

QL
939
A4x

THE HISTOCHEMICAL LOCALIZATION OF
ACETYLCHOLINESTERASE IN THE THORACIC
DORSAL ROOT GANGLIA OF THE CAT

A Thesis

Presented to
the Faculty of the Department of Biology
East Carolina University

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts in Biology

by
Wallace Witherell Ambrose

July 1976

6

Wallace Withere11 Ambrose. THE HISTOCHEMICAL LOCALIZATION OF ACETYLCHOLINESTERASE IN THE THORACIC DORSAL ROOT GANGLIA OF THE CAT (Under the direction of M. Evelyn McNeill) East Carolina University Department of Biology, July, 1976.

Thoracic spinal ganglia were studied morphologically and enzymatically in an attempt to correlate the position of the various sized cells within the ganglia with their degree of staining for acetylcholinesterase (AChE). This correlation could reveal a relationship between the stain uptake and sensory modality subserved by individual neurons.

Dorsal root ganglia were taken from the fourth and fifth thoracic spinal levels of 6 cats ranging in age from 6 to 16 months. Frozen longitudinal and cross sections were collected serially and stained with cresyl violet for total cell counts of the ganglia. The average number found at these levels was 3500 cells. Cresyl violet studies were on ganglia from the left side of the animals. The ganglia from the right side of the animals were stained for AChE after the method of El Badawi and Schenk (1967). All the cell fragments were counted in every fourth section. Also the diameter of each fragment was recorded. The cells of each ganglia were divided into 4 groups according to the amount of enzyme demonstrated. These groups, B1 (high activity - 29%), B2 (lesser but distinct activity - 52%), B3 (about equal demonstration of enzyme and counterstain - 15%), and BL (negligible enzyme shown - 4%), were represented graphically.

The study showed an inverse correlation between the size of the neurons and the amount of stain uptake. There was a size

gradation within the ganglia with the smaller fragments localized toward the center and largest at the periphery. The amount of enzyme demonstration was not strictly related to size in that there was a range of sizes in each of the four color classes. It was found that over half of the fragments displayed a medium activity (B2 cells), and 96% of the cells showed some AChE activity.

Ganglion cells have fibers that mediate different sensibilities (pain, touch, temperature, pressure and proprioception) depending on the size of the neuron. If enzymatic activity could be related to cell function, then a basis could be established for the study of various sensory pathways. The degree of activity could not be correlated by this study to any specific modality. There were indications that the presence of AChE and acetylcholine in dorsal root ganglion cells, where no synapses have been found, is localized in cells with small diameter (partly B1 cells), which have thinly or non-myelinated axons. The roles of acetylcholine alternative to membrane depolarization for the propagation of an action potential were discussed.

Dedicated to
my wife, Jeannie
for her patience
and love

THE HISTOCHEMICAL LOCALIZATION OF ACETYLCHOLINESTERASE

IN THE THORACIC DORSAL ROOT GANGLIA OF THE CAT

by

Wallace Witherell Ambrose

APPROVED BY:

SUPERVISOR OF THESIS

M. Evelyn McNeill
Dr. M. Evelyn McNeill

COMMITTEE MEMBER

James S. McDaniel
Dr. James S. McDaniel

COMMITTEE MEMBER

Charles E. Bland
Dr. Charles E. Bland

COMMITTEE MEMBER

Hubert W. Burden
Dr. Hubert W. Burden

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY

James S. McDaniel
Dr. James S. McDaniel

DEAN OF THE GRADUATE SCHOOL

Joseph G. Boyette
Dr. Joseph G. Boyette

ACKNOWLEDGMENT

Grateful acknowledgment is made to Professor M. Evelyn McNeill of the East Carolian University School of Medicine faculty for her kind and generous guidance and counsel. Her suggestions have made the completion of this thesis possible. The author thanks Drs. Charles Bland, Hubert Burden and James McDaniel for their assistance in reading the manuscript. Also special thanks is made to Dr. J. S. Hanker of the Dental Research Center of the University of North Carolina at Chapel Hill for making available his laboratory and support. Thanks go to Ms Irene Schwartz of the Learning Resources Center at the Dental School of the University of North Carolina for her technical skill in constructing graphs, charts and drawings. For typing the manuscript acknowledgment is made to Mss Wanda Wiseman, Shirley Nett, Jean Cochran and Virginia Bass.

TABLE OF CONTENTS

	PAGE
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
INTRODUCTION.....	1
Gross and Cellular Morphology.....	1
Cell Types and Function.....	2
Acetylcholinesterase.....	3
REVIEW OF LITERATURE.....	5
AChE Localization in Nervous Tissue.....	5
The Function of AChE.....	14
AChE Function and Localization in Dorsal Root Ganglia....	15
MATERIALS AND METHODS.....	19
RESULTS.....	22
Ganglion Morphology.....	22
AChE Localization in Spinal Ganglion Cells.....	23
DISCUSSION.....	26
Ganglion Morphology and Tissue Preparation.....	26
Color Categories as Shown By Enzymatic and Counter Staining.....	28
Functional Morphology of Sensory Ganglia.....	31
The Role of AChE in Sensory Ganglia.....	32
BIBLIOGRAPHY.....	36
APPENDIX A Acetylcholinesterase Staining Procedure.....	45
APPENDIX B Cresyl Violet Staining Procedure.....	47
APPENDIX C Combination Stain for Neurons and Their Fibers...	49
TABLES.....	50
FIGURES.....	53

LIST OF TABLES

TABLE		PAGE
1.	Vital Data of Study Animals and Actual Cell Numbers of T4 and T5 Spinal Ganglia as Counted by Nucleoli.....	50
2.	Analysis of the Number of Sections Taken in 12 Ganglia at the T4 and T5 Level in Cross or Longitudinal Section.....	51
3.	Comparison of Cell Types in AChE Stained Ganglia.....	52

LIST OF FIGURES

FIGURE		PAGE
1.	Diagram of spinal cord with dorsal and ventral roots as well as the dorsal root ganglion indicated. Also shown are some fiber ramifications from the respective rami.....	54
2.	Cat spinal cord with several spinal ganglia attached. Note the dimensions of the ganglia.....	56
3.	Study tissue stained with cresyl violet. a) longitudinal section of cat thoracic ganglion b) cross section through thoracic spinal ganglion of cat.....	58
4.	Thoracic spinal ganglia of the cat stained for AChE. a) longitudinal section b) cross section.....	60
5.	Comparison of the number of cell fragments in each section studied as to the color category in the 4 ganglia stained for AChE.....	62
6.	Comparison of the 4 color categories of the 3 ganglia sectioned longitudinally to the number of cell fragments counted per section. AChE stain.....	64
7.	Comparison of the number of cell fragments in each color category to their diameter in the 4 ganglia stained for AChE.....	66
8.	Comparison of the number of cell fragments of 4 ganglia stained for AChE as to their diameter and individual color category.....	68
9.	Neurons of thoracic spinal ganglia stain with cresyl violet, while the myelin surrounding the axons is	

- selectively stained with luxol fast blue (after Kluver-Barrera, 1957)..... 70
10. Sketch of a dorsal root ganglion with attached proximal and distal stumps. Note that the ventral root is closely apposed to the underside of the ganglion. It is possible to determine from where a section came by analyzing the relation of the ganglion to the ventral root..... 72
11. High magnifications of the four cell types found when stained for AChE. a) B1 cell b) B2 cell c) B3 cell d) BL cell..... 74
12. Comparison of the affinity of the spinal cord and the dorsal and ventral roots for AChE. a) The high degree of stain uptake in anterior motor horn cells of the spinal cord. Note the high activity in the neuropil. b) Spinal cord at the thoracic level. Note the sensory and motor horns are not extremely large. Also note the intermediolateral horn of the spinal gray..... 76
13. Control preparations of cat thoracic spinal ganglia using the AChE medium. a) AChE medium without substrate but with counterstain b) Incubation medium with substrate and counterstain but without the reversible nonspecific cholinesterase inhibitor (iso-OMPA). Note the peripheral staining of the cells due to the presence of nonspecific cholinesterase..... 78

INTRODUCTION

The aim of this study was to coordinate gross morphological features with a quantitative analysis of the graded acetylcholinesterase content in thoracic spinal ganglia. The localization of this enzyme was used in an attempt to characterize the small, medium and large cells and the sensory modalities they subserve. Concurrently, the importance of ACh in roles alternative to membrane depolarization and the propagation of an action potential was emphasized.

Gross and Cellular Morphology

There are 38 pairs of spinal ganglia in the cat: 8 cervical, 13 thoracic, 7 lumbar, 3 sacral and 7 caudal (Reighard, 1966). As part of the peripheral nervous system, the cell bodies and fibers of the spinal ganglia lie segmentally between adjacent vertebrae, passing through the intervertebral foramina just beneath each transverse process. Embryologically these ganglia differentiate from ectodermal neural crest cells. Each ovate ganglion is covered with a connective tissue capsule, while the individual neurons are surrounded by a Schwann-satellite cell sheath, which arose from undifferentiated neuroblasts (Crosby, et al., 1962). Thoracic ganglia in the cat measure about one by two millimeters. Cervical and lumbar ganglia range from one and a half to two or more times larger than thoracic ganglia, with those at the cervical levels being largest.

Ten to twelve nerve rootlets extend from the spinal cord at each level. Half of these from the dorsolateral sulcus of the spinal cord join into two bundles before entering the spinal ganglia as the dorsal root. Rootlets from the ventrolateral sulcus of the spinal cord

join to become the ventral root (Reighard, 1966). Ventral and dorsal root fibers converge just distal to the ganglia to become peripheral nerves. The oval ganglion cells are pseudounipolar. Their axons bifurcate into T-shaped processes. In sensory neurons of dog, cat, rabbit, and guinea pig, Dogiel (1896) found thick peripheral fibers and thin central ones. Ultrastructural research (Ha, 1969) has shown that some cells demonstrate myelinated peripheral fibers and unmyelinated central processes.

Cell Types and Function

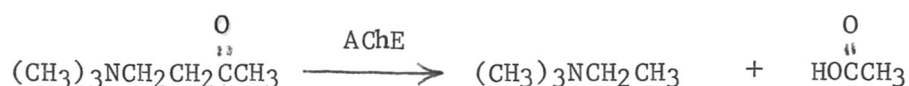
Degeneration and surgical studies have elucidated the sources of dorsal root fibers innervating various sensory end organs. Large clear cells are the origin of fibers to the skin, viscera and muscle with the largest fibers exclusively serving muscle spindles. Some of the above fibers also give rise to somatic and splanchnic nerves. The usual function of small, medium and large ganglion cells are well known (Crosby, et al., 1962). Over the lateral dorsal root bundle course small and medium diameter fibers leading to cell bodies of the same respective sizes. The small neurons mediate peripheral pain; medium sized cells conduct touch and temperature as well as some visceral sensation. The more specialized large dorsal root neurons evolved more recently. Their fibers enter the ganglion through a medial bundle. These neurons mediate proprioception through highly developed tactile endings (Crosby, et al., loc. cit.; Brodal, 1969).

The early studies of the spinal ganglia by Dogiel, Cajal (1897 and 1905-1906 respectively as cited in Ranson, 1912) and Lugaro (1903 as cited in Warrington and Griffith, 1904) were attempts to describe the staining variability in these specialized cells. All of the above

experimenters reported two types of cells, "light" and "dark", and many visualized more types according to the size, shape, uptake of stain, amount of neurofilaments and "chromophile" (Nissl substance) and position of the nucleus (Orr and Rows, 1901; Turner, 1903; Warrington and Griffith, 1904; Ranson, 1912). It did not take long to reveal that many of these "complex" cell types were artifactual (Ranson, 1914). However, Emery and Singhal (1973) have retained the criteria of cell size and depth of Nissl staining after Warrington and Griffith (1904) and Lugaro (1900-1903 as cited in Emery and Singhal, loc. cit.) in quantitating cell types in infants. Most recent studies ~~climit~~ the number of cell types to two or four on the basis of size, amount of Nissl substance, or the variation in stain uptake (Palay and Palade, 1955; Kokko, 1965; Bunge, et al., 1967; Peach, 1972).

Acetylcholinesterase

Histological localization of acetylcholinesterase (AChE) has been taken as a good, although not absolute, indication of the presence of acetylcholine (ACh). The molecular weight of AChE is 124,000 (Bernsohn, et al., 1962) of which there are several isoenzymes (Loewenstein, 1971). Acetylcholine is known to be the neurotransmitter in autonomic ganglia, postganglionic parasympathetic terminals, certain postganglionic sympathetic terminals (sweat glands, vasodilators) and in motor neurons and collateral Renshaw cell synapses (Brobeck, 1973). These are called cholinergic neurons. Acetylcholinesterase is present at terminals to hydrolyze ACh after its release from vesicles and subsequent passage across the synaptic space. The reaction is



However, there are neurons that display varying degrees of AChE activity. These may be called non-cholinergic or cholinceptive neurons. Chief among these are the spinal ganglia. Although these sensory nerve cell bodies contain a graded AChE content from negligible to quite high, most of their axons are almost completely devoid of AChE activity (Gruber and Zenker, 1973). The search for a definitive neurotransmitter in sensory cells has been fruitless, although, Helawer and Umrath (1948) and Holton and Perry (1951) have reported some type of transmitter. Koelle and his cohorts in 1962 postulated that ACh may serve to trigger another transmitter. It does not seem out of the question that there may be no neurotransmitter in sensory nerves because first order fiber firing could be evoked by mechanical stimulation of a receptor. It is recognized that some sort of transmitter is required in the nuclei cuneatus and gracilis for relay of an impulse to the second order neurons. This transmitter has not been revealed.

This study determined the actual number of cells in several ganglia, as well as their size. The AChE activity range and number of cells in each of four predetermined color categories were demonstrated after being stained with AChE and a counterstain.

REVIEW OF LITERATURE

AChE Localization in Nervous Tissue

Acetylcholine (ACh) and acetylcholinesterase (AChE) have been studied extensively in the nervous system where they are widely distributed in varying concentrations. Early experimentation established that most efferent tracts and many autonomic ganglia were cholinergic. High AChE activity was demonstrated in the cerebral cortex but low activity occurred in the pyramidal tracts (MacIntosh, 1941). Feldberg and Mann (1948) showed that very little ACh was synthesized in the afferent fibers of the optic nerve and dorsal roots of spinal nerves, but, there was high activity in the ventral roots. The highest activity in the central nervous system of dogs was "where cranial nerves originate" and in anterior motor horn cells, but even this was about 70% of the activity found in the ventral root fibers (Feldberg and Vogt, 1948). Areas of lowest activity were, in ascending order, sensory nerves and tracts, pyramidal fibers and the cerebellar cortex. These areas contain essentially no synthesizing ability for ACh. Choline acetylase (ChAc) is the enzyme that transfers an acetyl group from acetyl coenzyme A to choline (Roskoski, 1973). In rabbits, McCaman and Hunt (1965) found high concentrations of ChAc in the cerebral cortex and medulla, while there were very high concentrations in the pons, hippocampus and caudate nucleus. Low ChAc concentrations were found in the same areas where little ACh and AChE were located, such as in corpus callosum, cerebellum, spinal and trigeminal ganglia and optic tracts. An intermediate amount was reported in the olfactory bulb and dorsal gray column of the spinal cord. The positive correlation among AChE, ACh and ChAc localization has been demonstrated by Eranko

(1967), Silver (1971) and Silver and Wolstencroft (1971). Kokko (1965) showed positive correlation between AChE and acid phosphatase in rat spinal ganglion cells. In homogenized ruptured guinea pig brain synaptosomes (end terminal fraction) ACh was localized in the synaptic vesicles; AChE was localized in the microsome fraction (endoplasmic reticulum); while ChAc was found in the soluble fraction not membrane bound (Koelle, 1959; Toschi, 1959 as cited in Brzin, et al., 1966; Michealson, 1967). Brzin suggested that AChE is either synthesized in the rough endoplasmic reticulum and migrates through the cell independently, or that the organelle where AChE is produced may itself travel through the cell cisternae by axoplasmic flow with the enzyme still attached to the cell membrane and processes (Weiss and Hiscoe, 1948; Lasek, 1968). A third possibility is that AChE is synthesized in the axon proper, although, measurements of axonal RNA (as well as the synthesizing enzymes) are very low.

Feldberg and Vogt (1948) recognized that the ability to synthesize ACh in the central nervous system decreases in more "highly developed brains". They attributed this to the increased amount of supporting tissue found in more evolved brains.

