Acetylcholinesterase Activity in the Cornea of the Developing Chick Embryo

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I. ACKNOWLEDGEMENTS

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II. ACETYLCHOLINESTERASE ACTIVITY IN THE CORNEA OF THE DEVELOPING CHICK EMBRYO

INTRODUCTION

The cornea contains very high concentrations of the neurotransmitter, acetylcholine (von Brucke et al., 1949, Williams and Cooper, 1965), as well as acetylcholinesterase (Peterson et al., 1965, GHadinger et al., 1967, Howard, et al., 1973) and choline acetyltransferase (van Alphen, 1957, GHadinger et al., 1973). Surprisingly enough, the cornea appears to lack cholinergic receptors (Olsen and Neufeld, 1979). To date, no definitive physiological role for the cholinergic system has been defined in the cornea. There are, however, a number of suggested functions, including: maintenance of corneal transparency (Williams et al., 1965, van Alphen, 1957, Howard et al., 1973, Peterson et al., 1965); regulation of ion transport (Peterson et al., 1965); and transmission of neural information (Fitzgerald and Cooper, 1971).

In the mammalian cornea, the degradative enzyme, acetylcholinesterase, has been localized in both the epithelium and stroma (Peterson, 1965). Most acetylcholinesterase activity resides in the epithelium and is considered to be true acetylcholinesterase, whereas the stromal enzyme is thought to be pseudocholinesterase (Howard, 1975). Some acetylcholinesterase activity in the stroma has been localized to stromal nerves (Howard et al., 1975), whereas the epithelial enzyme appears to be concentrated in intercellular spaces, which appear devoid of corneal nerves (Howard, 1975, Tervo et al., 1978). This suggests that a large proportion of true corneal acetylcholinesterase is unrelated to nerve supply and may be produced by the corneal epithelium cells themselves (GHadinger et al., 1967).

Although the embryonic development of the avian cornea has been intensively studied for many years (Coulombre and Coulombre, 1961, Hay,

1980), the enzymes of acetylcholine biosynthesis and degradation have not been previously assayed during development in this tissue.

In the present study, we correlated the well-defined developmental changes in the avian cornea with changes in the specific activity of both true acetylcholinesterase and of non-specific (butyryl) cholinesterase. Enzyme activities were measured in corneal homogenates from day 7 through day 20 of incubation, as well as at three ages after hatching. The results indicate a striking 60-fold transient increase in true corneal acetylcholinesterase specific activity during embryonic development. This increase begins before stainable nerves are present in the cornea (Bee, 1982), reaches a peak as the embryonic cornea is just beginning to become transparent (Coulombre and Coulombre, 1958a), and during the time when the corneal epithelium become extensively innervated (Bee, 1982), and then falls sharply before hatching to a level maintained into adulthood. This pattern closely parallels the transient appearance of highly sialylated gangliosides in developing chick corneas (Rintoul et al., 1984).

MATERIALS AND METHODS

Fertilized White Leghorn (Gallus domesticus) chicken eggs were obtained through a local hatchery and incubated at 37 ± 0.5°C in a forced draft incubator. Developmental stages were assessed at the time of dissection according to Hamburger and Hamilton (1951).

Chemicals were procured as follows: 1,4,-bis-2-(5-Phenyloxazolyl)-Benzene (POPOP), 2,5-Diphenyloxazole (PPO), and Triton X-100 were obtained from Packard Instruments (Downers Grove, Illinois). One dram Opticlear borosilicate glass vials, 15 mm O.D. x 45 mm long, with open bottom polyethylene stoppers were purchased from Kimble (Toledo, Ohio). Iso-amyl alcohol, dimethyldichlorosilane, bovine serum albumin, and choline iodide were obtained from Sigma Chemicals (Saint Louis, Missouri). Toluene, mono- and dibasic sodium phosphate, HCl, and acetic acid were all reagent grade and obtained from Fisher Chemicals (Fairlawn, New Jersey). The cholinesterase inhibitors, tetraisopropylpyrophosphoramide (iso-OMPA) and 1:5-bis-(4-allyldimethyl-ammonium phenylpentane-3-one) (BW284c51) were acquired from Sigma Chemicals and Burroughs-Wellcome (Greenville, North Carolina), respectively. The acetylcholine iodide (acetyl-^{3}H) was purchased from New England Nuclear (Boston, Massachusetts).

Tissue Preparation

Unless otherwise stated, all processing was done on ice. Corneal homogenates were assayed immediately after their preparation. All embryos and hatchlings were killed by decapitation. Dissections were performed with fine iridectomy scissors and jeweler's forceps. In younger embryos, before day 12 of incubation, it was necessary to remove

the front part of the eye, including cornea, aqueous humor, and the lens before removing the cornea. These dissections were carried out with the aid of a dissection microscope. At the other ages, on and after 12 days of incubation, the eyelids and nictating membrane were removed before the cornea and scleral ring were dissected. Pigmented iris that adhered to the corneas was removed with a gentle sweeping motion of the dissected cornea and ring over a dry Kimwipe tissue. Great care was taken to avoid pinching the cornea itself.

Corneal diameters (ignoring corneal curvature) were measured at different ages during embryonic development using a calibrated eyepiece and a stage micrometer (data not shown). These measurements were made from eyes previously stained by Bee (1982) and stored in methyl salicylate to reveal the corneal nerves and the pericorneal nerve ring from which they arise. For the present study, it was important to ascertain that the corneal tissue removed for the enzyme assay did not contain portions of the pericorneal nerve ring. The corneal diameters obtained were then compared with those of Coulombre and Coulombre (1955), as well as with those obtained from the photomicrographs previously published by Bee (1982). These three sets of values were very similar when compared to one another at a given embryonic age (± 0.1 mm). Stainless steel tubing of appropriate inner diameter (I.D., 1.0-3.2 mm) was sharpened at one end and employed as corneal punches (penetrating trephines (Conrad and Woo, 1980)) to remove corneal tissue cleanly from embryos of days 7 through 20 of incubation.

Eyefronts, containing corneas to be dissected, were placed in plastic Petri dishes containing Saline G on ice, in groups of ten, with the corneal epithelium side up. This last step was done to ensure minimal disruption of the epithelial cell layer, the tissue layer

containing the majority of true acetylcholinesterase activity in mammals (GHadinger et al., 1967, Howard et al., 1975, and Williams et al., 1965). The corneas then were removed from the scleral rings and pericorneal nerve ring by bringing the steel trephine punch down perpendicularly and pushing against the dish bottom. The punch was first pressed straight downward and then pressed at an angle around the corneal edges without rolling the punch. This method proved to be extremely successful in dissecting corneas free of contaminating non-corneal tissue, including the pericorneal nerve ring. After each set of ten corneas had been punched, it was placed on a tared piece of Parafilm, excess saline was removed quickly by careful blotting, and wet weights were determined. At each embryonic age, the acetylcholinesterase activities detected in the homogenate fractions from each of three such replicate ten-cornea sets were averaged. Homogenates were prepared from each ten-cornea set as follows.

Corneas were transferred to a conical 0.5 ml microcentrifuge tube containing 200 µl of a phosphate buffer (50 mM NaH_2PO_4, 0.2 mM EDTA, 0.5% Triton X-100, pH 7.3). Samples from embryos of 7-11 days of incubation were not minced prior to sonication; however, for samples from embryos of 12 days of incubation and older, corneas were mechanically minced into minute particles with fine scissors while the corneas were still in the microcentrifuge tubes, on ice. In preparation for sonication (samples from embryos of all ages), the microcentrifuge tubes were capped, securely taped to the bottom of a 1 liter plastic beaker, and immediately covered with a suspension of crushed ice and water, in preparation for sonication. Sonication was performed with a Cell Disruptor sonifier (model W185, Branson Sonic Power Co., Plainview, New York), using a blunt tipped probe, power setting of 7 and two

sonication periods of 30 seconds each for each tube, separated by 60 seconds to allow cooling, approximate power output during each period was 100 watts. During sonication, the side of each tube was stroked with the blunt end of the probe while the tubes were kept continuously submerged in the ice suspension (Richardson and Spooner, 1976).

