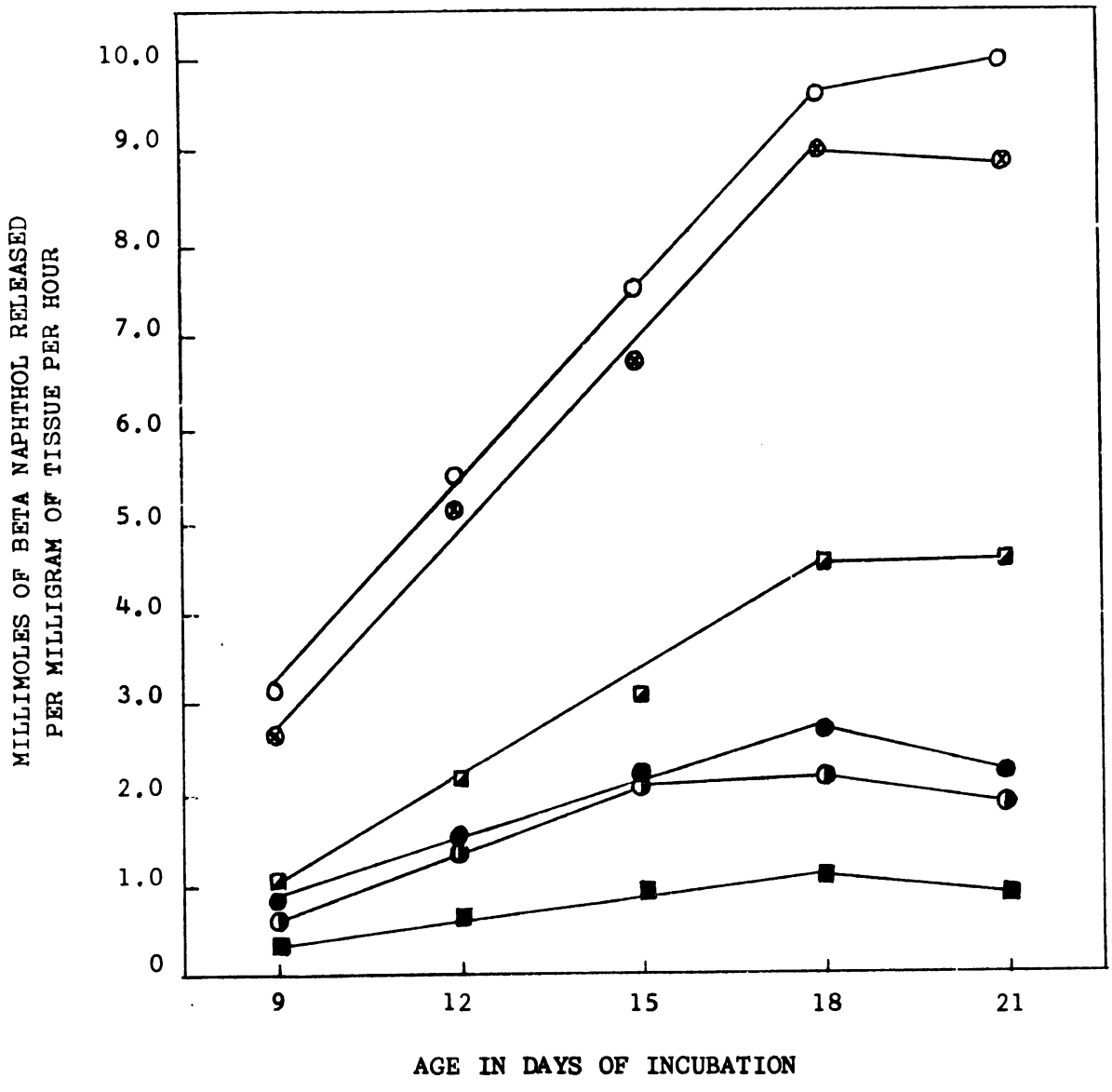

MILLIMOLES OF BETA NAPHTHOL RELEASED
PER MILLIGRAM OF TISSUE PER HOUR
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
9
12
15
18
21
AGE IN DAYS OF INCUBATION

TABLE 3

Percent transmittance of the azo dye solution following the incubation of liver homogenate with beta naphthyl laurate and the addition of Diazo Blue B.

Inhibitor	Age in days of incubation				
	9	12	15	18	21
Control	84.6	76.8	68.7	64.0	58.1
Sodium arsanilate	84.6	81.7	82.7	86.7	87.0
Benzaldehyde	86.7	79.2	72.3	67.3	62.7
Eserine sulfate	97.7	94.6	89.3	95.3	96.3
Sodium fluoride	96.1	97.1	95.8	98.0	98.6
Sodium taurocholate	85.9	79.2	71.5	67.3	66.2

TABLE 4

Esterase activity in liver homogenate using beta naphthyl laurate. Activity is expressed in millimoles of beta naphthol released per milligram of tissue per hour.

Inhibitor	Age in days of incubation				
	9	12	15	18	21
Control	0.071	0.113	0.157	0.188	0.228
Sodium arsanilate	0.008	0.026	0.016	0	0
Benzaldehyde	0	0.033	0.073	0.102	0.131
Eserine sulfate	0.011	0.023	0.047	0.019	0.016
Sodium fluoride	0.011	0.052	0.011	0	0
Sodium taurocholate	0	0.037	0.089	0.115	0.123

FIGURE 5

Esterase activity of liver homogenates of developing chicks at 3-day intervals from 9 days through 21 days of incubation using beta naphthyl laurate as a substrate expressed in millimoles of beta naphthol released per milligram of wet weight of tissue per hour of reaction time. Each point at 9 days represents the average of 5 tests; at 12-18 days, 6 tests; at 21 days, 4 tests. Ordinates, millimoles of beta naphthol; abscissae, age in days of incubation.

Legend:

- ○ Uninhibited control
- ● Sodium arsanilate 8×10^{-2} M
- ⊗ Benzaldehyde 1.6×10^{-3} M
- ◑ Eserine sulfate 8×10^{-3} M
- ■ Sodium fluoride 1×10^{-1} M
- ◪ Sodium taurocholate 2×10^{-2} M

FIGURE 5

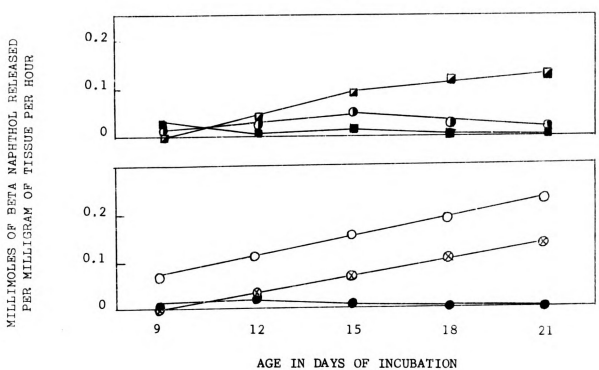

MILLIMOLES OF BETA NAPHTHOL RELEASED PER MILLIGRAM OF TISSUE PER HOUR
0.2
0.1
0
0.2
0.1
0
9
12
15
18
21
AGE IN DAYS OF INCUBATION

TABLE 5

Percent transmittance of the azo dye solution following the incubation of brain homogenate with beta naphthyl acetate and the addition of Diazo Blue B.