Early determinations of ACh and AChE were made in various nervous tissues manometrically. This method is still being used along with cell fractionation methods. The most precise method of localizing enzymatic activity is cytological histochemistry (Koelle, 1957). The ideal histochemical method produces a precipitate from a naturally occurring substrate (Holmstedt, 1971). The thiocholine technique first developed by Koelle and Friedenwald (1949) seemed to be the one which came closest to forming a "natural" product. This method involves

the incubation of frozen cryostat sections in acetylthiocholine, copper glycinate, copper thiocholine and ammonium sulfate to precipitate copper sulfate at loci of AChE activity. A histochemical method has never been developed which is specific for ACh, however, the localization of AChE has been accepted as a reasonable intracellular indicator of ACh. The Koelle-Friedenwald technique was based on the earlier work of Gomori (1948) who visualized AChE activity with a precipitate which could withstand the subsequent rigors of fixation and embedding. The problems of sensitivity and specificity of localization still took years to be worked out using various inhibitors and substrates. Lewis (1960) varied the time, temperature and pH of the Koelle method to determine optimal ranges of activity. A very specific modification by Karnovsky and Roots (1964) employed a ferricyanide reduced by acetylthiocholine to ferrocyanide, which when combined with Cu^{++} ions precipitated as a fine copper ferricyanide (Hatchett's brown) directly in areas where AChE is located (Karnovsky, 1964; Broderson, et al., 1974). This eliminated the large cupric sulfate crystals of the original Koelle-Friedenwald method, which made exact enzyme localization at the light microscopic level difficult and impossible at the electron microscopic level. Other modifications of the Koelle technique were worked out for demonstrating enzymatic activity in specific areas or structures (Coupland and Holmes, 1957), and for obtaining greater specificity (Hanker, et al., 1973). Some modifications included varying the types and concentration of selective reversible inhibitors of AChE such as diisopropyl fluorophosphate (DFP), eserine (physostigmine) and BW 284 C51 (Holmstedt, 1957a; 1957b; Koelle, 1960).

Nonspecific esterases are also found in nervous tissue. When acetylthiocholine is used as a substrate, the endproduct is the localization of specific (acetyl-, true) cholinesterase (AChE). Butyrylthiocholine produces a precipitate at sites of non-specific (butyryl-, pseudo-) cholinesterase (BuChE) (Koelle, 1954; 1955; 1957). Sites of non-specific ChE included the cytoplasm of fibrous astrocytes and other glial cells, capillary walls, smooth muscle of large blood vessels, (Navartham and Lewis, 1970; Holmstedt, 1971), and a few neurons. Cauna and Naik (1971) did not find BuChE in sympathetic neurons, but the enzyme was present at motor endplates along with AChE. Recently BuChE was demonstrated in processes of autonomic ganglia by Koelle and his co-workers (1974). Eranko (1967) has also found non-specific ChE in nervous and other tissues. Adams, et al. (1967) found BuChE and AChE in postganglionic fibers but only BuChE activity in unmyelinated fibers of non-cholinergic sensory nerves. Myelinated axons positive for AChE did not contain non-specific ChE. The great variability of enzymatic activity between species was shown by Silver and Wolstencroft (1971). In rat spinal cord, incubated for 14 hours, BuChE activity was noted in the walls of blood vessels and in both gray and white matter; whereas, in cats similar vessels were unstained, but there was pronounced staining of the white matter, neuropil of the ventral horn, and a few small cells in the intermediate and dorsal horns. An explanation for this could be that the non-specific ChE in the rat is the propriono- rather than the butyryl- form (Koelle, 1954; Holmstedt, 1971). These last two researchers saw areas of similar positive activity in the two species in white fiber tracts and glial cells, around the supraorbital nucleus and the third ventricle in the region of the

paraventricular nucleus, and in capillaries and blood vessel walls. Non-specific ChE, not found in most ganglia, showed high activity in Auerbach's plexus in the ileum (Koelle and Koelle, 1959). Non-nervous areas of BuChE activity include the carotid bodies, hepatic cells, muscularis mucosa of the intestinal canal and zona glomerulosa cells of the adrenal cortex.

The AChE hydrolyzes acetylthiocholine at a higher rate than acetylcholine, however, it hydrolyzes butyrylthiocholine as fast as BuCh. Non-specific ChE hydrolyzed both AcThCh and BuThCh in rats (Koelle, 1954; Giacobini, 1956; Clithrew, 1963). This demonstrates the specificity of AChE and the generalized function of BuChE.

Non-specific ChE is probably involved with permeability changes in capillary walls (Koelle, 1954). Clithrew, et al. (1963) suggested that the enzyme is concerned with slow nerve conduction and that non-specific ChE in the blood may be important in limiting responses to hydrolyzable cholinergic agents. He also stated that butyryl-choline (as well as other forms) may be a liver ester by-product that would be toxic if not hydrolyzed by BuChE.

Specific-ChE is attributed to AChE; however, electrophoretic studies by Bernsohn, et al. (1962) and Barron and Bernsohn (1968) showed several bands from homogenates of human lenticular formation. This suggests several molecular conformations or species of AChE, each of which could have a separate function.

Areas of Specific-ChE (AChE) activity were demonstrated by Koelle (1954) and Eranko (1967) at the neuronal membrane along axons and dendrites as well as at synapses of cholinergic neurons. High activities have been reported at nodes of Ranvier (Hanker, et al., 1973). Non-cholinergic

neurons were devoid of activity. Kokko (1965) described a wide variation of activity in rat spinal ganglia (purported to be non-cholinergic). Koelle and Koelle (1959) have substantiated the presence of an "internal" or "reserve" AChE and an "external" or functional" AChE. Internal AChE was a bound fraction on or in cytomembranes, whereas the external AChE was outside the cells along axons and at presynaptic terminals where its primary function of hydrolyzing liberated ACh is implemented. Similar results have been reported for AChE localization in squid axons (Villegas and Villegas, 1974). Koelle and Fukuda (1959) demonstrated that AChE activity in the cat corresponded closely with the distribution of Nissl substance after reversible inhibition of activity with DFP in vivo. The reappearance of the enzyme in these preparations was first noticed in the Nissl substance and later at the perikaryon and neuronal projections. This indicated a cytoplasmic origin of the enzyme with subsequent transport (internal) to the cell surface and axon (external). Tewari and Bourne (1962) considered the variability of AChE in sensory ganglion cells to be due to stages in the metabolic cycle involved in the production of the enzyme. Ritter, et al. (1974) used a thiocholine method of Lewis and Shute (1964, 1969) and the ferricyanide method of Karnovsky (1964) to show the ultrastructural localization of AChE in the rat central nervous system (hippocampal formation) and peripheral nervous system (sympathetic ganglia of rabbit and rat adrenal). The enzyme was demonstrated at membranes of all areas studied including perikaryon (endoplasmic reticulum and nuclear envelope), preterminal and terminal parts (axonal membranes) and axosomatic and axodendritic synapses of the Central Nervous System (presynaptic membranes and synaptic clefts). The postsynaptic membrane was always negative. Presynaptic activity was

of the vesicles present. Non-myelinated axons showed stronger activity than myelinated ones. Although the histochemical criterion for cholinergic nerves is AChE presence in the axon membrane, the best criterion is its demonstration at the terminals.

Microchemical methods became valuable to cell biologists when the ability to study single neurons was developed. The cartesian diver method of Giacobini (1959) showed the spatial specificity of the enzyme content in isolated spinal ganglion cells by microgasometric analysis. Brzin, et al. (1966) used a histochemical procedure as well as microgasometric analysis with a magnetic diver to locate AChE activity on the inner surface of the endoplasmic reticulum, nuclear envelope, subsurface cisternae, the agranular reticulum of the perikarya and axons of frog sympathetic and dorsal root ganglia. Inhibitors for these studies included the irreversible non-specific cholinesterase inhibitor, ISO-OMPA (EL Badawi and Schenk, 1967), and the reversible inhibitors of AChE, eserine and BW 284 C51. It was found that damaged neurons exhibited more activity than intact ones. The internal AChE on the endoplasmic reticulum is less sensitive to eserine inhibition than the enzyme on the neuron-cell sheath interface. This indicates a permeability barrier to bound AChE which is substantiated by the fact that quaternary ammonium compounds like ACh and the very large enzyme AChE (mol. wt. 65,000-250,000) are insoluble in lipids (Koelle and Koelle, 1959). Therefore, they must move through the cell membrane by way of pores or cisternae. Brzin (1966) disagreed with Giacobini's findings that sympathetic elements showed unmeasurable AChE and stated that Giacobini's experiments lacked sensitivity. Giacobini (1956) reported low to moderate AChE content with 5-10% of the cells showing

a high activity in autonomic ganglia. The activity in the spinal cord was high because of lack of a protective capsule around the neurons. Rat adrenergic neurons (superior cervical and stellate ganglia) showed generally faint staining for AChE. The ciliary ganglia showed high activity in both pre- and post-ganglionic fibers. This cholinergic nature was demonstrated by Cauna and Naik (1974) and Holmstedt (1971). The mixed (motor and sensory) nodose ganglion of the vagus showed heavily and intermediately stained types. Dorsal root ganglion cells showed intermediate staining with much variability. Auerbach's plexus in the cat ileum had highly stained cells. Studies of AChE in various areas of the nervous system helped to elucidate cholinergic tracts (Shute and Lewis, 1961; 1963) and by studies of cholinergic cutaneous receptors, to attempt to find the neurotransmitter in sensory nerves (Cauna and Naik, 1963). These latter studies revealed ChE-positivity in the phylogenetically young encapsulated meissner's corpuscle (discriminative touch receptors) and all tactile hairs. In the unencapsulated phylogenetically old merkel's corpuscles (touch) in man and cat, there was no AChE activity. No sensory modality could be associated with AChE. Feldberg and Vogt (1948) and Koelle (1954) noticed an alternating AChE-positive and AChE-negative arrangement in sensory tracts. The primary peripheral sensory fibers and the funiculus gracilis and cuneatus were AChE-negative. Secondary neurons from the nucleus gracilis and cuneatis showed moderate enzyme activity and the tertiary afferents showed a moderate to high AChE activity.

Rat myelinated axons of the mostly motor genitofemoral nerve exhibited AChE activity but the sensory auricular nerve showed only about 5 per cent activity (Adams, et al., 1967). In the mixed sciatic

nerve, a third of the myelinated axons were AChE positive. Myelinated AChE positive axons did not contain non-specific ChE. Conversely, unmyelinated sensory nerves exhibited only non-specific ChE. Unmyelinated fibers in the motor and mixed nerves contained both AChE and BuChE. Postganglionic parasympathetic fibers contained both enzymes with AChE predominant. Post-ganglionic sympathetic fibers contained non-specific ChE but often there was some AChE activity also. Eranko (1967) listed some areas of activity that are in agreement with most researchers: (a) some sympathetic fibers have catacholamines present but not that of AChE; (b) some sympathetic fibers have only AChE activity; (c) fibers appearing single under light microscopy may actually be a bundle of very small fibers with both catacholamine and AChE activity; and (d) liberation of catacholamine through a cholinergic link is possible. Navartham and Lewis (1970) showed AChE activity in the rat spinal cord to be almost entirely in gray matter as did Holmstedt (1971) in the rat and cat spinal cord. Cells in the intermediolateral and medial columns in the ventral horn have high activity. Though the substantia gelatinosa appears to have high AChE content, the activity is due to the neuropil (supporting cells and fibers). In cats, Silver and Wolstencroft (1971) found more activity in the neuropil of the adult cats than in kittens. They also demonstrated in cat spinal cord a positive correlation between demarcation of activity and laminae I, II and III of Rexed (1953). Clark's column had a few lightly stained cells (40-50 μ). The large cells were moderate to weak in activity and the neuropil was negative. In the ventral horn, histochemical investigations agree with electrophysiological, fluorescent dye and degeneration studies that the spinocerebellar tract may be cholinergic (Silver and

Wolstencroft, 1971). This seems to deviate from accepted dogma that only motor tracts are cholinergic and sensory and adrenergic nerves contain little AChE (i.e., non-cholinergic). This tract, however, is one of the few ascending tracts in the ventral horn (second order). The determination of a high, moderate, and low enzyme content is rather arbitrary and varies with the researcher as well as the organisms and sites within the organism studied. Many investigators consider moderate or weak staining neural areas are not cholinergic.

The Function of AChE

External AChE may function in preventing post-synaptically activated ionic liberation of ACh. It may also protect presynaptic terminals and prevent the accumulation and spread of ACh after a preganglionic series of volleys (Koelle and Koelle, 1959). The presence of AChE in non-cholinergic fibers could be an initial catalyst for the release of other neurotransmitters (Koelle, 1962), since ACh or other transmitters do not participate directly in the propagation of an impulse. Cauna (1961) demonstrated AChE in cutaneous receptors. Koelle (loc. cit.) reflected that ACh probably evolved in non-nervous tissue but has been retained through specialization because of its efficiency at the synapse. Acetylcholine has been found in cilia of protozoa, invertebrate nervous tissue and in all vertebrates. In each case cited, the enzyme caused membrane permeability changes in excitable tissue. Some AChE is also found in smooth muscle, bronchioles, urinary bladder, effector cells of salivary glands and in the blood of cats and rats (Holmstedt, 1971). In the autonomic nervous system AChE acts as a hormone to maintain rhythm and excitability in smooth and cardiac muscle. Shute and Lewis (1963) indicated an arousal mechanism

between the fornix pathways and cerebellar cortex. Brizn, et al. (1966) suggested the close apposition of the subsurface cisternae to the neurilemma could somehow coordinate excitation and intracellular metabolic processes. In the sarcoplasmic reticulum AChE may be linked to ion permeabilities and serve as the mechanism of transport in lieu of a plasmalemma. Blood AChE serves as a useful index of the absorption of anticholinesterase insecticides like parathion (loc. cit.).

AChE Function and Localization in Dorsal Root Ganglia

Great variability in AChE activity has been demonstrated in spinal ganglia of cat, squirrel monkey (Shanta, 1967) and bovine (Matsura, 1967) spinal ganglia. Giacobini (1956) and Galabov (1959) reported in rats and rabbits that 10 to 15% of the dorsal root ganglion cells in rats and rabbits had very high concentrations while the rest had very little activity. Giacobini (1959) confirmed these findings with biochemical assay procedures of individual ganglion cells in rats. He found that 70% of all the cells of the ganglia showed AChE positivity, and that the variability among some cells was up to 100 times. The amount of activity demonstrated is dependent on the length of incubation and this may be the source of discrepancy in some results (Koelle, 1955). Cauna and Naik (1963) found activity in all ganglion cells after long incubations. Giacobini (1959) estimated the absolute activity of dorsal root ganglion cells to be 10 to 20 times lower than that of anterior motor horn cells. There seems to be a negative correlation between AChE activity and cell diameter (Giacobini, 1959; Kokko, 1965). This may indicate a special cholinergic (autonomic?)

pathway in the smaller cells. The distribution of AChE activity in spinal ganglia seems to be very similar to enzyme localization in the trigeminal (Mazza, et al., 1973) and nodose (Cauna and Naik, 1963) ganglia of mouse, rat, cat, guinea pig, mole and man. Afferent nerve fibers to the ganglion gave a negative response to AChE in the cat and guinea pig but a weak to moderate reaction in the rat, mole, and man. A controversy has developed over the activity in myelinated and unmyelinated fibers. Novikoff, et al. (1966) found activity at the axon surface in myelinated and unmyelinated fibers in the dorsal roots of rat, rabbit, and frog, but Schlaepfer (1968) found activity in the rat only in the small unmyelinated fibers which are the axonal processes of the small cell bodies in the ganglion (also stained). In contrast, the large and small myelinated fibers serving extrafusal and intrafusal muscles respectively, showed a consistent activity, although there was relatively higher activity in groups of myelinated and unmyelinated fibers which were believed to be preganglionic sympathetic nerves. Gwynn and Flummerfelt (1971) found in glutaraldehyde fixed rat myelinated dorsal root fibers only moderate activity and no activity in unmyelinated fibers. Koelle (1955) found slight activity in peripheral afferents. Gruber and Zenker (1973) demonstrated that 18% of ventral root fibers were unstained and 8.9% of sensory fibers of dorsal roots showed stain after very long incubation at 6° C. The stained dorsal root fibers were not thought to be preganglionic. These fibers were of medium and large caliber, therefore, it was unlikely that they were visceral efferents. At present the general consensus is that these fibers were not visceral efferents since they were of medium and large caliber (McNeill, 1972). The low activity found by Schlaepfer (1968) is attributed to short incubation times.