Sonicated samples were centrifuged (15,000 rpm, 26,890 x g, 30 min, 4°C) in a Sorvall SS-34 rotor using a Sorvall RC-5B centrifuge. The supernatant solutions from each of three ten-cornea tubes were pooled and set aside on ice (supernatant S_1). The pellet was resuspended in another 200 µl of the phosphate buffer, with resuspension achieved by repeated mincing with fine scissors. Mincing was followed by repeating the sonication and centrifugation steps as described above. The supernatants from the second centrifugation, referred to as S_2, were combined with S_1. The pooled $S_1 + S_2$ combined supernatant fraction was used for measurement of soluble protein and enzyme activity at each day of embryonic development. This procedure completely solubilizes corneas from embryos younger than day 12 of incubation (i.e. no pellets are present after S_1 and S_2 are obtained). Corneal samples from embryos of 12 days of incubation and older displayed progressively larger pellets, but the $S_1 + S_2$ supernatant fraction was found to contain approximately 90% of all cholinesterase activity when compared to that still remaining in the pellet (data not shown). Nevertheless, acetylcholinesterase activity was measured in the pellets on alternate days after day 12 of incubation.

Substrate Preparation

Radioactively labelled acetylcholine substrate was obtained from New England Nuclear (acetylcholine iodide (acetyl-^{3}H-), lot number 1516, specific activity:67 mCi/mmole). One mCi of the ^{3}H-acetylcholine iodide

was dissolved in 300 µl of glass distilled water (approximate pH 5-6) and freed from products of spontaneous hydrolysis by chromatography on a column (0.5 x 6.0 cm) containing anion exchange resin (Dowex, Bio-Rad AG 1-X2, 200-400 mesh, chloride form, Bio-Rad, Richmond, California). The column had been rinsed previously with 10 ml volumes of: glass distilled water, 1.0 N HCl, and sufficient glass distilled water to remove excess Cl^- from the column, as judged by $AgNO_3$ precipitation. After application of the acetylcholine (together with 300 µl aliquots of glass distilled water used to rinse the original isotope vial), the column was washed with repeated 300 µl volumes of glass distilled water coupled with collection of eluent in corresponding 300 µl aliquots into 1.5 ml conical microcentrifuge tube already containing 100 µl of 1.0 mM citric acid, pH 3.5 (citrate buffer). The total volume in each sequential tube after collection of eluent therefore was 400 µl. A small volume (2 µl) was removed from each fraction for determination of radioactivity. Fractions with the highest radioactivity were pooled, and the isotope was diluted with additional citrate buffer so that 10 µl would contain 100,000 cpm. After dilution, the isotope was dispensed into tubes in aliquots of 0.5 ml each (sufficient for fifty individual assays) and stored at -70°C, conditions which held the level of spontaneous decay to less than 2% during this study.

Acetylcholinesterase Assay

The standard assay procedure was derived from Johnson and Russell (1975). The rationale for assaying acetylcholinesterase activity, as described below, at substrate (acetylcholine) concentrations well below the K_m of the enzyme has been presented in detail for choline acetyltransferase (see Rand and Johnson, 1981).

Each assay was carried out entirely in silanized 1 dram glass vials that served first as reaction vessels and then as counting vials. Silanization of the glass vials was achieved as follows: A solution of dimethyldichlorosilane and glass distilled water (1:100 v:v) was prepared immediately before use and added to the glass vials. The vials were allowed to sit 5 minutes at room temperature, rinsed briefly with warm soapy water followed by glass distilled water, and allowed to dry overnight at approximately 140°C.

Assays were conducted at 26°C. Each assay vial received the following, in order: 60 µl of the phosphate buffer described above for enzyme solubilization, 10 µl of 10^{-4} M iso-OMPA stock solution made fresh daily in the phosphate buffer, and 20 µl of the $S_1 + S_2$ corneal homogenate. Tubes then were pre-incubated for 20 min at ambient temperature in the presence of iso-OMPA to allow inactivation of non-specific cholinesterases. Labelled substrate (10 µl containing 10^5 cpm of ^{3}H-acetylcholine iodide) was added and the reaction was allowed to proceed at 26°C. Final reaction volume therefore was 100 µl and final concentration of added acetylcholine was 0.04 mM. Assay tubes were stopped (see below) in triplicate at each of four incubation times (1,3,5, and 7 min), and an average cpm value was determined for each time. A duplicate set of assay tubes was prepared by pre-incubating for 20 min in the absence of iso-OMPA, (10 µl of phosphate buffer alone was substituted for the iso-OMPA) and assayed as above in order to measure total cholinesterase activity (acetylcholinesterase + non-specific cholinesterase). Subtraction of these two sets of data therefore yielded the activity of the non-specific cholinesterase.

Incubations were stopped by addition of 100 µl of an acidic stopping solution containing 1.0 M monochloroacetic acid, 2.0 M NaCl,

and 0.5 M NaOH, pH 2.5. Four ml of a toluene-based scintillation fluid (90% toluene, 10% iso-amyl alcohol, 0.5% PPO, 0.03% POPOP) then were added. Each tube was capped and vortexed for 5 seconds at a medium setting. The vials were placed inside glass scintillation vials (25 min O.D. x 60 mm long) and counted in a Packard Tri-carb 4530 liquid scintillation counting system for one minute each. Counting efficiency was experimentally determined to be 37% in quenched and unquenched samples, as determined by addition of known amounts of a ^{3}H-toluene standard. The acidic stopping solution not only halts the reaction but also protonates the product of the enzyme reaction (acetate) to ensure its extraction into the organic phase during the extraction step, thus allowing its detection by the liquid scintillation counter (Johnson and Russell, 1975).

Controls were included during each assay to correct for spontaneous hydrolysis of the labelled substrate. Such tubes contained 80 µl of the phosphate buffer, 10 µl of the iso-OMPA 10^{-4} M stock solution, and 10 µl (10^{5} cpm) of the ^{3}H-acetylcholine. The reaction was incubated for 20 min at 26°C, stopped with 100 µl of the stopping solution, and mixed with 4 ml scintillation fluid. Radioactivity observed represented the maximum amount of ^{3}H-acetylcholine that underwent spontaneous hydrolysis in the experimental vials. The radioactivity detected in three such vials was averaged and subsequently subtracted from the radioactivity detected in vials containing enzyme. Throughout this study, spontaneous hydrolysis was equal to or less than 2% of releasable ^{3}H-acetate.

Controls also were included during each assay to allow direct determination of the total releasable ^{3}H- contained in 10 µl of the ^{3}H-acetylcholine used in that experiment. For this control, 10 µl of labelled acetylcholine was mixed with 20 µl of 2.0 N NaOH and incubated

for 20 min at 26°C in order to hydrolyze the substrate entirely to free ^{3}H-acetate. Forty microliters of 1.0 N HCl then was added to neutralize the solution, followed by 100 µl stopping solution and 4 ml scintillation fluid. Radioactivity detected in these tubes of labelled substrate represented the total amount of ^{3}H releasable from a 10 µl aliquot of labelled substrate when totally converted into ^{3}H-acetate.

Protein determinations were performed on the $S_1 + S_2$ supernatant fractions and pellets by the method of Lowry, as modified by Peterson (1977) to allow inclusion of Triton X-100, and even sodium dodecylsulfate. Corneal homogenate pellets were solubilized by adding 20 µl of 1.0 N NaOH and incubating at 56°C in a circulating water bath for 30 min before protein concentrations were determined. Protein standards were made from bovine serum albumin. A separate set of standards containing NaOH was used to determine protein concentrations of the pellets.

