Studies Pertaining to the Anatomy, Histology and Nervous Organization and Transmission in the Tunicate Ciona Intestinalis

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STUDIES PERTAINING TO THE ANATOMY, HISTOLOGY AND NERVOUS ORGANIZATION AND TRANSMISSION IN THE TUNICATE GIONA INTESTINALIS

by

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A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

June
1964
Charles L. Scudder was born in Milwaukee, Wisconsin, on October 6, 1926.

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I would like to express my gratitude to Dr. Alexander G. Karczmar for introducing me to the baffling problem of cholinergicity of the tunicates, as well as for his guidance and wise patience while acting as my advisor during the preparation of this work; to Dr. Thomas K. Akers for his helpful suggestions; and to Dr. G. M. Everett for his understanding and encouragement during recent years.
A dark field exposure of a 10 μm section of the tunicate ovary stained with Alizarine Red S. The highly refractile bodies in the chorion cells of stage three oocytes are visible bordering each ovum.
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The following abbreviations are employed in the text of this thesis.

RNA.................ribose nucleic acid
AChE.................true or acetylcholinesterase
BuChE.................pseudo or butyrylcholinesterase
MAO..................monoamine oxidase
DFP..................di-isopropyl phosphofluoridate
OOD..................open to open duration
CHAPTER I
INTRODUCTION
HISTORY, DISTRIBUTION AND GENERAL ANATOMY OF CIONA INTESTINALIS

A. HISTORY OF TUNICATE RESEARCH

Tunicates have been studied by each new generation of scientists. The reasons for this periodic renewal of interest in such esoteric animals have varied and have been related to the changing scientific attitudes and focus of each generation.

Aristotle classified the solitary ascidians calling them Thalia and the animal Tethys described by him may have been the same genus Ciona, which is used in the present studies (Berrill, 1950). Redi described several tunicates in the seventeenth century. They were classified by the great eighteenth century taxonomist Pellas, Forskål, Müller, as well as Cuvier, who placed the tunicates with the molluscs Lamellibranchs because of their sessile way of life and their paired siphons. The separate class Tunicata was established by Lamarck who referred to them as "animaux apathiques" and this class was further subdivided and brought to order by Chamiso, Milne Edwards and P. J. van Beneden. Interest in tunicates may have slackened at this point; but when Kovalewsky called attention to the chordate affinities of tunicate larvae at a time when zoological interest was concerned with
entelechy, teleology, and vitalism under the auspices of Goethe and Driesch, there was a tremendous surge of interest in the cleavage patterns and the larval anatomy of many tunicate species. These studies clearly set apart the Tunicata (or Urochordata) as a subphylum of the Chordata.

The subphylum Tunicata is subdivided into three classes which contain a total of over a thousand species. These classes are (1) the Larveacea which are pelagic, solitary, pedogenic forms, (2) the Thaleacea which are pelagic colonial forms, and (3) the Ascydeacea which are sessile, solitary zooids whose larvae are free swimming. Ciona, on the evidence presented by its hematology (Webb, 1939) and its egg cytology (Berrill, 1955), is now considered the most primitive genus of this latter class.

As a life form the tunicates are of immense antiquity (van Beneden, 1887). Because of the larval notochord, gill slits, and dorsal tubular nervous system, the tunicates are closely related to the protochordate stock. However, since the existence during the early Paleozoic of the ancestor now recapitulated in the ontology of the present-day tunicate, the urochordates have continued to evolve quite separate from the chordates, and the contemporary adults may not possess either in their anatomy or their biochemistry many features of the remotely related present-day chordates.

Tunicate eggs are strongly determinative; chordate eggs generally are not. Early in cleavage the adult nervous system is
set apart from that of the larva as a separate primordium. The adult brain resembles certain insect structures which are structureless before metamorphosis, but which suddenly emerge complex, complete, and unique (Garstang, 1928). Grave (1934, 1935, 1938) strongly emphasized this point of difference between the tunicates and other chordates. He considered the tunicate larva as a dual action system composed of two incompatible chemical systems or life forms: one, the ascidiozooid (adult) action system consists of the oral and atrial siphon, alimentary canal, ganglion, hypophyseal duct, neural gland, pericardium, and heart; the other, the larval action system, comprises various chordate-like apparatus such as the central nervous system, sense organs, various ganglia, the notochord, and the muscles of the tail. Both systems develop simultaneously from the mosaic egg, and for a time the adult system is inhibited in its development, which is fortunate because the way of life of the adult and that of the larva are incompatible. The balance between the two is precarious and may be upset by many diverse agents, leading to metamorphosis at which time no larval structures are visibly maintained by the adult.

The larva is a complex unstable system similar to the virgin egg. Both may be triggered into a sequence of developmental stages by non-specific agents (mechanical injury, light crowding, thyroid extract, heavy metals, etc.). One aspect of the recent revival of interest in tunicates is centered on this problem of
metamorphosis and has been reviewed by Lynch (1961). It is thought that some enzyme system must be put out of balance to produce the changes seen at metamorphosis, and a large part of contemporary tunicate studies is biochemically oriented. Thus, the effects of nucleosides (Cusimano, 1961), ribonuclease (Mancuso, 1961), para-aminobenzoic acid, 8-hydroxy-quinoline, para-hydroxy propiophenone (Minganti, 1957), cytochrome oxidase inhibition by KCN, malonate and selenite (Riverberi, 1957; Patricolo, 1959), aconitase inhibition by fluoroacetate (Patricolo, 1961) have been observed on eggs and larvae. These investigators have tacitly assumed the universal occurrence of the enzyme systems and the specificity of the inhibitors they have employed. However, no studies on the metabolic pathways of tunicate tissues have been made. Also, since these workers worked on different genera of tunicates, the results are difficult to interpret. Much of this contemporary biochemically oriented research on tunicates suffers from generalizations carried over from work on the vertebrates and applied to the urochordates without due consideration of the zoological insight which could be gained from earlier work.

B. THE CLASSIFICATION AND DISTRIBUTION OF CIONA

Ciona belongs to the class Ascideaceae and the order Enterogona (Berrill, 1950). This class comprises animals which possess an unpaired gonad lying within a loop of the intestine, bear an oviduct and spermatoduct which follow the course of the rectum to the anus, have the neural gland situated ventral to the
ganglion, bear tentacles which are simple, and have an atrial siphon which forms from a secondary fusing of two lateral and independent ectodermal sacs. *Ciona* belongs to the suborder of the Enterogona, the phlebotranchiata (Hartmeyer, 1926; Berrill, 1927), in which there is no division of the body into a thorax and an abdomen, and there are no folds in the branchial sac.

The family Cionidae is composed of solitary forms with a horizontal digestive loop posterior to the branchial sac, with elaborate pharyngeal papillae, with a V-shaped heart between the stomach and the base of the endostyle, and with the openings of the gonducts near the anus. The *Cionidae* are oviparous, and the epicardial cavities are enlarged to form two large sacs enveloping the viscera and communicating to branchial cavity (Newstead, 1894). This latter characteristic is quite unique and has been described in considerable detail (Damas, 1900a, 1900b). Berrill (1950) assigns only a single genus and species, *Ciona intestinalis*, to the Cionidae. However, earlier investigators tended to further subdivide the family. Undoubtedly, considerable diversity of form exists. Hardman (1899) recognized seven species and Hartmeyer (1915), nine species.

*Ciona* is a cosmopolitan genus. It has been reported from Suez, Singapore, Tasmania, and Yokohama. A single specimen has been dredged up near Grand Manan, and *Ciona* is not uncommon in the Bay of Fundy (Huntsman, 1912). It is not found in quantity in Departure Bay, and it is not mentioned among the ascidians of
of Puget Sound (Ritter, 1900) or Bermuda (Berrill, 1932). It is commonplace on both the East and West Coasts of the United States.

The American variety of Ciona seems to have somewhat fewer muscle bands (10) compared to the European variety (12-14). In England, the species occurs in several color patterns: (1) transparent forms with pale green and white stripes, (2) orange forms with greenish villi, (3) green-grey opaque forms (Millar, 1953). Animals with intermediate color patterns also occur. The cause or genetic basis of these patterns is unknown. Physiologically different types are also reported. These are induced to ovulate each at a different water temperature (Millar, 1953). Millar recognizes three morphologically different varieties of Ciona on the basis of pharyngeal size differences.

The extreme age and genetic diversity of the cosmopolitan genus Ciona may account for some of the conflicting results of investigators.

C. THE GROSS APPEARANCE AND ANATOMY OF CIONA

Ciona is a large, sessile tunicate, elongated (Fig. 1) and generally colored grey-green, brown or orange. It is found in relatively quiet salt water, usually attached by special adhesive, constantly-growing stolongs to, particularly, under surfaces of logs, pilings, stones, etc. The animal is usually oriented with the siphons facing the light, suggesting some positive phototropism (Millar, 1953, see also VI A). Ciona is essentially a biannual organism although specimens in aquaria may live for more
than two years. Usually, **Ciona** grows and matures during the first year and breeds and dies during the second.

Externally, the entire organism is enclosed (except for the oral siphon and the tip of the atrial siphon) in a tunic or test composed of tunicin, an unusual animal substance in that it is composed of 60.34 per cent cellulose (Berrill, 1950). The tunic is protective and phagocytic, as it is permeated by phagocytic, mesodermal cells (Berrill, 1955). It is constantly growing. It is non-sensory. New villi can be formed by it for adhesive re-attachment of the animal at any time in its life, but the power of stolon formation by the tunic and asexual reproduction through budding of the tunic has been lost by **Ciona**. Generally, the width of the tunic at the level of the anus of a relaxed animal is logarithmically proportional to its length (Millar, 1953):

\[ W = 0.383 \times L^{0.642} \]

The histological details of various organs of the animal are presented later so that only brief descriptions of the general adult organ systems need be discussed here (see Fig. 2 for the gross anatomical relationships of **Ciona**).

1. Feeding and Digestion

Water currents, impelled by the ciliary action of cells lining the pharynx, carry planctonic food through the oral siphon and into the pharyngeal cavity where the particles adhere to mucus sheets, while the water flows out of the pharynx through tiny stigmata which pierce it and passes into atrial cavity.
This cavity communicates by way of the atrial siphon to the outside of the animal. The food and the mucus rope bearing it are impelled by ciliary action through a short esophagus to a stomach which bears a pyloric gland and leads to a double coiled intestine, a rectum, and an anus. The latter empties into the exhaled water of the atrial cavity which serves as a cloaca as well as part of the feeding-filtering apparatus.

2. The Muscular System

The pharynx bears five to seven pairs of longitudinal smooth muscle bands which run from the base of the animal to the lobes of the siphons. Siphons contain poorly developed sphincter systems. The anatomy of the muscular pharynx is exceedingly complex. This complexity of pharyngeal design as well as Ciona's large size and absence of asexual reproduction are the only advanced biological traits shown by this fundamentally primitive tunicate genus.

3. The Reproductive System

The ovary of Ciona is a pear-shaped organ lying on the left side of the loops of the intestine. The testis is a much more diffuse structure wrapping around the stomach and the proximal intestine. From each gonad a gonoduct follows the intestine and the rectum to empty through a gonopore near the anus into the atrial cavity.

Ciona is oviparous and breeds throughout the year. Self fertilization may occur.

4. The Circulatory System
Circulation is accomplished by the action of the pharyngeal muscle bands and a simple V-shaped tubular heart of striated muscle which is unique in that it periodically reverses its direction of beat. The heart leads to branching, definite vessels which ramify in the body, form lacunae, and lead back to the heart again.

5. The Nervous System

The adult nervous system consists of a single ganglion situated between the siphons and bearing two dorsal, two ventral, and one visceral nerve.
FIGURE 1

Semi-diagramatic lateral view of the gross appearance of Ciona intestinalis (after Millar, 1953).

Or. s.......... oral siphon
Oc.............. ocellus
Ga............. ganglion
At. s.......... atrial siphon
Lon. mu........ longitudinal muscles
Vi.............. villi
Vis. re.......... visceral region
Br. re.......... branchial region
FIGURE 2

A cut-away lateral view showing the gross anatomy of *Cliona intestinalis* (after Millar, 1953).