In the cat the ratio of AChE activity in sensory to motor roots is 1:100; in dogs, 1:30; and in bullfrogs, 1:20-30. The ratio of choline acetylase in the cat is 1:400 and in bullfrogs, 1:100 (Gruber and Zenker, 1973). This agrees with studies by Galabov, et al. (1964) that dorsal root RNA, and; therefore, AChE synthesizing ability is low.

Acetylcholinesterase seems to be dispersed in the large and medium sized ganglion cells and dense in the medium-sized "multiangular" cells of the rabbit. The absence of choline acetyltransferase as well as ACh absence in dorsal roots obviates much possibility that sensory nerves are cholinergic, even though lesion and axoplasmic flow studies show a build up of AChE proximal to the lesion (Lasek, 1968; Schlaepfer, 1968; Gwynn and Flummerfelt, 1971). Schlaepfer's conclusions fit well with Bishop's (1959 as cited in Schlaepfer, 1968) postulation that the mammalian peripheral unmyelinated afferent fibers represent vestiges of a primitive sensory system over which the more elaborate and specific modalities have evolved. At the same time the larger myelinated afferent fibers, which conceivably could also have evolved as an alternate mechanism (i.e. transmitter) for impulse transmission, could have evolved.

Novikoff, et al. (1966) localized non-specific cholinesterase at Schwann cell-axon interfaces of unmyelinated fibers and sheath cell-perikaryon interfaces on myelinated axons. Cauna and Naik (1963) found non-specific ChE only in the spinal ganglia of cat and guinea pig. In human sensory ganglia the enzyme was found only in fetal life.

In the trigeminal ganglion, Mazza, et al. (1973) found BuChE in the intracellular channels between nerve fibers and satellite cells. It is found intracellularly in association with mitochondria, endoplasmic reticulum and the nuclear envelope of satellite cells and in mitochondria of neurons.

Tewari and Bourne (1962) localized AChE at the nucleolus and areas associated with RNA synthesis (nucleolar AChE activity has not been reported by others). Reaction product was deposited in the cisternae of rough endoplasmic reticulum and the intramembranous space of the nuclear envelope (Gwynn and Flummerfelt, 1971). Tewari and Bourne (loc. cit.) have suggested a continuous metabolic cycle of AChE and ACh production in the endoplasmic reticulum with their subsequent transport to the peripheral areas. Though this theory could generally explain the variability of activity in the spinal ganglion, Schlaepfer (1968) believed these cells to be several functional groups and notes that even Tewari and Bourne stated that the smallest cells have the highest activity.

MATERIALS AND METHODS

The fourth and fifth thoracic (T4 and T5) dorsal root ganglia (DRG) were obtained from 12 cats ranging in age from 6 to about 16 months. The animals were calmed with chloroform prior to intraperitoneal injection with sodium pentobarital (Nembutal) in dosages of 30 to 50 mg/kg. Deep surgical anesthesia was achieved in 20 to 30 minutes. During this time the animal's back was shaved. The spinal ganglia were removed bilaterally under aseptic conditions. After gently scraping and separating the paravertebral muscles of the back from the spinous and transverse processes of the vertebrae, bilateral laminectomies were performed to expose the spinal cord. To avoid damaging the ganglia, the transverse processes were carefully cut away. The ganglia could then be removed with ample proximal and distal roots attached. Vertebrae T4 and T5 were identified by first palpating the prominent seventh cervical spinous process and counting caudally to the desired level. All but four of the animals remained alive during the surgery. When they did not, the ganglia were removed within 30 minutes after death. The animals ranged in weight from 1.7 to 4 kilograms.

After each ganglion was removed, it was rinsed in physiological saline and cleaned of connective tissue including the extensive venous plexus surrounding it. The meninges were left intact. Each ganglion was immersed in a BEEM (Better Equipment for Electron Microscopy) capsule filled with Tissue Tek, a low temperature mounting medium, and oriented to achieve either a longitudinal or a cross section. The time for these steps was held to a minimum, and did not exceed three minutes. The BEEM capsule was plunged into a small beaker containing cold isopentane (-65°C to -70°C). The beaker floated in a dewar flask filled with

isopentane and dry ice which prevented contamination or damage due to carbonation. The tissue was quenched and left in the coolant five to 30 minutes. Excess isopentane was drained from the BEEM capsules after quenching the last ganglion. The labeled caps were snapped shut and the capsules were placed on the heat sink of a cryostat set at -25°C to equilibrate prior to sectioning. Although sectioning could be begun within five to ten minutes, the blocks could also be stored several days without apparent damage as long as thawing and refreezing did not occur. Most of the tissue was sectioned and stained within two to three days of quenching.

Ganglia from the right side of the animal were processed for histochemical staining for AChE. Ganglia from the left side were stained with cresyl violet (Nissl stain) for overall morphology and gross cell counts. Longitudinal sections were taken of the right T5 ganglia and cross sections of the right T4 spinal ganglia. Ganglia contralateral to the above were sectioned in the opposite plane to serve as controls. Since the T4 and T5 ganglia were seemingly uniform in size and similar in function, it was assumed that information obtained from one level could be applied to the other.

The ganglia were sectioned on a cryostat (American Optical Company). Tissue was mounted on a chuck and sectioned at approximately 16 micra. The sections were collected serially and mounted on slides pretreated with a gelatin and chrome alum adhesive. The serial sections of the entire ganglia were air dried ten to 20 minutes prior to being stained.

Sections to be histochemically stained were fixed in four percent neutral formalin for 15 to 20 minutes, washed and incubated after the basic Koelle-Friedenwald (1949) cholinesterase technique. This method

was modified by Karnovsky and Roots (1964) to increase the sharpness of precipitation at areas of AChE enzymatic activity, and also by El-Badawi and Schenk (1967) to be specific for acetylcholinesterase by the addition of ISO-OMPA (tetraisopropylpyrophosphoramidate), an inhibitor of butyryl- or non-specific cholinesterase activity. The AChE regimen is outlined in APPENDIX A. Three hour incubation times were used in this study.

Sections were stained with a cresyl violet method of Fernstrom (1958). Fresh sections on slides were placed in 95 percent ethyl alcohol and carried through the schedule as outlined in APPENDIX B. Graduated cylinders & staining trays for AChE incubation media were acid cleaned before each use. Double distilled water was used throughout.

The gross number of neurons was counted in serial sections of ganglia stained with cresyl violet. The sections were viewed either under a microscope with a grided ocular or with a BioScope at X200. The number of cells present in each section was enumerated by counting visible nucleoli.

The BioScope was used to examine every fourth section of the AChE-stained tissue. The amount of enzymatic activity and the diameter of each cell or cell fragment in the section was recorded. These cells were classified visually by the color demonstrated as B1 (brown), B2 (light brown), B3 (blue-brown) and BL (blue).

RESULTS

Tissue was studied grossly for ganglion morphology (figs. 1&2) and microscopically for a quantitative analysis of enzyme localization (figs. 5-8).

Ganglion Morphology

To determine the various cell types and their location within spinal ganglia, an initial investigation of the general morphology of the ganglia was undertaken. This included many dissections of thoracic as well as cervical and lumbar spinal ganglia from mouse, rat, cat, and cadaver material. The thoracic ganglia were sectioned on different planes for comparison when stained histologically or histochemically (Figs. 3 & 4). A myelin-Nissl method (Klüver and Barrera, 1953) was employed to examine fiber-cell body relationships of the dorsal root ganglia and the fibers of the ventral root at the same level (Fig. 9). The staining routine for this method is in Appendix C. With the knowledge gained, it was possible to determine from which quarter of a ganglion a section had been taken (Fig.10).

The results from ganglion morphology studies are shown in Table 1. The total number of cells in eight ganglia stained with cresyl violet were enumerated by counting visible nucleoli in each section. These ganglia were taken from the left side of the spinal cords, four at the fourth thoracic (T4) level and four at the fifth thoracic (T5) level. Three of the ganglia were cross sectioned while the other five were cut longitudinally. An average number of 3375 cells was found in the eight ganglia. The mean cell number for the T4 ganglia was 3053 and for the T5 ganglia 3699 cells. This indicates an appreciable increase in the number of cells from one thoracic level to the next. The means for

cross-sectioned and longitudinally sectioned ganglia were 3476 and 3314 cells respectively.

The number of serial sections in the four ganglia sectioned transversely ranged from 99 to 154 with a mean of 127 sections (Table 2). Ganglia sectioned longitudinally ranged from 84 to 116 sections or 1.3 to 1.8 mm wide. Therefore, the average dimensions of the spinal ganglia at the T4 and T5 level were 1.5 millimeters by 2.0 millimeters.

AChE Localization in Spinal Ganglion Cells

Four ganglia were used to demonstrate the localization of acetylcholinesterase (AChE) (Table 3). The range of the cell fragments numbered from 4309 to 5567 with a mean of 4975 and a standard deviation of 449 fragments. The cells were grouped into four categories according to their degree of stain uptake (Fig. 11a-d).

The B1 cells had the most intense accumulation of Hatchett's brown and appeared bright brown. The range in diameters of B1 fragments was 5 to 100 microns with a mean size of 36 micra. This type made up 29 percent of the total fragments in the ganglia studied. The bulk of these cells were at the lower end of the range. Generally, the smallest cells exhibited the heaviest staining. These intensely stained cells closely approached the degree of uptake shown throughout the anterior motor horn cells. However, the small aggregations of AChE in large B1 fragments, though still demonstrating high intensity, falls short of the activity of the anterior motor horn cells of the spinal cord (Fig. 12). In all the cell types there was clumpy deposition of the enzyme in smaller cells and a finer deposition in the medium and large cells. There seemed to be more of the small fragments of this group centrally located in the ganglia.

The B2 cells made up 52 percent of all the fragments counted. The mean size of this type was 41 micra with a range of 5 to 110 micra. These cells exhibited a finer deposition of AChE than B1 cells, and therefore, they appeared light brown. These cells seemed randomly dispersed throughout the ganglia.

B3 fragments showed approximately equal uptake of enzyme and counterstain. These blue-brown to purple cells made up 15 percent of the fragments counted in four ganglia. The size of these fragments ranged from 10 to 95 micra. Though the mean was the same as B2 cells (41 micra) the graphs of fragments were markedly more skewed toward the lower end of their range than the B2 cells. The enzyme was very finely deposited in these cells. These cells also seemed to be evenly distributed in the ganglia.

The BL cells made up 4 percent of the counted fragments in four ganglia. These cells contained no demonstrable AChE activity and were stained light blue with the counterstain. This type had a size range of about 10 to 90 microns with a mean diameter of 48 microns. These cells, though not the largest observed in the ganglia, were consistently large in a given section. The BL fragments fell close to normal distribution curves although there was some variation in the position of the curve from ganglion to ganglion. On inspection of sections, these cells were found routinely closer to the periphery.

A definite negative correlation was demonstrated between the size of the cells and the amount of AChE uptake. This agrees with the results of Kokko (1965). The cell fragments measured in the ganglia stained for AChE ranged in size from 5 to 120 micra. The same range was found in cresyl violet stained ganglia. Control preparations

included incubations without substrate, without counterstain and without non-specific cholinesterase inhibitor (Fig. 13).

Though the size of individual ganglia varies markedly, the relation between the cell types are in about the same proportion.

The one cross-sectioned ganglion showed no difference other than the larger section size, though one ganglion sectioned this way is not conclusive.

The graphs indicate that each type of cell, though having distinct mean sizes, is not limited to a small size range which could have been indicative of a definitive sensory modality.

DISCUSSION

Thoracic ganglia were selected for study because of their accessibility and their smaller size in relation to cervical or lumbar spinal ganglia. The importance of these ganglia lies in their role in general visceral sensation, and the fact that they have been little studied. It is known that fetal and young animals have different morphology and enzyme distribution than adult animals (Youngstrom, 1941; Weschler, et al., 1967), therefore, the test animals were at least six months old.

An increase in the mean number of cells with visible nucleoli in T5 ganglia over the T4 level is indicated. An increase of 6% in the number of cells at the T5 level should result in an increase in the size of the T5 ganglia. This size change is not revealed in the comparisons of the total longitudinal sections. A series of cross sectioned ganglia might reflect this size increase.

Though there have been many studies on the morphology of the spinal ganglia and also much clinical research on diseases affecting these ganglia, there are few studies correlating functional aspects of sensory neurons with the general morphology of sensory ganglia. Therefore, to attempt an analysis of this type, the dimensions of the study tissue were ascertained.

Fixation of tissue to be histochemically treated can be critical. It is important that the fixative be strong enough to properly curtail cellular processes without interfering with the localization of the enzyme. A fixative must be suited to the enzyme to be studied. A prime criterion of histochemical study is that the least fixation is the best

(Taxi, 1952); Burstone, 1959). This is because of the cytological changes due to any fixative, which concomitantly disrupts the localization and general distribution of an enzyme. Acetylcholinesterase in the cisternae of the Nissl substance or rough endoplasmic reticulum of neurons is relatively stable to most fixatives. Short formalin fixation after quenching was chosen to accommodate these last criteria, although, it necessitated special care in dissection to avoid tissue damage. Tissue fixed by perfusion or immersion prior to dissection would be better able to withstand the rigors of scalpel and probe. The problems arising with this type of fixation are manifested by the possibility of enzyme diffusion and the breakdown of cellular components by the fixative. Good preservation of reaction product does not guarantee the same measure of morphological preservation. The best preservation of tissue for histochemistry is a compromise between fixation and processing to obtain adequate cellular morphology while preserving the localization of enzymes.

The method of counting and measuring each cell or fragment present in a section was used because of morphological considerations. The ideal way to count cells in serial sections is by nucleoli (Ebbesson, 1972). Since nucleoli of neurons are usually no more than five micra in diameter, each cell would only be counted once in the sixteen micra sections. In this way split nucleoli counting error was minimized. Also the sections were thin enough to visualize all the nucleoli in a section. Nucleoli do not split readily (Ebbesson, 1970), rather, they tend to roll off one side of the knife or the other. However, there is a controversy over split nucleoli error (Ebbesson, 1970;

Offord, et al., 1974). Multiple nucleoli appear to be common in mouse neurons (personal observation), but in spinal ganglion cells of the cat they are as infrequent as 1% (Ebbesson, 1970), and 4% in human fetuses (McKinnis, 1936). In neurons containing double nucleoli, only one was counted. The method of counting cells by nucleoli was utilized in ganglia stained with cresyl violet. The numbers of cells in spinal ganglia as reported by others for humans (McKinnis, 1936; Ohta, 1974), cats, rabbits and dogs (Davenport and Ranson, 1931), rabbits (Hinsey, et al., 1937), rats (Hatai, 1902) and tadpoles (Prestige, 1965) indicate that there is a gradual decrease in the number of neurons in the lower cervical into the thoracic regions and the same type of increase of cells from thoracic to lumbar. Probably since T4 through about T8 has no involvement with a limb, the studies on these ganglia have been very sparse. The present study may show that the number of ganglion cells reaches its lowest in T4 and begins to increase at the T5 level.

In sections stained for acetylcholinesterase (AChE), it was possible to preserve enzyme localization for definite colorimetric evaluation even if the nuclei and nucleoli were disrupted. Therefore, all the cell fragments present were counted. The associated error was presumed to be random in all four color classes selected so that simple statistical parameters could be applied to the results. Kokko (1965) compared this method with photometric analyses and found a close correlation in results. A comparison between AChE stained ganglion cells counted by nucleoli and those enumerated by counting the cell fragments present also showed fair agreement, indicating that the method was valid. Gomori (1952) discussed the value of visual

quantitation over the use of photomicrographic methods after examining stain dispersions and reception of incident light by a photocell as a function of density. The only problem in visual analysis is the strict maintenance of visual criteria from one ganglion to the next.

Color Categories as Shown by Enzymatic and Counter Staining

The color categories were chosen arbitrarily, though there were definite limits which could be readily visualized with a microscope. The cells having a definite AChE activity can be divided into two groups: B1 with intense activity, and B2 having slightly less activity. With a counter stain the cells possessing little or no AChE reaction product (BL cells) stained blue. Hematoxylin staining also allowed the differentiation of another group of cells with a low degree of AChE activity. These cells showed about equal uptake of hematoxylin as AChE and exhibited a blue-brown or purple color (B3 cells). A question should be raised concerning the localization of these two stains within a neuron. Control sections stained with hematoxylin only, displayed a fine deposition of dye in all the cells. Some were slightly darker than others but none differed as markedly as when stained for AChE. The exact localization of this basic dye is not certain but it does have a great affinity for nucleic and ribonucleic acids. Since the differences were not great in the blue color from cell to cell, this was considered a homogeneous background over which the localization of Hatchett's brown could be superimposed. It is conceded that the B3 staining may be due, in part, to the darker cells mentioned above. These may be darker because of closer packed Nissl substance in these cells. Even by staining with AChE incubation medium minus the substrate, acetylthiocholine iodide, there was a light yellow-brown background staining in

which some cells were also slightly darker than others. This background is attributable to the copper-iron deposition in the cells.

