THE APPEARANCE OF ACETYLCHOLINESTERASE IN THE DORSAL ROOT NEUROBLAST OF THE RABBIT EMBRYO

A Study by Electron Microscope Cytochemistry and Microgasometric Analysis with the Magnetic Diver

VIRGINIA M. TENNYSON and MIRO BRZIN

From the Department of Pathology, Division of Neuropathology and Neurology, College of Physicians and Surgeons, Columbia University, New York, New York 10032. Dr. Brzin's permanent address is Institute of Pathophysiology, University of Ljubljana, Ljubljana, Yugoslavia

ABSTRACT

In the nine day old embryo, acetylcholinesterase (AChE) is found in the reticulum, i.e. the nuclear envelope, endoplasmic reticulum, and Golgi complex, of a few cells in the neural crest. When the neurite first enters the neural tube, reticulum-bound enzyme is present also in the varicosity of the growth cone of the bipolar neuroblast. At later stages, AChE in the neuroblast has a dual distribution; in addition to the reticulum, activity also appears at the axolemmal surface. The axolemmal activity is found initially on the distal portions of axons in the posterior fasciculus and then progressively appears along the nerve roots in a distal to proximal direction. Very little reticulum-bound enzyme is present within the axon proper. After the 13th day the levels of AChE activity in the posterior fasciculus greatly exceed those in the dorsal root or in the ganglion. Enzymatic activity in the dorsal root equals or exceeds that in the posterior fasciculus by day 16, and both areas are considerably more active than the ganglion.

INTRODUCTION

Acetylcholinesterase (AChE) appears very early in the embryonic nervous system, and it increases in quantity and distribution as the embryo matures and develops its morphological characteristics and functional activity (Nachmansohn, 1938, 1939, 1940; Youngstrom, 1938, 1941; Sawyer, 1943; Boell and Shen, 1944, 1950; Metzler and Humm, 1951; Wenger, 1951; Himwich and Aprison, 1955; Shen et al., 1956; Karczmar, 1963; and Turbow and Burkhalter, 1968). The appearance of acetyl-cholinesterase activity at sequential stages of development has been studied in chick embryonic neural tube (Zacks, 1954; Bonichon, 1958; Bonichon and Gerebtzoff, 1958; Gerebtzoff, 1959) and dorsal root ganglion (Strumia and Baima-Bollone, 1964). The enzyme appears in the ventro-lateral neural tube, where it develops a marked degree of activity by day 4, when the spinal ganglia first exhibits histochemical staining (Strumia and Baima-Bollone, 1964). Shortly thereafter, AChE

is found in the posterior fasciculus and later in the dorsal roots (Gerebtzoff, 1959).

The presence of a high level of AChE activity in the posterior fasciculus prior to its appearance in the dorsal roots (Gerebtzoff, 1959) raises a question concerning the origin of the axonal enzyme. It has been postulated, from studies of adult ciliary ganglion (Fukuda and Koelle, 1959), that AChE is synthesized in the perikaryon and reaches the distal axonal membrane by axoplasmic flow. If this were the case in the embryo, one might have expected to find considerable enzyme activity in the dorsal roots before it is found in the posterior fasciculus, unless submicroscopic amounts of the enzyme are traversing the nerve fibers continually and rapidly. To examine this question we studied different segments of the dorsal root neuroblast by electron microscopic cytochemistry at sequential periods of development. We also estimated the enzymatic activity which was present at different stages of development in each segment of the neuroblast by analyzing microgasometrically selected areas of the nervous system. Preliminary reports of these studies have appeared (Tennyson et al., 1966, 1967 a, b; Brzin and Tennyson, 1967; and Tennyson and Brzin, 1968).

MATERIALS AND METHODS

Histochemical Studies

Numerous types of fixatives and incubation media were tested in an attempt to overcome the disruptive effect of the cytochemical procedures on delicate embryonic tissues. Portions of neural tube and dorsal root ganglia from 269 Dutch rabbit embryos ranging in age from day 9 to day 16 of gestation were used for the cytochemical studies. The best preservation of structure and enzyme activity was obtained by perfusion fixation in situ (Tennyson, 1970) with 1% phosphate-buffered glutaraldehyde (Sabatini et al., 1963), or a mixture of aldehydes. Following fixation, the tissue was washed in several changes of phosphate buffer for 1 hr or more. The brachial level of the embryo was embedded in 7% agar and cut into 60 μ sections on a Smith-Farquhar microchopper. To facilitate handling of the sections, they were placed in baskets developed in this laboratory by Dr. Adam Bender. The baskets were made from B.E.E.M. capsules (Better Equipment for Electron Microscopy, Inc. Bronx, New York). The closed pyramid was removed from the capsule to form a cylinder open at both ends. The central portion of the cap was cut away, leaving only the rim. A small square of nylon mesh was placed between the cap and the cylinder forming a porous base for the basket. The sections

were transferred in these baskets through the cytochemical procedure, postfixation, and early dehydration stages.

A modification by Brzin et al. (1966) of the copper thiocholine technique for the localization of acetylcholinesterase (Koelle and Friedenwald, 1949) was used in these studies. The specimens were incubated at pH 6 for 15–60 min at 4°C in a freshly prepared medium containing 3.46 $\times$ 10^{-3} M acetylthiocholine iodide (Sigma Chemical Co., St. Louis, Mo.), 0.04 M glycine, 0.008 M copper sulfate, 0.12 M sodium chloride, and 0.03 M magnesium sulfate in a 0.02 M sodium hydrogen maleate buffer. Prior to incubation the tissue was preincubated for 30 min in the copper glycinate medium lacking the substrate.

In control studies the following inhibitors were added to the preincubation and incubation medium: BW 284 C 51 (1,5-bis-[4-allyl-dimethylammonium-phenyl]pentan-3-one dibromide) 2 $\times$ 10^{-4} M (Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.)., an inhibitor of AChE, and eserine (physostigmine sulfate) 1 $\times$ 10^{-4} M, (K + K Laboratories, Plainview, N. Y.), an inhibitor of cholinesterases (ChE) in general. To eliminate the possibility of hydrolysis of AThCh by butyrylcholinesterase (BuChE), iso-OMPA (tetraisopropyl pyrophosphortetramide) 2 $\times$ 10^{-4} M (Pierce Chemical Co., Rockford, Ill.), an inhibitor of BuChE, was used in some experiments. To determine whether sites of nonspecific ChE activity were present, butyrylthiocholine iodide (BuThCh) 3.1 $\times$ 10^{-3} M (K + K Laboratories), with or without the inhibitor BW 284 C 51, was used.

Following incubation, the tissue was washed briefly in cold distilled water and then postfixed in a freshly prepared solution of 3% potassium permanganate for 30–60 min. The tissue was rapidly dehydrated and embedded in Durcupan (International Chemical & Nuclear Corporation, Burbank, Calif.).

Thick sections for phase microscopy and thin sections for electron microscopy were cut with glass or diamond knives on a Porter-Blum microtome (Ivan Sorvall Inc., Norwalk, Conn.). The thin sections were placed on 200 mesh copper grids and immediately blotted dry. The sections were stained with uranyl acetate for 5 min (Watson, 1958), and then with lead citrate for 30 min (Reynolds, 1963). Electron micrographs were taken with a Siemens Elmiskop I.