T. pos............region of atrial siphon
La...............pharyngeal lappets
C. cav............the atrial chamber
Gen. du...........genital duct
An..................anus
Re..................rectum
Ge..................oesophagus
St..................stomach
Ov.................ovary
I...................intestine
Ht..................heart
Es. ap.............endostylar appendage
Ph. w.............pharynx
Es...............endostyle
Per. bd...........perivisceral band
Pre. z............pre-oral zone
Ten...............tentacles
CHAPTER II

ANATOMY AND HISTOLOGICAL DETAILS OF THE ORGAN SYSTEMS OF CIONA INTESTINALIS

A. THE NERVOUS SYSTEM AND SENSE ORGANS OF CIONA

1. The Ganglion

   a. Descriptions from the literature

   A satisfactory stain for nervous tissue in tunicates has not been found. The neurons are small and are patterned into nerves for only a part of their path. Non-the-less, early morphologists had no trouble localizing the ganglion which stands out as an easily accessible, glistening white patch between siphons. Hancock (1868) first described this single ganglion in A. intestinalis (Ciona). This fusiform body was described as lying in a blood sinus between the two "respiratory tubes." Hancock considered it homologous to the branchial ganglion of the lamellibranchs. Kupffer (1870) described in detail the larval nervous system of Ciona, but made no mention of the adult. Van Beneden (van Beneden and Julin, 1884) described the appearance of the central nerve band of the adult and claimed to be able to trace ganglion cells in a "cordon ganglionaire", but Ciona was not the genus he studied. Metcalf (1900) worked on the intersiphalonal organs of tunicates and mentioned Ciona. The intersiphalonal organs comprise the ganglion, the neural gland, the ciliated
funnel, and the structures (nervous and glandular) of the dorsal raphe. Metcalf pictured the Ciona ganglion as oval, twice as long as broad, and bifurcated on either end where the siphonal nerves arise. He recognized that the ganglion was composed histologically of an outer cellular cortex and an inner medulla of fibrous substance. He noted the large size of the neuron nuclei.

Metcalf also described the neural gland, which lies beneath the brain and is closely applied to it, as having a distinct lumen, non-glandular cuboidal walls, and opening by way of the ciliated funnel located in the region of the dorsal raphe of the branchial sac into the pharyngeal chamber. This communication of the lumen of the neural gland directly with the pharyngeal cavity will be seen to be of significance (see III) in that material from the pharynx may lodge in the neural gland and act as contaminant in biochemical studies of the ganglion.

The most recent and complete description of the Ciona ganglion in the literature is by Millar (1953), who stated that the cortex was composed of cells of two sizes. These cortical cells were only three to four layers deep. The larger cells were one-fifth as numerous as the smaller ones. The large cells were from 9 microns to 18 microns in diameter, with a nuclear size of 5.25 microns to 6.0 microns. The smaller neurons were from 5 to 7 microns and have 3.5 microns nuclei. Although Herdman (1899) found bipolar and multipolar cells in the ganglion, Millar found all ganglion cells to be unipolar with a tapering axon leading
into the medulla and bearing visible neurofibrillae. Because of their complexity and number of fibers, the course of these axons cannot be traced.

b. Observations from this laboratory

The methods employed in this laboratory for the general cytological studies were as follows. Whole organs or organ pieces were removed from the living adult tunicate and fixed in formalin alcohol. The tissues which were studied were ovary, heart (within the pericardium), the ganglion, the oral siphon, and a 2 mm piece of the intestine from the first loop adjacent to the stomach. These tissues were then dehydrated, cleared, sectioned, and appropriately stained with the following reagents: Harris' hematoxylin-eosin, Gresyl fast violet-toluidine blue-thionine, and urea-silver nitrate. The sections were mounted with Sargent's mounting fluid, dried, and photographed as they were studied with a Cooke, Troughton, and Simms camera attachment.

Our findings on the ganglion histology differ only slightly from Millar's account (see Plates 2 and 3). No clear cut division of the neurons into two distinct sizes was found. Instead, an entire range of sizes was observed. Furthermore, although unipolar cells were present, it was not uncommon to find bipolar and multipolar cells.

2. The Nerves of Ciona

a. General observations

There are two anterior nerves, two posterior nerves, and one
unpaired visceral (posterior) nerve coming from the ganglion. Each anterior nerve gives off one anterior (oral) siphon nerve and one trunk nerve to the ventral pharynx. Each posterior nerve gives off a posterior (atrial) siphon nerve and a trunk nerve to the dorsal pharynx. The nerves lie in ill-defined cellular tubules of unknown function. The nerves are believed to terminate in a fine network about the viscera and the smooth muscle bundles. The nerve net has never been clearly described in the literature.

b. Specific findings from this laboratory

A finding of interest in our laboratory was that very light staining with Giemsa-Wright stain of cross sections of the siphon musculature revealed tiny cells and strands between the muscle fibers which may represent the nerve net of this region (see Plate 4).

3. The Dorsal Strand

The dorsal strand was described by van Beneden (van Beneden and Julin, 1884) and Herdman (1899) as having a nervous function. In Ciona recent evidence indicates that this tissue is only a non-nervous extension of the neural gland (Millar, 1953). It is difficult to assign a nervous function to such tunicate tissues on the basis of cytology alone. The function of the neural gland itself is unknown; it may be neurohormonal in nature.

4. Sense Organs, the Ocelli, and a Discussion of the Neural Gland and the Ciliated Funnel
Sense organs probably do not exist in Ciona. Although Hancock (1868) ascribed an auditory or visual function to the ocelli, we have found no evidence for photoreception (see VI-A and Plates 54, 55, 56). Sensory cells have been found on the inner and outer epidermis of the siphons. There is disagreement whether or not they occur elsewhere (Millar, 1953).

The neural gland and its associated ciliary funnel have been assigned an olfactory or gustatory function (Metcalf, 1900). The neural gland is a ventral extension of the wall of the lumen of the neuro-hypophyseal tube opposite to the ganglion. The neural gland is occasionally filled with phagocytes and stellate cells. Millar (1953) states that its only clear function seems to be to produce an "escape route for unwanted cells." These pass by way of the hypophyseal canal and ciliated funnel into the pharynx where they are caught up and digested. The cilia of the ciliated funnel are long and immotile. The purpose of the entire apparatus is obscure.

5. Discussion

The ganglion of the tunicate in relation to the brains of other chordates is considered more or less homologous to the pituitary (Berrill, 1950). Embryologically, after closure of the neuropore and separation of the neural tube from the superficial ectoderm, the anterior part of the neural tube divides. The right side of the division forms the sensory vesicle of the larva; the left is the primordium of the hypophyseal duct of the adult. The
walls of the latter proliferate both the ganglion and the neural gland.

All nervous differentiation in the tunicate depends upon an evocator released from endodermal cells. If no presumptive chordal or endodermal cells are present, no brain develops. Thus, if the two vegetal cells, destined to be endoderm, are removed from the eight-celled morula, no brain develops (Riverberi et al., 1961). Since lithium ion damages endodermal cells, treatment with lithium at an early stage can lead to malformation of the nervous system at later stages (Riverberi and Farinella-Faruzza, 1961).

If the neural gland and the ganglion are pituitary homologues, their function may be, at least in part, hormonal. They may direct and control somatic activity by secreting neurohumors into the large blood sinus about them; or, perhaps, the cells released by the neural gland may bear chemical messages to direct and stabilize homeostatic activity of other distant cells within the animal.

B. THE ALIMENTARY CANAL OF CIONA

Feeding and digestion in Ciona intestinalis occurs as follows. Food particles (the basic food is the diatom Nitzchia, cf. Berrill, 1947) are swept into the inhalent or oral siphon. The rate of feeding is related to three factors: (1) the state of contraction of the pharyngeal and siphon muscles, (2) the rate of ciliary beat, and (3) the rate of mucus secretion. All three
FIGURE 3
The ganglion and major nerves of Ciona intestinalis (after Millar, 1953).

Pos. n. s........posterior (atrial) siphonal nerves
Pos. n. tr........posterior (visceral) nerves
Ant. n. tr........anterior (visceral) nerves
Ant. n. s..........anterior (oral) siphonal nerves
Ga................ganglion
Plate 2. A ten micron longitudinal section of the tunicate brain stained with Harris' hematoxylin and eosin and photographed with low power magnification (80X). Visible are the cortex, the medulla, and a portion of the ventral nerve trunk (to the far right), the median blood sinus, and the neural gland containing a few scattered orange blood cells.

Plate 3. The tunicate brain sectioned and treated as in plate 2, but photographed with a green filter for greater clarity. Note the close proximity of the neurons of the ventral cortex to the neural gland.
An oil immersion photomicrograph of an eight-micron section of the smooth muscle of the oral siphon stained lightly with Giemsa-Wright stain. The small blue bodies with branching dendritic processes may be components of the neural net. These cells measure about 1.2 microns in diameter.
parameters may be under nervous control. A six to seven cm. Ciona filters approximately 552-750 ml. of water per hour and picks up particles as small as two microns. Ciona is said to feed faster than other ascidians (Orton, 1913). Carmine particles have been found to adhere to the pharyngeal wall but not to the mucus secreting ventrally located endostyle nor to the dorsal ciliated funnel. The strands of mucus, containing particles of food, pass across the branchial sac to the dorsal lamina, hastened by movement of the longitudinal gill bars or pharyngeal papillae. This observation made by Hecht (1918b), was denied by Millar (1953). Pharyngeal-cloacal slits as well as an egestion reflex serve to clear the pharynx of irritant substances and to prevent congestion.

Food is passed through the short esophagus to the stomach which is clearly differentiated from the rest of the gut of the adult. The stomach is composed of a single layer of absorptive and secretory cells similar to those found in the intestine (see Plates 5, 6). All gut cells are ciliated. The secretory cells secrete a powerful amylase, an invertase, no maltase or lactase, a lipase and a weak protease (Berrill, 1929). The gut contents also contain carotenoids (Fox, 1953).

The various absorptive cells take up the products of digestion. They have a great affinity for both iodine and vanadium. The former is utilized in the synthesis of thyroglobulins, not so much concentrated in the endostyle which may be a thyroid homologue, but in the mantle which has a high level of iodinated
proteins (Roche, 1962).

The function and location of the vanadium absorbed by *Ciona* is not yet known although it comprises 0.04 per cent of the organic dry weight. In higher tunicates vanadium is concentrated in the vanadocytes of the blood along with high concentrations of sulfuric acid. *Ciona* contains no vanadocytes (see later discussion on the hematology of *Ciona*), but, none-the-less, it concentrates vanadium in its tissues from sea water, where it is present to the amount of only 0.3 to 0.6 mg./cu. meter (Webb, 1939).

*Ciona* was long thought to have no associated digestive glands like those found in higher ascidians (Winnewarter, 1895). However, Berrill (1947) has shown that there exists a simple vestigial pyloric gland, and a digestive caecum. The former is trapped during development in the epicardial mesentary and appears in the adult as part of the gonad. Its function, as best as it can be determined from the concretions which it contains, is probably excretory. The digestive caecum is only present in young animals and bears a histology identical to that of the stomach.

The cells of the epithelium of the intestine are also believed to be of two types, absorbtive and secretory. A gregarine parasite, *Lankasteria asciidiae*, infects many of these cells and has the appearance as a large swollen vacuole (see Plate 5).

Wrapped about the single layered epithelium which composes the intestine are the tubules of the testis and various vascular lacunae (see Plate 6). There are no distinct muscle layers
present about the intestine, nor has a nerve plexus or net been identified. The entire digestive tube is suspended by folds of the epicardium. The lumen of the epicardium forms a perivisceral sinus and communicates with the pharyngeal chamber. This peculiarity allows the sea water of the environment to bathe almost all the internal tissues of the tunicate. This is an important anatomical detail in relation to the drug studies (in Chapter VI).