Nuclei are lightly stained with hematoxylin, whereas, the nucleoli manifest a dark blue. There was no nuclear staining with AChE in any cells.

The cells with a high AChE activity (B1) showed the enzyme clumped evenly throughout the cytoplasm. In the cells demonstrating less AChE activities, (B2, B3, BL), a decrease in clumping of enzyme was seen. In BL cells the color due to hematoxylin is very finely dispersed throughout with negligible AChE demonstrated. This lends support to the rationale that these two stains do not necessarily compete for the same binding sites. Hematoxylin binds to sites of nucleic and ribonucleic acids, that is, nuclei and rough endoplasmic reticulum. The graded amount of enzyme product demonstrated in B1, B2, and B3 cells is due to the comparable gradation in the number of binding sites in the Nissl cisternae and associated polyribosomes. As a counter stain hematoxylin can only bind at sites unfilled by AChE. The variation in the amount of hematoxylin demonstrated is due to the similar gradation in the number of AChE active sites in each cell. By virtue of their agglomeration, the clumps of Hatchett's brown effectively block common or adjacent areas from hematoxylin penetration.

Satellite cells are seen with great difficulty, having very little cytoplasmic staining in light microscope preparations. Their sparse cytoplasm have very few Nissl aggregations. Therefore, only the nuclei stain with dyes (Wyburn, 1958). These supporting cells also contain no demonstrable specific cholinesterase activity. It was not hard to distinguish neuronal nuclei from satellite cell nuclei, although, they are approximately comparable in size and color. The former have a homogeneous matrix, whereas, the chromatin of the latter exhibits a substructure of dark spots.

Each spinal ganglion cell is ensheathed by a rather rigid satellite

cell capsule. The "perisomatic gliocytes" (Ortiz-Picon, 1955) with some exceptions completely separate one neuron from another. Therefore, the distance between a neuron and the nearest vascular element must necessarily be at least the width of an interceding satellite cell. They must be very important in the nourishment of nerve cells as well as in insulation from extraneous depolarizations from neighboring neurons (Pannese, 1969).

Dorsal root fibers both proximal and distal to the ganglia are usually unstained for AChE. Gruber and Zenker (1974) showed 8 to 9 percent of dorsal root fibers to be AChE active. On the other hand they reported that most of the ventral root fibers (85%) show high activity.

Functional Morphology of Sensory Ganglia

Dorsal root ganglia at all spinal levels develop proportionately in early development though not at the same time. Neuroblasts at limb levels eventually react to the "trophic" influences of the limb and differentiate to innervate these more expanded and complex areas (Hughes, et al., 1958). Hamberger and Levi-Montalcini (1949, as cited in Swanson, et al., 1965) in tracing the proliferation and differentiation of chick spinal ganglia, found two groups of cells developing from neural crest cells. The first of these were large cells mediating exteroceptive, tactile and visceral sensations. The second group, developing a week later, were composed of large and small cells mediating proprioception, pain and temperature respectively. Ranson (1904) nicely characterized the function of large, medium and small neurons and their processes. He also postulated that the large proprioceptive neurons (pain, touch and temperature) did not develop until organisms became so complex as to warrant detailed information about the position of the limbs, tail and head, which moved independently of the trunk. As added evidence Strumia and Baima-Ballone (1964) reported

two cell types in chick embryos with differences in the temporal appearance of AChE. It was first seen in large anterolaterally located ganglion cells and later in small dorso-medially located neurons. The "emergence of enzyme activity was strictly correlated in time with neurofibrillary differentiation" (Strumia and Baima-Ballone, 1964). Soon afterward the cells intermingled and, due to the heterogeneity of AChE activity, the two groups were obscured.

This correlates with my observation that the large BL cells, virtually unreactive for AChE, were located peripherally in the ganglia. Also, that the smaller high AChE active cells (Bl), clustered loosely in the center of the ganglia, may show some correlation to early morphological studies. These small cells with their small diameter and thinly or unmyelinated axons may be the neurons mediating pain.

How these anatomical studies compare with enzymatic studies may also have some basis in development. It is known that developing neuroblasts are different from mature neurons, both anatomically (Esterman, 1927; Tennyson, 1965; Weschler, et al., 1967) and enzymatically (Kaveler and Kimel, 1952; Youngstrom, 1941). In the chick embryo AChE was present in spinal ganglion cells at four days of incubation (Pannese, et al., 1971). The enzyme activity in motor areas was present earlier, the 60th hour in the spinal cord and the 72nd hour in the myotomes (Strumia and Baima-Ballone, 1964). This early demonstration of AChE in spinal ganglia predates the appearance of motor (synaptic) function by three days (Pannese, loc. cit., 1971). Hogg and Humphrey (1969) reported that reflex responses could still be elicited in human fetuses that had not yet developed discreet sensory endings, and further, had not developed sensory nerves to the periphery.

The demonstration of AChE in developing animals occurs, as with many enzymes, cranially to caudally in axons of the central nervous system (Strumia and Baima-Ballone, 1964) and distally to proximally in peripheral

neurons (Tennyson, 1965; Tennyson and Brzin, 1970). In the former case, neurons begin AChE production as they reach a certain maturational stage, while the peripheral axons show buildup due to axoplasmic flow.

The Role of AChE in Sensory Ganglia

The function of AChE in sensory ganglia is unclear. Several hypotheses have been made. Koelle (1957) apologetically suggested that AChE presence may be due to the failure of the cell to suppress its production after differentiation. Though it is improbable that this type of gross inefficiency would occur, the present study indicates that part of the answer may lie in this theory. Certainly, ubiquitous substances like ACh and AChE could have roles in neurons other than in impulse transmission. The structure of complex lipids, like phosphoglycerides and glycolipids (Lehninger, 1970) are very important to membrane formation. Sphingomyelin and phosphatidyl choline (lecithin) are very abundant in both plant and animal tissues, especially in nervous tissue. Both substances contain choline, a cleavage product of ACh. The leftover acetate, $\text{HO}-\overset{\text{O}}{\underset{\text{||}}{\text{C}}}-\text{CH}_3$, is a building block of lipids (Lehninger, 1970; Conn and Stumpf, 1963).

Nervous tissues are especially dependent on their membranes for carrying out their innate function in chemico-electrical transmission. It is only reasonable, in highly organized associations of lipid and protein where molecular turnover and repair are critical, that very complex and coordinated processes must take place for the optimal production and maintenance of membrane potentials. The selective ion permeabilities on either side of a 200 Å axonal membrane exemplifies this complexity.

If an afferent impulse slows down when it reaches the ganglion cell body, the presence of ACh and AChE might facilitate depolarization

across the cell body. Concurrently, the presence of ACh and its hydrolyzing enzyme might provide ready sources of products for the constant preservation of membrane polarity. If this were the case, more AChE would be necessary in small cells (as in B1 cells) to overcome the restraints to transmission induced by both the slow conduction across unmyelinated fibers and the high cable resistance due to limited surface area. An explanation for the range of sizes in B1 cells might be that they are in a transitional period phylogenetically, in which the presence of enzyme is "vestigial" (Koelle, 1957) since the advent of myelination. Some ganglion cells do not have a myelin sheath, therefore, the enzyme mechanism prevents leakage of depolarization from one neuron to another (Silver, 1971), or by any ACh liberated from adjacent sites, such as the blood stream (Koelle, 1957). This type of relationship could also apply to the other cell color categories in regard to cell size ranges. Myelination of nerves does not occur until the time of birth (Peach, 1973). Dixon (1963) saw this as a response to meet the demands of the environment. Subsequent maturation of the myelin sheath involves myelin breakdown and turnover (Berthold, 1973). The presence of AChE could be critical at that time.

Ventral roots have a high AChE activity; they transport ACh to the myoneural junctions. There it is ready to be released into the synaptic space.

It has been suggested that differential AChE activities are localized in sensory neurons in varying stages of differentiation, maturation and metabolism (Tewari and Bourne, 1962). Alternatively the various modalities subserved by cells of varying size has been postulated as a reason for multiple degrees of enzyme activity (Dixon, 1963). This, however, was not shown by my results since each color class has a large size range close

to the limits of nerve cells in general. Koelle and his cohorts (1962) postulated that in non-cholinergic neurons, ACh may be initially liberated to permit the release of another transmitter. They stress the fact that it is important in membrane permeabilities.

As organisms ventured into more varied surroundings, they evolved more complex systems of sampling and interpreting their environment. These changes came to encompass not only sensory stimuli external to the body, but also those from within the body. This internal stimuli yielded positional and highly discriminatory balance data (by coupling with the vestibular system) to animals too large to be controlled by one or a few simple elicited response systems. In higher animals and man, protective voluntary responses can be initiated with little or no overt external visualization of it (vocalization, facial expression, blushing and conscious control of any response, that is, "keeping it inside"). The degree of complexity and plasticity of higher brains is exhibited by subconscious response (vascular constriction and dilation, adaptation, accommodation, memory, learning). In other words response to stimuli has evolved from a diffuse reaction in lower animals to a very discriminant response, with a wide array of sensation and categorization of stimuli, in higher animals. The segmental spinal ganglia were retained because of their proximity to the periphery in carrying out their role in "unthinking" reflexes. This may be an oversimplification in light of the complex integration of sensory information from one spinal level to the next. Comcomitantly, AChE has persisted in spinal ganglion cells to act, like myelin in fast conducting axons, to "eliminate the element of diffusion so characteristic of the more primitive sensations" (Ranson, 1915). As large proprioception mediating cells evolved (partly BL cells), they naturally superimposed over the existing neurons and therefore are found more peripherally in the ganglia.

The large myelinated fibers of these cells precluded the need for AChE.

In conclusion, it was found there was an inverse correlation between the size of the neurons and the intensity of the AChE reaction product. Also there seemed to be a size gradation within the ganglia with the smaller fragments localized towards the center and the largest at the periphery. The amount of staining was not strictly correlated to size in that there was a range of sizes in each of the four color classes. It was found that over half of the fragments displayed medium activity (B2 cells). Only 4 percent of all the fragments studied showed negligible staining.

BIBLIOGRAPHY

- Adams, C. W. M., R. T. Grant and O. B. Bayliss]1967. Cholinesterases in the peripheral nervous system. *Brain Research*. 5:366-376.
- Albrecht, M. H.]1954. Mounting frozen sections with gelatin. *Stain Tech*. 29:89-90.
- Bacsich, P. and G. M. Wyburn]1953. Formalin sensitive cells in spinal ganglia. *Quart. J. Mic. Sci.* 94:89-92.
- Barron, K. D. and J. Bernsohn]1968. Esterases of developing human brain. *J. Neurochem.*]5:273-284.
- Bernsohn, J. K. D. Barron and A. R. Hess 1962. Multiple nature of acetylcholinesterase in nerve tissue. *Nature* 195:285-286.
- Brodal, A. 1969. *Neurological Anatomy*. 2nd ed. Oxford University, New York. 9m
451
- Broderson, S. H., L. E. Westrum and A. E. Sutton 1974. Studies of the direct coloring thiocholine method for localizing cholinesterase activity. *Histochemistry* 40:13-23.
- Brzin, M., V. M. Tennyson and P. E. Duffy 1966. Acetylcholinesterase in frog sympathetic and dorsal root ganglia. *J. Cell Biol.* 31:215-242.
- Bunge, M. B., R. P. Bunge, E. R. Peterson and M. R. Murray 1967. A light and EM study of long term organized cultures of rat dorsal root ganglia. *J. Cell Biol.* 32:439-466.
- Burstone, M. S. 1959. The relationship between the fixation techniques for the histochemical localization of hydrolytic enzymes. *J. Histochem. Cytochem.* 6:322-339.
- Carmel, D. W. and B. M. Stein 1969. Cell changes in sensory ganglia following proximal and distal nerve section in the monkey. *J. Comp. Neurol.* 135:145-165.
- Cauna, N. 1961. Cholinesterase activity in cutaneous receptors of man and of some quadrupeds. *Histochemistry of Cholinesterase, Symposium Basel. Bibl. Anat. vol. 2*, 128-138.
- Cauna, N. and N. T. Naik 1963. The distribution of cholinesterases in the sensory ganglia of man and some mammals. *J. Histochem. Cytochem.* 11:129-138.
- Clitherow, J. W., M. Mitchard and N. J. Harper 1963. The possible biological function of pseudocholinesterase. *Nature* 199:1000-1001.

QM
451

- Crosby, E. C., T. Humphrey and E. W. Lauer 1962. Correlative Anatomy of the Nervous System. MacMillan Company, New York, 731 pp.
- Coupland, R. E., and R. L. Holmes 1957. The use of the cholinesterase techniques for the demonstration of peripheral nervous structures. Quart. J. Mic. Sci. 98:327-330.
- Davenport, H. A. and S. W. Ranson 1931. Ratios of cells to fibers and of myelinated to unmyelinated fibers in spinal nerve roots. Am. J. Anat. 49:193-207.
- Davenport, H. A., S. W. Ranson and E. H. Terwilliger 1931. Artifacts in dorsal root ganglion cells. Anat. Rec. 48:251-255.
- Davenport, H. A. and R. T. Bothe 1931. Cells and fibers in spinal nerves, II. J. Comp. Neurol. 59:167-174.
- Dogiel, V. A. S. 1896. Der bau der Spinalganglien bei den Säugetieren. Anat. Anz. 12:140-152.
- Duncan, D. and L. L. Keyser 1936. Some determinations of the ratio of nerve fibers to nerve cells in the thoracic dorsal root and ganglia of the cat. J. Comp. Neurol. 64:303-311.
- El-Badawi, A. and E. A. Schenk 1967. Histochemical methods for the separate, consecutive and simultaneous demonstration of acetylcholinesterase and norepinephrine in cryostat sections. J. Histochem. Cytochem. 16:580-588.
- Emery, J. L. and R. Singhal 1973. Changes associated with growth in the cells of the dorsal root ganglion in children. Devel. Med. Child Neurol. 15:460-466.
- Eranko, O. 1967. Histochemistry of nervous tissues: catecholamines and cholinesterases. Ann. Rev. Pharmacol. 7:203-222.
- Esternam, B. and A. J. Gitlitz 1927. Notes on the golgi apparatus in spinal ganglion cells of the cat. Anat. Rec. 36:319-324.
- Feldberg, W. and T. Mann 1946. Properties and distribution of the enzyme system which synthesizes acetylcholine in nervous tissue. J. Physiol. 104:411-425.
- Feldberg, W. and M. Vogt 1948. Acetylcholine synthesis in different regions of the central nervous system. J. Physiol. 107:372-381.
- Friede, R. L. 1966. Topographic Brain Mechanisms. Academic Press Inc., New York. pp. 237-279.
- Fekuda, T. and G. B. Koelle 1959. The cytological localization of intracellular neuronal acetylcholinesterase. J. Biophys. Biochem. Cytol. 5:433-440.