The Taylor series approximation for the variance of a ratio was applied to all enzyme activity data reported in this paper (Kendall and Stuart, 1977). Enzyme activities are represented as the fraction of substrate that is converted into product (fractional conversion, F_c). In many figures, the calculation of standard errors using this methods were too small to represent graphically (i.e. error bars were smaller than the data points).

Calculation of Specific Activity

To increase sensitivity of this assay, substrate concentrations were deliberately kept well below the K_m for acetylcholinesterase (Rand and Johnson 1981). The experimental velocities are not represented at V_{max} values, but the individual assays can be compared to one another

provided that they have the same K_m. Under these conditions, the instantaneous velocity is directly proportional to the substrate concentration and therefore declines as the assay proceeds due to depletion of the substrate. The measured fraction of substrate converted to product at a given time is defined as the observed fractional conversion (F_{obs}). Rand and Johnson (1981) defined the F_{obs} as:

$$F_{obs} = \frac{\text{cpm with enzyme - cpm blank}}{\text{cpm "total conversion" - cpm blank}}$$

In the present study, cpm with enzyme was defined as the cpm obtained after completion of the respective incubation time (as outlined above). The cpm blank and cpm total conversion were defined as the spontaneous hydrolysis background control and the total ^3H-releasable control, respectively.

The F_{obs} values were transformed into corrected fractional conversion values (F_c) to correct for substrate depletion. Corrected fractional conversion is defined by Rand and Johnson (1981) as:

$$F_c = -\ln(1-F_{obs})$$

Fractional conversion is a measure of V_{max}/K_m in units of reciprocal time per volume of enzyme or per milligram of protein present in the assay. All values of enzyme activity presented in this paper are presented in the form of corrected fractional conversion, derived from the observed fractional conversion. See Rand and Johnson (1981) for a more detailed discussion of this method.

Calculation of the specific activity of acetylcholinesterase for each day of embryonic development was as follows:

$$\text{acetylcholinesterase activity} = \frac{(F_c/\text{min})\ (0.1\ \text{ml})\ (K_m)}{\mu\text{g Protein present in 20 } \mu\text{l of } S_1 + S_2}$$

$$= \frac{\mu\text{moles acetylcholine hydrolyzed/min}}{\mu\text{g Protein present in 20 } \mu\text{l of } S_1 + S_2}$$

K_m was experimentally determined (see Results) to be 1.1 mM at the three embryonic ages measured.

RESULTS

Standardization of the Assay:

Control assays were performed in order to define the assay requirements for the avian embryonic system used in this study. This demonstrates the validity of comparing acetylcholinesterase activities between corneas from different age chick embryos. All controls were performed at three embryonic ages (unless otherwise stated) which represent early (7 day), middle (14 day), and late (20 day) stages in embryonic corneal development. Normal chick embryos that were left undisturbed hatched at day 21 of incubation.

To determine the effect on acetylcholinesterase activity of varying assay incubation times, true acetylcholinesterase activity (hereafter referred to as acetylcholinesterase activity) from corneal homogenate, was measured as a function of increasing incubation time (see Fig. 1). This demonstrated that acetylcholinesterase activity increased proportionally with increased incubation time. In Fig. 1, the time dependency of acetylcholinesterase activity is shown at 7, 14, and 20 days of embryonic incubation. Enzyme activity is represented as the fractional conversion (F_c) which is defined as the fraction of substrate converted to product at the indicated incubation time (see Materials and Methods). These results confirm that acetylcholinesterase activity does increase proportionally as assay incubation time increases. This relationship exists in 7 and 14 day embryonic enzyme for assay incubation times from 0 through 20 min; in 20 day embryonic enzyme, this proportional relationship exists from 0 to 10 min of incubation. We therefore chose standard incubation times of 1, 3, 5, and 7 min for the acetylcholinesterase assay to ensure linear proportionality between acetylcholinesterase activity and incubation time.

Acetylcholinesterase activity was measured as a function of increasing volume of corneal homogenate (see Fig. 2). This experiment showed that enzyme activity increased linearly with increased amounts of corneal homogenate present in the assay. Based on this data, a corneal homogenate volume of 20 µl was used to assay acetylcholinesterase activity for all ages in the embryonic series.

The relative contributions of true acetylcholinesterase, non-specific cholinesterase, and other cholinesterases to total cholinesterase activity was measured at 7, 14, and 20 days of embryonic incubation (see Fig. 3). True acetylcholinesterase activity was measured as the enzyme activity observed in the presence of the non-specific cholinesterase inhibitor iso-OMPA. Non-specific cholinesterase activity was measured as the enzyme activity observed in the presence of the true acetylcholinesterase inhibitor BW284c51. Other cholinesterase activity was measured as the activity observed in the presence of both true acetylcholinesterase and non-specific cholinesterase inhibitors (iso-OMPA and BW284c51). As shown in Fig. 3, non-specific cholinesterase inhibitor (iso-OMPA) inhibited approximately 26% of total cholinesterase activity. True acetylcholinesterase inhibitor (BW284c51) inhibited approximately 84% of total cholinesterase activity. Little to no cholinesterase activity was measurable in assays containing both iso-OMPA and BW284c51. The relative amounts of true acetylcholinesterase activity and non-specific cholinesterase activity remained similar at early, middle, and late stages of embryonic development.

The effect of varying concentrations of iso-OMPA on true acetylcholinesterase activity was measured and the results are shown in Fig. 4. There is a decrease in cholinesterase activity between the iso-OMPA concentrations of 0 and 1 µm. Subsequent addition of iso-OMPA had

little effect on cholinesterase activity; from 10 to 100 μm cholinesterase activity varied by 7% or less. A final standard acetylcholinesterase assay concentration of 10^{-5} M iso-OMPA was chosen based on the data in Fig. 4. This final concentration has been routinely used in studies of muscle acetylcholinesterase (Groswald and Dettbarn, 1983).

Fig. 5 shows the corneal acetylcholinesterase as a function of time exposed at 26°C. After pre-incubation for 20 min with iso-OMPA, assay vials with enzyme were allowed to sit at 26°C for varying lengths of time before substrate was added and the reaction was allowed to proceed for an incubation time of 10 min. As indicated in Fig. 5, the acetylcholinesterase activity remained stable through 3 hours of incubation at 26°C.

Different concentrations of NaCl were added to the extracting buffer to measure the effect of the salt on acetylcholinesterase activity. The dramatic results of this experiment are shown in Fig. 6. The top half of the graph represents the sensitivity of corneal acetylcholinesterase from 14 day embryos. The lower half represents the sensitivity of corneal acetylcholinesterase from 7 day embryos. The incubation time in both ages was 10 min. Based on the results of this experiment, the extraction buffer used contained no NaCl.

Experimental:

The apparent K_m of embryonic corneal acetylcholinesterase was measured at three stages of embryonic development, early (7 day), middle (14 day) and late (20 day) and is shown in Fig. 7. The apparent K_m was determined to be $1.1 \pm 0.3 \times 10^{-3}$ M and is the same at all three stages of embryonic development.

Daily acetylcholinesterase assays were performed on embryonic corneas from day 7 through day 20 of incubation. Acetylcholinesterase

assays were also done at 1 day, 1 week, and 1 year after hatching (see Fig. 8, solid line). As shown in this figure, a 60-fold increase in acetylcholinesterase specific activity occurs during embryonic development. This increase begins at day 7 and reaches a peak at day 15 of embryonic development. After day 15, acetylcholinesterase specific activity decreases to adult levels. The level of non-specific cholinesterase was also measured from day 7 through day 20 of embryonic incubation. These results are shown in Fig. 8 (dotted line). Non-specific cholinesterase activity also undergoes a transient increase which reaches a peak at days 17-18 of embryonic incubation and then declines to adult levels.