C. THE HEART OF CIONA

1. General Observations in the Literature

The heart of Ciona is a simple tube, one cell layer thick, and it may not be homologous to the hearts of higher vertebrates (Wiley, 1894). It is enclosed in a roughly triangular pericardium to which it is fastened by a raphe and which is held in place in the body by the walls of the enlarged epicardial cavities. The pericardial wall itself is a single sheet of hexagonal mesodermal cells with large vesicular nuclei. The cardiac tube arises embryologically by an invagination of the pericardium and remains attached to it along one side of its length by the pericardial raphe (Fig. 4).

The contractile wall of the heart consists of a single sheet of unusual striated muscle cells (see Plates 7 and 8) whose contractile extensions are wound as fibers in a tight helix almost perpendicular to the long axis of the heart. The surface of the fibers facing the lumen is covered by a layer of non-cellular substance (Millar, 1953). These cardiac cells are continuously
Plate 5. A low power photomicrograph of the tunicate intestine sectioned at 8 microns, stained with Cresyl fast violet-toluidine blue-thionine. The section shows the digestive epithelium with many vacuolate cells. The testis tubules and vascular lacunae are also shown.

Plate 6. A section of the intestine similar to Plate 5 but stained with Giemsa-Wright stain. The tubules of the testes are dark blue, the intestine proper is lighter blue, and secretions of the intestinal mucosa are lavender.
proliferating at the two ends of the heart; at the same time, mature cardiac cells degenerate and are cast off into the pericardial body.

The pericardial body is an unattached mass of cells moving freely with each heart beat in the fluid of the pericardium. In the past, it has been variously described as a protozoan parasite (Cardiosporidium oedemae), as agglutinated blood cells, or as a specialized gland.

Although some workers using methylene blue have claimed to have found ganglion cells in the heart (Hunter, 1902, 1903), recent work suggests that they mistook for neurons, cells of the undifferentiated line, which is composed of connective tissue and lies opposite the raphé. Recent work on the EKG of isolated hearts suggests that extrinsic innervation is unlikely (Scudder, Akers and Karczmar, 1963).

2. Interesting Cytological Details Observed in this Laboratory

In this laboratory it has been found that when the cardiac cells are appropriately stained with urea-silver nitrate in a technique specifically designed to show dendrites and nerve endings in mammalian tissue, they show neuron-like processes linking one to another (see Plates 9 and 10). The cells seem to be specialized into an inner contractile striated fiber running in a helical coil about the lumen of the heart, and an outer portion containing the cyton with its nucleus, which communicates with other cardiac cells by means of a neuron-like net work. The
FIGURE 4

Car.rh.

Ht.

Pc.

Pc. b.
FIGURE 4

A drawing of the heart of *Cliona intestinalis* (after Millar, 1953).

Pc.................. pericardial sinus
Po. b............... pericardial body
Ht.................. heart
Car. rh............. cardiac raphe
Plate 7. An oil immersion photomicrograph of the striations of the heart (800X); Harris' Hematoxylin and eosin stain. Each unit of the marking scale represents 1.1 micron. The heart striations average 1 micron where they are most clearly visible.

Plate 8. An oil immersion photomicrograph of an eight micron cross section through the heart wall. The cardiac cell bodies lie above their contractile fibers which form a helix about the lumen and are joined to them by a cytoplasmic neck.
Plate 9. This is a urea-silver nitrate stained section of the heart cut from the side of the cardiac tube. This is, therefore, a section which allows many fibers to be seen throughout their length. Striations on certain of the fibers are visible (oil immersion).

Plate 10. This is the same section and region photographed in Plate 9, but here the focal plane is above the fiber level showing the cardiac cell bodies and their dendrite-like interconnections which suggest a primitive Purkinje system.
implications of this finding are discussed in the context of effects of drugs on the tunicate electrocardiogram (see V).

D. THE BLOOD OF CIONA

1. General Observations in the Literature

There is a surprising amount of literature dealing with the histology of the blood of ascidians. The earlier work dealt largely with the blood pigments believed to be associated with respiration; much of this earlier work has not been confirmed. One of the most up-to-date reviews on the subject is by Webb (1939) who, along with Robertson (1939), was interested in the ion content of the body fluids of marine organisms. Webb introduced the term vanadocyte for certain blood cells.

The vanadocytes comprise sixty per cent of the blood elements of tunicates, and are characterized by a morula form, greenish color, and the ability to rapidly reduce osmium tetroxide on contact. True vanadocytes fitting the above description are not found in Ciona. The only brightly colored cells in Ciona blood are the orange cells described later in this section. Also absent from Ciona is a high concentration of sulfuric acid, which in other tunicates is found in the vanadocytes (1.38 normal or 9 per cent). This lack of true vanadocytes and of a high sulfuric acid concentration is considered by Webb to be a primitive trait, and he has constructed a phylogenetic tree of contemporary tunicate inter-relationships based on their hematology.
Because *Ciona* differs from other forms so radically in its haematology, the classical studies of Heeht (1918b) on *Ascidia* blood should not be generalized to include *Ciona*. His assumptions as to the respiratory function of acroglobin (the vanadium-bearing pigment found in vanadocytes) and with regards to blood color changes, which he observed in tunicates with varying states of oxidation, have not born the test of time. Indeed, statements that acroglobin is a respiratory pigment have been disproved (Winterstein, 1909 and Florkin, 1934). Many unusual pigments exist in tunicates (Herdman, 1913a, 1913b); they bear no proven relationship to respiration and may be quite varied in composition (Lederer, 1934). The respiratory rate of tunicates is very low as might be expected (Vernon, 1895). Alsberg (1914) and Malouf (1937) have both shown that even in marine aniamls with well developed respiratory pigments (*Limulus*) there may still be as much dependence on oxygen pressure for blood saturation as found in those forms which have no oxygen carriers.

2. Observations from this Laboratory

In our laboratory, we have been unable to confirm the green cells described by George (1926, 1934, 1939) and we have found that the orange cells, which he describes as scanty, are quite numerous.

Present data bear close agreement with the blood picture presented by Millar (1953). Rarely in fresh blood were cells found that did not fit into the classification (cf. Plate 14)
which follows:

1. Heamoblasts: 4.4 micron cells, basophilic, granular, often mitotic. These are the stem mother cells for all haematopoiesis.

2. Lymphocytes: 3.5 micron cells with a 2.2 micron nucleus, no nucleolus, formed from heamoblasts and differentiating into all other blood elements.

3. Vesicular cells: 5 to 7 micron cells, amoeboid, containing a vacuole which is acidophilic when small, but becomes steadily less so as it grows.

4. Acidophilic granulocytes: 5.25 to 6.25 micron cells which bear acidophilic cytoplasm and ovoid spherical granules.

5. Nephrocytes: 5.25 to 6.25 micron cells which bear rounded or irregular basophilic granules that stain with haematoxylin.

6. Orange pigment cells: 5.5 to 6.0 micron cells, which contain an undetermined pigment variously described as a carotene, uric acid, or melamine derivative.

7. Hyaline amoebocytes: 5 to 9 micron cells, highly amoeboid. Many filiform pseudopodia were observed in this laboratory although Millar (1953) has described these as having lobose pseudopodia.

The blood cells are formed around the gut, in the transverse vessels of the branchial sac, and in the body wall near the test vessels. In these locations, many cells of the same type are
often found differentiating.

Most of the tunicate blood cells are amoeboïd. These cells wander freely through the tissues and by their active aggregation they are responsible for the clotting of blood. The blood plasma is alkaline, isotonic with sea water, and does not gel.

The orange cells of the blood are believed to become lodged in the body tissues and are responsible for color patterning such as exemplified by the yellow stripe around the siphon. Whether the red pigmented cells of the ocelli, of the pigment spot occasionally found over the intersiphonal organs and the pigment in the region of the genital pore are also derived from orange cells has not been proven. Although morphologically similar, they stain quite differently (see Plates 16, 53, 54, 55).

Phagocytic cells continually leave the mantle and invade the tunic. They are also reportedly discharged from the neural gland (Millar, 1955). Their appearance gives little clue to their functions which may be respiratory, nutritive, or hormonal (Andrew, 1962; Ries, 1938). In budding individuals most of the new zooids are formed from wandering mesenchymal cells.

Only the outer epithelium of a bud is formed from an evagination of the epicardial wall. Since these totipotent, wandering, mesenchymal cells all look very much alike and may bear numerous tiny filipodia, they may easily be mistaken for neurons in fixed preparations (see Plates 11 and 12).
Oil immersion photographs of live tunicate blood cells.

Plates 11 and 12. Two hyaline amoebocytes with filiform pseudopodia.

Plate 13. This very clearly shows a vesicular cell—the most common of the colorless blood cells.

Plate 14. Two orange cells, an immature vesicular cell and a morula, which is a type of cell not described in the literature for Ciona but structured similar to the vanadocyte of higher forms.
Plate 15. An oil immersion photomicrograph of a Harris' haematoxylin and eosin preparation of the testes sectioned at ten microns showing fixed and lightly stained vesicular elements. Particularly clear are the granules of the orange cells, the vesicular cells and lymphocytes. The small dark cells are primary and secondary spermatocytes.

Plate 16. An oil immersion photomicrograph of an eight micron section through the ocellus. Giemsa-Wright stain. The orange cells of the blood appear unchanged but the red ocellar cells (green lower cells of the photograph) which resemble structurally the orange cells of the blood in size and plastid distribution, are intensely meta-chromatic staining deep emerald green with Giemsa-Wright stain.
E. THE OVARY, OVUM, TESTES AND SPERM OF CIONA

The pear-shaped ovary of Ciona lies in the intestinal loop displaced to the left. Its epithelium differentiates into two types, germinal and ciliated. The ova pass from the ovary along an oviduct following the course of the gut, and are released into the atrial chambers by way of a gonopore located within a yellow, orange, or red pigmented spot (Millar, 1953). The living eggs in the ovary may appear red or green. The significance of the color is not known, but young eggs early in the season tend to be green, while older eggs are red (Berrill, 1924).

Oogenesis and ovulation have both been studied in great detail. A recent review on the cytology of Ciona eggs is by Reverberi (1960). The ovum (150 - 170 microns in diameter) is released into the water along with various accessory structures: (1) an inner layer of test or chorion cells, (2) a chorion, and (3) an outer layer of pyramidal follicular cells unique to Ciona (Costello, 1957).

The oocyte at ovulation has a thin clear cortical layer and a medullary layer of colored granules (yolk); and it is released in the metaphase of the first division (see Plates 17 and 18).

The formation of the yolk during oogenesis was described by Herschler (1917) who elaborated on a series of transformations of a "yolk nucleus" which generated both the yolk and also the mitochondria. This structure was not found by earlier workers (Morgan, 1891) and denied by Parat (1926) and Harvey (1927), who
concluded that the test cells generate the yolk or, at least, aid in its production. Refractile granules easily seen in the test cells disappear as the yolk forms (see Plates 1 and 19).

The outermost cells about a mature and ovulated ovum are the follicle or nurse cells. They are cone-shaped, spread at the base forming a hexagonal coating over the chorion. They bear a distinct granule at their tips (see Plate 20). In the electron microscope, they appear without mitochondria, endoplasmic reticulum, or ribosomes (Mancuso, 1959b).

The chorion is a non-cellular, single-layered membrane. Its derivation is from either chorion, egg, or follicle cell.

The origin of the inner test or chorion cells is unknown. Various investigators (Kupffer, 1870; Metchnikoff, 1872) believed that they originated from the egg nucleus. Others (van Beneden, 1884, 1886, 1887; Morgan, 1891; Bancroft, 1903; Harvey, 1927) described their origin from primitive follicle cells. In any case, they appear to migrate into the interior of the egg, release some unknown substance, and then return to form a cellular test about the ovum. At ovulation, the test cells have a clear nucleus and contain many refringent granules (see Plate 19).