- Galabov, G., B. Penev and S. Manolov 1964. Morphological and histochemical investigation on the spinal ganglion cells. *Bolg. Akad. Nank.* 17:419-422.
- Gardner, E. 1940. Decrease in human neurons with age. *Anat. Rec.* 77:329-336.
- Giacobini, E. 1956. Histochemical demonstration of AChE activity in isolated nerve cells. 36:276-290.
- Giacobini, E. 1959. Quantitative determination of cholinesterase in individual spinal ganglion cells. *Acta Physiol. Scand.* 45:238-254.
- Glees, P. 1973. Age changes in the centrally and peripherally located sensory neurons in rat. *Z. Zellforsch. & Mik. Anat.* 141:285-298.
- Gomori, G. 1948. Histochemical demonstration of sites of cholinesterase activity. *Proc. Soc. Exptl. Bio. Med.* 68:354-358.
- Gruber, H. and W. Zenker 1973. Acetylcholinesterase: histochemical differentiation between motor and sensory fibers. *Brain Res.* 51:207-214.
- Gwynn, D. G. and B. A. Flumerfelt 1971. Acetylcholinesterase in non-cholinergic neurons: a histochemical study of dorsal root ganglion cells in the rat. *Brain Res.* 34:193-198.
- Ha, H. 1969. Fine structure of the spinal ganglion of the cat. *Anat. Rec.* 163:192-193.
- Hanker, J. S., L. P. Thornburg, P. E. Yates and H. G. Moore 1973. The demonstration of cholinesterases by the formation of osmium blacks at the sites of Hatchett's brown. *Histochemie* 37:223-242.
- Hatai, S. 1902. Number and size of the spinal ganglion cells and dorsal foot fibers in the white rat at different ages. *J. Comp. Neurol.* 12:107-124.
- Hess, A. 1955. The fine structure of young and old spinal ganglia. *Anat. Rec.* 123:399-412.
- Hinsey, J. C., M. A. Krupp and W. T. Lhamon 1937. Reaction of spinal ganglion cells to section of dorsal roots. *J. Comp. Neurol.* 67:205-214.
- Hogg, I. D. and J. W. Bryant 1969. The development of sensory innervation in the mouth and pharynx of the albino norway rat (Mus norvegicus albinus). *J. Comp. Neurol.* 136:33-56.
- Holmstedt, B. 1957a. A modification of the thiocholine method for the determination of cholinesterase. I. Biochemical evaluation of selective inhibitors. *Acta Physiol. Scand.* 40:321-337.

- Holmstedt, B. 1957b. A modification of the thiocholine method for the determination of cholinesterase. II. Histochemical application. Acta Physiol. Scand. 40:331-337.
- Holmstedt, B. 1971. Distribution of cholinesterases in mammals. Bulletin Wld. Hlth. Org. 44:99-107.
- Hughes, A and P. A. Tschumi 1958. The factors controlling the development of the dorsal root ganglia and ventral horn in Xenopus laevis (Daud.). J. Anat. 92:498-527.
- Karnovsky, M. J. 1964. The localization of cholinesterase activity in rat cardiac muscle. J. Cell Biol. 23:217-232.
- Karnovsky, M. J. and L. Roots 1964. A "direct coloring" thiocholine method for cholinesterases. J. Histochem. Cytochem. 12:219-221.
- Kavalier, F. and V. M. Kimel 1952. Biochemical and physiological differentiation during morphogenesis. XV AChE activity in the motor cortex of the fetal guinea pig. J. Comp. Neurol. 96: 113-119.
- Kiss, F. 1932. Sympathetic elements in the cranial and spinal ganglia. J. Anat. 66:488-498.
- Klüver, H. and E. Barrera 1953. A method for the combined staining of cells and fibers in the nervous system. J. Neuropath. Exp. Neurol. 12:400-403.
- Koelle, G. B. 1954. The histochemical localization of cholinesterases in the central nervous system of the rat. J. Comp. Neurol. 100:211-228.
- Koelle, G. B. 1955. The histochemical identification of acetylcholinesterase in cholinergic, adrenergic and sensory neurons. J. Pharmacol. Exp. Therap. 114:167-184.
- Koelle, G. B. 1957. Histochemical localization of acetylcholinesterase in nervous tissue, in New Research Techniques of Neuroanatomy, (William F. Windle, Ed.). Charles C. Thomas, Springfield, Illinois. pp.62-69.
- Koelle, G. B. 1962. A new general concept of the neurohumoral functions of acetylcholine and acetylcholinesterase. J. Pharm. Pharmacol. 14:65-90.
- Koelle, G. B. 1963. Cytological distribution and physiological functions of cholinesterases, in Handbuch der Experimentellen Pharmakologie, (G. B. Koelle, Ed.). Berlin, Springer-Verlag.
- Koelle, G. B., R. Davis and W. A. Koelle 1974. Effects of aldehyde fixation and of preganglionic denervation of acetylcholinesterase and butyrylcholinesterase of cat autonomic ganglia. J. Histochem. Cytochem. 22:244-251.

- Koelle, W. A. and G. B. Koelle 1959. The localization of external or functional acetylcholinesterase at the synapse of autonomic ganglia. *J. Pharmacol.* 126:1-8.
- Koelle, G. B. and J. S. Friedenwald 1949. A histochemical method for localizing cholinesterase activity. *Proc Soc. Wxp. Bio. Med.* 70:617-622.
- Kokko, A. 1965. Histochemical and cytophotometric observations on esterases in the spinal ganglia of the rat. *Acta Physiol. Scand.* 66, supp 261:1-73.
- Lasek, R. 1968. Axoplasmic transport in cat dorsal root ganglion cells as studied with [H]-L-Leucine. *Brain Res.* 7:360-377.
- Lewis, P. R. 1960. The effect of varying the conditions in the Koelle technique. *Biblio. Anat.* 2:11-20.
- Loewi, O. 1956. On the intraneural state of acetylcholine. *Experientia* 12:331-333.
- Loewy, A. D. 1970. A study of neuronal types in Clark's column in the adult cat. *J. Comp. Neurol.* 139:53-80.
- Lumb, W. V. 1963. *Small Animal Anesthesia.* Lea & Febiger. Phila. pp 420.
- Luna, L. G. (Ed.) 1968. *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, 3rd ed. McGraw-Hill, New York. pp 258.
- MacIntosh, F. C. 1941. The distribution of acetylcholine in the peripheral and central nervous system. *J. Physiol.* 99:436-442.
- Manocha, S. L. 1973. Experimental protein malnutrition in primates: histochemical studies of the dorsal root ganglion cells of healthy and malnourished squirrel monkeys (*Saimiri sciureus*). *Acta Histochem.* 47:220-232.
- Margolis, G. and J. P. Pickett 1956. New application of the luxol fast blue stain. *Lab. Invest.* 5:459-474.
- Matsura, H. 1967. Histochemical observation of bovine spinal ganglia. *Histochemie* 11:152-160.
- Mazza, J. P., J. S. Hanker and A. D. Dixon 1973. Ultrastructural localization of cholinesterase activity in the trigeminal ganglion of the rat. *J. Anat.* 115:65-78.
- McCaman, R. E. and J. M. Hunt 1965. Microdetermination of choline acetylase in nervous tissue. *J. Neurochem.* 12:253-259.

- McKinnis, M. 1936. The number of ganglion cells in the dorsal root ganglia of the second and third cervical nerves in human fetuses of various ages. *Anat. Rec.* 65:255-259.
- Michealson, A. 1967. The subcellular distribution of acetylcholine, choline acetyltransferase and acetylcholinesterase in nerve tissue. *Annals of the New York Academy of Science* 144:387-410.
- Nachmansson, D. 1971. Acetylcholine and the acetylcholine receptor. in *Handbook of Sensory Physiology* vol. 1 (W. R. Loewenstein Ed.). Springer-Verlag, New York. pp 18-102.
- Navaratnam, V. and P. R. Lewis 1970. Cholinesterase containing neurons in the spinal cord of the rat. *Brain Res.* 18:411-425.
- Novikoff, A. B., N. Quintana, H. Villaverde and R. Forschirm 1966. Nucleoside phosphatase and cholinesterase activities in dorsal root ganglion and peripheral nerve. *J. Cell Biol.* 29:525-545.
- Offord, K., R. F. Oenning and P. J. Dyck 1974. Method of morphometric evaluation of spinal and autonomic ganglia. *J. Neurol. Sci.* 22:65-71.
- Ohta, M., K. Offord and P. J. Dyck 1974. Morphometric evaluation of first sacral ganglia in man. *J. Neurol. Sci.* 22:73-82.
- Orr, D. and R. G. Rows 1901. The nerve cells of the human posterior root ganglia and their changes in general paralysis of the insane. *Brain* 24:286-298.
- Palay, S. L. and G. E. Palade 1955. The fine structure of neurons. *J. Biophys. Biochem. Cytol.* 1:69.
- Pannese, E., L. Luciano, S. Iurato and E. Reale 1971. Cholinesterase activity in spinal ganglia neuroblasts; a histochemical study at the electron microscope. 36:46-67.
- Peach, R. 1972. Fine structural features of light and dark cells in the trigeminal ganglion of the rat. *J. Neurocyt.* 1:151-160.
- Peach, R. 1973. Development of the trigeminal ganglia. in *Oral Sensation and Perception* (J. F. Bosmer Ed.). pp 53-74.
- Pearse, A. G. E. 1960. Carboxylic esterases. in *Histochemistry, Theoretical and Applied* (A. G. E. Pearce Ed.). Little, Brown and Co., Boston. pp 456.
- Prestige, M. C. 1965. Cell turnover in the spinal ganglia of Xenopus laevis tadpoles. *J. Embryo. Exp. Morph.* 13:63-72.
- Ranson, S. W. 1912. The structure of the spinal ganglia and of the spinal nerves. *J. Comp. Neurol.* 22:159-175.

- Ranson, S. W. 1914. Transplantation of the spinal ganglion, with observations on the significance of the complex types of spinal ganglion cells. *J. Comp. Neurol.* 24:547-558.
- Ranson, S. W. 1928. The role of dorsal roots in muscle tone. *Arch. Neurol. Psych.* 19:201.
- Ranson, S. W. and H. K. Davenport 1931. Sensory unmyelinated fibers in the spinal nerves. *Am. J. Anat.* 48:331-353.
- Reighard, J., H. S. Jennings and R. Elliott 1966. *Anatomy of the Cat*, 3rd ed., Holt, Reinhart and Winston, New York. pp 486.
- Rexed, B. 1954. A cytoarchitectonic atlas of the spinal cord in the cat. *J. Comp. Neurol.* 100:297-379.
- Ritter, V. J., J. Wenze., H. Daniel and G. Duwe 1974. Electron microscopical demonstration acetylcholinesterase in neurons and synapses of the central and peripheral nervous system (in German). *Acta Histochem.* 49:176-203.
- Robain, O. and L. Jardin 1972. Histoenzymologie du ganglion spinal du lapin. *J. Neurol. Sci.* 17:419-433.
- Roskoski, R. Jr. 1973. Choline acetyltransferase, evidence for an acetyl enzyme reaction intermediate. *Biochem.* 12:3709-3714.
- Schlaepfer, W. W. 1968. Acetylcholinesterase activity of motor and sensory nerve fibers in the spinal nerve roots of the rat. *Zeit. Zellforsch.* 88:441-456.
- Scott, D., E. Gutmann and P. Horsky 1966. Regeneration in spinal neurons following nerve growth factor administration. *Science* 152:787-788.
- Sechrist, J. W. 1969. Neurofibrillar changes in motor neurons undergoing diffuse versus central chromatolysis: a light and E. M. study. *Anat. Rec.* 163:259.
- Shanta, T. R. 1967. Enzyme histochemistry of the mesenteric and dorsal root ganglion cells of cat and squirrel monkey. *Histochemie* 10:234-245.
- Shute, C. C. D. and P. R. Lewis 1961. Cholinergic nervous pathways in the forebrain. *Nature* 189:332-333.
- Shute, C. C. D. and P. R. Lewis 1963. Cholinesterase containing systems of the brain of the rat. *Nature* 199:1160-1164.
- Silver, A. 1971. The significance of cholinesterase in the developing nervous system. in *Progress in Brain Research* (O. Eranko Ed.). vol. 34, Histochemistry of nervous transmission. Elsevier Publishing Co., New York. pp345-355.

- Silver, A. and J. H. Wolstencroft 1971. The distribution of cholinesterase in relation to the structure of the spinal cord in the cat. *Brain Res.* 34:205-227.
- Stevens, P. F. and S. H. Bensley 1955. Histological studies of the adrenal glands after repeated injections of histamine in albino mice. *Anat. Rec.* 123:227-234.
- Strumia, E. and P. L. Baima-Ballone 1964. AChE activity in the spinal ganglia of the chick embryo during development. *Acta Anat.* 57:281-293.
- Swanson, A. G., G. C. Buchan and E. C. Alvord 1965. Anatomical changes in congenital insensitivity to pain. *Arch. Neurol.* 12:12-17.
- Taxi, J. 1952. Action du forol sur l'activite de diverses preparations des cholinesterases. *J. Physiol.* 44:595-597.
- Tennyson, V. M. 1965. Electron microscopic study of the developing neuroblast of the dorsal root ganglion of the rabbit embryo. *J. Comp. Neurol.* 124:267-318.
- Tennyson, V. M. and M. Brzin 1970. The appearance of AChE in dorsal root neuroblasts of the rabbit embryo (EM and micro-gasometric analysis). *J. Cell Biol.* 46:64-80.
- Tewari, H. B. and G. H. Bourne 1962. Histochemical evidence of metabolic cycles in spinal ganglion cells of the rat. *J. Histochem. Cytochem.* 10:42-64.
- Truex, R. C. and M. B. Carpenter 1969. *Human Neuroanatomy*, 6th ed. Williams and Wilkins, Baltimore. pp 673.
- Turner, J. 1903. An account of the nerve cells in thirty three cases of insanity, with special reference to those of the spinal ganglia. *Brain* 26:27-72.
- Villegas, G. M. and J. Villegas 1974. Acetylcholinesterase localization in the giant nerve fiber of the squid. *J. Ultrastructural Res.* 46:149-163.
- Walker, W. F. 1967. *A Study of the Cat*. W. B. Saunders, Philadelphia. pp 10-12, 85. GL
812
- Warrington, W. B. and F. Griffith 1904. On cells of the spinal ganglia and on the relationship of their histological structure to axonal distribution. *Brain* 27:297-326.
- Wechsler, W. 1967. Electron microscopic investigation of the development of autonomic (sympathetic trunk) and spinal ganglia in Gallus domesticus. *Acta Neuroveg.* 30:427-444.

- Willis, W. D. Jr. and R. G. Grossman 1973. Medical Neurobiology.
The C. B. Mosby Co., New York. pp 5-50.
- Wyburn, G. M. 1958. The capsule of spinal ganglion cells. J.
Anat. 92:527-533.
- Youngstrom, K. A. 1941. AChE concentration during development of the
human fetus. J. Neurophysiol. 4:473-477.
- Zsoldos, V. M. 1963. Multipolare Zellen im Spinalganglion.
Anat. Anz. 122:37-46.

APPENDIX A

Acetylcholinesterase Staining Procedure (after El-Badawi and Schenk 1967)

1. Fix air-dried sections in ice-cold (-4 to +4 C) 4% neutral formalin, 15 to 25 minutes.
2. Wash thoroughly in 2 changes of deionized distilled water (used throughout this procedure), 1 minute each.
3. Place sections in freshly prepared incubation medium*. Pipettes, glassware, and staining dishes used in this step must all be chemically clean. Incubate at 37 C until cholinergic cells and fibers exhibit desired degree of staining. This step is critical; sections should be tested under the microscope. Tissue with high concentrations of AChE stains brown. Incubation time was prolonged 3- 10 hours in this experiment (rather than 0.5 to 2 hours as suggested) in an attempt to force staining of tissues known to have low concentrations of AChE. This long duration in the incubation medium necessitated the use of pretreated slides. Tissue sections floated off if mounted on clean slides.
4. Rinse in distilled water, 1 minute.
5. Counterstain nuclei in freshly filtered Harris' hematoxylin** 10 to 15 seconds.
6. Wash in running distilled water. Thorough washing is necessary because hematoxylin adheres to the gelatinized slides.
7. Blue in 1% aqueous lithium carbonate, 1 minute.
8. Wash in distilled water, 1 minute.
9. Begin dehydration in 70% alcohol, 5 minutes.
10. Continue dehydration in 1 change of 80% alcohol, 5 minutes.
11. Continue dehydration in 1 change each of 95% and absolute alcohol, 5 minutes each.
12. Leave in equal parts absolute alcohol and xylene, 5 minutes.
13. Clear in 3 changes of xylene, 5 minutes each.
14. Mount with Permount and cover.

*Incubation Medium

Acetylthiocholine iodide.	12.5 mg
0.06 N (0.82%) Sodium acetate.	15.8 ml
0.1 N (0.6%) Acetic acid.	0.5 ml
0.1 M (2.94%) Sodium citrate.	1.2 ml
30 mM (0.75%) Cupric sulfate.	2.5 ml
4 mM (0.137%) Iso-OMPA.	0.5 ml
5 mM (0.165%) Potassium Ferricyanide.	2.5 ml
Deionized distilled water.	2.0 ml

The medium is prepared no earlier than 30 minutes before use by adding to the substrate (acetylthiocholine iodide) the solutions in the order in which they are listed. Distilled deionized water is used throughout the experiment. Pipettes, glassware, and staining dishes must be chemically clean (acid dichromate solution). The medium has a clear green color and a pH of 5.5 to 5.6. A cloudy green is indicative of contamination. Individual solutions may be kept in stock in the refrigerator at 4 C.