Microgasometric Procedure

The embryos used for microgasometric analysis were first perfused with saline to remove all blood prior to dissection, since preliminary cytochemical studies showed that developing blood cells contained considerable amounts of AChE. Most embryos were dissected in the fresh condition, but a few were perfused with the same fixative used for the cytochemical
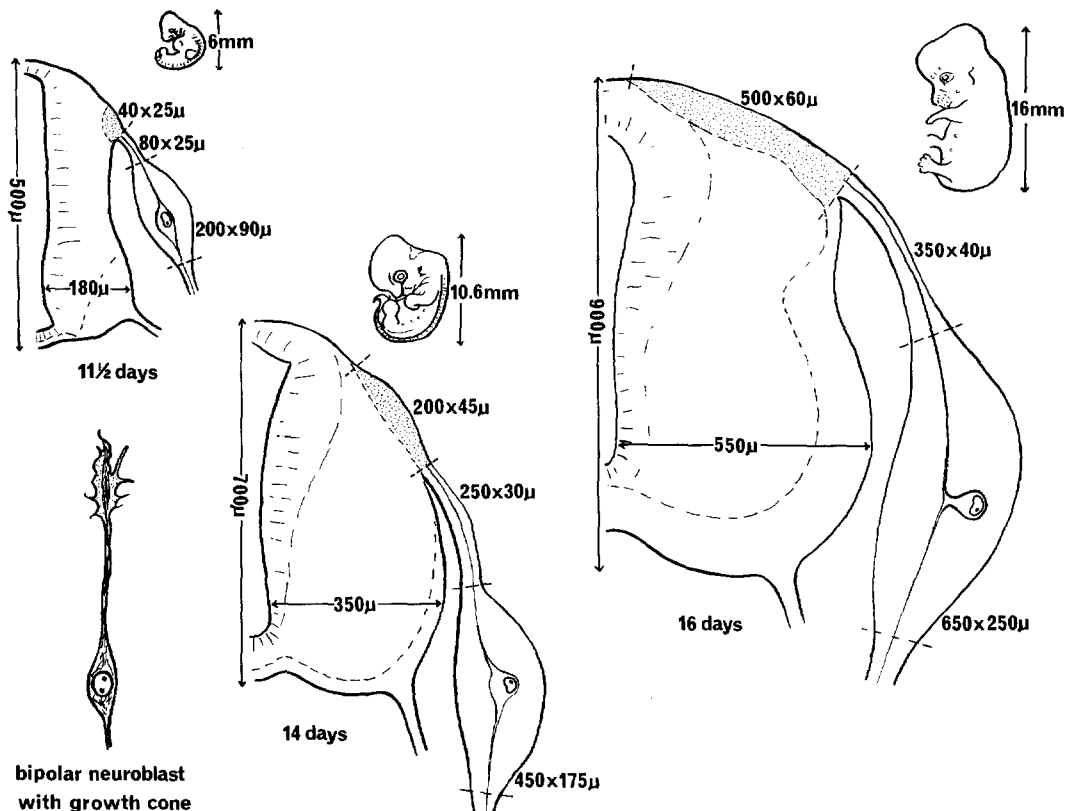
FIGURE 1 A diagram showing the stage of development and the approximate cross-sectional dimensions of typical samples of the posterior fasciculus (stippled), dorsal roots, and dorsal root ganglia isolated from the brachial level of the spinal cord during day 11½, 14, and 16. Both fresh tissue and phase microscopy thick sections were used for this information. The nerve cells (not drawn to scale) illustrate the stage of differentiation reached by some of the more mature cells in the ganglion. Information for the gross images of the embryos was taken from Minot and Taylor (1905). A bipolar dorsal root neuroblast having a growth cone with a thickened fibrillar central core and a delicate veillike ectoplasm is illustrated.

studies to determine the effect of fixation on AChE activity. The tissue was isolated in cold saline by free hand technique with the aid of a dissecting microscope. Specimens of neural tube, separated from the adjacent somites at levels 6 to 17 from two 9-day embryos, were selected for study. The following specimens isolated from the brachial level of 49 embryos ranging in age from day 11 1/2 to day 16 were also examined: the dorsal root ganglion, the dorsal roots (when long enough for accurate dissection), and the posterior fasciculus. An attempt was made to remove the small amount of mesenchymal ensheathment present by teasing it away with fine forceps. The areas examined with typical cross-sectional dimensions are represented in the diagram in Fig. 1. The length of the brachial region ranged from 0.5 to 2.0 mm depending on the age of the embryo. This region was selected because it is

readily identifiable grossly and provides an adequate amount of tissue for dissection of the posterior fasciculus. The isolated specimens were usually cut into small rectangles for more accurate measurement of their volume. The size of the specimen was estimated by measuring the length, width, and thickness with an ocular micrometer. The specimens were kept in saline at 4°C prior to microgasometric analysis, and then were transferred to a bicarbonate medium containing acetylcholine iodide (ACh) (3×10^{-3} M) (Calbiochem, Los Angeles, Calif.) and introduced into the ampulla of the diver (Zajicek and Zeuthen, 1961). Measurements were performed in an atmosphere of 95% nitrogen and 5% carbon dioxide. The same procedure was followed as described previously (Brzin et al., 1965). In control experiments, the following inhibitors were used: BW 284 C 51 (2×10^{-5} M) iso-OMPA (2×10^{-5} M), and eserine (1×10^{-5} M).
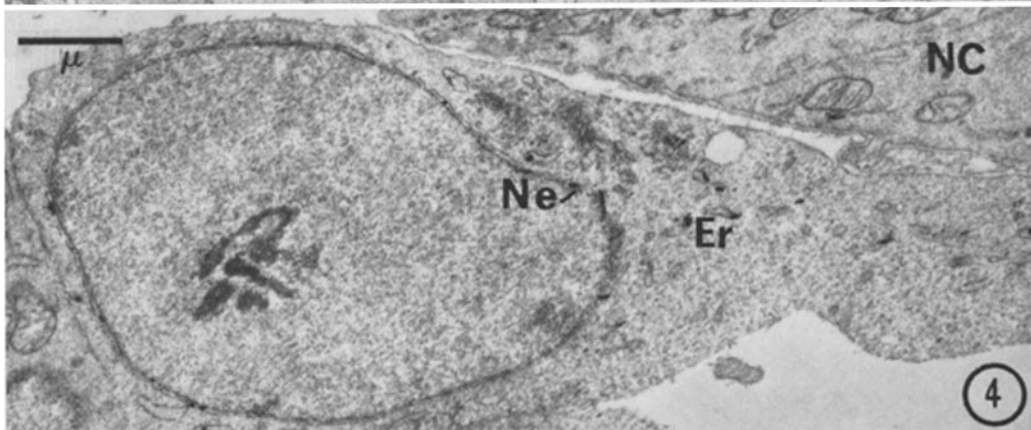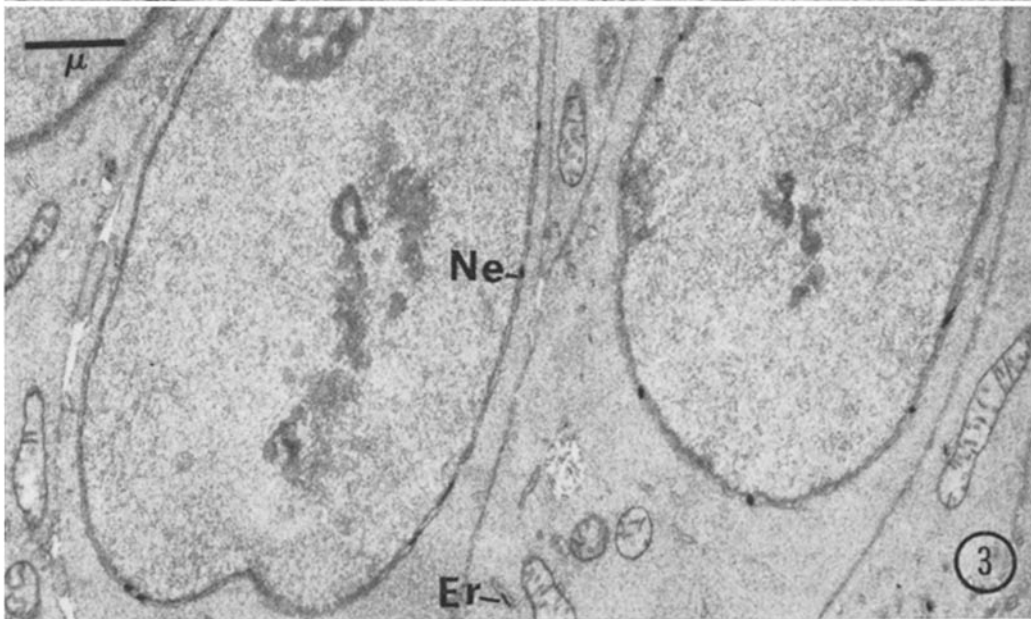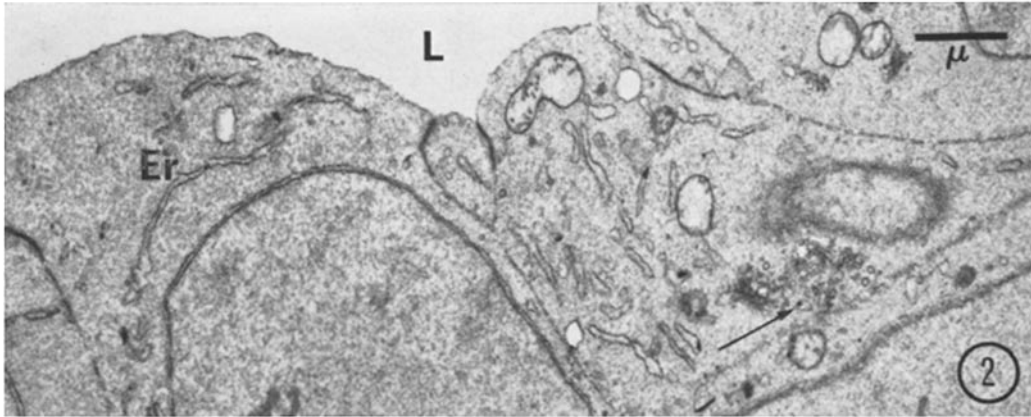
FIGURE 2 Cells at the luminal (L) surface of the neural crest have end product in the endoplasmic reticulum (Er) and Golgi complex (arrow). 9 day embryo. AThCh 60 min. Scale marker: 1μ. × 12,400.