Mancuso (1959b) centrifuged and stained Ciona eggs. He found five distinct layers in a centrifuged egg: (1) a lipoid layer, (2) a centripetal hyaline layer containing RNA, (3) a mitochondrial layer, (4) a yolk granule layer, and (5) a centrifugal layer containing RNA.
Such centrifuged eggs are still capable of normal mosaic development (La Spina, 1958). Eggs will develop under many diverse conditions. Small fragments, slightly over half the ovum size, will develop normally if they contain a nucleus (Ortolani, 1958). Even the hyaline fragment may cleave a few times (La Spina, 1960). Abate (1961) tried to determine if the cortex or the medulla contained the factors regulating development; and he concluded that the egg is a totipotent, self-organizing system, and that any endoplasmic vesicle, either from the cortex or the medullary ooplasm, is the equivalent of a complete ovum.

Oocyte development within the ovaries of Ascidiida and Ecteinascidia has been studied by Crowden (1961a, b). He cautions against generalizing from one species to another, but our observations on Ciona have allowed us to use his classification of egg stages. The first stage consists of small, large-nucleated, strongly basophilic eggs. The second stage is characterised by eggs with nucleoli much larger than nucleoli of stage I eggs. Finally, eggs of stage III have less cytoplasmic basophilia, nucleoli with smaller vacuoles, protein and yolk granules in the ooplasm and bear large test cells containing refringent vesicles.

Crowden suggests that the decreased basophilia of the maturing ovum represents a decreased RNA synthesis. The RNA of a mosaic egg is presumably set up early. The RNA distribution of C. intestinalis eggs has actually been studied from the egg to the tadpole stage (Mancuso, 1959a; Crowden, 1961a). Using the
intensity of Feulgen reaction of interphase nuclei as a criterion of RNA concentration, no new RNA appears before metamorphosis in Ciona except for local accumulations in some mesenchymal cells and in the adhesive papillae. Supporting the hypothesis that all the RNA of Ciona is set up early in development is the fact that the uptake of radioactive adenine, phenylalanine, and methionine, is highest during the early cleavages.

Studies on the testis are much less numerous than those on the ovary. The testis is a diffuse gland wrapped about the endothelium of the stomach and proximal intestine. Sperm are produced by mitotic division of cells of the epithelium of the terminal chambers of the testes tubules. They pass along a system of anastomosing vasa efferentia which lead to two main channels, one from the gut and the other from the stomach. These two tubules join to form one vas deferens which follows a course along the intestine and the rectum, next to the oviduct, to an opening into the atrial chamber. The sperm have a three micron head and a fifty micron tail. The head has a central deep staining rod containing chromatin and a somewhat irregular cytoplasmic mass around it (Millar, 1953; also see Plate 22).

F. THE MUSCLES OF CIONA

In contrast to other tunicate genera, Ciona has relatively scanty and simple musculature. All muscles except the heart are smooth. They are arranged about the barrel-like body in two
Plates 17 and 18. Live tunicate eggs removed from the oviduct and placed in sea water. Low power photomicrograph. Unstained. Plate 17 - dark field; Plate 18 - transmitted light. In both plates it is possible to see the cortex and medulla of the ovum, the chorion, the chorion cells, the nurse cells. The terminal granules of the nurse cells are visible in some cases.
PLATES 19 - 21

Stained middle power views of tunicate ova.

Plate 19. Basic fuchsin-methylene blue stain. This plate shows clearly the complex internal structure of the nucleolus and the many glistening refringent bodies in the chorion cells of this stage.

Plate 20. Giemsa-Wright stain. This plate illustrates the heterochromatic staining of the highly alveolar cytoplasm of the nurse cells.

Plate 21. Cresyl fast violet-toluidine blue-thionine stain. Complex subdivision of the nucleolus is shown clearly.
Plate 22. Middle power photomicrograph of an eight micron section of a testes tubule of tunicate. Harris' haematoxylin and eosin. Note orange blood cell in upper right. Differentiating sperm can be seen in lower left of the photograph.
layers, an outer series of longitudinal bands and an inner group of circular muscles (Fig. 5). The latter are much reduced except where they occur as sphincters of the siphons; even in this site they form a diffuse net, not a clear band of tissue.

There are usually five longitudinal muscle bands on either side of the pharynx running from the base of the animal to the siphon edges. They have been numbered by Millar (1953) as bands L1 to L5 as they lie on both sides of the animal counting from the most ventral to the most dorsal band. Pairs L1 to L3 serve the oral siphon with L3 split at the level of the tentacles to form L3a and L3b, and present a total of eight muscle bands to the eight oral siphon lobes. L4 and L5 bend in one anterior-dorsal direction. L5 splits into two bands, L5a and L5b, and these furnish the six lobes of the atrial siphon with sixty-four retractor muscle bands.

There are no longitudinal muscles in the ocellar region of the siphon. In the oral siphon at the level of the tentacles many longitudinal muscles move inward so that they may lie under the circular muscle band layers (see Plates 23, 24 and Fig. 5).

Each muscle band is composed of strands and each of these contains from four to twelve (usually eight) smooth muscle fibers arranged radially and interspersed with connective tissue and nerve cells (see Plate 25). The exact innervation of the muscles is accomplished by a nerve net. No clear cut plexi, ganglia, or nerve terminations have been found (see IIB; Plate 4).
Plate 23. Low power photomicrograph of a 10 micron cross section of tunicate smooth muscle in the region of the atrium. The stain is Alizarine Red S. Both longitudinal and circular muscles are visible, the former appear as clusters of fibers.

Plate 24. Low power photomicrograph of an oblique section of tunicate smooth muscle fibers in the region of the siphon stained with Giemsa-Wright stain and sectioned at 12 micron.
Plate 25. A middle power photomicrograph of tunicate smooth muscle fibers showing the arrangement of the longitudinal muscles into bundles. Between the bundles lie the tiny neuron-like structures shown in Plate 4. Giemsa-Wright stain.
FIGURE 5

A drawing (from Millar, 1953) illustrating the arrangement of the muscles of the oral siphon.

Oc..................ocellus
Per. bd..............peripharyngeal band
L.1, 2, 3a, 3b......longitudinal muscles
V. lon. mu...........ventral longitudinal muscles
Ant. es..............anterior endostyle
Ten. mu..............sphinctor muscles of the tentacles
Cir. mu...............circular muscle bands
CHAPTER III
THE DISTRIBUTION AND SIGNIFICANCE OF ACETYL-
CHOLINESTERASE IN CIONA INTESTINALIS

A. INTRODUCTION

Few workers have investigated acetylcholine, acetylcholinesterase, and cholinergic transmission in tunicates, although investigators studying the heart and its presumed autonomic innervation were concerned with this problem indirectly (Scudder, 1963). Only Bacq (1935a, b, c; 1937a, b; 1939, 1947), Durante (1956, 1957, 1958, 1959) and Florey (1963) have worked on this cholinergic problem directly.

Durante's effects were concentrated almost entirely on the larvae and embryos of Ciona, using the Gomori (1952) modification of the Koelle technique. Durante found cholinesterase to appear in the morula; then, as the embryo developed, the esterase was localized in the muscles of the tail and was completely lost at metamorphosis when the tail and larval action system disintegrated. Cholinesterase inhibitors of varying concentration such as eserine (0.004 - 0.008 per cent) and neostigmine (0.24 - 0.086 per cent) caused a progressive weakening of the larvae. Diisopropyl phosphofluoridate (DFP) had unusual effects in that although increasing doses gradually depressed the larvae and
caused paralysis, the effect was reversible after half an hour in sea water. DFP had no effect on the staining of the larvae by the Gomori technique.

Bacq extracted acetylcholine from adult Cliona by the method of Chang and Gaddum (Chang, 1933) and assayed the extracts on a leech muscle strip with completely negative results. He also exposed known acetylcholine dilutions to the blood and tissues of Cliona and assayed the acetylcholine on a leech muscle strip to determine the cholinesterase activity. He found none in the blood and a minimum (+ out of a possible ++++) in other tissues. On the basis of muscle strip studies (see VI) Bacq (1937) concluded that the concentration of acetylcholine necessary for contraction was so high that the measure of cholinergic nerves in the adult Cliona seemed unlikely.

Recently Florey has rechecked Bacq's negative findings on tunicates using Cliona adults from the same locality in which Bacq obtained his specimens. In his experiments, Florey excised the ganglia together with the neural glands and then dissected them free. Heated homogenates of these ganglia tested on a leech muscle strip gave a range of values from 22 to 120 µg acetylcholine per gram wet weight of tissue. Unboiled homogenates lost activity in ninety minutes, but eserine prevented this deterioration. Enzymatic destruction of acetylcholine was also prevented if the homogenates were made in sea water. Florey also found 0.01 to 0.2 micrograms of acetylcholine per gram wet weight of the body.
wall without the tunic which he assumed to indicate a very large amount of acetylcholine concentrated in the nerves.

B. METHOD

In this laboratory the histochemical method of Koelle (1951) with the modification introduced by Koelle in 1955 was employed to differentiate butyrylcholinesterase (BuChE) from acetylcholinesterase (AChE) in tunicate tissues. Living *Ciona* intestinalis from the Marine Biological Laboratory at Wood's Hole were used for these experiments. The following tissues were examined: ovary, heart (within the pericardium), the ganglion, the oral siphon, and a two mm piece, of the intestine from the first loop adjacent to the stomach. Control tissues (mouse brain, cat tibialis muscle, and frog olfactory bulb) were also used (see Plates 26 and 27). Each of these was frozen at minus 35°C and sectioned eight to fourteen microns. Since tunicate tissues have a very high water content, they are difficult to section from the frozen state. Embedding the tissues in 1, 5 or 10 per cent gelatin solution did not improve the sectioning.

The sections were placed on a clean glass slide and allowed to dry a few seconds depending on the amount of water present in the sections. The sections were then either pre-treated with inhibitor for one half hour at 38°C or placed directly into an incubation solution. The inhibitors, dissolved in 24 per cent *Na₂SO₄*, were as follows: physostigmine (3 x 10⁻⁶ g/ml),
neostigmine (10^{-6} \text{ g/ml}), \text{DPP} (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} \text{ g/ml}), \text{TEPP} (10^{-4} \text{ g/ml}), \text{BW 284} (3 \times 10^{-4} \text{ g/ml})^{1}, \text{and NU 683} (6 \times 10^{-7}, 6 \times 10^{-7} \text{ g/ml})^{2}.

After a half hour treatment with the inhibitors at 38{\textdegree}C the slides were placed in incubation solutions containing either butyryl thiocholine or acetylthiocholine as substrates and copper ion, magnesium ion, NA_{2}SO_{4}, copper thiocholine, and water in the amounts recommended by Koelle. The incubation solutions were run at 16{\textdegree}, 25{\textdegree}, and 38{\textdegree}C. The incubation times were varied from five minutes to twenty-four hours. Slides were also studied with no incubation.

After incubation the slides were briefly washed free of sulfate in copper thiocholine saturated solutions and were then developed in aqueous or alcoholic developer (Bull, 1951). The latter was found preferable. The developer was saturated with H_{2}S prior to development by bubbling through the solution, generating the gas by means of slightly warming an acidic solution of thioacetamide (Walton, 1959).

After development for two minutes the slides were dehydrated in absolute alcohol. At this time they were usually counterstained with alcoholic eosine. They were then cleared in xylol

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^{1}\text{This compound was obtained through the courtesy of Dr. H. T. Openshaw, Welcome Research Laboratory, Tuckahoe 7, New York.}

^{2}\text{This compound was obtained through the courtesy of Dr. John L. Schmidt, Hoffman-La Roche Inc., 120 S. La Salle Street, Chicago, Illinois.}
and mounted for study.

This technique on mammalian tissues serves to distinguish AChE from BuChE in the following way. Slides run without pre-treatment with any inhibitors stain when incubated with acetylthiocholine if either AChE or BuChE are present. Slides not pre-treated with inhibitors but incubated with butyryl thiocholine, stain if BuChE is present. Slides pre-treated with suitable concentrations of DFP which destroys BuChE much more rapidly than AChE, and incubated with acetylthiocholine, stain only if AChE is present. Slides pre-treated with the same concentration of DFP, but incubated with butyryl thiocholine, do not stain and serve as controls (Koelle, 1955).