** Harris' Hematoxylin

Hematoxylin crystals.	5.0 gm
Alcohol, 100%.	50.0 ml
Aluminum potassium sulfate.	100.0 gm
Distilled water.	1000.0 ml
Mercuric oxide (red).	2.5 gm

Dissolve the hematoxylin in the alcohol, the alum in the water by the aid of heat. Remove from heat and mix the two solutions. Bring to a boil rapidly (use glass beads for safety). Limit heat to less than 1 minute and stir often. Remove from heat and add the mercuric oxide slowly. Reheat to a simmer until it becomes dark purple, remove from heat immediately and plunge the vessel into a basin of cold water until cool. The stain is ready for use as soon as it cools. Adding 2 to 4 ml of glacial acetic acid per 100 ml of solution increased the precision of the nuclear stain. Filter before use.

APPENDIX B

Cresyl Violet Staining Procedure (after Fernstrom 1958)

1. Mount sections on pretreated slides*.
2. Place in 95% alcohol, 5 minutes.
3. Transfer to absolute alcohol, 5 minutes.
4. Soak in equal parts absolute alcohol and chloroform, 20 minutes.
5. Hydrate in 80% alcohol, 5 minutes.
6. Continue hydration in 70% alcohol, 5 minutes.
7. Wash in distilled water, 5 minutes (or longer, if stopping time is required).
8. Stain in 0.5% aqueous cresyl violet for 20 minutes. Add 4 drops glacial acetic acid per 100 ml, filter and preheat to 56 to 60 C for 20 minutes just before using.
9. Rinse briefly in distilled water.
10. Rinse in 70% alcohol a few seconds.
11. Rinse in 95% alcohol, 1 minute.
12. Soak in chloroform, 20 minutes.
13. Transfer to 95% alcohol, 1 minute.
14. Differentiate by agitating in 95% alcohol. Add 1 ml glacial acetic acid per 200 ml of alcohol. This step is critical in determining the desired color. Thin sections require a few seconds; thick sections take longer. Test with naked eye or with microscope. Neurons and glia stain pink to violet; white matter remains essentially colorless.
15. Further differentiate in 3 changes of 95% alcohol, 5 minutes each.
16. Clear in 2 changes terpeneol, 5 minutes each.
17. Clear in 2 changes xylene, 5 minutes each.
18. Mount with Permount and cover.

*Aqueous Gelatin for Pretreatment of Slides

Gelatin. 3.0 gm
Distilled water. 600.0 ml

Dissolve thoroughly by placing solution in the oven
at 56 to 60°C for 10 to 20 minutes.

Chromium potassium sulfate. 300.0 mg

Dip slides in solution, place wet slides upright in a
staining tray and dry in oven at 56 to 60°C for one
hour.

APPENDIX C

Combination Stain for Neurons and Their Fibers (after Kluver and Barrera 1953)

1. Let mounted cryostat sections dry.
2. Rinse in 95% alcohol.
3. Stain overnight (10 to 16 hours) in Luxol fast blue* at 57 C.
4. Wash in distilled water.
5. Begin differentiation by quick immersion in 0.05% lithium carbonate**.
6. Continue differentiation in 70% alcohol until gray and white matters can be distinguished.
7. Wash in distilled water.
8. Finish differentiation by rinsing briefly in 0.05% lithium carbonate and then putting through several changes of 70% alcohol until the greenish blue of the white matter contrasts sharply with the colorless gray matter.
9. Wash thoroughly in distilled water.
10. Stain for 6 minutes in warm cresyl violet solution***.
11. Differentiate in several changes of 95% alcohol.
12. Dehydrate in absolute alcohol, clear in xylene and mount.

*0.1% Luxol fast blue solution

Luxol fast blue.	0.05 gm
Alcohol, 95%	100.0 ml

**Lithium carbonate solution

Lithium carbonate	0.05 gm
Distilled water.	100.0 ml

***0.1% Cresyl violet solution

Cresyl echt violet.	0.1 gm
Distilled water.	100.0 ml

TABLE 1

VITAL DATA OF STUDY ANIMALS AND ACTUAL CELL NUMBERS OF T₄ AND T₅ SPINAL
GANGLIA AS COUNTED BY NUCLEOLI

Cat	Sex	Age	Weight(Kg)	Level	No. of Sections	Type of Section	Total Cells
11	♂	16 mo.	4.0	T ₅ L	154	C.S.	3941
12	♂	7 mo.	1.7	T ₄ L	114	L.S.	2583
13	♂	6 mo.	1.9	T ₄ L	99	L.S.	3706
13				T ₅ R	92	L.S.	----
13				T ₅ L	99	C.S.	3414
15	♂	12 mo.	3.8	T ₄ L	84	L.S.	2997
16	♂	12 mo.	3.6	T ₅ L	114	C.S.	3075
16				T ₅ R	116	L.S.	----
17	♀	12 mo.	4.0	T ₄ L	101	L.S.	2924
17				T ₄ R	140	C.S.	----
17				T ₅ L	103	L.S.	4364
17				T ₅ R	84	L.S.	----
$\bar{X} = 3365, S = 656.7, S_{\bar{X}} = 231.2$							Total = 27004

TABLE 2 ANALYSIS OF THE NUMBER OF SECTIONS TAKEN IN 12 GANGLIA
AT THE T_4 OR T_5 LEVEL IN CROSS OR LONGITUDINAL SECTION

	<u>Cross Section</u>			<u>Longitudinal Section</u>			<u>Σ Sections</u>
	No. of ganglia	No. of sections	$\bar{X}$	No. of ganglia	No. of sections	$\bar{X}$	
T_4	1	140	140	4	398	99	762
T_5	3	367	122	4	395	99	538
Σ Sections		507			793		

TABLE 3

COMPARISON OF CELL TYPES IN AChE STAINED GANGLIA

Cat	Level	Type of Section	No. of Section	No. of Fragment		% B ₁ ($\bar{X}$)	% B ₂ ($\bar{X}$)	% B ₃ ($\bar{X}$)	% BL ($\bar{X}$)
13	T ₅ R	L.S.	23	4932		34 (33)	52 (36)	14 (41)	1 (44)
16	T ₅ R	L.S.	29	5567		18 (39)	55 (45)	18 (38)	9.4 (51)
17	T ₄ R	C.S.	35	5092		34 (35)	47 (41)	15 (42)	2 (46)
17	T ₅ R	L.S.	21	4309		30 (36)	55 (42)	11 (44)	2 (50)
Total				19,900	Ave.	29 (36)	52 (41)	15 (41)	4 (48)

$$\begin{aligned}\bar{X} &= 4975 \\ S &= 449.3 \\ S_{\bar{X}} &= 224.6\end{aligned}$$

FIGURE 1. DIAGRAM OF SPINAL CORD WITH DORSAL AND VENTRAL ROOTS
AS WELL AS THE DORSAL ROOT GANGLION INDICATED. ALSO
SHOWN ARE SOME FIBER RAMIFICATIONS FROM THE RESPECTIVE
RAMI.

FIGURE 2. CAT SPINAL CORD WITH SEVERAL SPINAL GANGLIA ATTACHED.

NOTE THE DIMENSIONS OF THE GANGLIA.

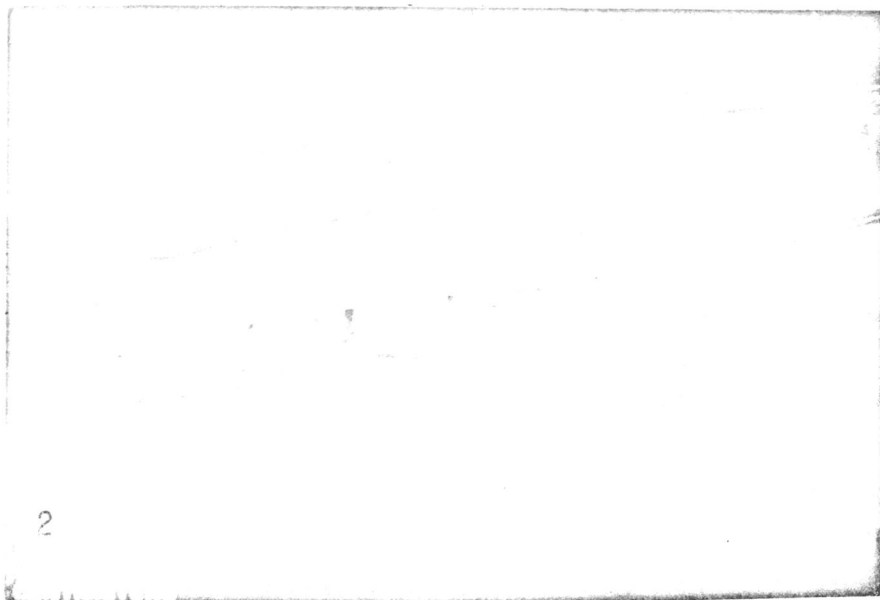


FIGURE 3. STUDY TISSUE STAINED WITH CRESYL VIOLET. a) LONGI-
TUDINAL SECTION OF CAT THORACIC GANGLION b) CROSS
SECTION THROUGH THORACIC SPINAL GANGLION OF CAT. 50X

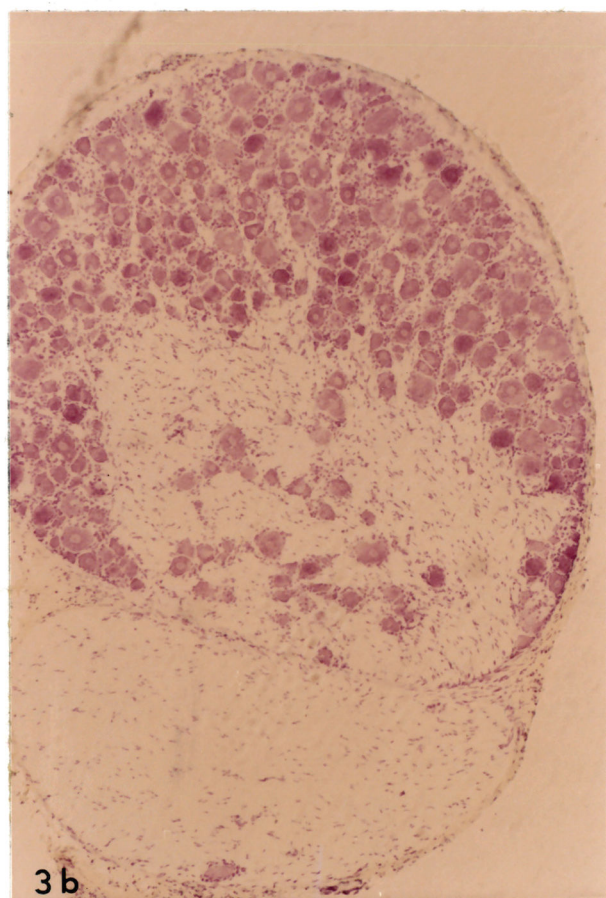
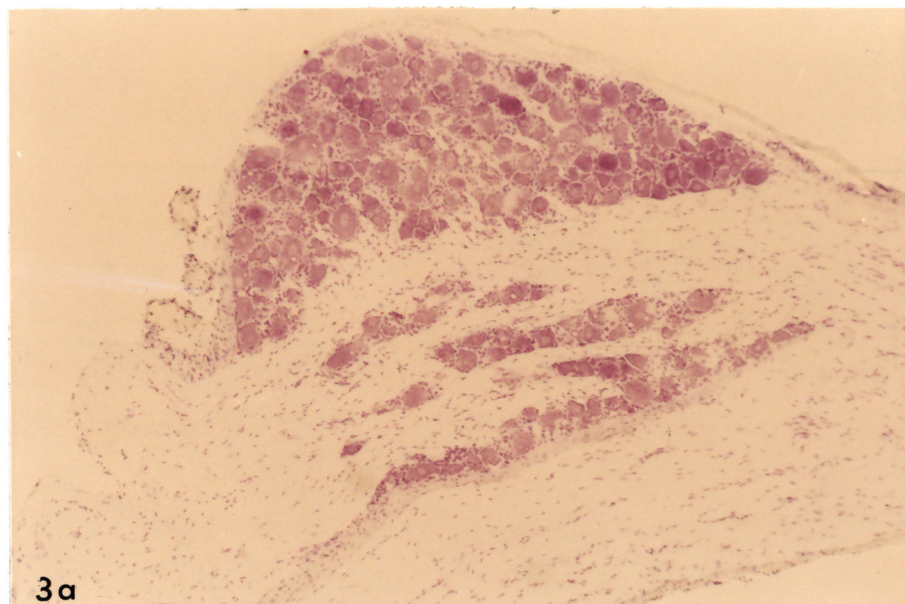


FIGURE 4. THORACIC SPINAL GANGLIA OF THE CAT STAINED FOR AChE.

a) LONGITUDINAL SECTION b) CROSS SECTION. 50X

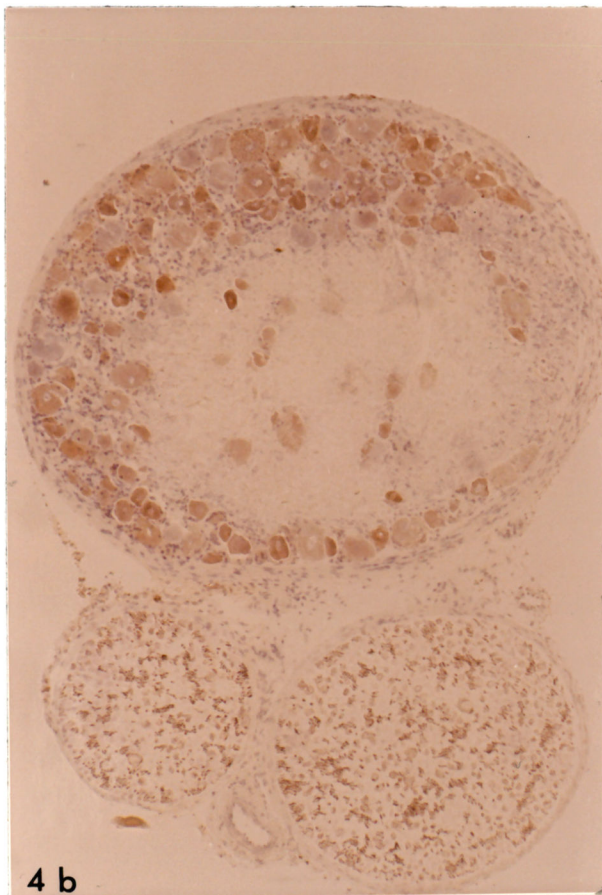
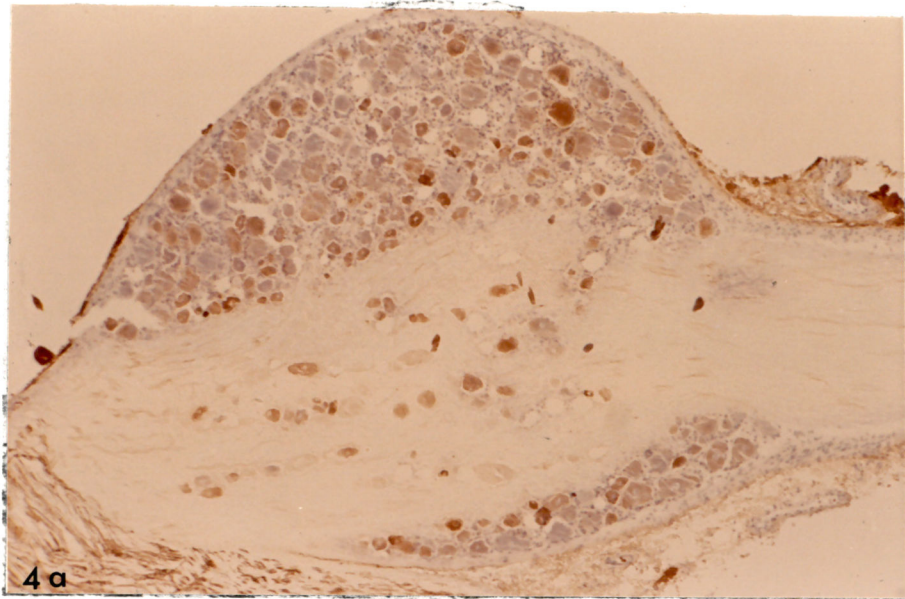


FIGURE 5. COMPARISON OF THE NUMBER OF CELL FRAGMENTS IN EACH SECTION STUDIED AS TO THE COLOR CATEGORY IN THE 4 GANGLIA STAINED FOR AChE.