Inhibitor	Age in days of incubation				
	9	12	15	18	21
Control	78.5	75.2	70.0	58.2	45.0
Sodium arsanilate	70.2	66.6	63.3	53.2	42.4
Benzaldehyde	82.0	78.5	75.2	64.7	52.0
Eserine sulfate	94.7	95.0	91.0	89.0	83.5
Sodium fluoride	98.1	99.5	96.8	97.2	96.0
Sodium taurocholate	84.0	84.6	82.2	79.8	77.3

TABLE 6

Esterase activity in brain homogenate using beta naphthyl acetate. Activity is expressed in millimoles of beta naphthol released per milligram of tissue per hour.

Inhibitor	Age in days of incubation				
	9	12	15	18	21
Control	0.200	0.242	0.301	0.458	0.673
Sodium arsanilate	0.170	0.221	0.252	0.399	0.599
Benzaldehyde	0.042	0.074	0.116	0.240	0.420
Eserine sulfate	0.042	0.042	0.040	0.095	0.147
Sodium fluoride	0	0	0.017	0.011	0.021
Sodium taurocholate	0.021	0.017	0.042	0.074	0.095

FIGURE 6

Esterase activity of brain homogenates of developing chicks at 3-day intervals from 9 days through 21 days of incubation using beta naphthyl acetate as a substrate expressed in millimoles of beta naphthol released per milligram of wet weight of tissue per hour of reaction time. Each point represents the average of 6 tests. Ordinates, millimoles of beta naphthol; abscissae, age in days of incubation.

Legend:

- ○ Uninhibited control
- ● Sodium arsanilate 8×10^{-2} M
- ⊗ Benzaldehyde 1.6×10^{-3} M
- ◑ Eserine sulfate 8×10^{-3} M
- ■ Sodium fluoride 1×10^{-1} M
- ◪ Sodium taurocholate 2×10^{-2} M

FIGURE 6

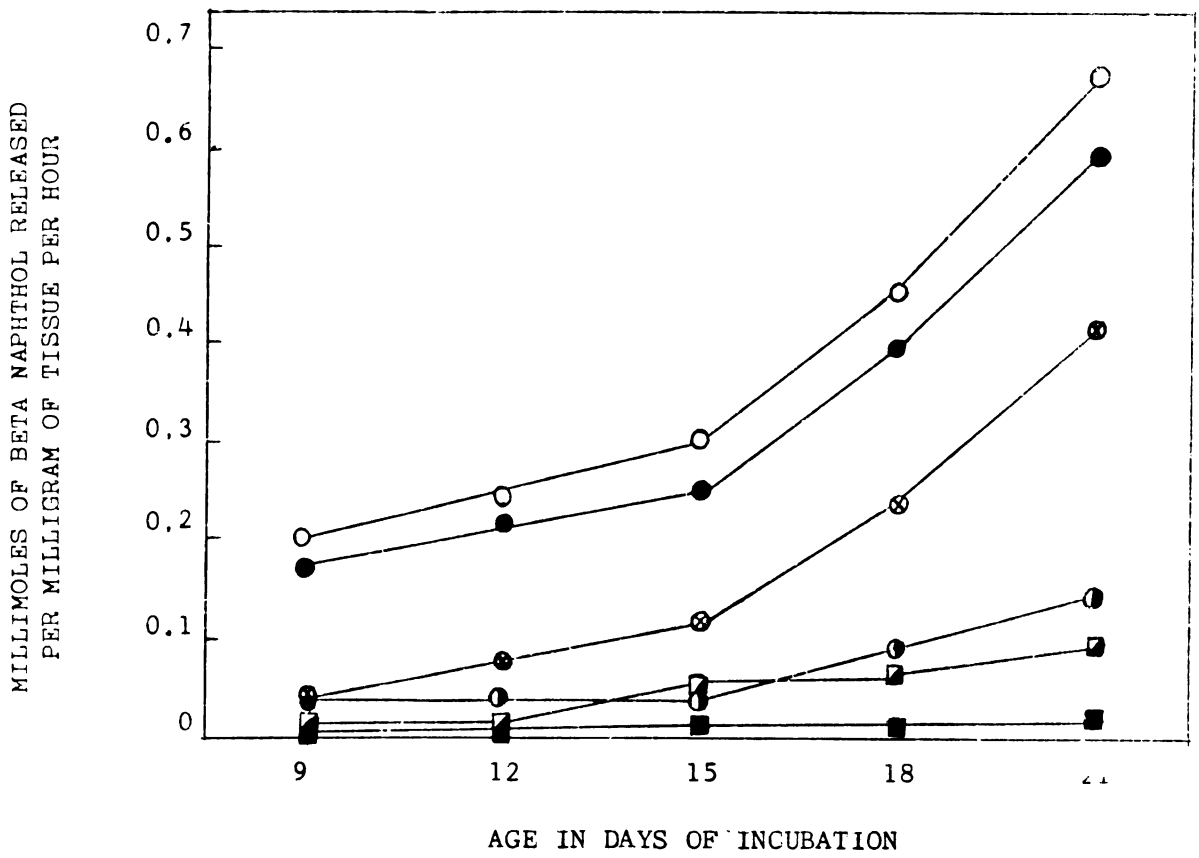

MILLIMOLES OF BETA NAPHTHOL RELEASED PER MILLIGRAM OF TISSUE PER HOUR
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
9
12
15
18
AGE IN DAYS OF INCUBATION

TABLE 7

Percent transmittance of the azo dye solution following the incubation of brain homogenate with beta naphthyl laurate and the addition of Diazo Blue B.

Inhibitor	Age in days of incubation				
	9	12	15	18	21
Control	88.2	84.4	83.8	80.0	75.0
Sodium arsanilate	79.9	76.0	76.2	76.0	72.0
Benzaldehyde	90.8	87.9	89.3	84.0	83.0
Eserine sulfate	94.3	90.4	86.2	86.0	82.0
Sodium fluoride	92.0	90.2	87.5	86.5	84.0
Sodium taurocholate	90.4	92.2	91.4	91.0	93.0

TABLE 8

Esterase activity in brain homogenate using beta naphthyl laurate. Activity is expressed in millimoles of beta naphthol released per milligram of tissue per hour.