DISCUSSION

Acetylcholinesterase activity is more readily assayed than other components of the cholinergic triad (i.e acetylcholine and choline acetyltransferase) and was used as a marker of possible cholinergic activity in this study. The cornea is known to contain high levels of acetylcholine (von Brucke et al., 1949, Williams and Cooper, 1965) but lacks cholinergic receptors (Olsen and Neufeld, 1979). This disparity lead researchers to postulate a possible non-neuronal function for acetylcholine in the cornea (Peterson et al., 1965, Williams et al., 1965, van Alphen, 1957, Howard et al., 1973). The cornea is also known to contain high levels of acetylcholinesterase (Peterson et al., Gnadinger et al., 1967, Howard et al., 1973). If acetylcholinesterase activity indicates the presence of acetylcholine, then the results obtained in this study support the idea that acetylcholine may have a non-neuronal function in the developing chick cornea.

A radiometric assay was used to monitor the specific activity of corneal acetylcholinesterase during day 7 through day 20 of chick development. The results reveal a dramatic transitory increase in enzyme activity which begins at day 7, reaches a maximum at day 15, and then decreases to levels maintained into adulthood.

Days 12-14 of corneal development are considered critical and mark the beginning of corneal maturation in the chick (Hay, 1980). Maturation is completed by day 19 (Hay, 1980). Acetylcholinesterase activity reached a maximum during the end of the critical period and before maturation. Consideration of concurrent developmental events provides a perspective for interpreting the transitory increase of acetylcholinesterase activity.

Chick corneal innervation begins on day 12 with nerves entering the stromal periphery. By day 15, extensive innervation has occurred and nerves have entered the epithelium from the stroma. Migrating nerves reach the center of the epithelium by day 18 (Bee, 1982). Bee observed (1982) the appearance of mottled loci by Bodian silver staining one day before the peak acetylcholinesterase activity (day 14); these loci disappeared by day 16.

Several other significant developmental events occur during this period. Corneal transparency is acquired between day 14 and day 19 of development (Coulombre and Coulombre, 1958). The thyroid hormone thyroxine elicits this change (Coulombre and Coulombre, 1964). Dehydration of the cornea occurs starting at day 14 and continues until day 19 of development (Coulombre and Coulombre, 1964). Other processes occurring from day 13 through day 19 include: epithelial cell stratification, formation of Descemet's and Bowman's membranes (Hay, 1980), thyroxine induced increased synthesis of 3'-phosphoadenosine-5' phosphosulfate (PAPS) (Beckenhauer and Conrad, 1981), increased sulfation of glycosaminoglycans (Hart, 1976), and eyelid closure on day 14.

The transitory increase in acetylcholinesterase activity parallels the transitory appearance of highly sialylated gangliosides in developing chick corneas on days 14-16 of development (Rintoul et al., 1984). The significance of the appearance of these gangliosides remains undetermined. Burton and co-workers have suggested that gangliosides play a functional role in the transport of acetylcholine from synaptic vesicles, through the presynaptic membrane, and into the synaptic cleft (Burton et al., 1963, Burton and Howard, 1967). Burton reported synaptic vesicle subcellular fractions from rat brain that contained

high levels of gangliosides also contained high levels of acetylcholine. Other researchers reported that synaptic vesicle subcellular fractions isolated from rat brain were devoid of gangliosides and acetylcholine but that both these compounds were present in abundance in the same synaptic membrane subcellular fractions (Lapetina et al., 1966). This latter observation led Lapetina's group to postulate that gangliosides may act as acetylcholine receptors. Subcutaneous application of exogenous ganglioside to neonatated rats purportedly facilitates learning behavior (acquisition and retention) and levels of cortical acetylcholinesterase activity (Karpiak et al., 1984).

Further research is required to clarify the functional significance of the transitory increase in acetylcholinesterase activity during chick corneal development.

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III. APPENDIX

APPENDIX - Definition and Derivation of Fractional Conversion (F)

Fractional Conversion and Corrected fractional conversion were derived from the Michaelis-Menten equation by Rand and Johnson (1981) as follows.

$$\frac{d[P]}{dt} = \frac{Vmax[S]}{K_m{}^r[S]} \cong \frac{Vmax[S]}{K_m}$$

$$\frac{d[P]}{dt} = \frac{Vmax}{K_m}\,dt$$

Rand and Johnson defined the measured fraction of substrate converted to product as:

$$\text{"F"} = \frac{\text{cpm with enzyme - cpm blank}}{\text{cpm "Total conversion" - cpm blank}}$$

According to this theory, if S_o is the initial substrate concentration, then $[P] = S_o(F)$ and $[S] = S_o(1-F)$. By substitution:

$$\frac{dS_o(F)}{S_o(1-F)} = \frac{Vmax}{K_m}\,dt$$

and since S_o cancels,

$$\frac{dF}{1-F} = \frac{Vmax}{K_m}\,dt$$

and $dF = -d(1-F)$, then,

$$\frac{-d(1-F)}{1-F} = \frac{Vmax}{K_m}\,dt \qquad , \quad -\int_{1}^{1-F}\frac{d(1-F)}{1-F} = \int_{0}^{t}\frac{Vmax}{K_m}\,dt$$

therefore, $-\ln(1-F) = Vmax/K_m \cdot t$

Thus $-\ln(1-F)$ increases linearly with time. The corrected fractional conversion is defined as:

$$F_c = -\ln(1-F)$$

F_c is a measurement of $Vmax/K_m$ in units of reciprocal time per volume of enzyme or per milligrams of protein present in the assay. F_c will increase linearly as the reaction progresses provided that the enzyme obeys Michaelis-Menten kinetics and provided that the initial concentration of labelled substrate is well below the K_m.

All figures in this study have been plotted as F_c values.

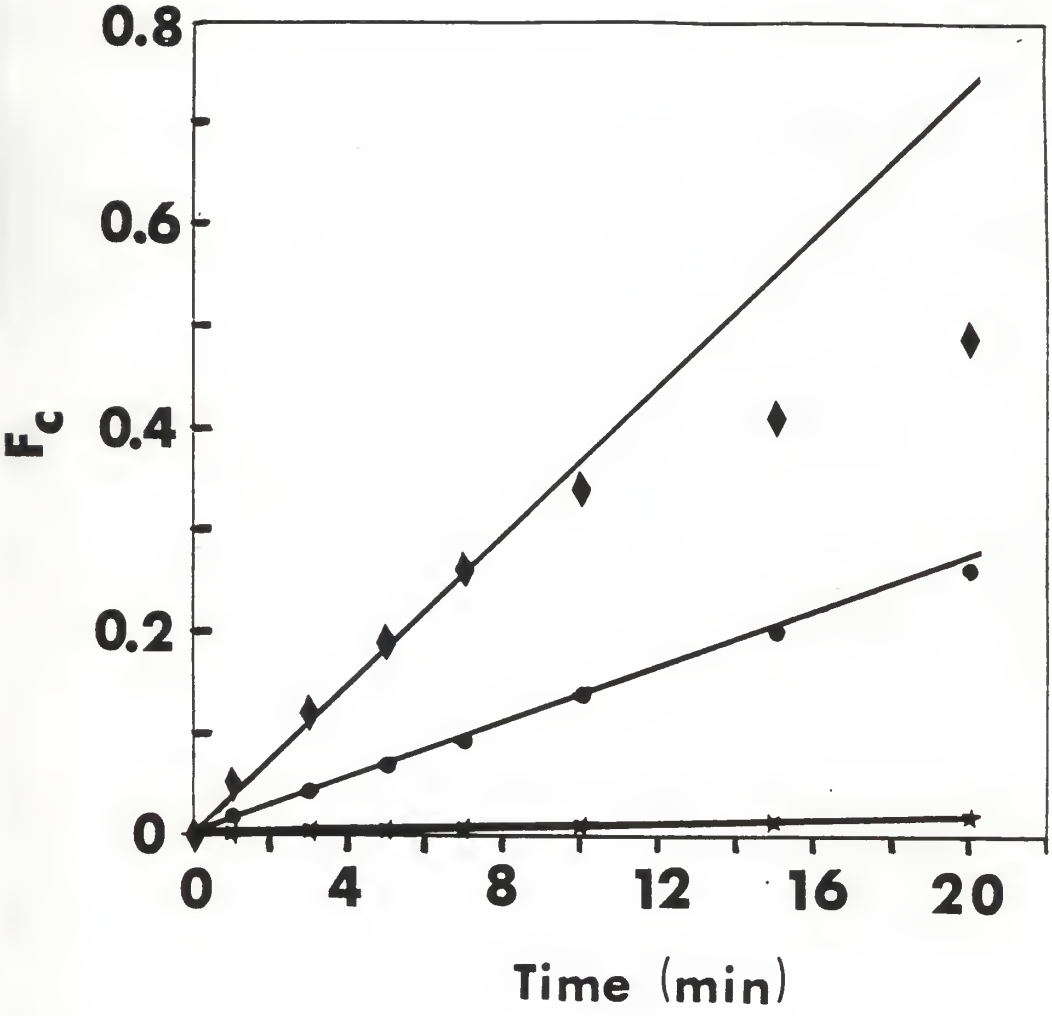
0.8
0.6
0.4
0.2
0
F_c
0
4
8
12
16
20
Time (min)