FIGURE 3 Cells in the basal region of the neural crest have end product in the nuclear envelope (Ne) and in a few cisternae of the endoplasmic reticulum (Er). 9 day embryo. AThCh 60 min. Scale marker: 1 μ. × 13,000.

FIGURE 4 A presumptive neuroblast in the mesenchyme adjacent to the neural crest (NC) has end product in the nuclear envelope (Ne), endoplasmic reticulum (Er), and in the Golgi region. 9 day embryo. AThCh 60 min. Scale marker: 1 μ. × 13,200.

OBSERVATIONS

The ultrastructural characteristics of the neural crest and the differentiating dorsal root neuroblasts including their axons and growth cones have been described in previous studies (Tennyson, 1965, 1970 a, 1970 b); therefore, they will not be considered in detail here. It should be pointed out that the cytoplasm of these cells is filled with clusters of ribosomes, although these particles are

migration, but the dorsal root ganglion has not formed. Microgasometric examination showed measurable AChE activity. Two samples of neural tube had values of 18.6 and 22.1 μl CO_2 × 10^{-2}/hr per mm^3 of fresh tissue, respectively. Cytochemical studies of the neural tube show that enzyme activity is located in only a few cells in the neural crest (Figs. 2, 3) or in the presumptive ventral horn. The rest of the neuroepithelial cells are nega-

TABLE I

Acetylcholinesterase Activity of Embryonic Dorsal Root Neuroblasts and their Axons Expressed in
*μl CO_2 × 10^{-2}/hr per mm^3 Fresh Tissue ($\pm$SD)**

Gestation day	11 ½	12	13	14	16
Dorsal root ganglion	65.6 ± 13.4 (n = 4)	69.2 ± 20.4 (n = 3)	126.6 ± 32.3 (n = 8)	115.0 ± 19.5 (n = 4)	349.0 ± 183.8 (n = 6)
Dorsal root			222.5 ± 44.7 (n = 7)	576.0‡ 640.0‡	1437.9 ± 337.3 (n = 4)
Posterior fasciculus	10.9 ± 3.6 (n = 3)	44.5 ± 20.6 (n = 3)	842.7 ± 196.8 (n = 9)	1071 ± 196.8 (n = 4)	1023.0 ± 262.2 (n = 7)

* In control divers containing sample without substrate no change of gas volume could be measured during 60 min of experiment.
‡ Individual results of two experiments.

not readily demonstrable in specimens which have been treated with potassium permanganate. The rest of the cell and its organelles are recognizable, but they are not optimally preserved after undergoing the cytochemical procedure.

Neural Crest

The earliest embryos studied were at the 11 to 17 somite stage during day 9 of gestation, when the neural tube consists of radially arranged neuroepithelial cells with nuclei concentrated in three or four levels. The neural crest shows evidence of cell

tive. Some neural crest cells and cells apparently migrating from the neural crest to form the dorsal root ganglion (Fig. 4) have end product of AChE activity in the endoplasmic reticulum (Figs. 2–4, Er) and nuclear envelope (Figs. 3, 4, Ne), as well as in elements of the Golgi complex (Fig. 2, arrow).

The number of cells showing AChE activity in these areas increases during the next 2 days, but since the ultrastructural distribution of end product remains the same, they will not be illustrated. Other tissues, such as the surface ectoderm, somites (Slotwiner and Tennyson, 1969), notochord, and

FIGURE 5 A bipolar dorsal root neuroblast has cytochemical activity in the nuclear envelope (Ne), endoplasmic reticulum (Er), and Golgi complex (G). 11½ embryo. AThCh 35 min. Scale marker: 1 μ. × 10,800. Inset. End product is present in the agranular reticulum (arrow) of the varicosity of a growth cone in the dorsal root. 12 day embryo. AThCh 30 min. × 22,400.

FIGURE 6 End product is present at the surface of cross sectional profiles of axons (Ax), and also of the filopodia (F) and varicosities (V) of growth cones. Deposits can be seen in the agranular reticulum of some varicosities (arrow), but most of the agranular reticulum (Ar) lacks end product. Junction of the dorsal root (bottom) with the posterior fasciculus. 11¾ day embryo. AThCh 47 min. Scale marker: 1 μ. × 11,600.
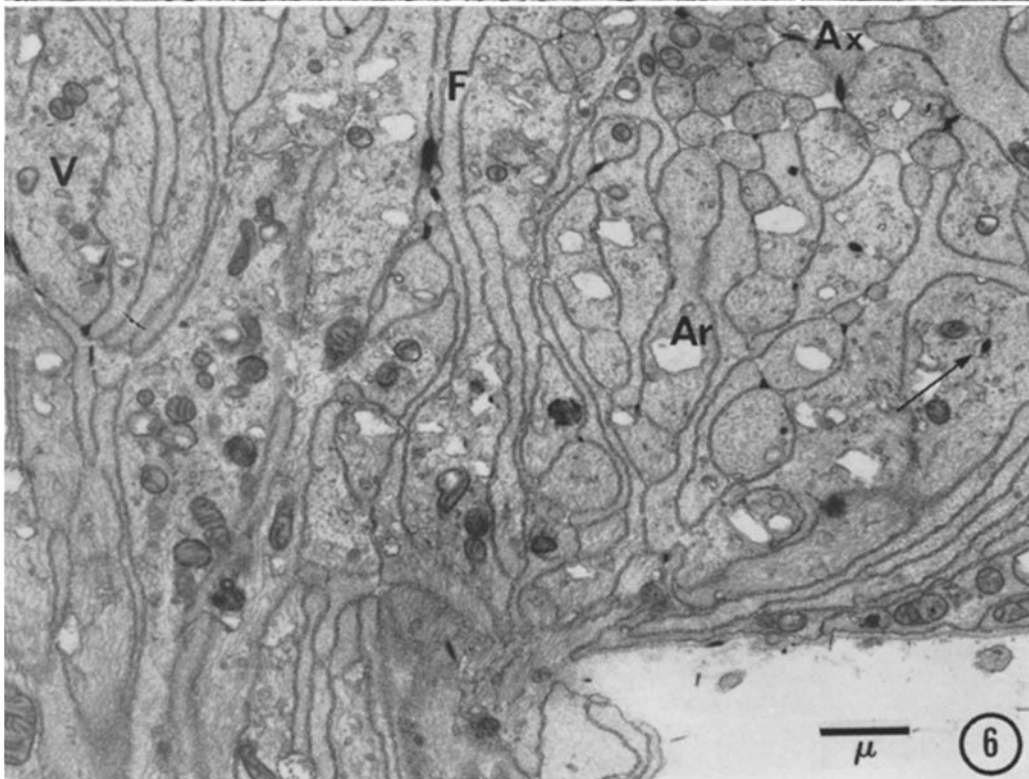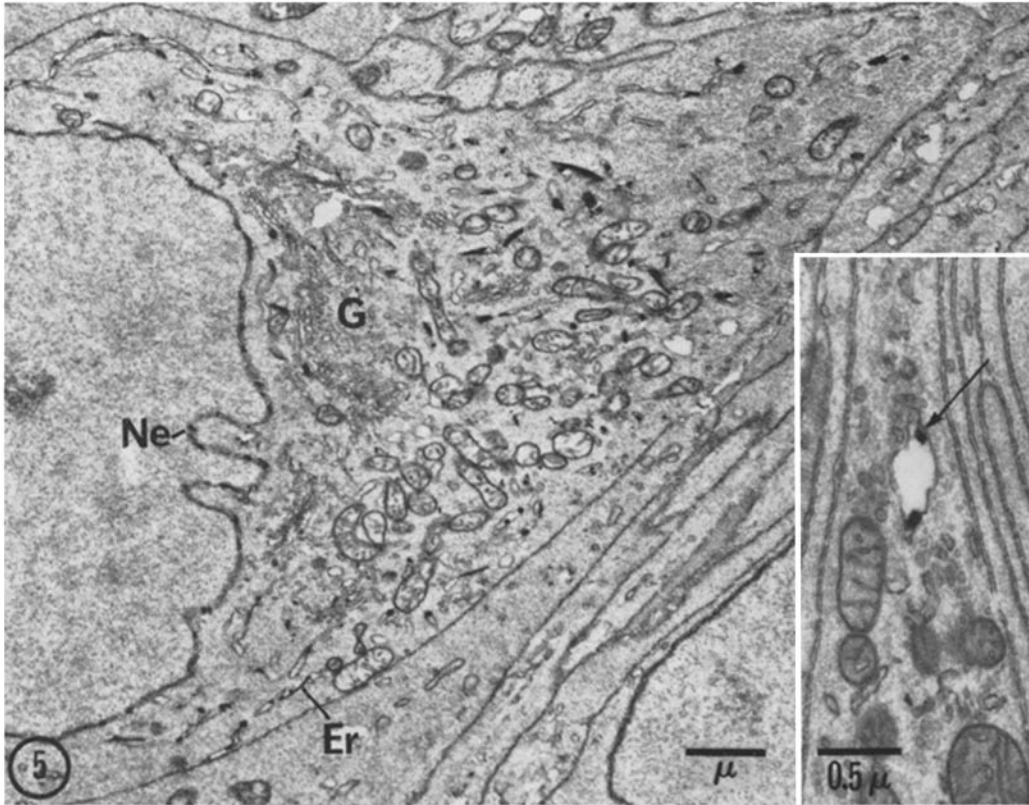
developing blood cells and heart (Tennyson et al., 1968; Hagopian et al., 1970) also have end product in the nuclear envelope, endoplasmic reticulum, and Golgi complex during this early developmental period. Nucleated red blood cells having very little endoplasmic reticulum exhibit end product randomly distributed in their cytoplasmic matrix.