The selective inhibitors BW284 and NU683 were employed as an added attempt to differentiate the AChE. These agents have been found at these concentrations to affect specifically the true cholinesterase in the peripheral ganglia of cat, monkey, and rabbit. Slides pre-treated with these agents and incubated with acetylthiocholine should be blank or at least somewhat inhibited if mammalian AChE were present.

C. RESULTS

All tunicate tissues incubated with butyrylthiocholine were blank.

Tissues incubated with acetylthiocholine with or without pre-treatment with inhibitors stained, but the pattern of staining
between the pre-treated and untreated groups varied in some organs. All tunicate tissues if incubated long enough were stained. Whole organs (such as the fragile heart) were almost black upon development (see Plate 28).

The effect of inhibitors was noted only in two places: (1) in the gut lumen and (2) in the cellular debris in the lumen of the neural gland. This cholinesterase which was rapid acting and affected by agents like physostigmine was probably not endogenous to the tunicate, but belonged to various crustacea and annelids which were either parasites or part of the tunicate's diet. The inhibitors had no effect on any of the staining observed elsewhere.

Sections of tunicate tissues incubated for only two hours were almost without stain, or, if staining occurred, it was diffuse throughout a tissue often appearing as a precipitate on the cells rather than intracellularly (see Plate 28). No evidence of neurons, endplate structures, or nerve nets could be found. The nuclei of stage three oocytes sometimes stained discretely as did certain of the vesicular cells (see Plates 28 and 30). On most of the slides the structureless plasma of the blood seemed to contain some esterase; and it was very difficult to be sure which cells had endogenous activity. Sections of ganglia were without much activity if left in incubation for less than two hours. If a thick smear of the ganglion was briefly dried and incubated, it showed a marked stain within two hours, but it was not localized
to the ganglion cells but rather the entire ganglion stained more or less evenly (see Plates 31, 32, 33 and 34).

As stated, the esterase of ganglia, blood or of oocytes was not inhibited by various inhibitors employed.

D. DISCUSSION

The above results indicate that rapid-acting, mammalian, AChE such as is inhibited by the classical inhibitors and found at the endplate in the cat or in the motor neurons of the mouse, is not present in the tunicate. What is present in Ciona is an acetylthiocholine-splitting agent which is not affected by inhibitors and which has an almost ubiquitous occurrence.

The control tissues run with the above technique showed clear staining of the AChE at the myoneural junction of the cat tibialis and tenuissimus muscles. Also, AChE was found in certain cells of the frog olfactory bulb and the motor nuclei of the mouse (see Plates 26 and 27). The staining of the mammalian tissues was very specific and discrete.

The existence of a cholinergic system in tunicate cannot be denied on the basis of these results, but such data, considered with other lines of investigation (see V and VI), makes it appear unlikely. This work tends to confirm the original conclusion of Bacq that cholinergic nerves do not exist in tunicates.

The work of Durante is also substantiated by our results in that sections of a young adult Ciona, unless incubated for a
considerable length of time show no cholinesterase. It should be stated that Durante (1956-1959) employed formalin fixation, which inhibits cholinesterases (Koelle, 1955, 1963; Karczmar, 1963). While it would be claimed that the absence of cholinesterase in Durante's preparation is due to his employment of formalin, the present study cannot be criticized on these grounds.

Florey's work led him to the opposite conclusions; namely, that there are large amounts of acetylcholine and cholinesterase present in tunicate ganglion and tissues. The reasons for his conclusions are discussed further in Ch. VI.

The findings of Florey that physostigmine did affect the concentration of acetylcholine which he measured in ganglion homogenated may have been due to contamination of his homogenates by foreign cells from the lumen of the neural gland where true cholinesterase may be found. It is almost impossible to free the ganglion completely from neural gland cells (see Ch. I, Plates 2 and 3).

The tunicate adult, unlike the larva, shows little morphological similarity to the chordates. The adult tunicate is a very old form and has evolved independent of the chordate line. The extreme antiquity of tunicates, the significance of their metamorphosis and body organization, and their behavior do not permit the assumption that adult tunicates should necessarily contain chemical systems similar to the chordates.

A central nervous system is necessary if an animal is to
possess large size and high mobility (Prosser, 1946). Presence of
AChE is, generally, implied by the presence of a central nervous
system and by mobility (Karczmar, 1963). Quite complex behavior
is possible, however, for small or sessile animals completely
without a central nervous system such as the coelenterata and
porifera. The amounts of cholinesterase in these forms need not
correlate directly with the amount of nervous tissue in these
forms (Karczmar, 1963). The behavior of Ciona (see Ch. VI) is
only slightly more complex than that of a sponge suggesting a very
simple nervous system. Finally, electrical transmission seems
possible in many simple forms, or even in certain restricted
synaptic areas of higher forms (Eccles, 1964).
Plate 26. Cholinesterase staining of a twelve micron section of *Fellis Tibialis* muscle endplates. Note the selective deposits of stain in this low power photomicrograph. The substrate was acetylthiocholine. The incubation time was two hours. The tissue was counterstained lightly with eosin.

Plate 27. A low power photomicrograph of a motor nucleus in an eight micron section of the medulla of *Mus* brain. The conditions and incubation time were similar to Plate 26.
Plate 28. A photomicrograph of the pericardium of the tunicate heart incubated six hours at 25°C in an acetylthiocholine substrate solution. Note the large, more or less hexagonal pericardial cells which are not in themselves highly stained. The copper sulfide is precipitated upon and between them. When whole pieces of any tunicate tissue are incubated for sufficient lengths of time, all showed some stain.
Plate 29. An oil immersion photomicrograph (800X) of the ovum of the tunicate from a twelve micron section of the ovary treated for two hours with acetylthiocholine. Slight staining occurred within bodies within the nucleus. In appearance these resembled chromosomes.

Plate 30. An oil immersion photomicrograph of blood cells of the tunicate exposed for two hours to acetylthiocholine substrate. Light staining of vascicular elements occurs.
Plate 31. An oil immersion photomicrograph of a smear of the ganglion of the tunicate. This thick preparation was incubated for two hours with acetylthiocholine. The cells here are difficult to identify.

Plate 32. Blood cells in a ganglion smear similar to Plate 31.
Plate 33. A low power photomicrograph of a whole ganglion placed upon a slide and teased. Such large pieces of tissue take a diffuse stain after two hours of incubation. This is not true of thin sections.

Plate 34. A low power photomicrograph of a thick (thirty microns) section of tunicate ganglion incubated six hours in acetylthiocholine. Staining is not particularly localized.
CHAPTER IV

ON THE HISTOCHEMICAL DETERMINATION OF MONOAMINE OXIDASE AND CATECHOLAMINES IN THE TISSUE OF CIONA INTESTINALIS

A. INTRODUCTION

Epinephrine accelerates the beat of the tunicate heart (Scudder, et al., 1963; see also V) and dopamine has been found in molluscan ganglia (Sweeney, 1963). Furthermore, tunicates observed in solutions of amphetamine and ephedrine seemed, in our laboratory, more reactive and turgid than controls suggesting that adrenergic transmission may exist in these animals.

On the basis of this evidence and the indications that true cholinesterase does not occur in tunicate tissues (Ch. III), that cholinergic agents and anticholinesterases do not affect the siphon withdrawal (Ch. VI), and that the heart is noncholinergic (Ch. V), we attempted to determine histochemically the distribution of monoamine oxidase and catecholamines in tunicate tissues.

B. MONOAMINE OXIDASE DETERMINATIONS

1. Methods

The procedure used for the histochemical determination of monoamine oxidase (MAO) in tissues was that described by Arioka (1957). Half millimeter pieces of tunicate brain, heart, siphon,
intestine, and ovary; equally small pieces of mouse brain (C57 Bl6 strain) were excised from adult specimens and used in this study.

The pieces were washed with m/15 phosphate buffer (pH 7.4) briefly and then incubated at 37°C for seven hours in an oxygenated incubation solution containing 5 mg/10 ml of 5-hydroxytryptamine creatinine sulfate at a pH of 7.4. Traces of copper and magnesium were added to the incubation fluid. Afterwards, the tissues were washed, dehydrated, cleared, and embedded in paraffin. Sections were cut at eight microns, de-paraffined in xylol, mounted without further treatment in Sargent's mounting fluid. To serve as controls, similar pieces of the same tissues were incubated without serotonin in the incubation solution and were also, in another experiment, incubated with serotonin after heating them for thirty minutes at 80°C in a pH 7.4 buffered solution.

Tissues of a tunicate exposed for four hours to a solution containing iproniazid (Marsilid), 25 mg/100 cc sea water, were also studied.

2. Results

All control tissues as well as the tissues from the Marsilid-treated animal were blank. The tissues which were not heated, but were subjected to incubation with serotonin, were quite visibly darkened. Tunicate tissues were stained by the incubation much more than were the mouse brain pieces.

The sectioned tunicate tissues showed staining in almost all cells. Tunicate tissues when not properly fixed undergo very
great distortions as they are dehydrated. However, evident staining had occurred in all cells, whereas in the mouse brain only the meninges and a few glia about vessels had stained.

**Siphon.** The smooth muscle of the siphon showed virtually no staining. In the same sections, however, the epidermis and outermost connective tissue about the muscle bands stained considerably (see Plate 35). The impression might lead to the conclusion that penetration of the substrate was incomplete, but with seven hours of incubation this appears unlikely.

**Ovary.** The chorion stained uniformly; nuclei of some eggs stained evenly. Yolky ooplasm was very pale. Test cells stained, but follicle cells were without stain. Scattered mesodermal cells and vascular cells stained heavily. In these slides as in the siphon sections it was the supportive mesentaries that stained most darkly. The oocyte was relatively free of MAO.

**Intestine.** The digestive endothelium was very faint. However, the cells of the tubules of the testes stained quite strongly. The cells which stained were not the sperm but cells lining the tubules. These are not germinal cells but connective in nature.

**Heart.** Cardiac cells stained quite darkly both in the heart and in the pericardium. The pericardium stained as intensely as the cardiac cells themselves.

**Ganglion.** The sections of the ganglia showed maximum staining in the connective tissue about the ganglion. There was a
slightly greater staining of the cortex as compared with the
medulla, but this was due perhaps to the fact that it is a more
dense tissue. The tissue of the epidermis and the lining of the
sinus about the brain was much darker, however, than any part of
the ganglion or neural gland.

3. Discussion

In applying this technique to tunicates the question arises
whether it is specific for monoamine oxidase. The staining of the
mouse brain slides resembled that described by Arioka (1957). The
color was concentrated about blood vessels and this led to Arioka's
statement that MAO was one of the components of the blood brain
barrier.

Obviously, the results in tunicates indicate that an oxidase
is present throughout the tunicate tissues which will oxidise
serotonin. Whether this is a true monoamine oxidase can be con-
cluded in part by its responses to inhibitors. Exposure of the
tunicate for four hours to a high concentration of Marsilid
caused disappearance of staining.

This strongly argues for the presence of an enzyme very
similar to MAO in the ganglion and other tunicate tissues. It may
even be an indication that adrenergic transmission exists in
tunicates; however, the distribution of this enzyme is in
connective tissue, the pericardium, the testes tubules, and not
localized to nervous structures. It may be functioning protec-
tively to prevent serotonin concentrations. The role of
Plate 35. Low power photomicrograph of the tunicate siphon stained for MAO using a serotonin substrate.
serotonin thus may be as a smooth muscle neurohumor with a diffuse activity.

C. THE CATECHOLAMINE HISTOCHEMISTRY STUDIES

It was hoped that the distribution of catechol amines could be determined histochemically by employing the recently published Falcoq technique (Falcoq, 1962, 1963; Carlson, 1961, 1962). This technique depends on the reaction with formaldehyde of the amines in freeze-dried tissues; intensely fluorescent isoquinoline derivatives are formed. The technique may not be entirely specific (personal communication from Dr. G. Koelle). For success of the technique, the tissues must not contain high amounts of auto-fluorescence.