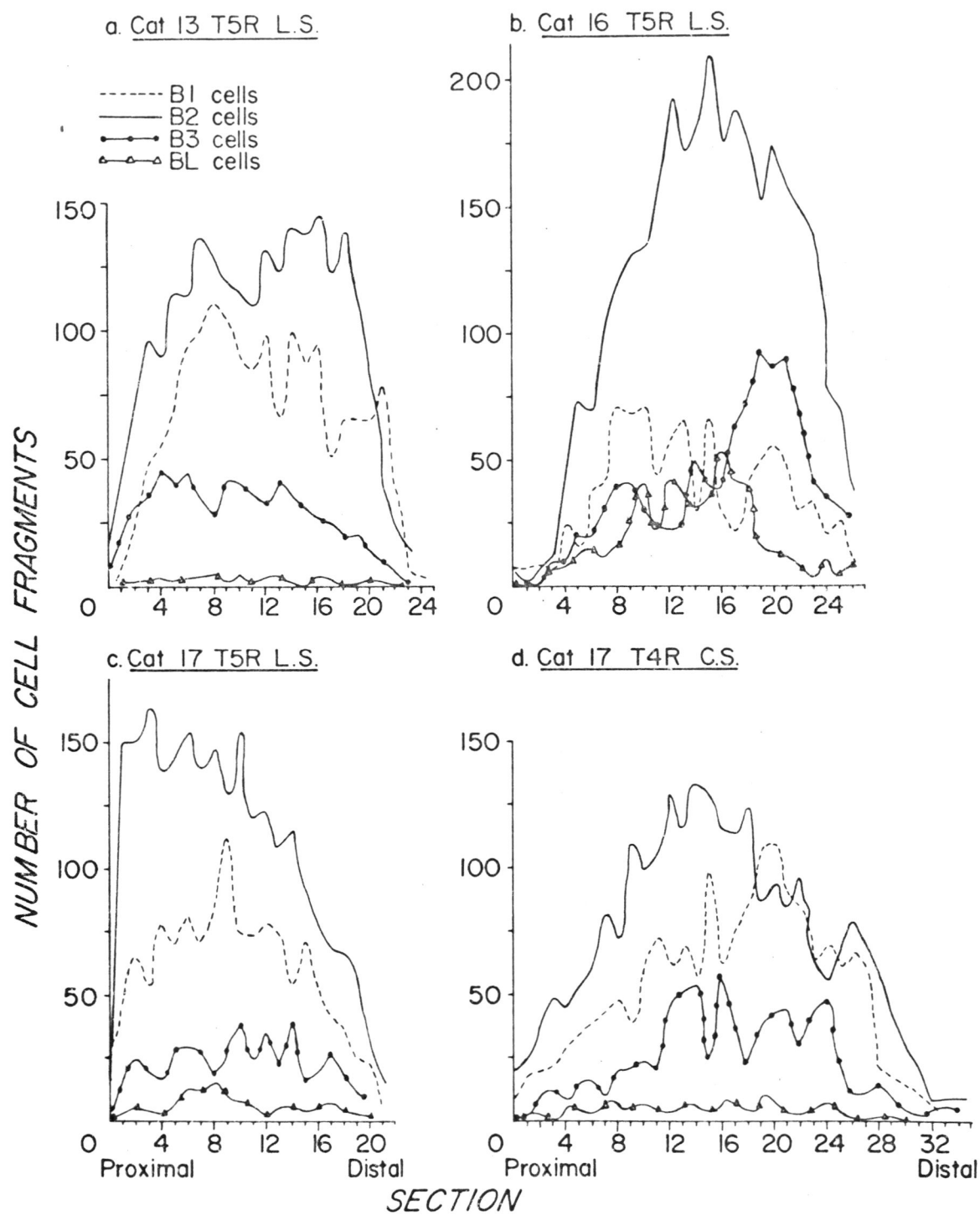


FIGURE 6. COMPARISON OF THE 4 COLOR CATEGORIES OF THE 3 CANGLIA
SECTIONED LONGITUDINALLY TO THE NUMBER OF CELL FRAGMENTS
COUNTED PER SECTION. AChE STAIN.

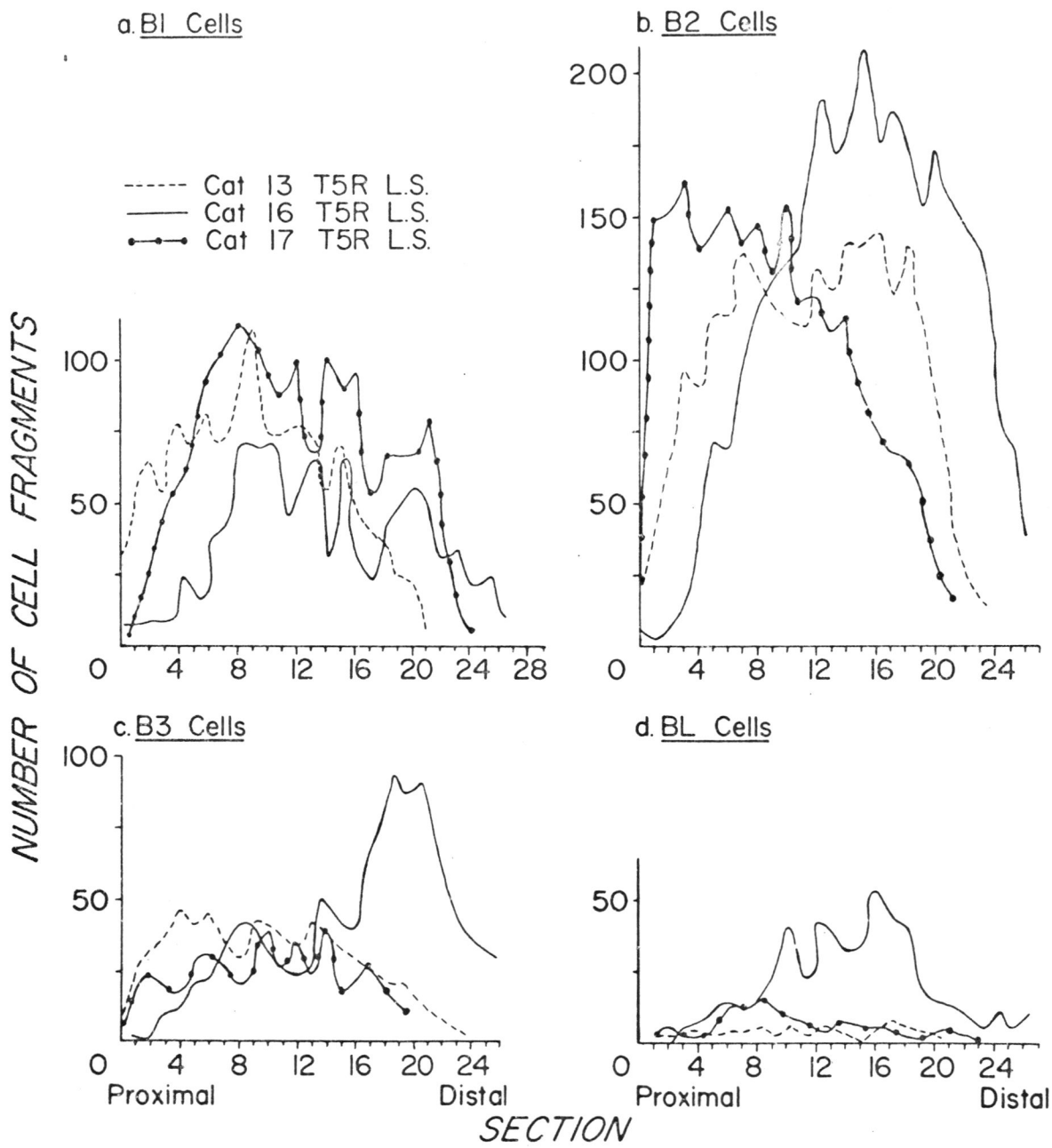


FIGURE 6

FIGURE 7. COMPARISON OF THE NUMBER OF CELL FRAGMENTS IN EACH
COLOR CATEGORY TO THEIR DIAMETER IN THE 4 GANGLIA
STAINED FOR AChE.

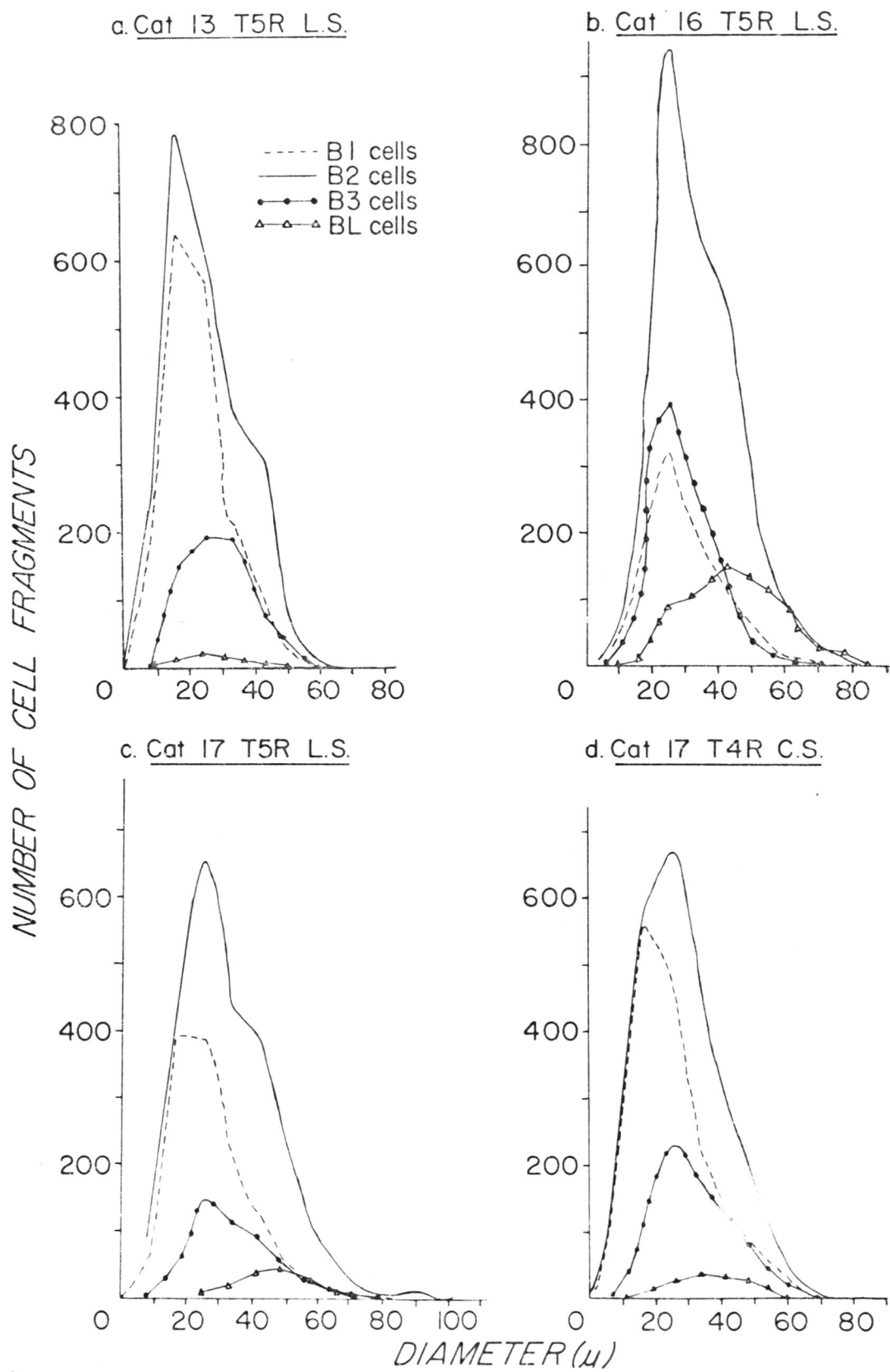


FIGURE 8. COMPARISON OF THE NUMBER OF CELL FRAGMENTS OF 4 GANGLIA
STAINED FOR AChE AS TO THEIR DIAMETER AND INDIVIDUAL
COLOR CATEGORY.

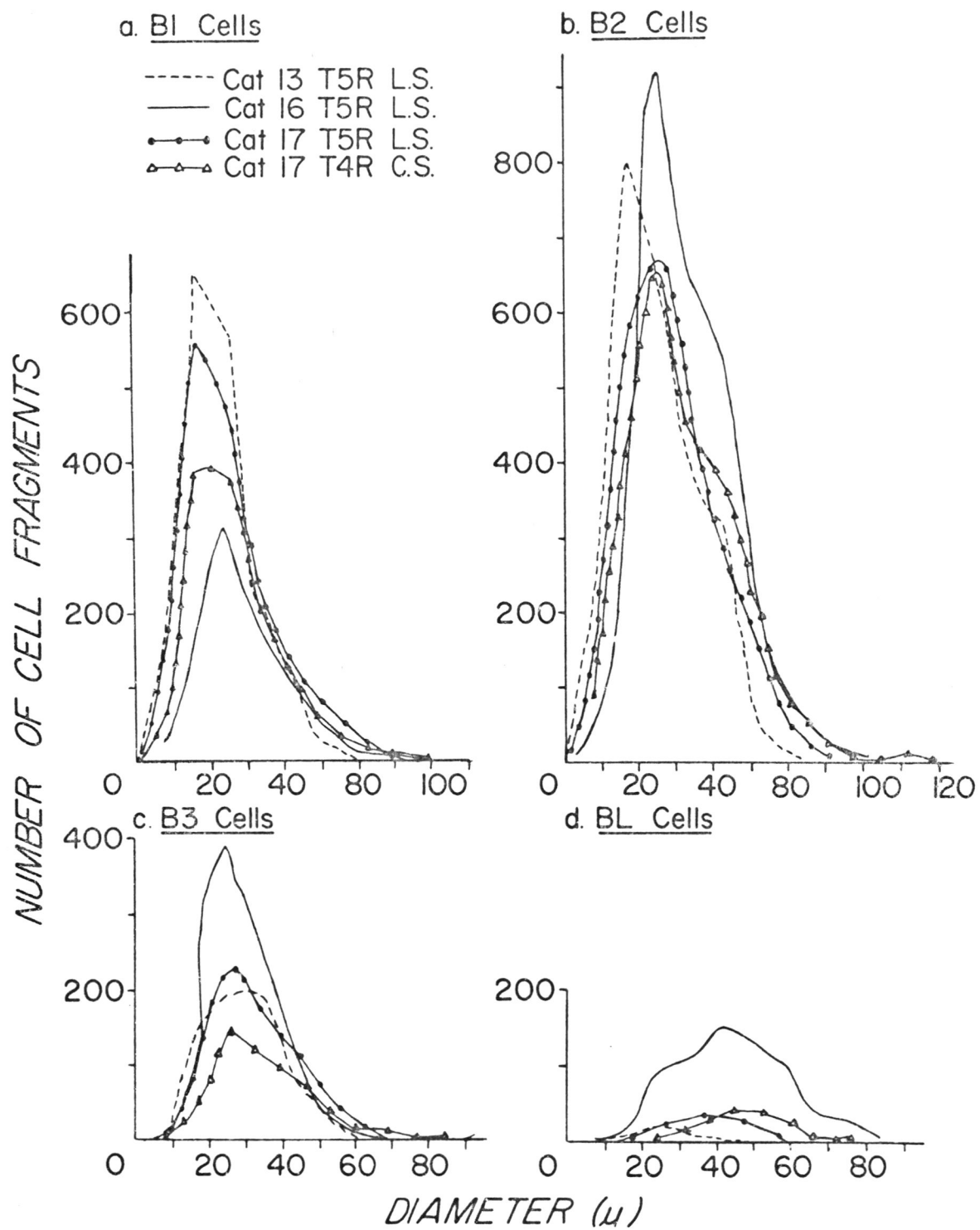


FIGURE 8

FIGURE 9. NEURONS OF THORACIC SPINAL GANGLIA STAIN WITH CRESYL VIOLET, WHILE THE MYELIN SURROUNDING THE AXONS IS SELECTIVELY STAINED WITH LUXOL FAST BLUE (AFTER KLUVER BARRERA, 1957). 150X



FIGURE 10. SKETCH OF A DORSAL ROOT GANGLION WITH ATTACHED PROXIMAL AND DISTAL STUMPS. NOTE THAT THE VENTRAL ROOT IS CLOSELY APPOSED TO THE UNDERSIDE OF THE GANGLION. IT IS POSSIBLE TO DETERMINE FROM WHERE A SECTION CAME BY ANALYZING THE RELATION OF THE GANGLION TO THE VENTRAL ROOT.