Neuroblast Differentiation and Formation of the Posterior Fasciculus

The gross appearance of the embryos, the general characteristics of the developing neural tube and ganglion, and the approximate dimensions of the areas dissected in the present study are illustrated in Fig. 1. The results of microgasometric analysis of the dorsal root ganglion and its processes from day 11½ through day 16 of gestation are presented in Table I. Day 11½ is the earliest period when samples of dorsal root ganglia and posterior fasciculus could be dissected. The small size and stickiness of the central processes during day 11½ and 12 prevented their isolation. The values in the age groups 12 to 16 days represent the spread of AChE activity in embryos whose ages range within the stated 24 hr period. Variations in development between litters of the same age and even among littermates make it impractical to attempt a more exact determination of age. Small differences in age and developmental stages, therefore, are factors which contribute to the standard deviation.

A significant amount of AChE activity can be measured in the dorsal root ganglion during day 11½ and 12 (Table I). Enzyme activity in the distal axonal processes of the dorsal root neuroblasts, which form a small posterior fasciculus, is just measurable during day 11½, but approaches the level of activity in the ganglion during day 12.

There is a twofold increase of AChE activity in the dorsal root ganglion during day 13 and 14, and a fivefold increase by day 16. During day 13, enzyme activity in the posterior fasciculus exceeds that in the dorsal root by a factor of about four, and exceeds the activity in the ganglion by a factor of about seven. A distal to proximal gradient of AChE activity is evident in the various portions of the dorsal root neuroblast during day 13 and 14. Enzyme activity in the dorsal roots equals or exceeds that in posterior fasciculus by day 16, and in both areas the activity of AChE is much higher than in the ganglion.

Cytochemical studies have shown that the AChE activity measured microgasometrically in the ganglion during day 11½ and 12 is attributable to only a few bipolar neuroblasts which have begun to differentiate. The less differentiated cells in the ganglion do not contain end product. The cytochemically active cells exhibit end product primarily in the nuclear envelope (Fig. 5, Ne) and endoplasmic reticulum (Er), although a small amount is associated with some tubular elements of the Golgi complex (G). Some end product may occasionally be found in the agranular reticulum of the proximal portions of the neurites, but this area usually exhibits less cytochemical activity than the growing tips (see Fig. 1 for a diagram of the varicosity and filopodia of the growth cone). The end product is often found in the agranular reticulum (Figs. 5, inset, and 6, arrow) of the varicosities and at the surface of the varicosities (Fig. 6, V)and filopodia (F). Cross-sectional profiles of longitudinally oriented axons (Ax), which are the major component of the posterior fasciculus, commonly have end product at their surfaces, but only rarely within the agranular reticulum (Ar).

A greater number of neuroblasts differentiate

FIGURE 7   There is very little cytochemical activity associated with the axons (Ax) of the proximal portion of the dorsal root, but a randomly disposed end product is present in the Schwann cells (S). 13 day fetus. AThCh 40 min. Scale marker: 1 μ. × 11,400.

FIGURE 8   There is a much greater amount of cytochemical activity associated with the axons of the distal portion of the dorsal root. Most of the end product is located at the axolemmal surface (arrow). The agranular reticulum (Ar) along the length of the axon is usually negative. Same fetus as in Fig. 7. Scale marker: 1 μ. × 11,400.

FIGURE 9   A large number of axons within the posterior fasciculus exhibit end product at their surface (arrow). The agranular reticulum (Ar) is usually negative. Same fetus as in Figs. 7 and 8. Scale marker: 1 μ. × 16,200.
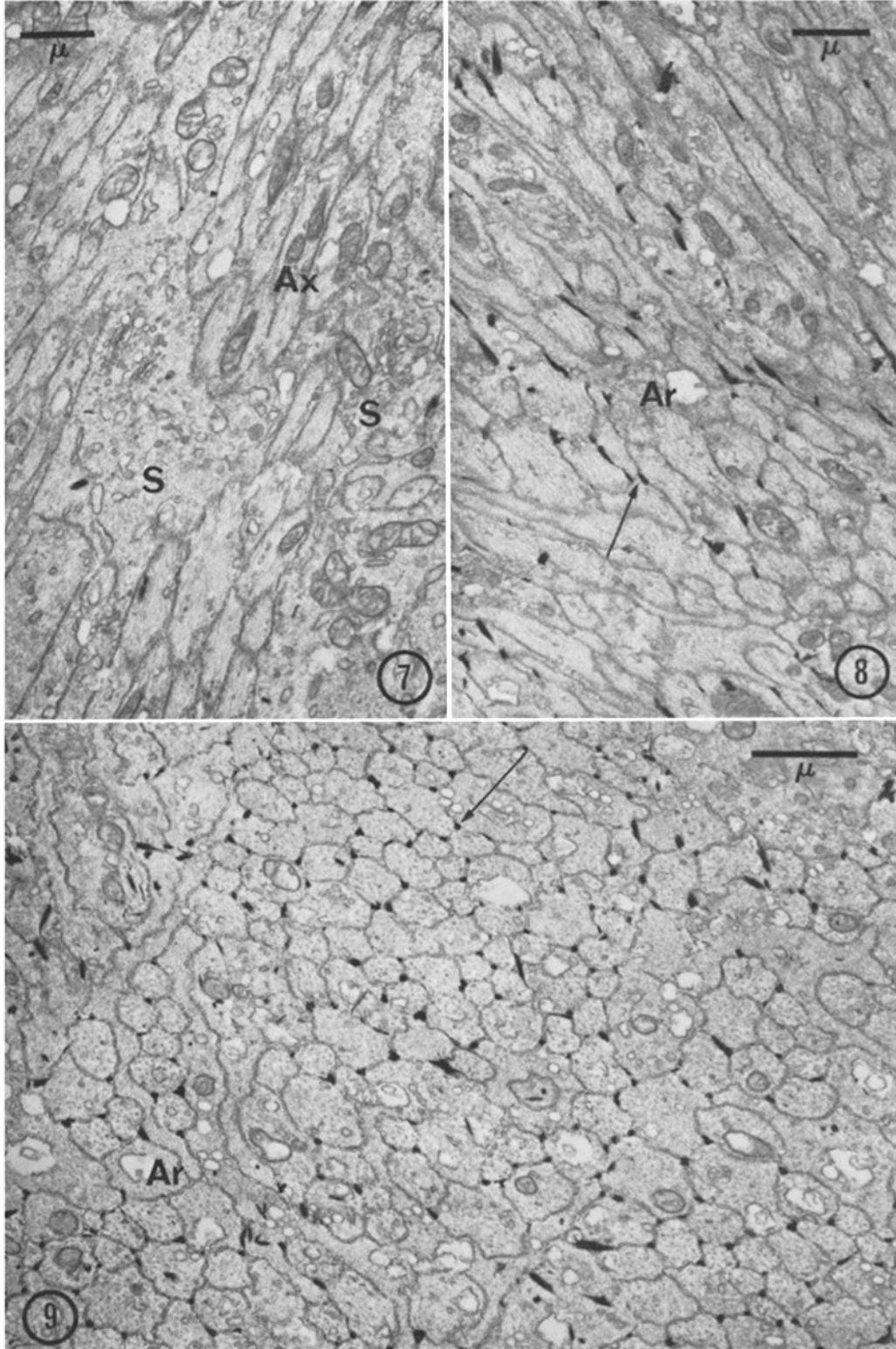
and develop AChE activity during day 13 and 14. The cells are more globular than those at day 12, but the enzyme localization is essentially the same. Studies of the dorsal roots during day 13 and 14 have indicated that cytochemical end product is not distributed evenly along their length. There seems to be a distal to proximal gradient of activity with respect to the axonal enzyme. This observation is based on a systematic study of near-serial sections of 46 nerve roots from 16 fetuses cut either longitudinally or in cross-section. Only a minimal amount of end product is found associated with the axons of the proximal portion of the dorsal root (Fig. 7, Ax), but considerably more cytochemical activity is found in the distal root close to its junction with the posterior fasciculus (Fig. 8, arrow). A large number of the axons within the posterior fasciculus at day 13 (Fig. 9, arrow) and day 14 (Fig. 15) exhibit AChE activity at their surface. The end product is rarely found in the agranular reticulum of axons in the dorsal root or posterior fasciculus unless growth cones are present. Schwann cells, which have previously been inactive, appear to develop cytochemical activity during this period (Fig. 7, S). The end product is rarely well localized to membranes but is randomly distributed in the cell, as in the case of the developing red blood cell.