1. Methods

Four procedures were run on pieces of tunicate ovary, siphon, ganglion, heart, and intestine, and on mouse spleen, adrenal, and mesentary proper.

a. Wet mounted tissues: Tissues were examined mounted wet either as frozen sections (cut at minus 35°C at 10 u) or as sheets spread out under a non-fluorescence. The fluorescent microscope used was equipped with non-fluorescent objectives and Kodac-Wratten filters (No. 15) in the ocular tubes. These filters have high absorption of light between 510 and 490 millimicrons, respectively. The exciting light was supplied by an Osram high-pressure mercury lamp and filtered through a narrow band pass
Wratten filter No. 43.

b. Dried tissues: Tissues were examined after drying on the slide without further treatment.

c. Freeze-dried, sectioned, and xylol-treated tissues: The tissues were examined after being freeze-dried in vacuo at minus 70°C and infiltrated with paraffin in vacuo. The paraffin was removed from these sections for microscopic examination by treatment with xylol.

d. Paraformaldehyde-treated tissues: The tissues were examined after the above treatment (see c. above), but with the added step of one hour's treatment in a closed reaction vessel at 80°C prior to embedding in paraffin in vacuo. The heated paraformaldehyde forms formaldehyde gas which reacts with the amines to form highly fluorescent compounds (Falcq technique).

2. Results

The results of the above studies were unexpected. In the literature survey no mention had been found of the high fluorescence of tunicate tissues. This autofluorescence is in the range of green to yellow, specific for the identification of catecholamines in mammalian tissues by the Falcq technique.

a. Wet mounted tissues: High autofluorescence was found in the intestinal contents of some tunicates, in all tissues investigated which contained blood cells, and in the cells of the yellow band of the siphon. Mild fluorescence occurred in the cortex of the egg and in the test cells, and there was high fluorescence in
the terminal vacuole at the tips of the follicle cells. Muscles, connective tissue, heart cells, and ocelli, were either invisible or reflected the strong fluorescence of the other cells. These tissues began to fluoresce as they dried on the slide (see Plates 36, 37, 38, 39, and 40).

b. Dried tissues: As the tunicate tissues dried on the slide, fluorescence began to appear in the connective tissue and the smooth muscles, and, generally, in almost all cells of the animal in varying degrees. The fluorescence was usually yellow or green and varied in intensity in different cells. Some brownish or reddish fluorescence was observed in the heart tube as it dried (see Plates 41 and 42).

c. Xylo! and paraformaldehyde-treated tissues: Slides dehydrated by freeze-drying or by the conventional treatment of alcohol concentrations followed by xylo! were strongly fluorescent. This fluorescence was as intense as that resulting from paraformaldehyde treatment, both in mammalian and tunicate tissues. In the mammalian slides, the nerve plexi about arterioles and venules was visible clearly in both formaldehyde-treated and xylo!-treated slides. On tunicate tissues the formaldehyde treatment tended to decrease fluorescence and clarity (see Plates 43 - 52).

It was found in the mammalian tissues that the Falco technique (paraformaldehyde) was difficult to reproduce. The results shown by photographs in his paper (Falco, 1962) were also seen in
this laboratory, but the clarity and amount of fluorescence was only slightly greater than that seen in frozen sections dried and treated with xylol. Since tunicate tissues were found to be so highly fluorescent, further investigations on the nature of artifacts resulting from Falcq's technique are necessary before any conclusions with regard to catechol amines of tunicate tissues are possible.

3. Conclusions

It was concluded from these studies that the Falcq technique was not applicable to tunicates because of their high auto-fluorescence when dried. This intense fluorescence appears as yellow or whitish-yellow in the muscles and certain blood and vacuolate cells. Most of the other tissues such as the neural gland, brain, connective tissue, ovary, various mesenteries, etc. fluoresced green when dry. No attempt has been made as yet to determine the cause for this fluorescence.
Plate 36. Autofluorescence of a live tunicate egg removed from the oviduct. Ooplasm is mildly fluorescent, chorion and chorion cells somewhat more fluorescent. The intense yellow spots about the egg are the terminal vacuoles of the follicle cells.

Plate 37. Autofluorescence of the yellow line around the termination of the oral siphon. Note the scattered yellow blood cells near the edge of the siphon. The red pigment spots of the ocelli did not fluoresce. This is a wet mount of fresh tissue under a cover slip.
Plate 38. Fluorescence of vascular elements posterior to the yellow margin of the oral siphon. Note that the layers of smooth muscle bands are not fluorescent in a wet mount like this.

Plate 39. Autofluorescence in a specimen of living tunicate blood from the heart. The fluorescent elements are primarily vesicular cells.

Plate 40. Autofluorescence of cells in the vascular incunae of the pharynx. Note that the fluorescence varies both in intensity and color. Smooth muscle and connective tissue do not fluoresce in the wet mount.
Plate 41. Fluorescence of the tunicate pericardium dried on a slide for two hours at room temperature.

Plate 42. The fluorescence of a pericardial body dried for four hours on a slide.
Plate 43. The Falcoq technique employed on the mouse spleen. The fluorescence of the capsule and occasional large cells is visible.

Plate 44. A low power photograph of the muscles of the tunicate siphon using daylight illumination with a ground glass blue filter. This tissue was frozen and dried at minus 70°C, in vacuo for two days, and then sectioned at eight microns after embedding in vacuo in degassed paraffin (Falcoq technique).

Plate 45. The same section of the siphon illustrated in Plate 44 seen under ultra violet light. All smooth muscle fibers fluoresce an intense yellow (Falcoq technique).
Plate 46. An eight micron section of tunicate siphon after treatment with the Falco technique. The fluorescence is not visibly changed from that of the dehydrated control.

Plate 47. Autofluorescence of an eight micron section of a freeze-dried tunicate brain. Note the blood sinus and the neural gland ventral to it (Falco technique).

Plate 48. A photomicrograph of an eight micron section through the ganglion (to the middle right), the neural gland (lower), and some smooth muscle fibers (intense fluorescence on the left) (Falco technique).

Plate 49. Autofluorescence of an eight micron section of a freeze-dried ganglion (upper left), neural gland (lower half of picture) and intensely fluorescing muscle (Falco technique).
Plate 50. Autofluorescence in a dried xylol-treated fragment of the tunicate heart (Falcq technique).

Plate 51. Autofluorescence of tunicate eggs from the ovary dried on a slide and treated with xylol. The chorion cells appear intensely fluorescent (Falcq technique).

Plate 52. This is a conventionally prepared slide of the ovary stained with basic fuchsin-methylene blue and photographed with a wide band-pass filter on the ultra violet light source. The yellow fluorescence of granules within the chorion cells is visible (see II, Plate 19) (Falcq technique).
A BRIEF INTRODUCTION TO CHAPTERS V AND VI

The peculiar lack of cholinesterase in tunicate tissues (Ch. II) suggests that tunicates may not be cholinergic. The following two chapters present two other lines of investigation that we have undertaken to help substantiate this hypothesis. Chapter V presents studies of the effects of pharmacological agents upon the control of the heart, and Chapter VI presents a study of the effects of drugs on the total behavior of the organism; in particular, on the contraction of the siphon.
CHAPTER V

THE EFFECTS OF DRUGS UPON THE ELECTROCARDIOGRAM
OF CIONA INTESTINALIS

A. INTRODUCTION

The tunicate heart is the only striated muscle found in the adult animal (see Ch. II). It was proposed that this organ, therefore, might serve to record the influence of cholinergic agents.

B. THE PHYSIOLOGY OF THE TUNICATE HEART

1. Its Origin and Unique Beat Reversal

The heart and circulation of the tunicate have been the subject of scientific investigation many times in the past, ever since it was observed that the heart could reverse its polarity and direction of beat. This phenomenon is not altogether unique in the animal kingdom. Embryonic hearts of the snails Limax and Planorbis, and also the arrow worm Phoronis reverse their beats periodically. Lepeoptheiris pectoralis (a parasitic copepod) shows beat reversal in the adult (Haywood, 1950).

Embryologically, the heart is probably entirely mesodermal. (van Beneden, 1887; Longchamp, 1901). Its origin is from the pericardium which in turn is derived from the earlier fusion of two mesodermal masses.
Roule (1882) described in detail the heart and circulation of *Ciona*. He divided the circulation into three main parts: the cardiosplanchnic, the splanchnobranchial, the branchiocardiac. These form a continuous closed circulatory system. Enrique (1904) placed oil drops in the vessels and found that they moved more often in relation to changes in the body pressure than to heart beat. He suggested, therefore, that the heart may not be important to circulation. Recently, Millar (1953) using latex injections in the vessels, has come to a different conclusion. Millar did not doubt the important function of the *Ciona* heart in circulation, and he suggested a circulation time as brief as one minute.

2. Nervous Control of the Tunicate Heart

The presence of nerves or of nervous control of the heart has been a subject of debate. Hunter (1902 and 1903) claimed to have seen nervous tissue in the *Molgula* heart stained with methylene blue. He localized this tissue in either end of the heart, and traced fibers leading from the nervous tissues to the dorsal nerve chain. He also supported his observations with evidence that nerve poisons "curari", nicotine, and muscarine affected the heart. On the other hand, Basq (1935a, 1935b) removed the cerebral ganglion of *Ciona* and found no change in heart rate.

Day (1921) found that cutting the animal and causing hemorrhage would increase the heart rate. This effect was
independent of the ganglion. He stated that the heart was free of innervation and responded only to changes in pressure.

Millar (1952) found no nervous differentiation in Ciona heart. From the experiments that follow it is obvious that the heart can function free of the body.

3. Intrinsic Pace-maker Activity of Tunicate Hearts

Von Skramlik (1926) believed the Ciona heart had two pace-makers, an abvisceral and an adviserial one. He postulated that these pace-makers were situated at the two ends of the heart where Hunter had seen ganglion cells.

Nicolae (1908) found that the rates of the two pace-makers varied in regard to their responses to temperature and drug effects.

Quincke (1932) suggested that any part of the heart could have a pace-maker function.

Bancroft (1903) postulated a theory of physiological polarization. He stated that no matter which end of the heart initiates the beat, all subsequent points along the heart relate to this end and the beat begins from its direction even when the heart is cut. He suggested that the cardiac cells are polarized in some way across the short axis of their fibers as they wind about the heart axis. Recently, work in our laboratory (Ch. I, Cardiac Histology) has revealed the cytological details whereby this cellular communication and polarization could be established.

4. Back Pressure Theory of Cardiac Reversal
Early workers such as Enrique (1904) suggested that the cause of tunicate heart reversal was due to a pressure build-up in the circulation. This theory is frequently quoted, but pressure measurements have never been made. The appearance of the anatomy does not suggest any reason why pressure should mount up with continued beating of the heart.

Haywood (1950) carried this back pressure theory, as it was called, to its limit. He developed long and complex formulae to relate beat reversal with pressure build-up. The system of Haywood, however, does not fit the physiological evidence such as it is (Krijgsman, 1955).

Recently, Krijgsman (1952, 1955, 1956) has published reviews on pace-maker mechanisms in invertebrates and has called attention to the peculiar properties of tunicate hearts; they are: its lack of nerves, unusually scanty musculature, unknown venous and arterial pressures, and unusual polarization and autonomy.

5. Temperature Effects on Ciona Heart

The effect of temperature on the Ciona heart was studied specifically by von Skramlik (1926) and by Wolf (1933). The former found the frequency lowest at 18°C and increasing both below and above this point. The latter found a steady increase in temperature from 10° to 30°C with slight fluctuations of the slope occurring at 10°, 15° and 20°C, which he concluded were temperature effects on three different fundamental chemical processes involved in the beat.
6. Drug Effects on Ciona Heart

Drug effects on tunicate hearts have been studied by several investigators (Krijgsman, 1956, 1959; Waterman, 1939, 1942, 1943; Bacq, 1935c).