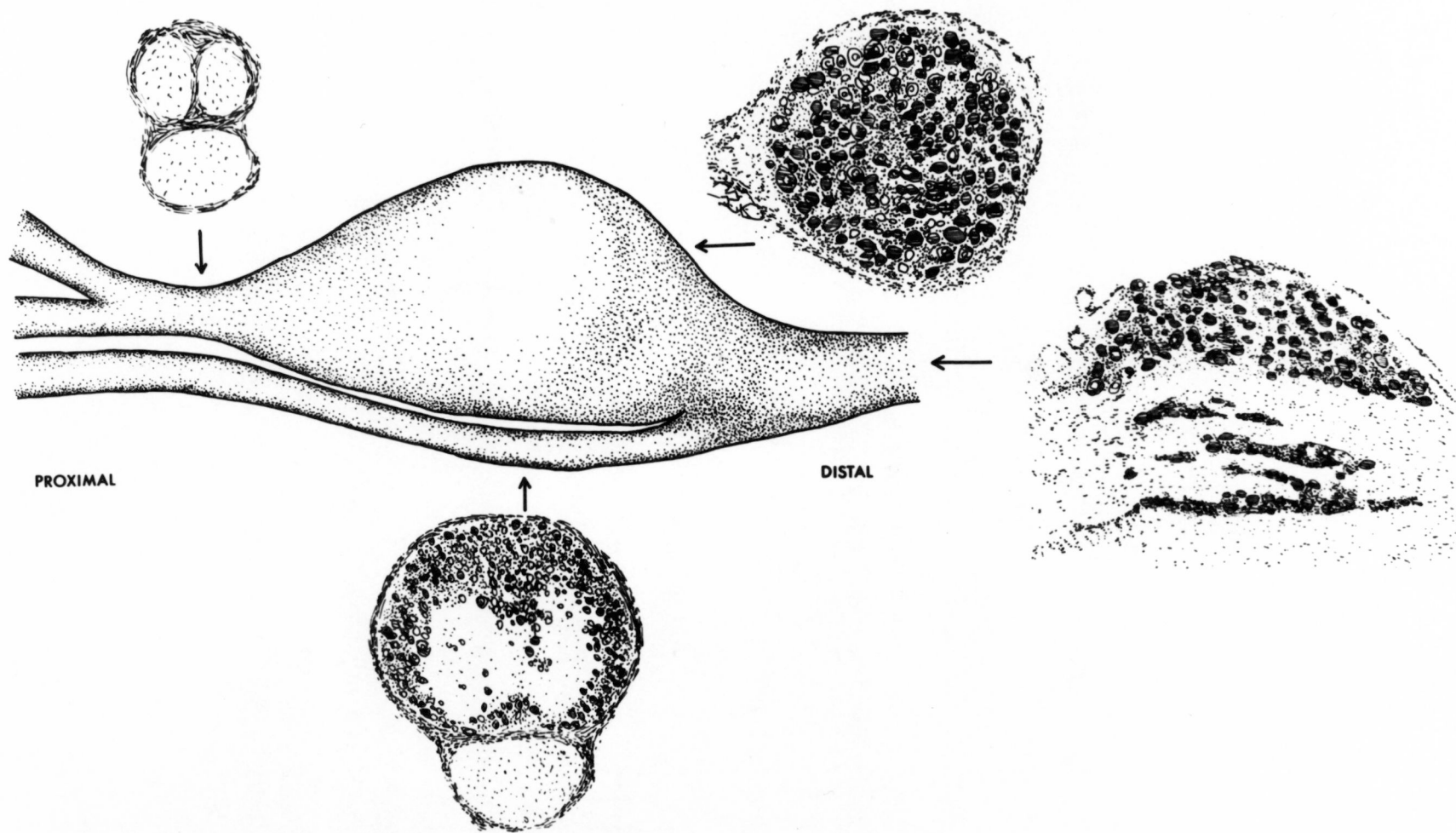


FIGURE 11. HIGH MAGNIFICATIONS OF THE FOUR CELL TYPES FOUND WHEN
GANGLIA ARE STAINED FOR AChE. a) B1 CELL b) B2
CELL c) B3 CELL d) BL CELL. 500X

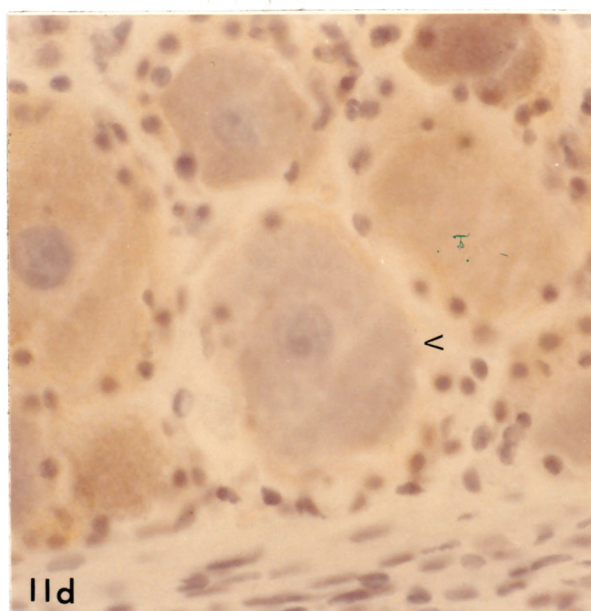
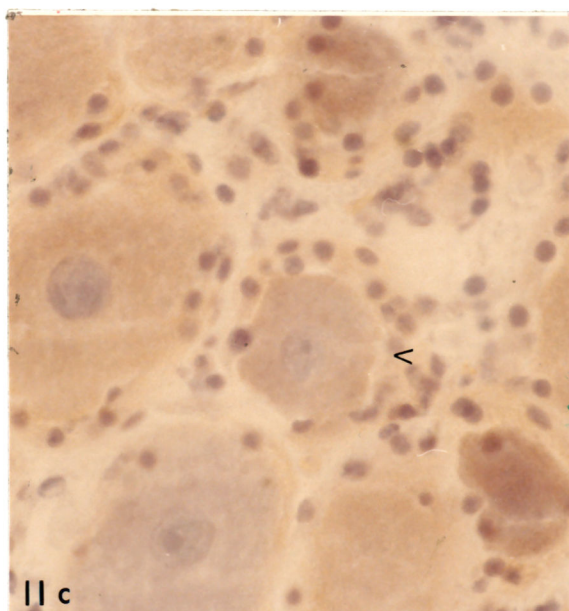
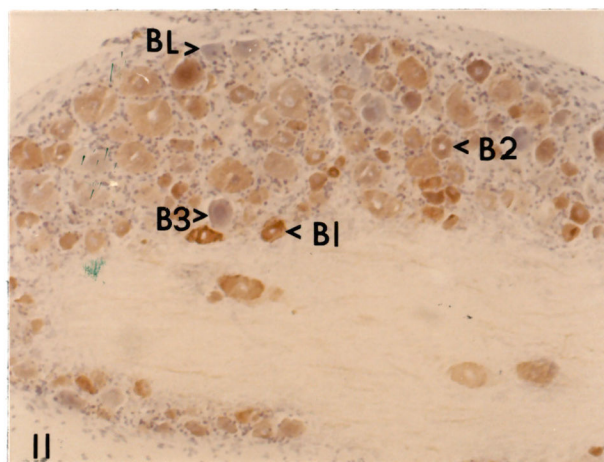
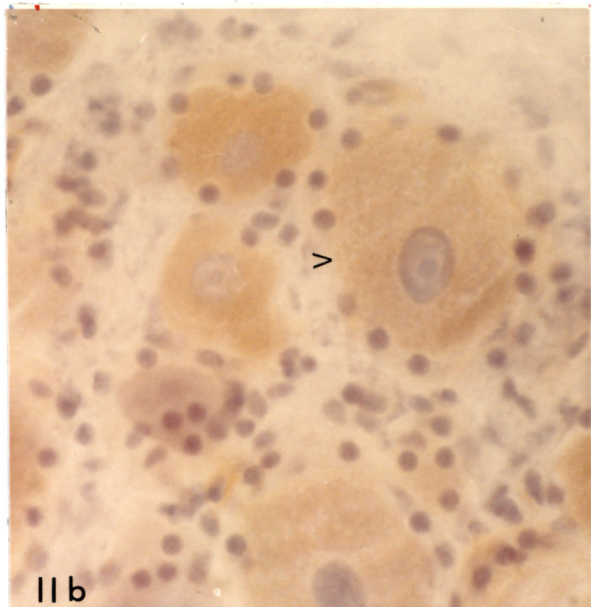
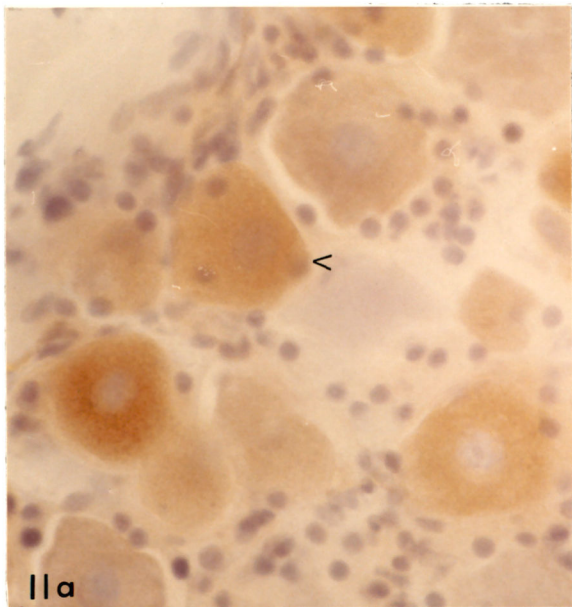


FIGURE 12. COMPARISON OF THE AFFINITY OF THE SPINAL CORD AND THE DORSAL AND VENTRAL ROOTS FOR AChE. a) THE HIGH DEGREE OF STAIN UPTAKE IN ANTERIOR MOTOR HORN CELLS OF THE SPINAL CORD. NOTE THE HIGH ACTIVITY IN THE NEUROPIL. b) SPINAL CORD AT THE THORACIC LEVEL. NOTE THE SENSORY AND MOTOR HORNS ARE NOT EXTREMELY LARGE. ALSO NOTE THE INTERMEDIOLATERAL HORN OF THE SPINAL GRAY. c) THE DORSAL ROOT IS ALMOST UNSTAINED WHILE THE VENTRAL ROOT IS RELATIVELY HIGHLY STAINED. a) 500X, b) 50X c) 100X

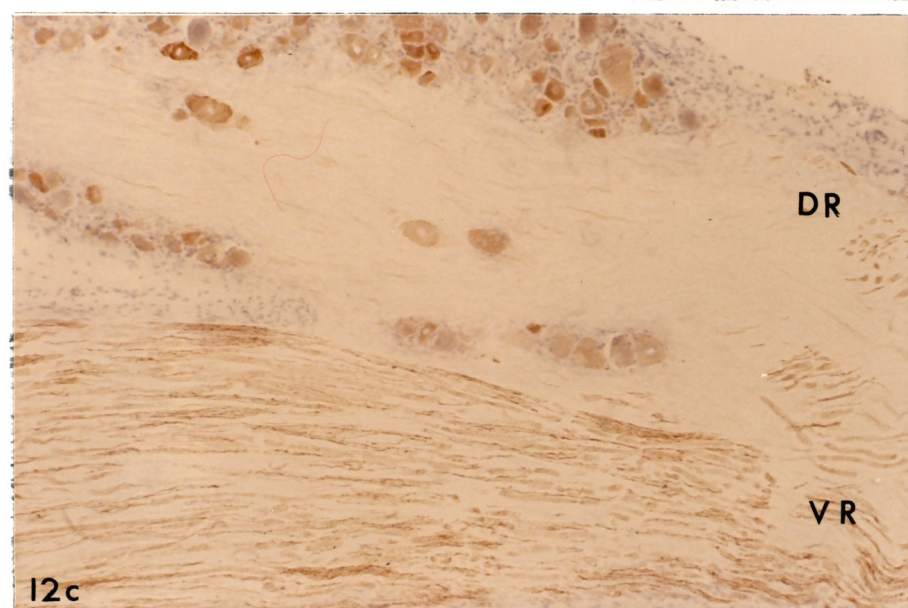
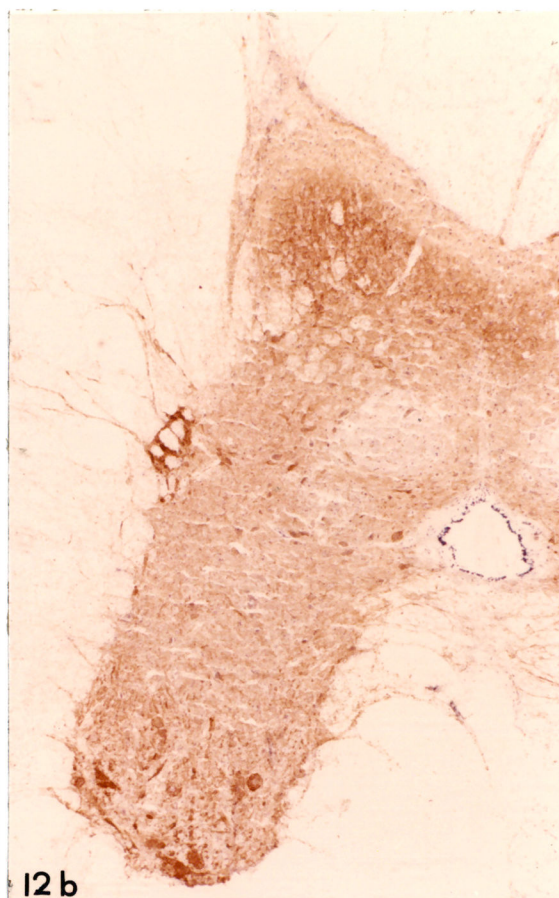
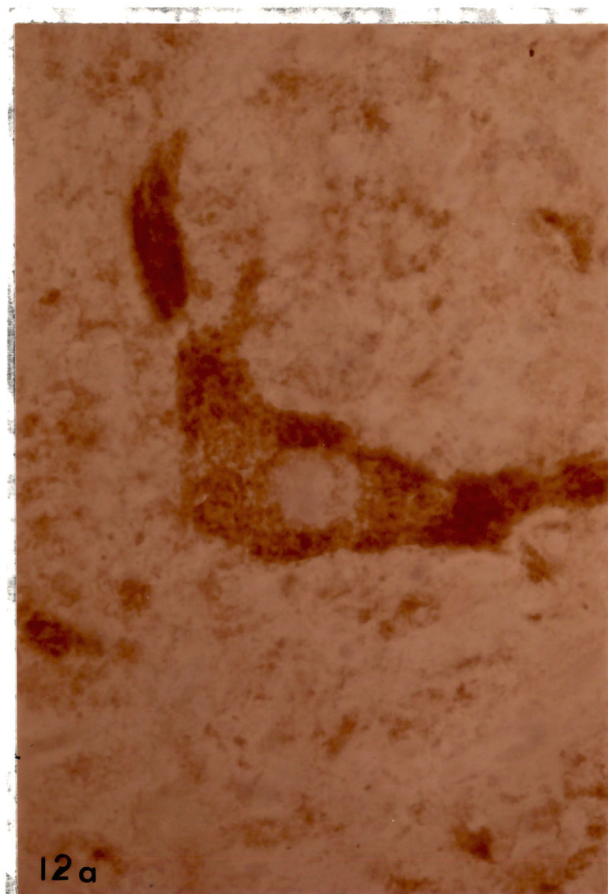


FIGURE 13. CONTROL PREPARATIONS OF CAT THORACIC SPINAL GANGLIA USING THE AChE MEDIUM. a) AChE MEDIUM WITHOUT SUBSTRATE BUT WITH COUNTERSTAIN. b) INCUBATION MEDIUM WITH SUBSTRATE AND COUNTERSTAIN BUT WITHOUT THE REVERSIBLE NONSPECIFIC CHOLINESTERASE INHIBITOR (iso-OMPA). NOTE THE PERIPHERAL STAINING OF THE CELLS DUE TO THE PRESENCE OF NONSPECIFIC CHOLINESTERASE. 50X