FIG. 1. Time dependency of the assay. Acetylcholinesterase activity (F_c) as a function of incubation time at three developmental stages in the embryonic chick cornea: early (7 day *), middle (14 day ●), and late (20 day ◆). Standard assay procedure was performed using 20 µl of $S_1 + S_2$ corneal homogenate enzyme at 26°C in the presence of 10^{-5} M iso-OMPA in a total reaction volume of 0.1 ml. Reactions were terminated by adding a standard stopping mixture and 4 ml of a toluene-based scintillation fluid. Each data point represents the average value from three identical assays.

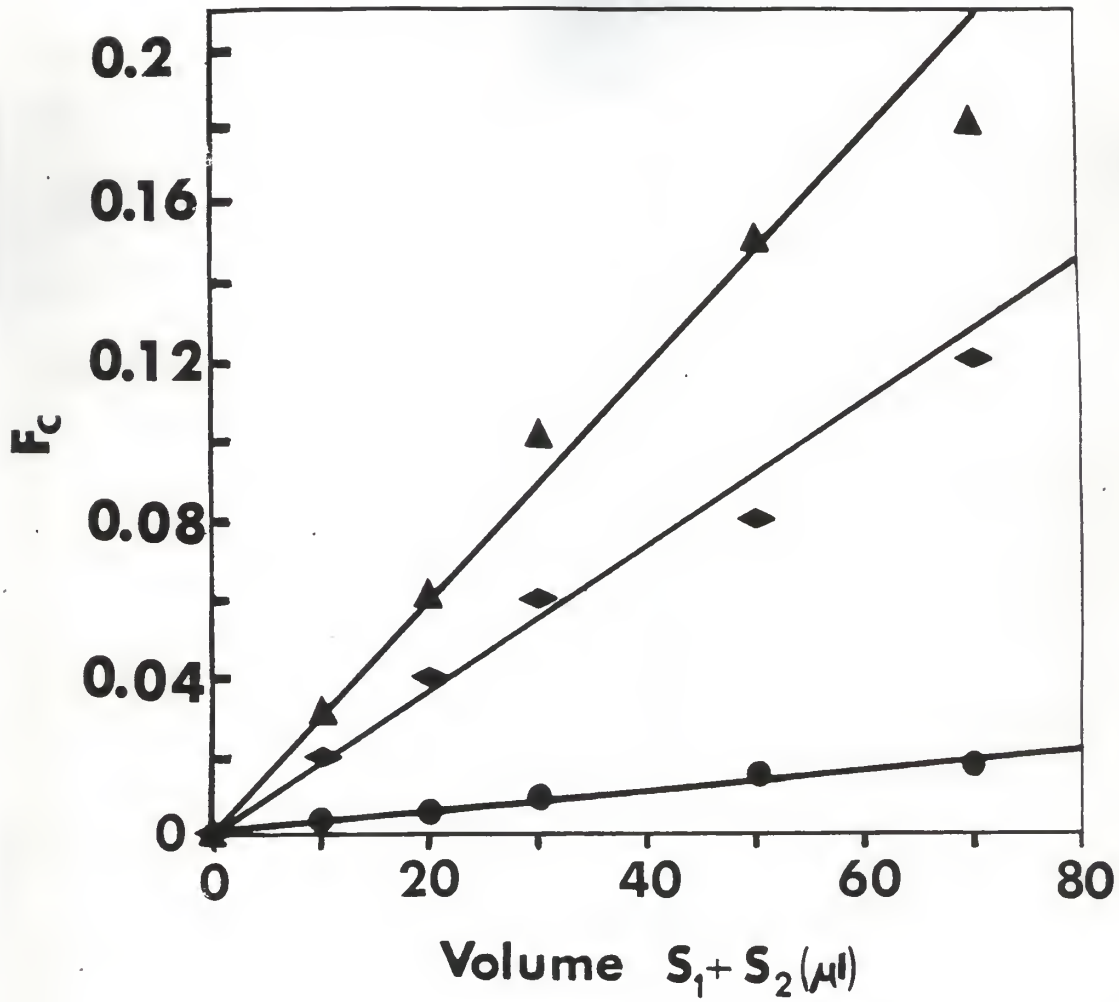
0.2
0.16
0.12
0.08
0.04
0
F_C
0
20
40
60
80
Volume $S_1 + S_2$ (µl)

FIG. 2. Concentration dependency of the assay. Enzyme activity (F_c) measured as a function of the volume of corneal homogenate enzyme present in the assay at three stages of corneal development: early (7 day ●), middle (14 day ◆), and late (20 day ▲). Assays were performed by standard method with the exception of the alteration in the volume of corneal homogenate enzyme added to the reaction vials. Reactions were terminated and counted in standard manner. Each data point represents an average value from three identical assays.