Waterman found that epinephrine accelerated Salpa hearts. Acetylcholine increased the rate and dominance of the abvisceral pace-maker at low doses and caused irregular beating at higher doses. Methylcholine, epinephrine, strychnine, colchicine, and atropine depressed both pace-makers. Physostigmine increased cardiac rate; nicotine and pilocarpine slowed it.

Bacq using Ciona found that epinephrine, acetylcholine, and ergotamine in concentrations of $10^{-4}$ to $10^{-6}$ g/ml did not change the frequency; and higher concentrations stopped the heart.

Krijgsman found that acetylcholine ($10^{-8}$ to $10^{-3}$ g/ml) had little noticeable affect. At $10^{-6}$ g/ml, slight stimulation occurred, whereas at $10^{-3}$ g/ml, there was weak inhibition. Physostigmine was without affect on the visually observed heart rate. Krijgsman could find no differential effect on the two "pace-makers."

All of the above studies on rates, temperature and drug effects on the Ciona heart have been carried out by visual observation. The contribution of the present research was to study the effects of drugs by means of the electrocardiogram, a method permitting greater quantitative accuracy in the analysis and control of ambient parameters.
C. METHODS

*Ciona intestinalis* collected at Wood's Hole Marine Laboratory were used for these experiments. Relatively large, mature animals about 10 cm long were placed in a Petri dish. The mantle was opened according to a technique described by von Skramlik (1926). The visceral and hypobranchial vessels were ligated and cut distal to the heart and pericardium; and the pericardial sac containing the heart was removed. The pericardial fluid functioned in this preparation as a volume conductor, and the electrocardiogram, recorded as described below, consisted of phases of depolarization and repolarization along the heart itself (see Fig. 6).

The pericardial sac was placed in a moist chamber made of transparent plastic and provided with two firmly anchored electrodes. The electrodes were parallel to one another, 3 mm apart, insulated except for their tips, and applied directly to the pericardium. The heart thus bridged the two electrodes. The electrodes led to a pen writing Grass oscillograph via a Grass EEG preamplifier and power amplifier. The noise level at the sensitivity employed was less than 0.1 microvolt (Fig. 6).

The chemical agents employed were dissolved in sea water and the solutions, kept at appropriate temperatures, were dripped on the pericardial sac. Washings were also made with sea water.

D. RESULTS

The isolated tunicate heart exhibited electrical activity
which could be described as follows (Figs. 6a and 7). The most prominent finding was that of a diphasic depolarization wave ("spike"). The amplitude of the spike was dependent on the positions of the electrodes; however, no well defined position produced consistent high amplitude spiking in all animals. In particular, the position of the raphe relative to that of the electrodes did not appear to influence the spike amplitude. The average amplitude of the spike varied between 30 and 50 microvolts. At 28°C the average interval between spikes was 1.9 seconds. Often the heart exhibited a series of high frequency spikes lasting for ten seconds. Additional low amplitude (10 to 20 microvolt) spikes, unrelated to the visually observed heart beat appeared frequently (Fig. 6a). Finally, the normal heart showed sustained, low amplitude (less than 10 microvolt) electrical activity (cf. for instance, Fig. 6a). This activity was found to fluctuate with the physiological state of the heart; it disappeared in a failing heart even when spikes were present.

An interesting finding pertains to a comparison between visually observed cardiac beats on the one hand and the electrical activity of the heart on the other. Generally, the visually observed cardiac beats corresponded with the 30 to 50 microvolt spikes. However, occasionally the heart appeared quiescent while good electrical activity, at the usual rate and unchanged in pattern, could be recorded. When, by visual observation, the origin of the beats appeared to reverse, alternating from
advisceral to abvisceral pace-maker, the reversal was not accompanied by changes in the electrocardiogram.

Acetylcholine \((2 \times 10^{-3} \text{ to } 2 \times 10^{-6} \text{ g/ml}; 20 \text{ hearts})\) caused no change in the amplitude and slight slowing in frequency of spikes. A slight increase in spike duration was occasionally noted with higher concentration of acetylcholine (Fig. 7). This finding confirms observations of Krijgsmann (1959) of the feeble action of acetylcholine upon the tunicate heart.

The electrical activity of the tunicate heart changed considerably when epinephrine \((1:1000; 20 \text{ hearts})\) was applied (Fig. 6B). The rate increased threefold and the amplitude of the spike was decreased to 10 microvolts, a three to five fold decrease from the control amplitude. In the case of hearts with an irregular pattern of electrical activity and of spike frequency, epinephrine frequently re-established regular rhythm.

The effects of physostigmine \((3 \times 10^{-3} \text{ g/ml}; 11 \text{ hearts})\), pilocarpine \((2 \times 10^{-3} \text{ g/ml}; 5 \text{ hearts})\) and procaine \((5 \times 10^{-2} \text{ g/ml}; 2 \text{ hearts})\) were essentially similar in that these drugs all produced an increase in spike duration. Pilocarpine and procaine in particular produced a pronounced spreading of the spike. Procaine also occasionally brought about a peculiar alternation of rapid bursts of spikes followed by periods of comparative electrical silence. This phenomenon may relate to the de-sensitizing properties of procaine which, at the concentration employed, effectively and reversibly anesthetized Ciona.
In additional experiments, isolated hearts were left in the apparatus for several hours. The hearts showed deterioration after a period of three hours; the spike frequency and the amplitude became highly variable. The regularity of spike amplitude and frequency could be restored by digitalis (0.2 mg/ml; 4 hearts; Fig. 6D).

D. DISCUSSION

The evaluation of the literature on the pharmacology of the tunicate heart is difficult (Prosser, 1961; and Krijgsman, 1956). There may be several reasons for this. In the first place, different species have been used, and it is quite possible that the actions of drugs is not similar in all genera of tunicates. Thus, pilocarpine depressed the heart of Perophora (Waterman, 1939, 1943), while in the present study of Ciona, pilocarpine, like procaine, did not depress the heart rate significantly although it did cause spreading of the spike. The argument for external innervation of the tunicate heart was based on the data of Hunter (1902, 1903), indicating that nicotine and "curari" may stop the Molgula heart. Yet the heart of Salpa is reported accelerated by nicotine (Krijgsman, 1956). Epinephrine has been reported to cause acceleration without block in the hearts of Salpa and Molgula (Nicolae, 1908), acceleration with subsequent block in Polycitor heart, and only block and disorganization of beat in Perophora (Waterman, 1939, 1942). Epinephrine has been reported
to block also the heart of Ciona (Bacq, 1947). These species differences may exist not only in regard to transmitter-like agents but in regard to other drugs too. For instance, Waterman (1939) found an inconsistent action of a digitalis-like agent in Perophora, while we found digitalis effective in Ciona.

The second reason for the differences which have been observed may be due to the fact that various investigators used widely different concentrations. Furthermore, frequently the concentrations were so high that they could easily cause toxic rather than pharmacological effects. It should be noted that the lack of response of Ciona heart to physostigmine is not surprising since the tunicate heart contains no AChE. On the other hand, other than AChE or BuChE, esterases are present in the heart which may be involved in cardiac function and which are not inhibited by AChE and BuChE inhibitors (see Ch. III).

In the third place, a significant source of disagreement may due to the fact that visual observation is not reliable with regard to the study of drug action on the tunicate beat.

Visual counting is difficult because of the possible occurrence of partial beats and irregular tremors of the endothelium, because of the reversal of the origin of beat, and because the appearance of the contractile wave varies from beat to beat. The present study indicated not only that regular spikes could be observed in the presence of many of the above activities, but also that spikes persisted in a visually quiescent heart.
Altogether, these results add dependable support to the general impression that acetylcholine has a feeble effect on the heart of Ciona. It is of interest that this non-innervated heart seems to be sensitive to another transmitter, epinephrine. In this context, our data do not agree with that of Baoq (1935a, b, c) who found that in Ciona epinephrine produced cardiac standstill at concentrations that in our preparations readily caused rate acceleration as well as regularization of the beat of a failing heart. Actually, epinephrine may decrease the amplitude of pulsations and this renders the visual observation difficult.

Another interesting aspect of the method employed at present is that it revealed an effect of several drugs on the shape of the spikes, and the pattern of electrical activity of the heart. Further studies along this line have not yet been carried out.
Electrocardiograms of isolated hearts of *Ciona intestinalis*. A, Electrocardiogram obtained from a control heart, maintained for $\frac{1}{2}$ hr in sea water (28°C). Notice 30–50 µV spikes; occasionally low amplitude spikes; and background activity (cf. also text). B, Effects of epinephrine, 1 : 1000 (28°C). C–D, Effect of digitalis, 0.2 mg/cm³, upon a failing heart (28°C).

**FIGURE 6**

The normal electrocardiogram of the tunicate and the effects of epinephrine and digitalis on the failing heart.
The effect of acetylcholine (ACH), $10^{-6}\text{ g/ml}$, on the normal electrocardiogram of *Ciona intestinalis*, at 28°C.

**FIGURE 7**

The effect of acetylcholine on the electrocardiogram of the tunicate.
CHAPTER VI

THE EFFECTS OF DRUGS ON THE BEHAVIOR

OF CIONA INTESTINALIS

A. INTRODUCTION

In a previous chapter we have shown that AChE does not appear to be present in the tissues of the tunicate adult (Ch. III) and that the striated muscle of the tunicate heart seems to be without innervation and not affected by cholinergic agent (Ch. V). The problem of whether the smooth muscle of tunicates is cholinergically innervated or not has recently become a matter of controversy. Early work (Bacq, 1935a, b, 1937a, b, c, 1939, 1941, etc.) led to the conclusion that cholinergic nerves did not exist in tunicates and that their tissues were free of acetylcholine and contained relatively little cholinesterase.

Bacq, using an electrical stimulus to induce a pharyngeal muscle strip to contract, showed that eserine produced no potentiation of the contraction and he found the muscle itself to be only weakly sensitive to acetylcholine.

Recently, these conclusions have been supported by Durante (1956, 1957, 1958, 1959) who found no cholinesterase at all in a newly metamorphosed adult Ciona. However, Florey (1963) found muscle strips quite sensitive to acetylcholine. He found tunicate
muscles responded easily to acetylcholine even at dilutions of $10^{-6}$ g/ml, but this effect was not potentiated by eserine. In his studies relating to the acetylcholine content of ganglia he found large variations (22 - 120 μ grams of acetylcholine per gram wet weight of tissue; see Ch. III). He concluded that cholinergic transmission was present in tunicates.

Visible movement in the adult tunicate is confined to the muscles of the pharynx and siphons which have been described previously (see Ch. II, F).

Nerve endings have not been clearly seen by other investigators, but a diffuse nerve net is presumed to exist (see Plate 4 and Fig. 5). The patterns of contraction of these muscles have been arbitrarily classified into reflexes. For example, in response to a stimulus on the oral siphon a tunicate may close the oral siphon only (an ipsilateral response) or it may close only the atrial siphon (a crossed response). If the stimulus is sufficient, both siphons may close. It is possible for a tunicate to leave its siphons open, close one, or close both and at the same time contract the muscles of the pharynx forcibly expelling water from the pharyngeal and cavities. Such an activity may clean debris from the siphons and the reflexes have been named oral egestion reflex, atrial egestion reflex, etc..

In this laboratory, we have not found these to be true reflexes in an all or none sense. The amount of closure of an electrically stimulated siphon increased with the parameters of
stimulation. With increasing stimulation more and more muscles were brought into play and a clear cut separation of different reflexes was not possible. These reflexes are, therefore, not true reflexes in an all or none sense.

Although there may be a slow growth of Ciona toward the light (Millar, 1953), and ovulation may be induced by light (Costello, 1957), no sudden response such as that described by Hecht (1918a) has been observed in this laboratory. The function of the ocelli as visual receptors has not been observed by us.

Studies in this laboratory have shown no innervation of the ocellus in agreement with recent workers (Millar, 1953).

The function of the ganglion of the tunicate has been disputed. Jordan (1908) stated that removal of the ganglion affected only the body tone and that "reflexes" were unaffected. However, Cate (1928) believed he had shown impairment of simultaneous closure, symmetry of closure, and co-ordination of closures following various incisions into the ganglion and the siphonal nerves. Frolich (1903) stated that the ganglion was necessary for prolonged closures and that without it the siphons opened immediately when they were closed.