AChE activity is found in most of the neuroblasts in the ganglion by day 16, and is located in the same sites as in earlier periods. The number of enzyme-active sites within the endoplasmic reticulum (Fig. 10, Er) increases concomitantly with the development of this organelle within the growing nerve cell. The relative number and distribution of active sites closely resembles that seen in some adult neurons (Torack and Barrnett, 1962; Koelle and Foroglou-Kerameos, 1965; Lewis and Shute, 1966; Novikoff et al., 1966; Brzin et al., 1966; Duffy et al., 1967; Eränkö et al., 1967; Kása and Csillik, 1968; Kokko et al., 1969).

Cytochemical activity can be found at the surface of many axons throughout the length of the dorsal root at day 16 (Figs. 11, 12), although there is still a tendency for a greater accumulation of end product in the distal portion of the root. Some axons appear to have less cytochemical activity than others. These fibers may be randomly dispersed among the enzyme-active fibers, or they may be grouped together (Fig. 12, Ax). Only a small amount of end product is present within the agranular reticulum (Fig. 11, Ar) of the axons in

the dorsal root. The Schwann cells appear to be less active than in the previous period.

Most of the axons within the posterior fasciculus show evidence of AChE activity at their surface (Fig. 13), but in some areas there are a greater number of axons which seem to have less cytochemical end product (Fig. 14, Ax). A regional distribution is beginning to be established between the active (Ax') and less active fibers. The fibers having little end product appear to enter the posterior fasciculus from the lateral portion of the dorsal root, as was found in an earlier histochemical study of the chick embryo (Bonichon and Gerebtzoff, 1958). In the posterior fasciculus of the 16 day gestation rabbit, these fibers are often concentrated beneath the pial surface near the junction with the dorsal root. A thin rim of these fibers also extends medially beneath the pial surface for a short distance. The remainder of the posterior fasciculus, i.e. the greater part of this tract of fibers, is composed of AChE-active axons. As in the earlier period, most of the enzyme activity is found at the surface of the axons, with only minimal evidence of AChE activity within the agranular reticulum.

Control Studies

Specimens of dorsal root ganglia, dorsal root, and posterior fasciculus from day 9 through day 16 of gestation gave similar cytochemical results when incubated in AThCh and the following inhibitors. Specimens treated with iso-OMPA (2×10^{-4} M), an inhibitor of nonspecific ChE (Fig. 15), show approximately the same distribution and number of deposits of cytochemical end product as specimens incubated in AThCh without inhibitors. There was inhibition of cytochemical activity when the tissue was treated with either BW 284 C 51 (2×10^{-4} M), an inhibitor of AChE (Fig. 16), or eserine (1×10^{-4} M), an inhibitor of both specific and nonspecific ChE (Fig. 17). Cytochemical end product was rarely observed when specimens were incubated in BuThCh (3.1×10^{-3} M) (Fig. 18). Occasional residual deposits are abolished by the use of BW 284 C 51.

Microgasometric measurements were made on specimens from day 13 embryos treated with inhibitors to verify the cytochemical results. With ACh as substrate, enzyme activity was not measurable in two samples, one of the posterior fasciculus and one of the dorsal root ganglion in the presence of BW 284 C 51 (2×10^{-5} M) or in two
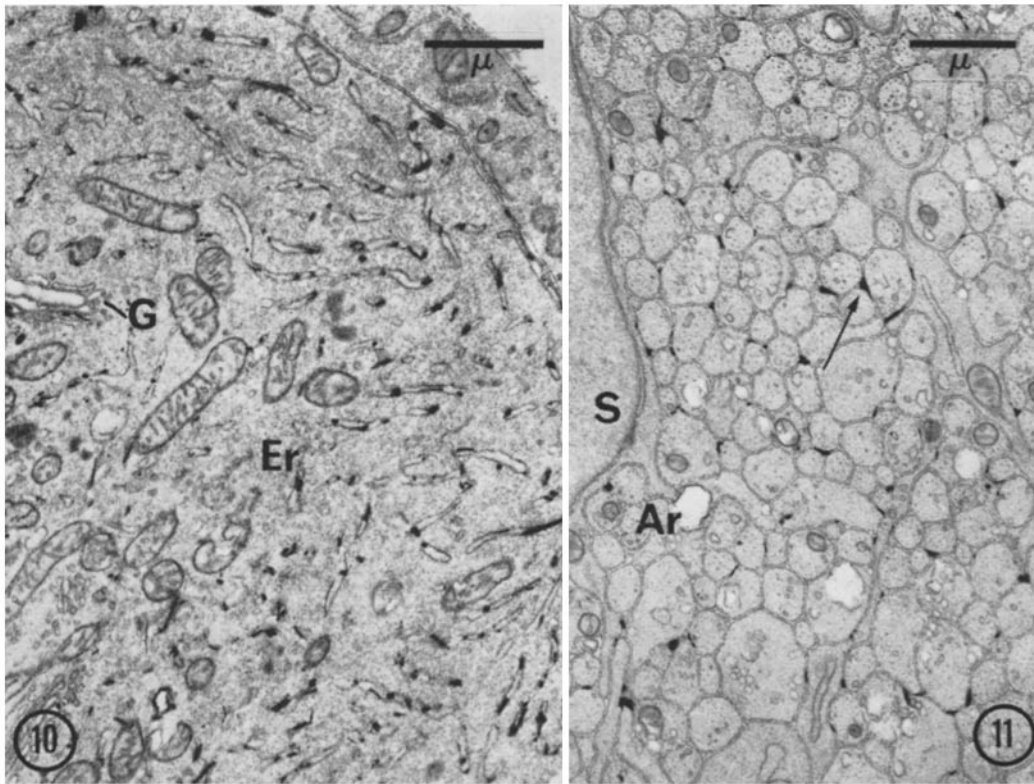
FIGURE 10   End product is found in most of the cisternae of the endoplasmic reticulum (*Er*) and in some elements of the Golgi complex (*G*) of the immature neuron. 16 day fetus. AThCh + iso-OMPA 30 min. Scale marker: 1 μ. $\times$ 16,000.

FIGURE 11   A large number of the axons within the proximal portion of the dorsal root exhibit end product at their surface (arrow), but very little is present within the agranular reticulum (*Ar*). Some end product is found between the Schwann cell (*S*) and the axons. 16 day fetus. AThCh 34 min. Scale marker: 1 μ. $\times$ 13,800.

samples of the posterior fasciculus in the presence of eserine (1 $\times$ 10^{-5} M). The effect of fixation in 1% glutaraldehyde on enzyme activity was also determined microgasometrically on specimens from day 13 embryos. Three samples of fixed dorsal root ganglia measured 106.2, 112.4, and 121.0 μl CO_2 $\times$ 10^{-2}/mm^3, as compared with 119.3 μl CO_2 $\times$ 10^2 in two unfixed specimens. Since all of these values are within the standard deviation shown in Table I for day 13 specimens, no significant decrease in enzyme activity due to fixation could be determined.

DISCUSSION

Acetylcholinesterase has been shown to be the cholinesterase present in the rabbit embryonic dorsal root neuroblast by the use of the substrates acetylcholine and acetylthiocholine in conjunction with selective inhibitors. A correlation of the results from cytochemistry and microgasometric analysis allows a more meaningful interpretation of the data on the development of this enzyme. The quantitative values given for the dorsal root ganglion during day 11½ and 12 (Table I) are a measure of specimens which cytochemically show only a few very active neuroblasts. Most of the cells in the ganglion have no cytochemical end product as yet. The quantitative values, therefore, indicate the amount of AChE present in the ganglion as a whole, but are not representative of the amount of enzyme present in individual cells. A greater number of neuroblasts contain AChE-active sites dur-

ing the subsequent days, but they tend to be concentrated in the ventrolateral portion of the ganglion, as was shown in an earlier histochemical study (Strumia and Baima-Bollone, 1964). The uneven distribution of these cells may partially account for the standard deviation, since the larger size of the ganglion at later stages necessitates its division into smaller samples for microgasometric analysis. It should be pointed out that AChE is present in Schwann cells during a period of their development. This contributes to the values measured for the dorsal roots. Factors such as permeability barriers imposed by partial ensheathment of the neuroblasts by developing satellite cells may also be responsible for the variability of biochemical measurement of fresh tissue. Brzin et al. (1966, 1967) showed that the intact neuroplasmalemma and satellite cells, which completely surround adult neurons, form at least a partial barrier to the penetration of the substrates, ACh and AThCh.