Inhibitor	Age in days of incubation				
	9	12	15	18	21
Control	0.013	0.018	0.018	0.024	0.030
Sodium arsanilate	0.008	0.013	0.013	0.013	0.018
Benzaldehyde	0	0	0	0.003	0.004
Eserine sulfate	0.006	0.011	0.017	0.017	0.021
Sodium fluoride	0.007	0.008	0.012	0.013	0.016
Sodium taurocholate	0	0	0	0	0

FIGURE 7

Esterase activity of brain homogenate of developing chicks at 3-day intervals from 9 days through 21 days of incubation using beta naphthyl laurate as a substrate expressed in millimoles of beta naphthol released per milligram of wet weight of tissue per hour of reaction time. Each point at 9 days represents the average of 5 tests; at 12-18 days, 6 tests; at 21 days, 4 tests. Ordinates, millimoles of beta naphthol; abscissae, age in days of incubation.

Legend:

- ○ Uninhibited control
- ● Sodium arsanilate 8×10^{-2} M
- ⊗ Benzaldehyde 1.6×10^{-3} M
- ◑ Eserine sulfate 8×10^{-3} M
- ■ Sodium fluoride 1×10^{-1} M
- ◪ Sodium taurocholate 2×10^{-2} M

FIGURE 7

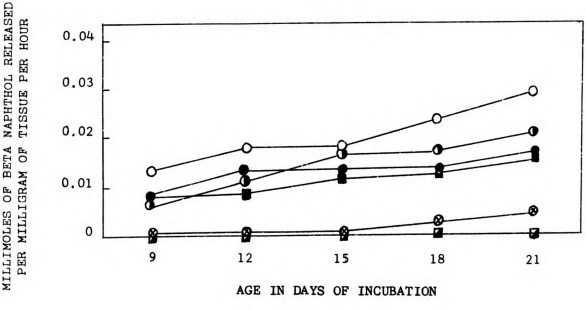

MILLIMOLES OF BETA NAPHTHOL RELEASED PER MILLIGRAM OF TISSUE PER HOUR
0.04
0.03
0.02
0.01
0
9
12
15
18
21
AGE IN DAYS OF INCUBATION

TABLE 9

Intensity of esterase activity of liver tissue in zymograms using alpha naphthyl acetate substrate and Fast Garnet GBC. The numbers indicate the relative intensity from 0 where no band was visible to 4 where the reaction was the most intense.

Inhibitor	Age in days of incubation	Activity in bands using starch insert			Activity in bands using filter paper insert		
		A	B	C	A	B	C
Control	9	2	0	0	2	0	1
	12	4	0	1	3	0	4
	15	4	1	1	4	1	4
	18	4	3	3	4	1	4
	21	4	3	2	4	1	4
Eserine sulfate	9	2	0	0	2	0	1
	12	4	0	1	3	0	4
	15	4	1	1	4	1	4
	18	4	3	3	4	1	4
	21	4	3	2	4	1	4
Sodium fluoride	9	0	0	0	0	0	0
	12	0	0	0	0	0	0
	15	0	0	0	0	0	0
	18	0	0	0	0	0	0
	21	0	0	0	0	0	0
Sodium taurocholate	9	1	0	0	2	0	1
	12	4	0	1	3	0	4
	15	4	1	1	4	1	4
	18	4	3	3	4	1	4
	21	4	3	2	4	1	4
Sodium arsanilate	9	0	0	0	0	0	0
	12	0	0	0	0	0	0
	15	0	0	0	0	0	0
	18	0	0	0	0	0	0
	21	0	0	0	0	0	0
Benzaldehyde	9	2	0	0	2	0	1
	12	3	0	1	3	0	4
	15	4	1	1	4	1	4
	18	4	3	3	4	1	4
	21	4	3	2	4	1	4

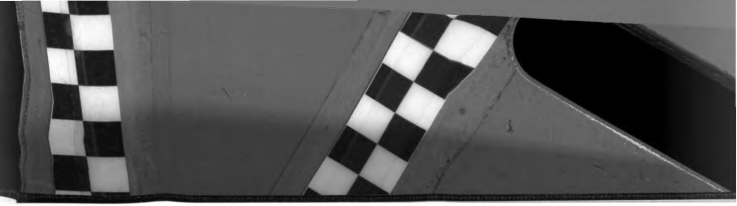


TABLE 10

Intensity of esterase activity of brain tissue in zymograms using alpha naphthyl acetate substrate and Fast Garnet GBC. The numbers indicate the relative intensity from 0 where no band was visible to 4 where the reaction was the most intense.

Inhibitor	Age in days of incubation	Activity in bands using starch insert				Activity in bands using filter paper insert			
		A	B	C	D	A	B	C	D
Control	9	1	1	0	0	1	2	2	2
	12	1	1	0	0	1	1	1	2
	15	1	1	0	0	0	0	1	2
	18	1	1	0	0	0	0	2	3
	21	1	1	0	0	0	0	2	4
Eserine sulfate	9	1	1	0	0	1	1	0	0
	12	1	1	0	0	1	1	0	0
	15	1	1	0	0	0	0	0	0
	18	1	1	0	0	0	0	0	0
	21	1	1	0	0	0	0	0	0
Sodium fluoride	9	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0
	18	0	0	0	0	0	0	0	0
	21	0	0	0	0	0	0	0	0
Sodium taurocholate	9	1	1	0	0	1	2	2	2
	12	0	0	0	0	1	1	1	2
	15	1	1	0	0	0	0	1	2
	18	1	1	0	0	0	0	2	3
	21	1	1	0	0	0	0	2	4
Sodium arsanilate	9	1	1	0	0	1	1	1	2
	12	1	1	0	0	1	1	1	2
	15	0	0	0	0	0	0	1	2
	18	0	0	0	0	0	0	2	3
	21	0	0	0	0	0	0	2	4
Benzaldehyde	9	1	1	0	0	2	2	1	2
	12	1	1	0	0	1	1	1	2
	15	1	1	0	0	0	0	1	2
	18	1	1	0	0	0	0	2	3
	21	1	1	0	0	0	0	2	4

FIGURE 8

Esterase bands obtained with brain and liver homogenates using the disc electrophoresis procedure. Migration was accomplished with a current of 2 milliamperes per tube for 45 minutes. Esterase bands were developed using alpha naphthyl acetate substrate and Fast Garnet GBC. The insert end is at the bottom.

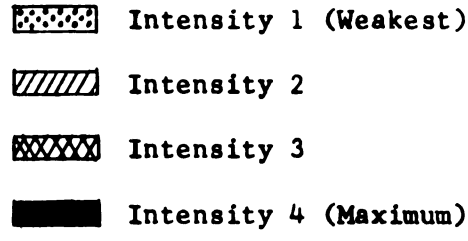
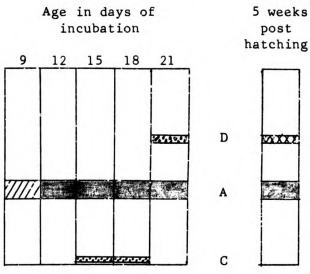


FIGURE 8

Liver Tissue



Brain Tissue

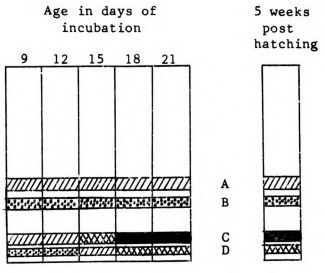


TABLE 11

Localization of esterase activity in brain and liver tissue using 5-bromoindoxyl acetate as a substrate. The amount of activity was estimated visually and represented by numbers from 0-8 where 0 indicates no activity visible and 8 indicates the maximum activity in uninhibited liver tissue. Control 1 was developed immediately following fixation and Control 2 was left in distilled water 30 minutes after fixing and before developing. Column I, control 1; II, control 2; III, sections inhibited with eserine sulfate 1×10^{-3} M; IV, sections inhibited with sodium fluoride 7.14×10^{-2} M; V, sections inhibited with silver nitrate 1×10^{-2} M; and VI, sections inhibited with copper sulfate 1×10^{-3} M.