In all the behavioral experiments involving whole tunicates, no quantitation of the stimulus was attempted by these workers, and visual observation alone had to suffice for their records. It was the object of our studies to develop a quantitative technique whereby we might study the activity of tunicate smooth muscle and
see the effects of ganglionectomy and various drugs upon it.

B. METHODS

Ciona intestinalis from the same locality and similar to the others used in this study were placed in the transparent plastic chamber of the apparatus shown in Fig. 8. The chamber held 50 cc of sea water and its temperature was monitored by means of a Waters needle thermistor which was placed close to the animal. A transparent heat absorbing shield was placed on top of the chamber to prevent heating of the animal and of the water by the photo-flood lamp several feet above it. Silver wire electrodes connected to a square wave stimulator were placed on either side of the siphon when the animal lay fully extended and at rest. Activation of the stimulator turned on both the signal light at the front of the chamber and a timer not shown in the figure.

The entire activity of the siphon was photographed by means of an 8 mm zoom lens camera with close-up lenses and a shutter speed of 24 frames/second. Fig. 9 shows a front view of the chamber as it would appear when photographed by the camera.

The parameters of stimulation were between 0 - 100 volts and 3 milliseconds, and 15 cycles per second, applied for two seconds. A threshold voltage which would produce complete siphon closure without contraction of the pharynx was established for each animal. The timer was allowed to run until the siphon returned to its original diameter. We defined this interval from the time of
stimulation until the return to the opened, resting state as the 0.0.0. ("open to open duration"). The stimulus latency was also measured.

Measurements were made from the photographic record by means of a Bell and Howell reader and a millimeter rule. Color film and a light purple paper background was employed to afford maximum color contrast with the yellow siphon for measurements of the diameter of the siphon from one ocellus to its opposite across the siphon. The first measurement was obtained from the last frame prior to the lighting of the stimulus signal lamp; the last measurement was taken at the point of maximum closure. The interval between frames was roughly 41 milliseconds. The siphon diameter measurements of successive frames were plotted against the time and the slope representing the rate of closure was obtained.

The ganglionectomy was carried out either surgically or by means of a Grass high frequency lesion apparatus.

All drugs were prepared fresh in sea water and were used immediately in the water bathing the animal.

C. RESULTS

1. Controls

Fig. 10 shows the typical results obtained from measuring three sequential closures of a single tunicate and plotting them on the same graph. It can be seen that the closure rate for any
one animal is constant and relatively unaffected by a small
temperature change. The stimulus latency and O.O.D. may be some-
what influenced by temperature, however.

Fig. 11 shows the average maximum rate of closure for 12
individuals as measured from the siphon diameter change.

2. Ganglionectomy

Immediately after ablation of the ganglion the animal is
flaccid, the siphons are crooked and limp, all responses are weak
or lacking, and there is a general loss of tone. After eight to
twenty-four hours the animal appears normal again to gross
observation.

Fig. 12 portrays the effect of ganglionectomy on closure.
These tests were conducted from one to three hours after removal
of the ganglion. The stimulation parameters had to be increased
tenfold to elicit a closure response. The ganglionectomized
animals closed slower and remained closed longer than the controls.
Contrary to observations by Frolich (1903), the control O.O.D. was
more than doubled by ganglionectomy.

3. Drug Effects

The drugs employed in these studies were; acetylcholine
\((2 \times 10^{-4} \text{ to } 10^{-2} \text{ g/cc})\), anticholinesterase agents (DFP, \(10^{-5} \text{ to } 10^{-3} \text{ g/cc}\); TEPP, \(10^{-3} \text{ g/cc}\); physostigmine, \(10^{-5} \text{ to } 10^{-3} \text{ g/cc}\);
neostigmine, \(10^{-5} \text{ to } 10^{-3} \text{ g/cc}\)); cholinergic blocking agents
(\(d\)-tubocurarine \(10^{-6}, 2 \times 10^{-5}, 2 \times 10^{-4} \text{ g/cc}\), scopolamine \(10^{-6} \text{ and }
2 \times 10^{-4} \text{ g/cc}\)); arecoline \((5 \times 10^{-5} \text{ g/cc})\); procaine (0.1 per cent).
and nicotine (10^{-6}, 10^{-3} \text{ and } 10^{-1} \text{ g/cc}). Acetylcholine, anti-
cholinesterases, and cholinergic blocking agents, were all without
effect on the O.O.D., the rate of closure, and the general
appearance of the animal. Acetylcholine seemed to irritate the
animal and produced a few spontaneous closures when the drug
arrived at the siphon of the animal. This may have been a chemotac
tic reflex. It is possibly the same effect observed in muscle
strip preparations that led Florey (1963) to conclude that the
strip was cholinergically innervated. It only occurred at higher
dose levels (1 \times 10^{-3} \text{ g/cc}). Injections into the vascular lacunae
of either acetylcholine or physostigmine had no effect of the
behavioral parameters of the animal.

Arecoline, nicotine and procaine did effect the animal.
Prolonged treatment with arecoline led first to an increase in the
closure speed followed by a reversible anesthesia. Nicotine at
low doses increased the closure rate also at first with many
spontaneous contractions, but then led to an irreversible paralysis
and death. Procaine anesthetized the animal reversibly with-
out any spontaneous contractions or increase in closure speed.

The results of drugs found to effect the tunicate are
summarized in Table 13.

D. DISCUSSION

By means of the technique described above results have been
obtained which indicate that a cholinergic system plays little or
no part in tunicate transmission. The results of Florey (1963) and Bacq (1935a) who found contractions of a pharyngeal muscle strip bathed in large doses of acetylcholine but not potentiated by eserine are probably due to the effect of the drug on chemo-sensitive sensory cells of the epidermis which trigger off responses in the neural net and bring about smooth muscle contraction. Such reflex effects have been observed at present in whole animal preparations; there is no reason, in view of the loose organization of the tunicate nervous system, that they would not occur autonomously in smaller pieces of the animal.

It may be suspected in view of our results that the drugs did not enter the tissues. Yet, a review of the anatomy of Ciona has shown that the perivisceral cavity is continuous with the pharynx so that all the organs are bathed in sea water separated from it only by mesentory and connective tissue. Also, injected cholinergic agents are without effect in Ciona. Finally, procaine would produce anesthesia.

E. GENERAL CONCLUDING REMARKS

On the basis of these siphon retraction studies and the negative findings in the histochemical investigation of cholinesterase, and the lack of cholinergic effects on the heart described in this thesis, it seems unlikely that tunicates employ cholinergic transmission. As has been stated, this evidence supports the original hypothesis of Bacq and the more recent data
of Durante. It is contrary to the interpretation which Florey has
given to his results, but an alternate explanation has been
offered here which makes his results quite plausible but which
does not favor cholinergic transmission in these forms.

Adrenergic transmission is a definite possibility and an
expansion of the techniques presented here to include a greater
variety of drugs might prove interesting. However, it may be that
the adult Ciona has evolved so far away from the chordate line
that it has developed a unique nervous system somewhat like the
interacting pace-maker hierarchies of a coelenterate (Passano,
1963), and conventional transmission systems may have been modified
or lost along with the notochord and the central nervous system.

It is also a possibility that transmission might be
primarily electrical (cf. Eccles, 1964) or ephaptic. Substance P
is found in tunicates and this may have a transmitter role
(Dahlstead, 1959). The mysteries of this system remain unsolved.
APPARATUS FOR THE STUDY OF THE TUNICATE SIPHON CLOSURE

- Photo flood lamp
- 8 mm. zoom camera with close up lens
- Light meter
- Mirror
- Electrodes to square wave stimulator
- Reflecting heat shield
- Tunicate
- Sea water
- Thermister and drain plug
- Plexiglas
- Signal light
- 10 cm.

FIGURE 8
FRONT VIEW OF APPARATUS

Tunicate
Oral siphon
Atrial siphon
Signal lamp
Cm. ruler

+-------------------+-------------------+
| 1 2 3 4 5         | 6 cm.             |
+-------------------+-------------------+
|       T           |
+-------------------+-------------------+
| Electrode         |
+-------------------+-------------------+
| 6 cm.             |
+-------------------+-------------------+
| Oral siphon       |
+-------------------+-------------------+
| A = diameter      |
FIGURE 9
CONTROL SIPHON CLOSURES OF
ONE CIONA INTESTINALIS
### AVERAGE CONTROL VALUES ± ONE STANDARD DEVIATION FOR CIONA INTESTINALIS

<table>
<thead>
<tr>
<th>Number of Animals</th>
<th>Strength of Stimulus Volts</th>
<th>Temperature °C</th>
<th>Latency secs.</th>
<th>Maximum Rate of Closure mm./sec.</th>
<th>Open to open Duration (OOD) sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>15 ± 10</td>
<td>20 ± 1.1</td>
<td>0.127 ± 0.074</td>
<td>4.7 ± 1.2</td>
<td>16.3 ± 3.3</td>
</tr>
</tbody>
</table>

**FIGURE 11**
THE EFFECT OF GANGLIONECTOMY ON THE SIPHON CLOSURE IN CIONA INTESTINALIS

- Control O.O.D. 20 sec. ave.
- Ganglionectomy O.O.D. 50 sec. ave.

FIGURE 12
<table>
<thead>
<tr>
<th></th>
<th>Maximum Slope</th>
<th>OOD</th>
<th>Other Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Acetylcholine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^{-4}$ to $10^{-2}$ gm/cc</td>
<td>(NC)</td>
<td>sl.† Spontaneous closures at high doses</td>
</tr>
<tr>
<td>2.</td>
<td>Anticholinesterase Agents</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>DFP $10^{-5}$, $10^{-3}$ gm/cc</td>
<td>NC</td>
<td>NC No visible or measurable response observed</td>
</tr>
<tr>
<td></td>
<td>TEPP $10^{-3}$ gm/cc</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phystostigmine $10^{-5}$, $10^{-3}$ gm/cc</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>Neostigmine $10^{-5}$, $10^{-3}$ gm/cc</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>3.</td>
<td>d-Tubocurarine</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>$10^{-5}$, $2 \times 10^{-5}$, $2 \times 10^{-4}$ gm/cc</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>4.</td>
<td>Scopolamine</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>$10^{-6}$, $2 \times 10^{-4}$ gm/cc</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>5.</td>
<td>Nicotine</td>
<td>NC</td>
<td>sl.† Spontaneous closures</td>
</tr>
<tr>
<td></td>
<td>$10^{-6}$ mg/cc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$ gm/cc</td>
<td></td>
<td>One contraction and death</td>
</tr>
<tr>
<td></td>
<td>$10^{-1}$ gm/cc</td>
<td></td>
<td>Instantaneous death</td>
</tr>
<tr>
<td>6.</td>
<td>Arsenolene</td>
<td>initially</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-5}$ gm/cc</td>
<td>later</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^{-4}$, $10^{-3}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Procaine</td>
<td></td>
<td>Reversible</td>
</tr>
<tr>
<td></td>
<td>0.1 per cent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
PLATES 53 - 55

Plate 53. Low power photomicrograph of a Giemsa-Wright stained 12 micron section of the oral siphon of the tunicate. The red pigment cells of the ocellus stain dark green.

Plate 54. A low power photomicrograph of a light Harris' Haematoxyline and eosin stained 10 micron section through the ocellus. The pigmented cells of the ocellus are dark orange.

Plate 55. A medium power photomicrograph of a 10 micron section through the ocellus stained with cresyl fast violet-toluidine blue-thionine. The ocellar cells are stained almost black while the orange cells of the blood near or among them are relatively unstained.

In all the sections through ocelli no evidence for differentiation of the outer epithelium or of nervous structures was found.
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Sweeney 1963


APPROVAL SHEET

The dissertation submitted by Charles L. Scudder has been read and approved by five members of the faculty of the Graduate School.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

5/20/1904

Signature of Adviser