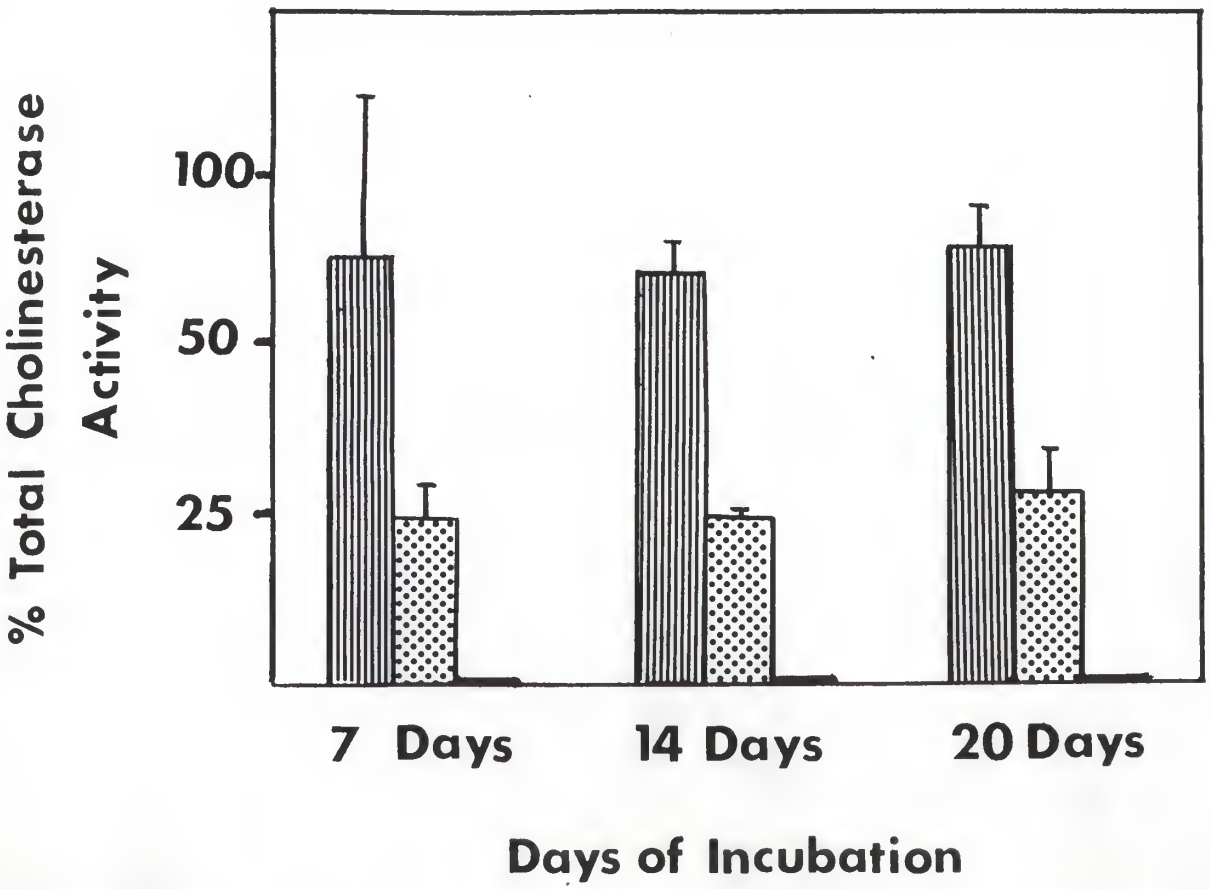
% Total Cholinesterase Activity
100
50
25
7 Days
14 Days
20 Days
Days of Incubation

FIG. 3. True acetylcholinesterase, non-specific cholinesterase, and other cholinesterase activity expressed as a percentage of total cholinesterase activity at three different stages of corneal development: early (7 day), middle (14 day), and late (20 day). Assays were performed by standard method, except that one or both cholinesterase inhibitors were added to discriminate between different enzyme activities. Non-specific cholinesterase inhibitor, iso-OMPA was present in one group of assays to determine the amount of activity of true acetylcholinesterase (stripes). True acetylcholinesterase inhibitor, BW284c51 was added to a separate set of assay vials to determine the amount of activity of the non-specific cholinesterase (dots). Very little activity was seen in the presence of both inhibitors (solid). Bars represent standard error from an average percent value using three identical assays.

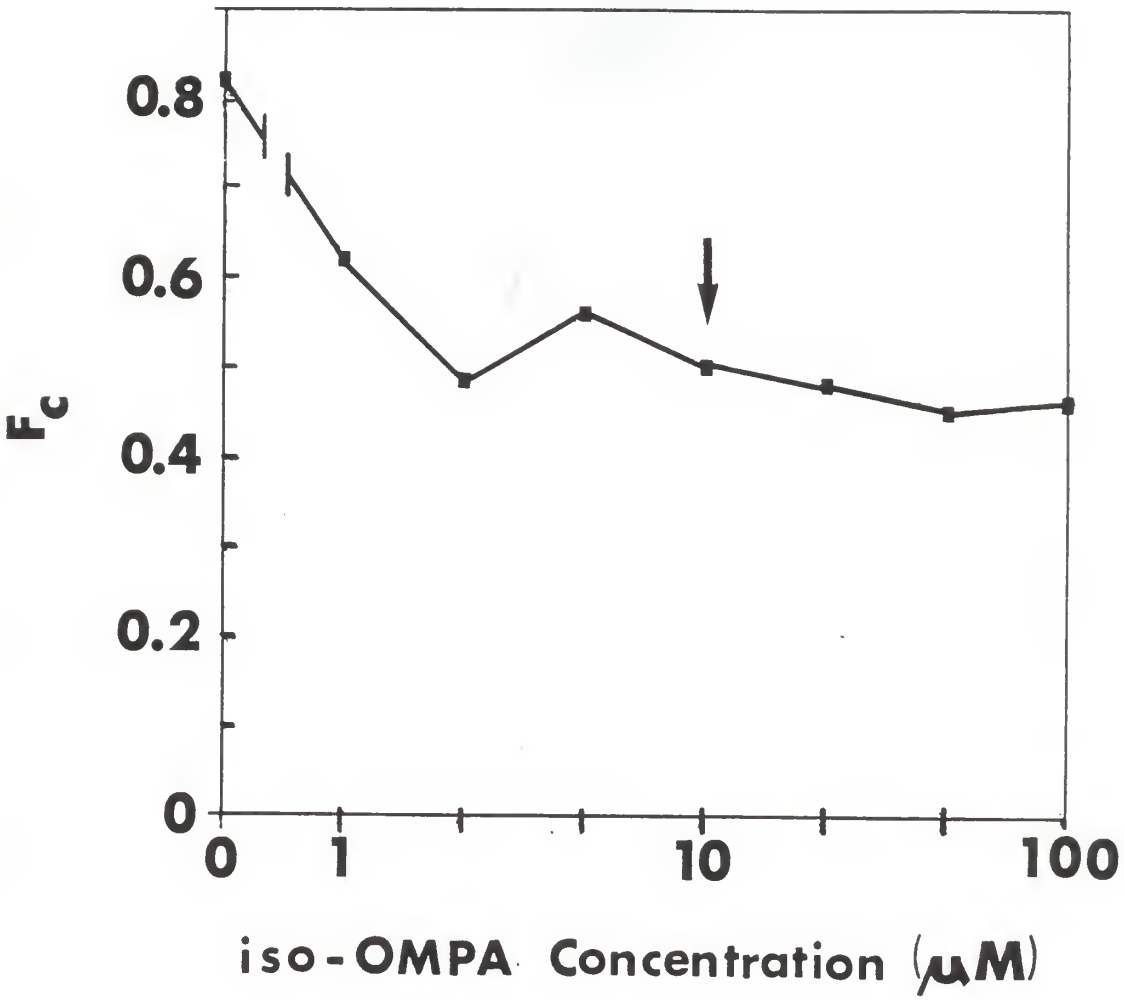
0.8
0.6
0.4
0.2
0
F_c
0
1
10
100
iso-OMPA Concentration (μM)

FIG. 4. Acetylcholinesterase activity (F_c) as a function of iso-OMPA concentration present during the assay. The effect of a change in the concentration of the non-specific cholinesterase inhibitor, iso-OMPA was measured over a hundred-fold range. Each data point represents an average value from three identical assays. Assays were performed using the standard method except for the alteration of iso-OMPA concentration. Incubation times were 10 min using 15 day embryos. Reactions were terminated in the standard manner. Arrow indicated iso-OMPA concentration used throughout the remainder of this study to allow measurement of acetylcholinesterase activity, specifically.