The problem of permeability barriers is less likely to be a contributing factor to the spread of values observed in the microgasometric studies of the posterior fasciculus, since these axons lack an ensheathment and the enzyme is localized primarily on the axolemmal surface. The lack of uniformity in the distribution of the cytochemically active axons more readily explains the large standard deviation, particularly at day 16. Bonichon and Gerebtzoff (1958) and Gerebtzoff (1959) also noted variations in the distribution of histochemically active axons in the posterior fasciculus with increasing age. Axons containing high AChE activity predominate in the earlier stages and are rather evenly distributed throughout the posterior fasciculus. Later there is an ingrowth of axons which do not exhibit histochemical activity. The active fibers remain in the lateral region forming

the tract of Lissauer. The change in the proportion of axons having high and low AChE activity during development may explain why Turbow and Burkhalter (1968) failed to find evidence of high enzyme activity in white matter in their study of AChE in the chick embryo.

In relation to our findings it is interesting to discuss possible sites of AChE synthesis in the neuroblast. Enzyme activity first appears associated with cisternal elements of neural crest cells. After the outgrowth of the axon, one should account for the dual distribution of the enzyme. The agranular reticulum and axolemmal surface exhibit the same cytochemical activity, but the possibility exists that the macromolecular structure surrounding the active-site of the enzyme may be different in these two different cellular membranes. The question of whether the enzyme in one site could have been derived from the other forms the basis of two alternative possibilities of the origin of axonal AChE, i.e., perikaryal synthesis as opposed to independent axonal synthesis. The concept of perikaryal synthesis proposes that the active enzyme formed in the endoplasmic reticulum of the perikaryon moves down the axon either bound to the agranular reticulum or free within it, and then is extruded or fuses with the surface to form the axolemmal surface enzyme (Fukuda and Koelle, 1959; Kása, 1968; Kása and Csillik, 1968). This proposal is supported by the presence of large concentrations of enzyme in the cell body and by the evidence of axoplasmic transport of AChE in nerve transection experiments (Lubińska, 1964; Niemierko and Lubińska, 1967; Kása, 1968). The fact that large concentrations of AChE are found in the endoplasmic reticulum does not necessarily imply transport of the enzyme to the axonal surface. The enzyme is found in the endoplasmic reticulum at an early period in a wide variety of

FIGURE 12   Many axons within the distal portion of the dorsal root have end product at their surface, but there is very little in the agranular reticulum. Some groups of axons (Ax) do not exhibit end product. S, Schwann cell. 16 day fetus. AThCh 34 min. Scale marker: 1 μ. X 16,200.

FIGURE 13   Most nerve fibers within the posterior fasciculus of the 16 day fetus exhibit axolemmal surface end product, even those beneath the pial surface (P). AThCh + iso-OMPA 20 min. Scale marker: 1 μ. X 6800.

FIGURE 14   At some levels of the posterior fasciculus of the 16 day fetus there is a zone of axons (Ax) beneath the pial surface (P) which exhibits less end product than is present in deeper lying axons (Ax'). AThCh 34 min. Scale marker: 1 μ. X 6800.
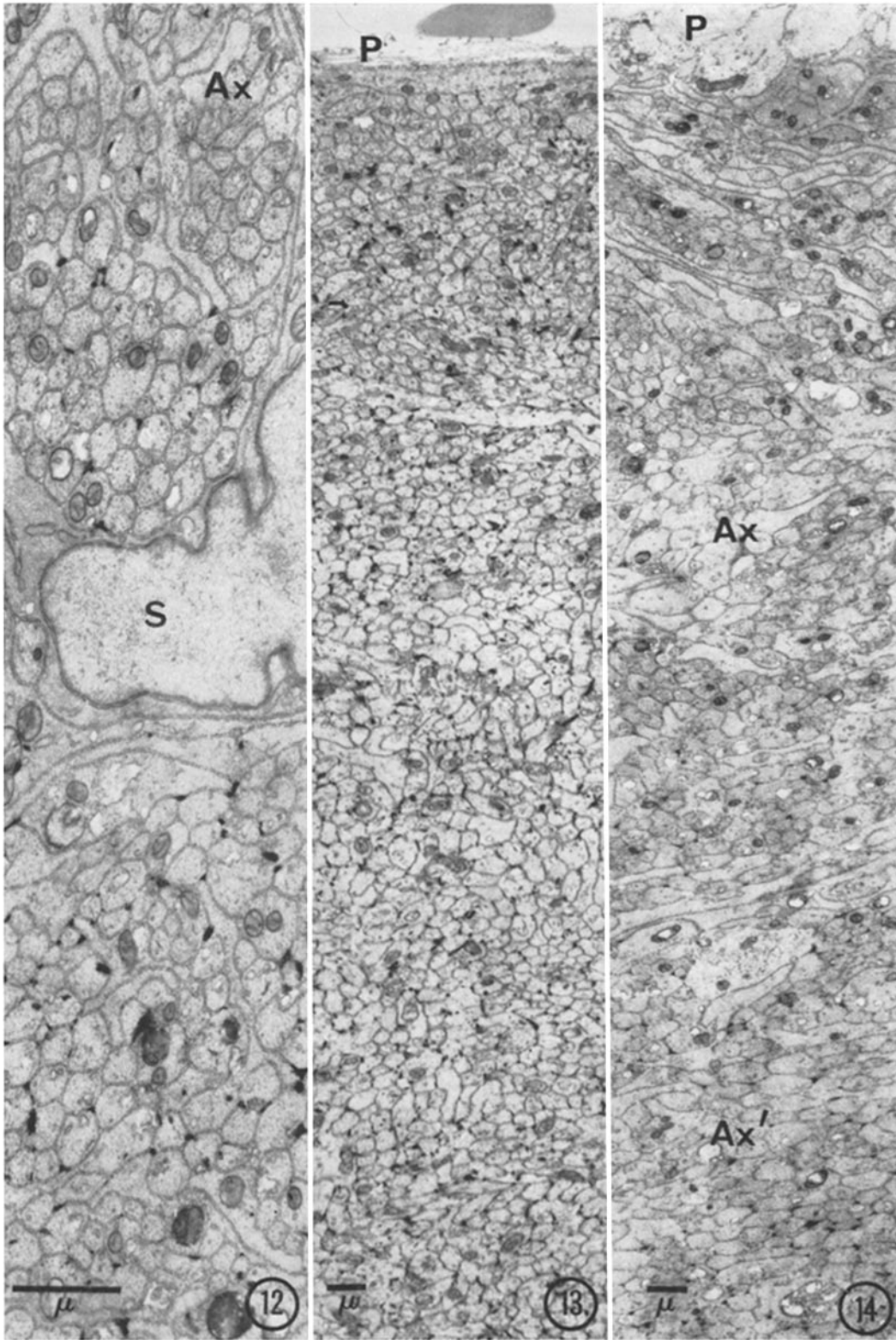
embryonic cells, such as surface ectoderm, noto-chord, and white blood cells, which do not develop AChE at their surfaces. Kása and Csillik (1968) and Kása (1968) have claimed to demonstrate the continuity of intracellular enzyme with the axonal surface, which they interpret as evidence for ex-trusion of enzyme from tubules to the surface of the axon. The possibility exists that enzyme-con-taining invaginations of the surface membrane may represent uptake rather than extrusion. In the study by Kása (1968), it was reported that these axons pinocytosed exogenously injected ferritin. The phenomenon of somatofugal axoplasmic flow of AChE (Lubińska, 1964) and other substances (Weiss and Hiscoe, 1948; Barondes, 1967; Lasek, 1968; McEwen and Grafstein, 1968) is well recog-nized, but it does not necessarily preclude the pos-sibility that some axonal components may be formed in the axon as well. The concept of a local axonal protein synthetic mechanism has been sup-ported in studies of the reappearance of AChE after its irreversible inactivation in axons (Koenig and Koelle, 1961; Clouet and Waelsch, 1961; and Koenig, 1965, 1967 a) and also in investigations using isotopes to study the incorporation of amino acids into protein in decentralized nerve (Koenig, 1967 b; Edström and Sjöstrand, 1969; Fischer and Litvak, 1967; Giuditta et al., 1968).