Tissue	Age in days of incubation	I	II	III	IV	V	VI
Liver	9	8	8	8	4	0	8
	12	8	8	8	6	0	8
	15	8	8	8	6	8	8
	18	8	8	8	6	0	8
	21	8	8	8	6	8	8
Cerebrum	9	2	2	0	0	0	0
	12	2	2	0	0	0	2
	15	3	2	1	0	2	2
	18	3	2	1	0	1	2
	21	2	3	1	0	2	2
Diencephalon	9	3	2	0	0	0	0
	12	3	3	0	0	2	2
	15	5	4	1	0	3	2
	18	5	4	1	0	2	3
	21	4	5	1	0	3	4
Optic lobe	9	3	2	0	0	1	1
	12	3	2	0	0	2	2
	15	5	3	1	0	3	2
	18	6	3	1	0	2	3
	21	3	4	1	0	2	4
Mesencephalon	9	4	2	0	0	0	1
	12	4	3	0	0	2	3
	15	6	4	1	0	3	3
	18	6	4	1	0	3	3
	21	4	5	1	0	4	5

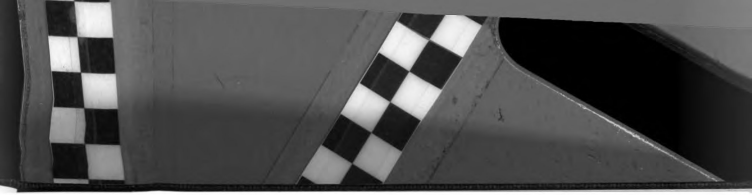


TABLE II (Contd.)

Tissue	Age in days of incubation	I	II	III	IV	V	VI
Cortex of cerebellum	9	2	2	0	0	0	1
	12	2	2	0	0	0	2
	15	4	2	1	0	2	1
	18	4	2	1	0	2	2
	21	3	4	1	0	2	4
Medulla of cerebellum	9	2	2	0	0	0	1
	12	4	3	0	0	2	3
	15	6	4	1	0	4	3
	18	6	4	1	0	3	3
	21	5	6	0	0	4	6
Medulla oblongata	9	4	4	0	0	2	2
	12	4	5	0	0	3	4
	15	6	6	1	0	4	4
	18	6	6	1	0	4	4
	21	5	6	0	0	4	6

TABLE 12

Localization of esterase activity in brain and liver tissue using naphthol AS acetate as a substrate. The amount of activity was estimated visually and represented by numbers from 0-8 where 0 indicates no activity visible and 8 indicates the maximum activity in uninhibited liver tissue. Column I, control; II, tissue sections inhibited with eserine sulfate 1 x 10^{-4} M; III, sections inhibited with silver nitrate 1 x 10^{-2} M; IV, sections inhibited with sodium fluoride 7.14 x 10^{-2} M.

Tissue	Age in days of incubation	I	II	III	IV
Liver	9	8	6	1	8
	12	8	6	1	8
	15	8	6	2	8
	18	8	6	4	8
	21	8	7	2	8
Cerebrum	9	4	2	1	2
	12	4	2	0	3
	15	4	2	1	2
	18	4	3	3	3
	21	5	4	2	4
Diencephalon	9	4	2	1	2
	12	5	2	0	3
	15	5	2	1	3
	18	6	3	4	4
	21	6	4	2	4
Optic lobe	9	4	2	1	2
	12	4	3	0	3
	15	5	3	1	3
	18	5	4	3	4
	21	6	4	2	4
Mesencephalon	9	4	2	1	2
	12	4	2	0	3
	15	5	2	1	3
	18	5	4	4	4
	21	6	4	2	4

TABLE 12 (Contd.)

Tissue	Age in days of incubation	I	II	III	IV
Cortex of cerebellum	9	4	2	1	2
	12	4	2	0	3
	15	4	2	1	2
	18	4	2	2	2
	21	5	4	2	4
Medulla of cerebellum	9	4	2	1	2
	12	4	3	0	3
	15	5	2	1	2
	18	5	3	4	4
	21	6	4	2	4
Medulla oblongata	9	5	2	1	2
	12	5	3	0	4
	15	6	4	1	4
	18	6	4	4	4
	21	6	4	2	5

TABLE 13

Localization of esterase activity in brain and liver tissue using alpha naphthyl acetate as a substrate. The amount of activity was estimated visually and represented by numbers from 0-8 where 0 indicates no activity was visible and 8 indicates the maximum activity in uninhibited liver tissue.

Tissue	Age in days of incubation	Control	Eserine sulfate 1×10^{-5} M
Liver	9	8	8
	12	6	6
	15	6	6
	18	6	6
	21	6	6
Cerebrum	9	1	1
	12	2	2
	15	2	2
	18	2	2
	21	4	2
Diencephalon	9	0	0
	12	2	2
	15	2	2
	18	3	2
	21	4	3
Optic lobe	9	2	2
	12	3	3
	15	3	3
	18	3	3
	21	4	3
Mesencephalon	9	1	1
	12	3	3
	15	3	3
	18	4	4
	21	5	4
Cortex of cerebellum	9	0	0
	12	2	2
	15	2	2
	18	2	2
	21	4	2

TABLE 13 (Contd.)

Tissue	Age in days of incubation	Control	Eserine sulfate 1×10^{-5} M
Medulla of cerebellum	9	0	0
	12	3	3
	15	4	3
	18	4	4
	21	6	4
Medulla oblongata	9	2	2
	12	4	4
	15	5	3
	18	5	4
	21	6	4

FIGURE 9

Calibration curve for protein nitrogen by the Folin-Ciocalteu colorimetric procedure using a graded series of bovine albumen obtained from the Armour Pharmaceutical Co. Ordinates, optical density; obscissae, micrograms of protein nitrogen per ml of solution. Wavelength, 660 mμ.