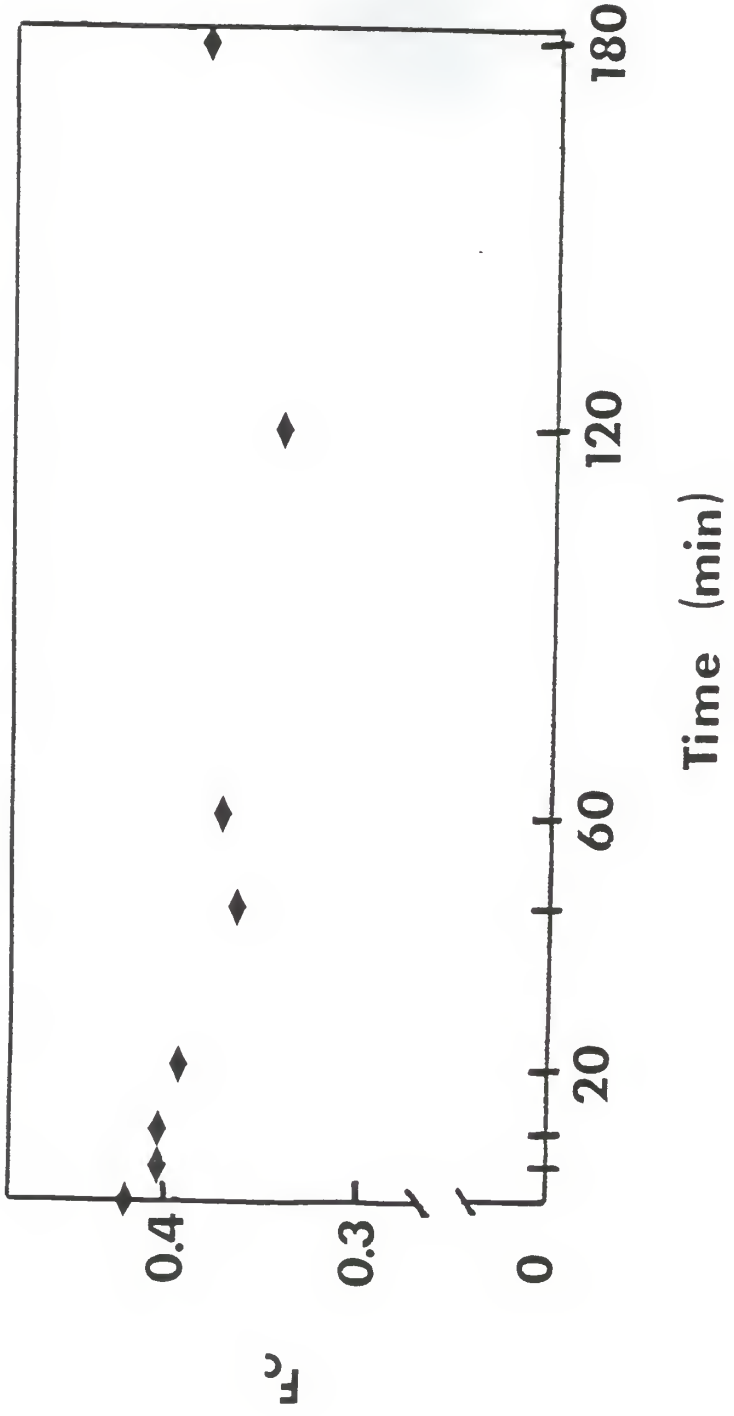
0.4
0.3
0
20
60
120
180
Time (min)
C_F

FIG. 5. Acetylcholinesterase activity as a function of time elapsed before addition of ^{3}H-acetylcholine. Assays were performed by standard method except that labelled substrate was added and reaction was allowed to proceed only after indicated times had elapsed (on abscissa). Corneas were from 18 day chick embryos. Reactions were allowed to proceed for 10 minutes. Assays were terminated and counted by standard method.

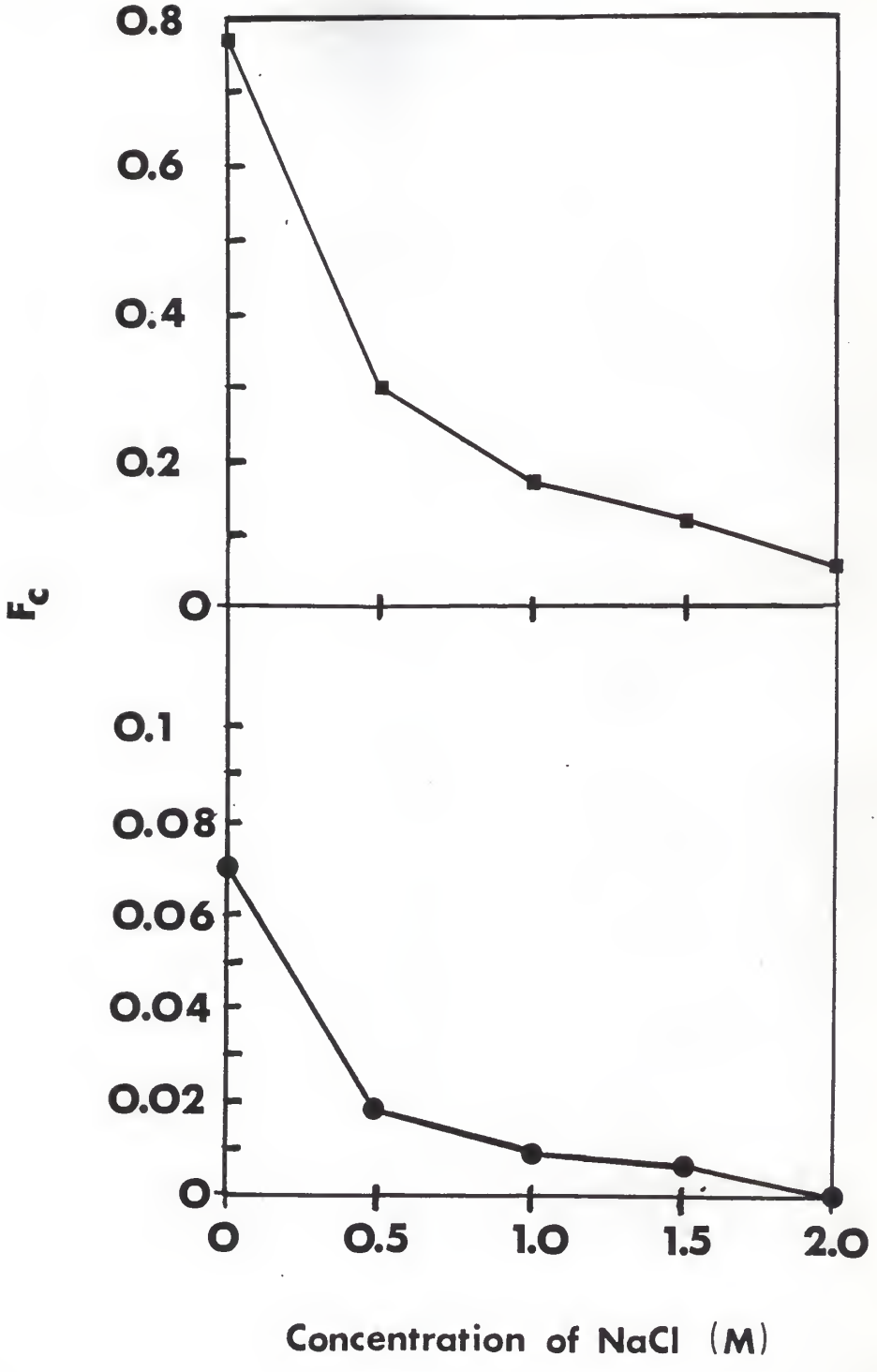
0.8
0.6
0.4
0.2
0
F_c
0.1
0.08
0.06
0.04
0.02
0
0
0.5
1.0
1.5
2.0
Concentration of NaCl (M)

FIG. 6. Sensitivity of acetylcholinesterase activity (F_c) as a function of NaCl concentration present during the assay. Assays were performed by standard method and in the presence of varying concentrations of NaCl. Early (7 day ●—●) and middle (14 day ■—■) stages of embryonic corneal development are shown. Incubation times were 10 minutes after which the assays were terminated and counted by standard method. Data points represent the average value from three identical assays for a given age.