Although AChE is found first in the perikaryon of the embryonic neuroblast bound to elements of the endoplasmic reticulum, this study cannot pro-vide conclusive evidence on the site of synthesis of axonal AChE. If a small amount of enzyme is formed in the perikaryon and is then transported very rapidly and continuously to the distal surfaces of the nerve fibers where it collects, the micro-gasometric and cytochemical results might be simi-lar to those shown in this study. Lubińska (personal communication) has estimated that AChE moves down a transected adult axon at the rate of at least 200 mm per day. The length of most of the centrally directed axons of the dorsal root neuro-blast is probably less than 200 μ at day 11½. On the other hand, the distal to proximal gradient of AChE activity in the nerve fibers and the paucity of enzyme-containing agranular reticulum during the period of rapid enzyme accumulation raises the question of a possible independent axonal syn-thesis. The small amount of reticulum-bound en-zyme that is found in the axon could have been incorporated by pinocytosis of enzyme-active sur-face membrane in the growth cone. Furthermore, ribosomes are present in embryonic axons and growth cones during the early outgrowth period (Tennyson, 1970 a). It should be pointed out, however, that axoplasmic ribosomes are greatly diminished by day 16, although mitochondrial ribosomes are still present. A compromise proposal involving both the perikaryon and the axon in the formation of axonal AChE could also explain the fiindings of this study. Enzymatcally inactive sub-units of AChE may be synthesized in the pari-karyon and transported to the axon. In this site the active enzyme may then be formed by the assembly of the subunits into the surface membrane.

FIGURE 15 Most of the axons exhibit the end product of AChE activity. Posterior fasciculus. 14 day fetus. AThCh + iso-OMPA 25 min. Scale marker: 1 μ. X 12,600.

FIGURE 16 Cytochemical activity is inhibited by treatment with BW 284 C 51. Posterior fasciculus. 14 day fetus. AThCh + BW 284 C 51 25 min. Scale marker: 1 μ. X 12,600.

FIGURE 17 Cytochemical activity is inhibited by treatment with eserine. Posterior fasciculus. 14 day fetus. AThCh + eserine 25 min. Scale marker: 1 μ. X 12,600.

FIGURE 18 No end product is present after incubation in BuThCh. Posterior fasciculus. 14 day fetus. BuThCh 25 min. Scale marker: 1 μ. X 12,600.
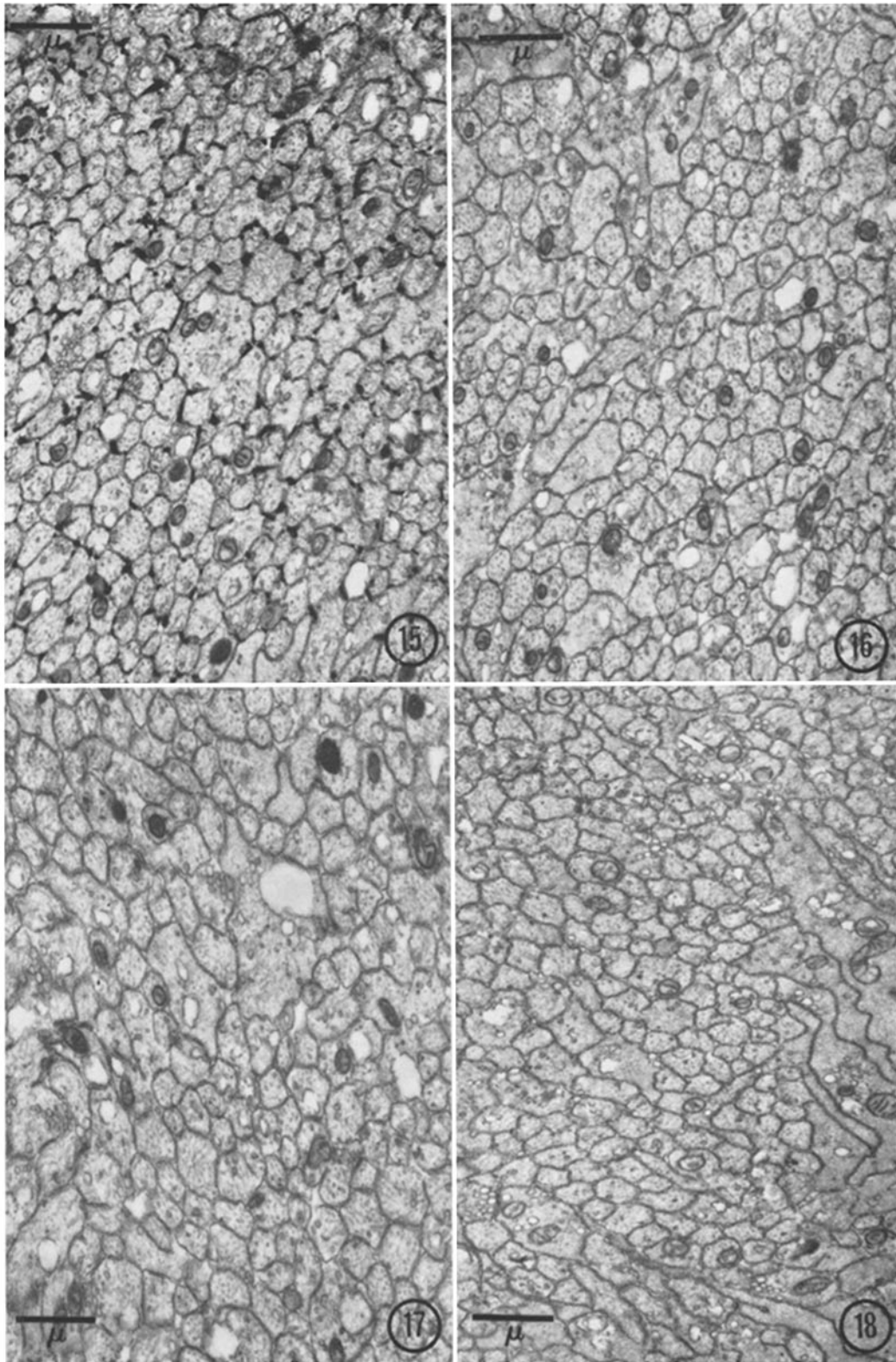
REFERENCES

BARONDES, S. H. 1967. Axoplasmic transport. *Neurosci. Res. Program Bull.* **5**:311.

BOELL, E. J., and S.-C. SHEN. 1944. Functional differentiation in embryonic development. I. Cholinesterase activity of induced neural structures in *Amblystoma Punctatum. J. Exp. Zool.* **97**:21.

BOELL, E. J., and S.-C. SHEN. 1950. Development of cholinesterase in the central nervous system of *Amblystoma Punctatum. J. Exp. Zool.* **113**:583.

BONICHON, A. 1958. L'acetylcholinesterase dans la cellule et la fibre nerveuse au cours du developpement. I. Differenciation biochimique précoce du neuroblaste. *Ann. Histochim.* **3**:85.

BONICHON, A., and M. A. GEREBTZOFF. 1958. L'acetylcholinesterase dans la cellule et la fibre neurveuse au cours du developement 2. Relation entre la localisation synaptique de l'enzyme et la myelinisation de la fibre. *Ann. Histochim.* **3**:171.

BRZIN, M., W.-D. DETTBARN, P. ROSENBERG, and D. NACHMANSOHN. 1965. Cholinesterase activity per unit surface area of conducting membranes. *J. Cell Biol.* **26**:353.

BRZIN, M., and V. TENNYSON. 1967. Microgasometric and ultracytochemical study of cholinesterase development in the embryonic and adult rabbit. *J. Cell Biol.* **35**:18A.

BRZIN, M., V. M. TENNYSON, and P. E. DUFFY. 1966. Acetylcholinesterase in frog sympathetic and dorsal root ganglia. A study by electron microscope cytochemistry and microgasometric analysis with the magnetic diver. *J. Cell Biol.* **31**:215.

BRZIN, M., V. M. TENNYSON, and P. E. DUFFY. 1967. Ultrastructural, cytochemical and microgasometric studies of acetylcholinesterase in isolated neurons of the frog. *Int. J. Neuropharmacol.* **6**:265.

CLOUET, D., and H. WAELSCH. 1961. Amino acid and protein metabolism of the brain VIII. The recovery of cholinesterase in the nervous system of the frog after inhibition. *J. Neurochem.* **8**:201.

DUFFY, P. E., V. M. TENNYSON, and M. BRZIN. 1967. Cholinesterase in adult and embryonic hypothalamus. A combined cytochemical electron microscopic study. *Arch. Neurol.* **16**:385.

EDSTRÖM, A., and J. SJÖSTRAND. 1969. Protein synthesis in the isolated Mauthner nerve fiber of goldfish. *J. Neurochem.* **16**:67.

ERÄNKÖ, O., L. RECHARDT, and L. HÄNNINEN. 1967. Electron microscopic demonstration of cholinesterases in nervous tissue. *Histochemie.* **8**:369.

FISCHER, S., and S. LITVAK. 1967. The incorporation of microinjected ^{14}C-amino acids into TCA insoluble fractions of the giant axon of the squid. *J. Cell. Physiol.* **70**:69.

FUKUDA, T., and G. B. KOELLE. 1959. The cytological localization of intracellular neuronal acetylcholinesterase. *J. Biophys. Biochem. Cytol.* **5**:433.