FIGURE 9

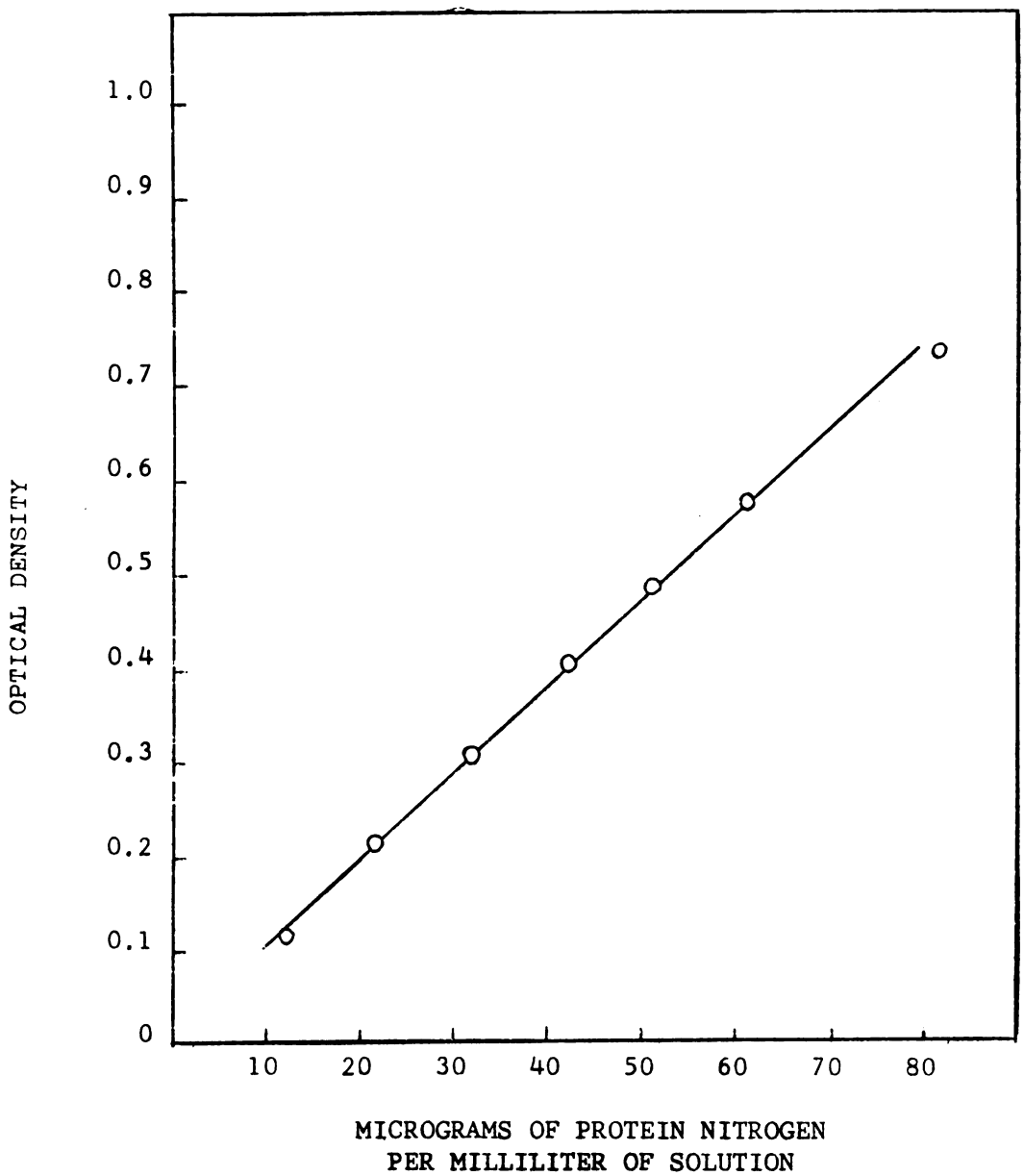

OPTICAL DENSITY
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
80
MICROGRAMS OF PROTEIN NITROGEN
PER MILLILITER OF SOLUTION

TABLE 14

The amount of protein nitrogen in liver and brain tissue calculated in milligrams per gram of wet weight of tissue of developing chicks at 3-day intervals from 9 to 21 days of incubation.

Tissue	Age in days of incubation	No. of tests	No. of embryos	Optical density			Mg of protein nitrogen
				Tests	Control	Corrected	
Liver	9	4	16	.3851	.0066	.3785	19.5
	12	3	14	.4260	.0057	.4703	22.0
	15	5	13	.4330	.0200	.4130	21.5
	18	3	8	.4647	.0448	.4199	22.0
	21	4	12	.4486	.0565	.3921	20.5
Brain	9	4	16	.2048			10.3
	12	3	14	.1938			9.5
	15	5	13	.1979			9.8
	18	3	8	.2262			11.3
	21	4	12	.2733			13.8

FIGURE 10

Amount of protein nitrogen per gram of wet weight of brain and liver tissue of developing chicks at 3-day intervals from the 9th day to the 21st day of incubation determined by the Folin-Ciocalteu colorimetric procedure. Each point is the average of the number of tests and chicks indicated in table 14. Ordinates, mg of protein nitrogen per gram of wet weight of tissue; abscissae, age of chicks in days of incubation. Wavelength 660 mμ.