V, moles/liter/min x 10^6
100
10
1
1
10
100
V/S, liters/min x 10^4

FIG. 7. K_m determination of corneal acetylcholinesterase from early, middle, and late stages of embryonic development in the chick. Velocity (V) was plotted as a function of the ratio of the velocity to substrate concentration (V/S). The result is an Eadie-Hofstee plot, in which the negative of the slope yields the K_m. The slope was calculated by linear regression and is indicated ± the standard deviation. Enzyme was assayed at eight different acetylcholine concentrations ranging from 1×10^{-2} M to 1×10^{-4} M. A constant amount of ^{3}H-acetylcholine substrate was added to each assay vial followed by varying amounts of unlabeled acetylcholine to achieve the final desired substrate concentration. Assay times were adjusted to achieve a fractional conversion (F_c) of approximately 0.2. Day of incubation: 7(◆), 14 (★), 20 (*).

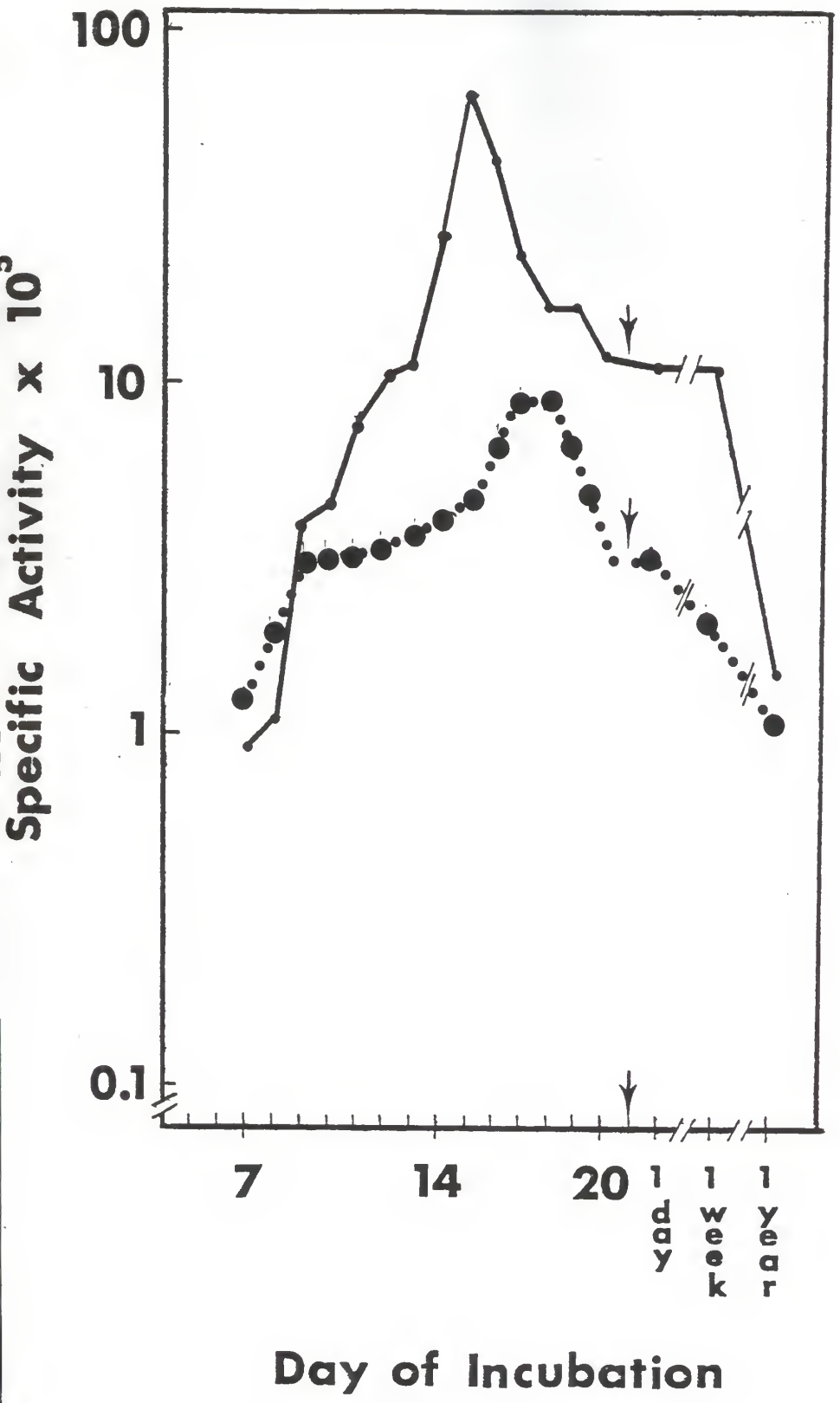
100
10
1
0.1
Specific Activity x 10^5
7
14
20
1 day
1 week
1 year
Day of Incubation

FIG. 8. Changes in the specific activity of corneal acetylcholinesterase (——) and non-specific (butyryl) cholinesterase (····) during embryonic development in the chick. Corneal homogenates were measured from day 7 through day 20 of embryonic development. Three post-hatch ages were also measured; at 1 day, 1 week, and at 1 year. Arrows indicate the day chicks hatched. As noted, the ordinate scale is logarithmic. (See Materials and Methods for calculations of F_c).

Acetylcholinesterase Activity in the Cornea of the Developing Chick Embryo

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B.A., Washington University in St. Louis, 1982

AN ABSTRACT OF A MASTER'S THESIS

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MASTER OF SCIENCE

Division of Biology

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1984

The specific activity of acetylcholinesterase and non-specific (butyryl) cholinesterase was measured in extracts from chick corneas, in a developmental series from day 7 through day 20 of incubation and at several ages after hatching. The activity of acetylcholinesterase was measured by the bi-phasic single-vial radiometric assay of Johnson and Russell which utilizes ^{3}H-acetylcholine as a substrate. After hydrolysis of the substrate by acetylcholinesterase, the ^{3}H-acetylcholine is converted into ^{3}H-acetate, which is extracted from an aqueous phase into an organic phase consisting of scintillation counting fluid. It is then quantified by a liquid scintillation spectrometer. In the present study, assay of acetylcholinesterase specifically was achieved by performing the assay in the presence of the inhibitor of the non-specific cholinesterase, tetraisopropyl-pyrophosphoramide (iso-OMPA). True acetylcholinesterase activity was verified by control assays run in the presence of both the non-specific cholinesterase inhibitor (iso-OMPA) and the true acetylcholinesterase inhibitor, 1:5-bis-(4-allyldimethylammonium phenylpentane-3-one diiodide (BW284c51). With both inhibitors present, no cholinesterase activity was measurable using this assay. The specific activity of the non-specific (butyryl) cholinesterase was obtained by determining the difference between total cholinesterase activity present in the corneal homogenate (no cholinesterase inhibitors present) and the activity of acetylcholinesterase (iso-OMPA present) in the same corneal homogenate for a given age. Additional controls indicated that: corneal acetylcholinesterase had an average K_m of 1.1 ± 0.3 mM at days 7, 14, and 20 of development and retained 91% activity even after 3 hours at 26°C; at least 90% of the total cholinesterase activity was solubilized by the Triton X-100 and sonication treatment used; activity decreased

dramatically with increasing concentrations of NaCl present in the assay; a 100-fold range of iso-OMPA concentrations gave the same degree of inhibition of non-specific (butyryl) cholinesterase.

Assay of corneal extracts indicated that a 60-fold transient increase in acetylcholinesterase specific activity occurs during the period from day 7 through day 20 of embryonic development. This increase begins on the first day measured (day 7), progresses steadily and rapidly during the subsequent week, reaches a peak at day 15, and then decreases prior to hatching to a level maintained into adulthood. A similar pattern was observed for non-specific cholinesterase, except that peak activity was not reached until day 17 and was then maintained until day 19 before a rapid decline began. The peak of acetylcholinesterase activity seen at day 15 of embryonic incubation coincides with the age at which a similarly transitory peak occurs in the concentration of highly sialylated gangliosides in the cornea.