GEREBTZOFF, M. A. 1959. Morphogenetic study of acetylcholinesterase in nervous system and skeletal muscle. *In* Cholinesterases. Pergamon Press Inc., New York. 36.

GIUDITTA, A., W.-D. DETTBARN, and M. BRZIN. 1968. Protein synthesis in the isolated giant axon of the squid. *Proc. Nat. Acad. Sci. U.S.A.* **59**:1284.

HAGOPIAN, M., V. M. TENNYSON, D. SPIRO. 1970. Cytochemical localization of cholinesterase in embryonic rabbit cardiac muscle. *J. Histochem. Cytochem.* **18**:38.

HIMWICH, H. E., and M. H. APRISON. 1955. The effect of age on cholinesterase activity of rabbit brain. *In* Biochemistry of the Developing Nervous System. H. Waelsch, editor. Academic Press Inc. New York. 301.

KARCZMAR, A. G. 1963. Ontogenesis of cholinesterases. *In* Handbuch der Experimentellen Pharmakologie. G. B. Koelle, editor, Springer-Verlag, Berlin. **15**:129.

KÁSA, P. 1968. Acetylcholinesterase transport in the central and peripheral nervous tissue. The role of tubules in the enzyme transport. *Nature (London).* **218**:1265.

KÁSA, P., and B. CSILLIK. 1968. AChE synthesis in cholinergic neurons: Electron histochemistry of enzyme translocation. *Histochemie.* **12**:175.

KOELLE, G. B., and C. FOROGLOU-KERAMEOS. 1965. Electron microscopic localization of cholinesterases in a sympathetic ganglion by a gold-thiolacetic acid method. *Life Sci.* **4**:417.

KOELLE, G. B., and J. S. FRIEDENWALD. 1949. A histochemical method for localizing cholinesterase activity. *Proc. Soc. Exp. Biol. Med.* **70**:617.

KOENIG, E., and G. B. KOELLE. 1961. Mode of regeneration of acetylcholinesterase in cholinergic neurons following irreversible inactivation. *J. Neurochem.* **8**:169.

KOENIG, E. 1965. Synthetic mechanisms in the axon. I. Local axonal synthesis of acetylcholinesterase. *J. Neurochem.* **12**:343.

KOENIG, E. 1967 *a.* Synthetic mechanisms in the

axon. III. Stimulation of acetylcholinesterase synthesis by actinomycin-D in the hypoglossal nerve. *J. Neurochem.* 14:429.

KOENIG, E. 1967 *b*. Synthetic mechanisms in the axon. IV. *In vitro* incorporation of ^{3}H precursors into axonal protein and RNA. *J. Neurochem.* 14:437.

KOKKO, A., H. G. MAUTNER, and R. J. BARRNETT. 1969. Fine structural localization of acetylcholinesterase using acetyl-α-methylthiocholine and acetylselenocholine as substrates. *J. Histochem. Cytochem.* 17:625.

LASEK, R. J. 1968. Axoplasmic transport in cat dorsal root ganglion cells: as studied with ^{3}H-L-leucine. *Brain Res.* 7:360.

LEWIS, P. R., and C. C. D. SHUTE. 1966. The distribution of cholinesterase in cholinergic neurons demonstrated with the electron microscope. *J. Cell Sci.* 1:381.

LUBIŃSKA, L. 1964. Axoplasmic streaming in regenerating and in normal nerve fibers. *In* Progress in Brain Research, Mechanisms of Neural Regeneration. M. Singer and J. P. Schadé, editors. Elsevier, Amsterdam. 13.

McEWEN, B. S., and B. GRAFSTEIN. 1968. Fast and slow components in axonal transport of protein. *J. Cell Biol.* 38:494.

METZLER, C. J., and D. G. HUMM. 1951. The determination of cholinesterase activity in whole brains of developing rats. *Science (Washington).* 113:382.

MINOT, C. S., and E. TAYLOR. 1905. Normal plates of the development of the rabbit (Lepus Cuniculus L.). *In* Normentafeln zur Entwicklungsgeschichte der Wirbelthiere. Fünftes Hefte. F. Keibel, editor. Gustav Fischer, Verlag, Jena, Germany.

NACHMANSOHN, D. 1938. Cholinestérase dans les tissus embryonnaires. *Comp. Rend. Soc. Biol.* 127:670.

NACHMANSOHN, D. 1939. Cholinestérase dans le systeme nerveux central. *Bull. Soc. Chim. Biol.* 21:761.

NACHMANSOHN, D. 1940. Choline esterase in brain and spinal cord of sheep embryo. *J. Neurophysiol.* 3:396.

NIEMIERKO, S., and LUBIŃSKA, L. 1967. Two fractions of axonal acetylcholinesterase exhibiting different behavior in severed nerves. *J. Neurochem.* 14:761.

NOVIKOFF, A. B., N. QUINTANA, H. VILLAVERDE, and R. FORSCHIRM. 1966. Nucleoside phosphatase and cholinesterase activities in dorsal root ganglia and peripheral nerve. *J. Cell Biol.* 29:525.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208.

SABATINI, D. D., K. BENSCH, and R. J. BARRNETT. 1963. Cytochemistry and electron micrsocopy. The preservation of ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* 17:19.

SAWYER, C. H. 1943. Cholinesterase and the behavior

problem in amblystoma. I. The relationship between the development of the enzyme and early motility. II. The effects of inhibiting cholinesterase. *J. Exp. Zool.* 92:1.

SHEN, S.-C., P. GREENFIELD, and E. J. BOELL. 1956. Localization of acetylcholinesterase in chick retina during histogenesis. *J. Comp. Neurol.* 106:433.

SLOTWINER, P., and V. TENNYSON. 1969. The development of acetylcholinesterase activity within skeletal muscle of the embryonic rabbit. *Anat. Rec.* 163:326.

STRUMIA, E., and P. L. BAIMA-BOLLONE. 1964. AChE activity in the spinal ganglia of the chick embryo during development. *Acta Anat.* 57:281.

TENNYSON, V. M. 1965. Electron microscopic study of the developing neuroblast of the dorsal root ganglion of the rabbit embryo. *J. Comp. Neurol.* 124:267.

TENNYSON, V. M. 1970 *a*. The fine structure of the developing nervous system. *In* Developmental Neurobiology. W. Himwich, editor. Charles C. Thomas Publisher, Springfield, Ill.

TENNYSON, V. M. 1970 *b*. The fine structure of the axon and growth cone of the dorsal root neuroblast of the rabbit embryo. *J. Cell Biol.* 44:62.

TENNYSON, V., and M. BRZIN. 1968. Electron microscopic and microgasometric studies of cholinesterase development in embryonic dorsal root neuroblasts. *Anat. Rec.* 160:440.

TENNYSON, V., M. BRZIN, and P. DUFFY. 1966. Cholinesterase localization in the developing nervous system of the rabbit embryo and human fetus by electron microscopic histochemistry. *Anat. Rec.* 154:432.

TENNYSON, V., M. BRZIN, and P. DUFFY. 1967 *a*. Ultracytochemical study of the onset of cholinesterase activity in dorsal root neuroblasts in the embryonic rabbit. *Anat. Rec.* 157:332.

TENNYSON, V. M., M. BRZIN, and P. DUFFY. 1967 *b*. Electron microscopic cytochemistry and microgasometric analysis of cholinesterase in the nervous system. *Progr. Brain Res.* 29:59.

TENNYSON, V. M., M. HAGOPIAN, and D. SPIRO. 1968. Ultracytochemical studies of cholinesterase in rabbit embryonic cardiac muscle. *J. Cell Biol.* 39:134a.

TORACK, R. M., and R. J. BARRNETT. 1962. Fine structural localization of cholinesterase activity in the rat brain stem. *Exp. Neurol.* 6:224.

TURBOW, M. M., and A. BURKHALTER. 1968. Acetylcholinesterase activity in the chick embryo spinal cords. *Develop. Biol.* 17:233.

WATSON, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. Biochem. Cytol.* 4:475.

WEISS, P., and H. B. HISCOE. 1948. Experiments on the mechanism of nerve growth. *J. Exp. Zool.* 107:315.

WENGER, B. S. 1951. Cholinesterase activity in

different spinal cord levels of the chick embryo. *Fed. Proc.* **10:**268.

YOUNGSTROM, K. A. 1938. On the relationship between cholinesterase and the development of behavior in amphibia. *J. Neurophysiol.* **1:**357.

YOUNGSTROM, K. A. 1941. Acetylcholine esterase concentration during the development of the human fetus. *J. Neurophysiol.* **4:**471.

ZACKS, S. I. 1954. Esterases in the early chick embryo. *Anat. Rec.* **118:**509.

ZAJICEK, J., and E. ZEUTHEN. 1961. Quantitative determination by a special "ampulla-diver" of cholinesterase activity in individual cells, with notes on other uses of the method. *Gen. Cytochem. Methods.* **2:**131.