Legend: ● Liver tissue

O Brain tissue

FIGURE 10

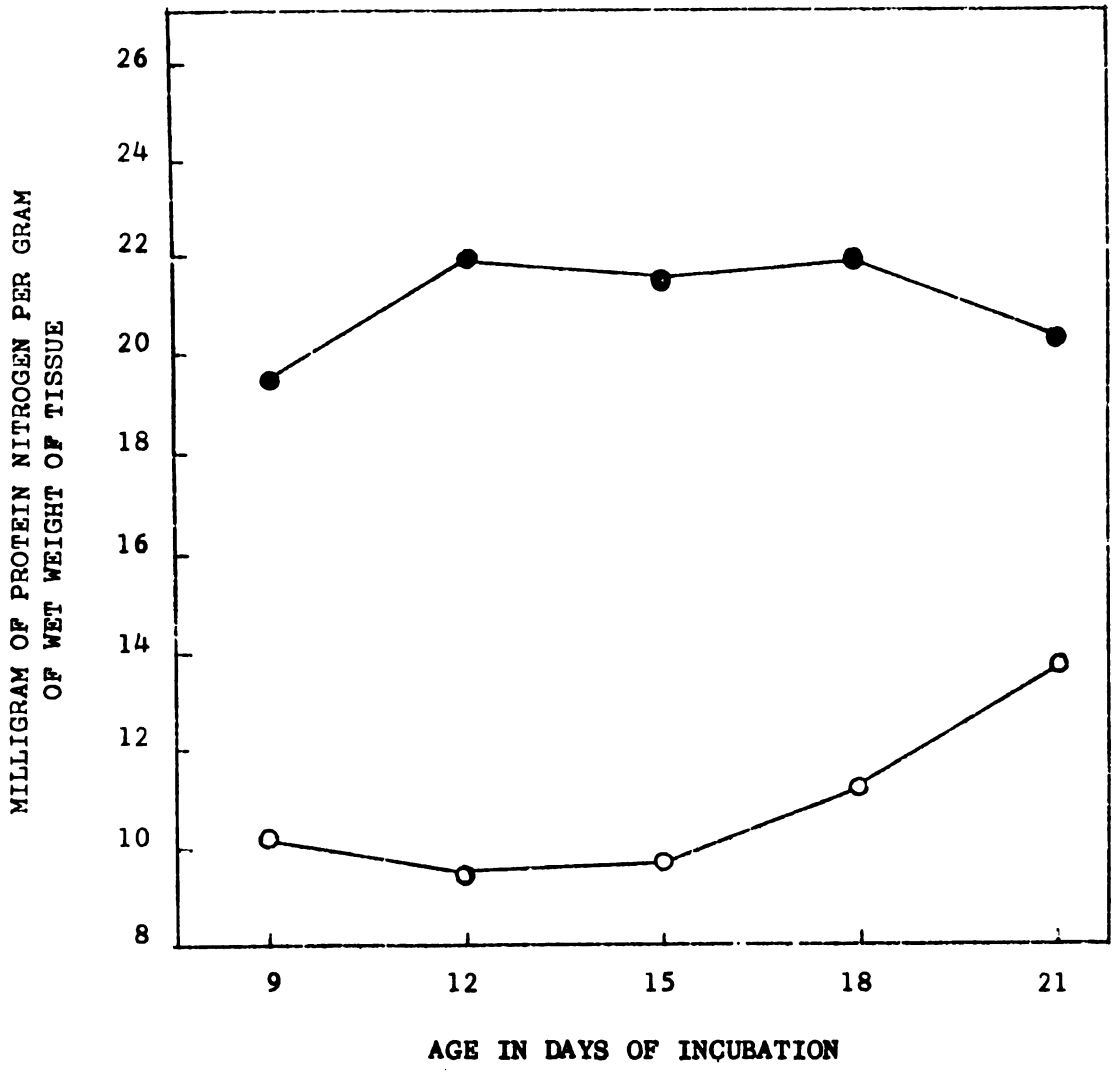

MILLIGRAM OF PROTEIN NITROGEN PER GRAM OF WET WEIGHT OF TISSUE
26
24
22
20
18
16
14
12
10
8
9
12
15
18
21
AGE IN DAYS OF INCUBATION

PLATE 1

EXPLANATION OF FIGURES

Zymograms showing esterase activity in liver tissue of developing chicks at 9 days of incubation. The bands were developed using alpha naphthyl acetate substrate and Fast Garnet GBC. The sample insert end is at the bottom. All bands migrated toward the anode. Upper figures, photographs and diagrams of bands using the starch insert; lower figures, using Whatman #1 filter paper insert.

Fig. 11. Inhibited with benzaldehyde 2.67 x 10^{-2} M.

Fig. 12. Inhibited with sodium arsanilate 1 x 10^{-1} M.

Fig. 13. Inhibited with sodium taurocholate 1.6 x 10^{-2} M.

Fig. 14. Inhibited with sodium fluoride 7.14 x 10^{-2} M.

Fig. 15. Inhibited with eserine sulfate 1 x 10^{-3} M.

Fig. 16. Uninhibited control.

Legend:

 Intensity 1 (Weakest)

 Intensity 2

 Intensity 3

 Intensity 4 (Maximum)

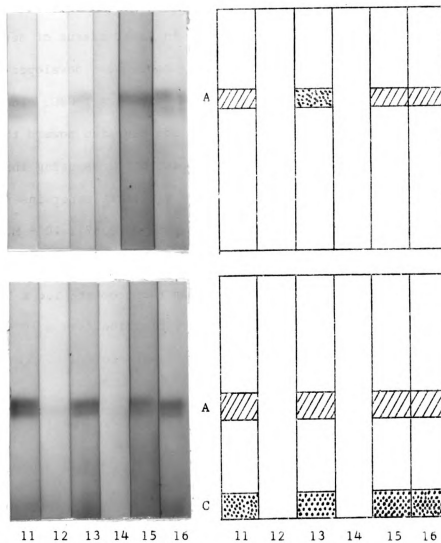

A
A
C
11 12 13 14 15 16
11 12 13 14 15 16

PLATE 2

EXPLANATION OF FIGURES

Zymograms showing esterase activity of liver tissue of developing chicks at 12 days of incubation. The bands were developed using alpha naphthyl acetate substrate and Fast Garnet GBC. The sample insert end is at the bottom. All bands migrated toward the anode. Upper figures, photographs and diagrams of bands using the starch insert; lower figures, using Whatman #1 filter paper insert.

Fig. 17. Inhibited with benzaldehyde 2.67×10^{-2} M.

Fig. 18. Inhibited with sodium arsanilate 1×10^{-1} M.

Fig. 19. Inhibited with sodium taurocholate 1.6×10^{-2} M.

Fig. 20. Inhibited with sodium fluoride 7.14×10^{-2} M.

Fig. 21. Inhibited with eserine sulfate 1×10^{-3} M.

Fig. 22. Uninhibited control.

Legend:

 Intensity 1 (Weakest)

 Intensity 2

 Intensity 3

 Intensity 4 (Maximum)

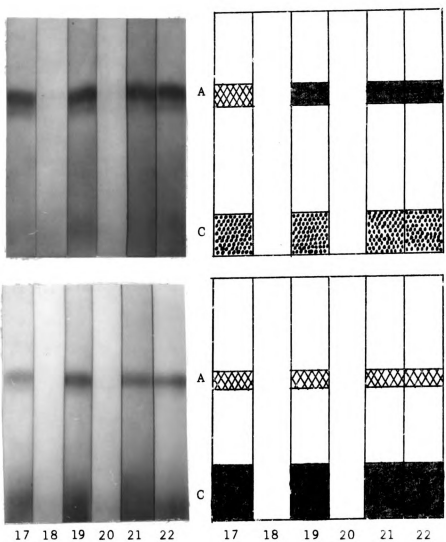

A
C
A
C
17 18 19 20 21 22
17 18 19 20 21 22

PLATE 3

EXPLANATION OF FIGURES

Zymograms showing esterase activity in liver tissue of developing chicks at 15 days of incubation. The bands were developed using alpha naphthyl acetate substrate and Fast Garnet GBC. The sample insert end is at the bottom. All bands migrated toward the anode. Upper figures, photographs and diagrams of band using the starch insert; lower figures, using Whatman #1 filter paper insert.

Fig. 23. Inhibited with benzaldehyde 2.67 x 10^{-2} M.

Fig. 24. Inhibited with sodium arsanilate 1 x 10^{-1} M.

Fig. 25. Inhibited with sodium taurocholate 1.6 x 10^{-2} M.

Fig. 26. Inhibited with sodium fluoride 7.14 x 10^{-2} M.

Fig. 27. Inhibited with eserine sulfate 1 x 10^{-3} M.

Fig. 28. Uninhibited control.

Legend:

 Intensity 1 (Weakest)

 Intensity 2

 Intensity 3

 Intensity 4 (Maximum)

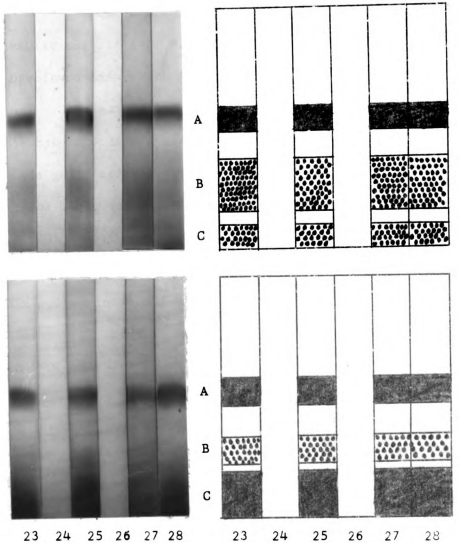

A
B
C
A
B
C
23 24 25 26 27 28
23 24 25 26 27 28

PLATE 4

EXPLANATION OF FIGURES

Zymograms showing esterase activity in liver tissue of developing chicks at 18 days of incubation. The bands were developed using alpha naphthyl acetate substrate and Fast Garnet GBC. The sample insert end is at the bottom. All bands migrated toward the anode. Upper figures, photographs and diagrams of bands using the starch insert; lower figures, using Whatman #1 filter paper insert.

Fig. 29. Inhibited with benzaldehyde 2.67×10^{-2} M.

Fig. 30. Inhibited with sodium arsanilate 1×10^{-1} M.

Fig. 31. Inhibited with sodium taurocholate 1.6×10^{-2} M.

Fig. 32. Inhibited with sodium fluoride 7.14×10^{-2} M.

Fig. 33. Inhibited with eserine sulfate 1×10^{-3} M.

Fig. 34. Uninhibited control.

Legend:

 Intensity 1 (Weakest)

 Intensity 2

 Intensity 3

 Intensity 4 (Maximum)

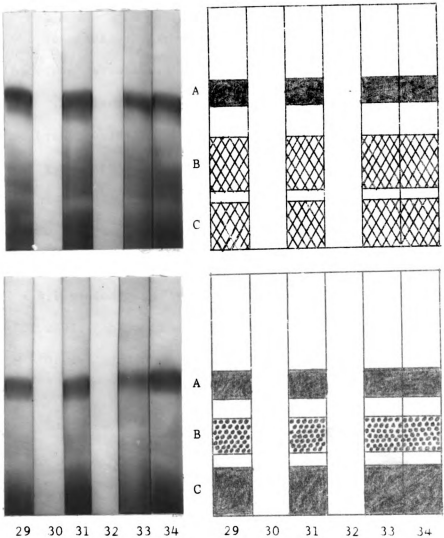

A
B
C
A
B
C
29 30 31 32 33 34
29 30 31 32 33 34

PLATE 5

EXPLANATION OF FIGURES

Zymograms showing esterase activity in liver tissue of developing chicks at 21 days of incubation. The bands were developed using alpha naphthyl acetate substrate and Fast Garnet GBC. The sample insert end is at the bottom. All bands migrated toward the anode. Upper figures, photographs and diagrams of bands using the starch insert; lower figures, using Whatman #1 filter paper insert.

Fig. 35. Inhibited with benzaldehyde 2.67 x 10^{-2} M.

Fig. 36. Inhibited with sodium arsanilate 1 x 10^{-1} M.

Fig. 37. Inhibited with sodium taurocholate 1.6 x 10^{-2} M.

Fig. 38. Inhibited with sodium fluoride 7.14 x 10^{-2} M.

Fig. 39. Inhibited with eserine sulfate 1 x 10^{-3} M.

Fig. 40. Uninhibited control.

Legend:

 Intensity 1 (Weakest)

 Intensity 2

 Intensity 3

 Intensity 4 (Maximum)

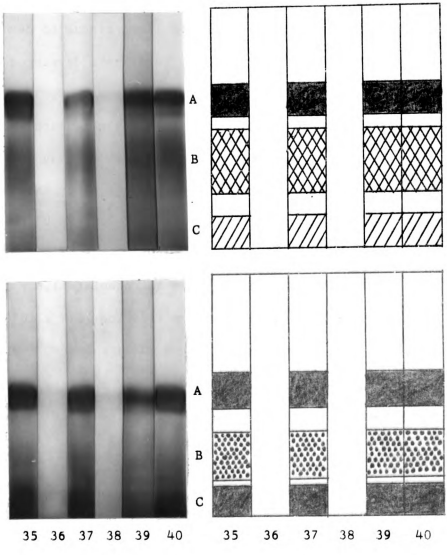

A
B
C
A
B
C
35 36 37 38 39 40
35 36 37 38 39 40

PLATE 6

EXPLANATION OF FIGURES

Zymograms showing esterase activity in brain tissue of developing chicks at 9 days of incubation. The bands were developed using alpha naphthyl acetate substrate and Fast Garnet GBC. The sample insert end is at the bottom. All bands migrated toward the anode. Upper figures, photographs and diagrams of bands using the starch insert; lower figures, using Whatman #3 filter paper insert.

Fig. 41. Inhibited with benzaldehyde 2.67×10^{-2} M.

Fig. 42. Inhibited with sodium arsanilate 1×10^{-1} M.

Fig. 43. Inhibited with sodium taurocholate 1.6×10^{-2} M.

Fig. 44. Inhibited with sodium fluoride 7.14×10^{-2} M.

Fig. 45. Inhibited with eserine sulfate 1×10^{-3} M.

Fig. 46. Uninhibited control.

Legend:


Intensity 1 (Weakest)


Intensity 2


Intensity 3


Intensity 4 (Maximum)

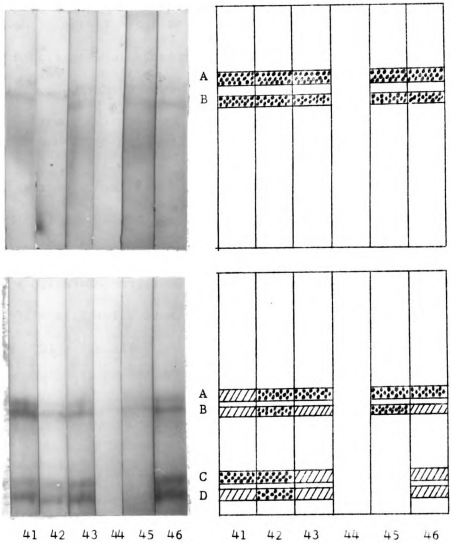

A
B
A
B
C
D
41 42 43 44 45 46
41 42 43 44 45 46

PLATE 7

EXPLANATION OF FIGURES

Zymograms showing esterase activity in brain tissue of developing chicks at 12 days of incubation. The bands were developed using alpha naphthyl acetate substrate and Fast Garnet GBC. The sample insert end is at the bottom. All bands migrated toward the anode. Upper figures, photographs and diagrams of bands using the starch insert; lower figures, using Whatman #3 filter paper insert.

Fig. 47. Inhibited with benzaldehyde 2.67×10^{-2} M.

Fig. 48. Inhibited with sodium arsanilate 1×10^{-1} M.

Fig. 49. Inhibited with sodium taurocholate 1.6×10^{-2} M.

Fig. 50. Inhibited with sodium fluoride 7.14×10^{-2} M.

Fig. 51. Inhibited with eserine sulfate 1×10^{-3} M.

Fig. 52. Uninhibited control.

Legend:

 Intensity 1 (Weakest)

 Intensity 2

 Intensity 3

 Intensity 4 (Maximum)

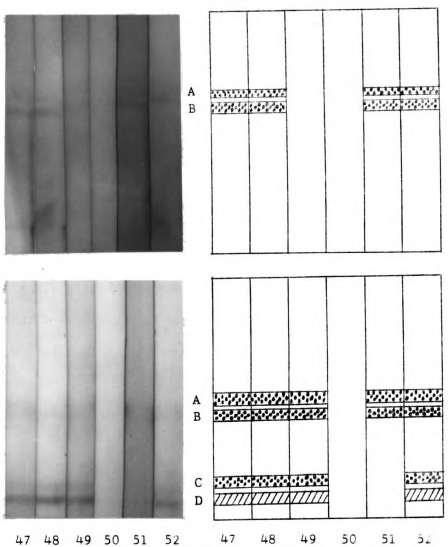

A
B
A
B
C
D
47 48 49 50 51 52
47 48 49 50 51 52

PLATE 8

EXPLANATION OF FIGURES

Zymograms showing esterase activity in brain tissue of developing chicks at 15 days of incubation. The bands were developed using alpha naphthyl acetate substrate and Fast Garnet GBC. The sample insert end is at the bottom. All bands migrated toward the anode. Upper figures, photographs and diagrams of bands using the starch insert; lower figures, using Whatman #3 filter paper insert.

Fig. 53. Inhibited with benzaldehyde 2.67 x 10^{-2} M.

Fig. 54. Inhibited with sodium arsanilate 1 x 10^{-1} M.

Fig. 55. Inhibited with sodium taurocholate 1.6 x 10^{-2} M.

Fig. 56. Inhibited with sodium fluoride 7.14 x 10^{-2} M.

Fig. 57. Inhibited with eserine sulfate 1 x 10^{-3} M.

Fig. 58. Uninhibited control.

Legend:

 Intensity 1 (Weakest)

 Intensity 2

 Intensity 3

 Intensity 4 (Maximum)

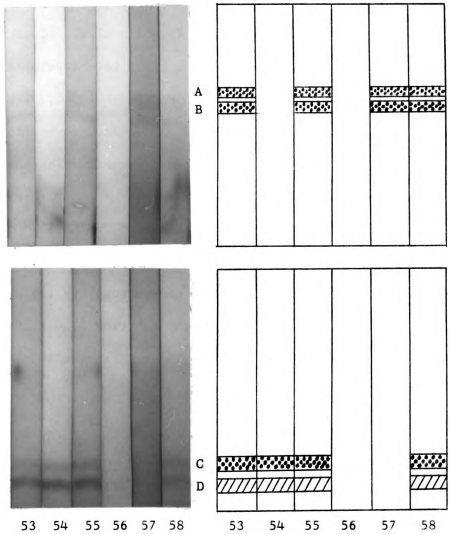

A
B
C
D
53 54 55 56 57 58
53 54 55 56 57 58

PLATE 9

EXPLANATION OF FIGURES

Zymograms showing esterase activity in brain tissue of developing chicks at 18 days of incubation. The bands were developed using alpha naphthyl acetate substrate and Fast Garnet GBC. The sample insert end is at the bottom. All bands migrated toward the anode. Upper figures, photographs and diagrams of bands using starch insert; lower figures, using Whatman #3 filter paper insert.

Fig. 59. Inhibited with benzaldehyde 2.67 x 10^{-2} M.

Fig. 60. Inhibited with sodium arsanilate 1 x 10^{-1} M.

Fig. 61. Inhibited with sodium taurocholate 1.6 x 10^{-2} M.

Fig. 62. Inhibited with sodium fluoride 7.14 x 10^{-2} M.

Fig. 63. Inhibited with eserine sulfate 1 x 10^{-3} M.

Fig. 64. Uninhibited control.

Legend:

Intensity 1 (Weakest)

Intensity 2

Intensity 3

Intensity 4 (Maximum)

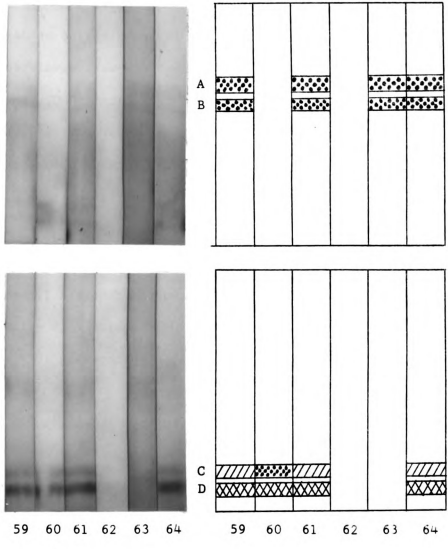

A
B
C
D
59 60 61 62 63 64
59 60 61 62 63 64

PLATE 10

EXPLANATION OF FIGURES

Zymograms showing esterase activity in brain tissue of developing chicks at 21 days of incubation. The bands were developed using alpha naphthyl acetate substrate and Fast Garnet GBC. The sample insert end is at the bottom. All bands migrated toward the anode. Upper figures, photographs and diagrams of bands using the starch insert; lower figures, using Whatman #3 filter paper insert.

Fig. 65. Inhibited with benzaldehyde 2.67 x 10^{-2} M.

Fig. 66 Inhibited with sodium arsanilate 1 x 10^{-1} M.

Fig. 67. Inhibited with sodium taurocholate 1.6 x 10^{-2} M.

Fig. 68. Inhibited with sodium fluoride 7.14 x 10^{-2} M.

Fig. 69. Inhibited with eserine sulfate 1 x 10^{-3} M.

Fig. 70. Uninhibited control.

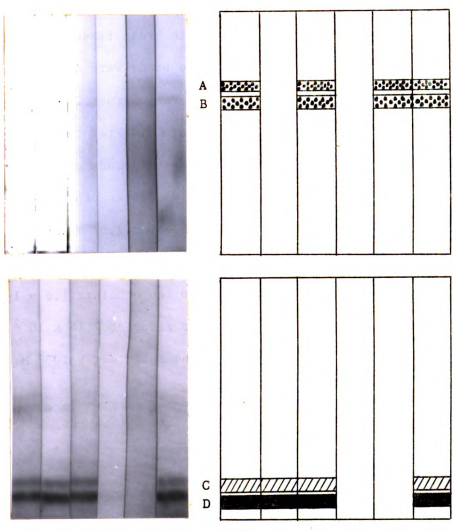
Legend:

 Intensity 1 (Weakest)

 Intensity 2

 Intensity 3

 Intensity 4 (Maximum)


A
B
C
D
65 66 67 68 69 70
65 66 67 68 69 70