MORPHOLOGICAL AND PHYSIOLOGICAL STUDIES OF THE LIMULUS POLYPHEMUS HEART: MICROSCOPIC ANATOMY OF THE CARDIAC GANGLION AND ROLE OF THE CARDIOREGULATORY NERVES

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ABSTRACT

MORPHOLOGICAL AND PHYSIOLOGICAL STUDIES OF THE *LIMULUS POLYPHEMUS* HEART: MICROSCOPIC ANATOMY OF THE CARDIAC GANGLION AND ROLE OF THE CARDIOREGULATORY NERVES

By

Charles Robert Bursey

The morphology of the cardiac ganglion of *Limulus polyphemus* (L) was examined by reconstructions from stained serial sections. This ganglion is composed of two distinct parts: a fiber tract extending the entire length of the heart and a cellular portion underlying the fiber tract. The cellular portion extends continuously from ostia III to the posterior terminus of the heart. The mean number of ganglion cell bodies is 231. Most of the ganglion cells are located among the glial elements of the cellular portion. The greatest density of cells is found in segments five and six. Six nerve cell types are recognized: 1) large pigmented unipolar cells approximately 120 u in diameter with distinct connective tissue capsules around them; 2) large pigmented bipolar cells approximately 120 u in length which are also encapsulated;

3) pigmented multipolar cells approximately 80 u in diameter which are free of capsules; 4) small pigmented bipolar cells approximately 40 u in length which are encapsulated but which are found exclusively within the fiber tract; 5) non-pigmented multipolar cells approximately 30 u in diameter which are scattered among the connective tissue elements of the cellular portion; and 6) small non-pigmented cells approximately 7-10 u in diameter.

The cardioregulatory nerves were studied by electrical stimulation. Nerves 7 and 8 are predominately inhibitory and nerve 8 contains a few acceleratory fibers. Nerves 9-11 are predominately acceleratory but they contain a few inhibitory fibers. Nerve 9 probably has more of these inhibitory fibers since inhibition produced by stimulation of nerve 9 is greater than that produced by stimulation of nerves 10 and 11. Stimulation of nerve 6 or the pericardial nerve does not affect heart rate. The cardioregulatory nerves enter the ganglion directly.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Many of the arthropod hearts that have been investigated have nerve cells which initiate the rhythmic contractions of the heart, while the cardiac muscle cells themselves possess no inherent rhythmicity. The nerve cells are located in a cardiac ganglion which lies on the heart and which receives nerves from the ventral ganglion that are capable of modifying the activity of these cells. This situation occurs in a number of Crustacea, in limulus and in several arachnids.

The crustacean heart has received the most attention and the morphology of many representative cardiac ganglia has been well described (see Bullock and Horridge, 1965, for review). The arachnids have not received as much attention and little morphological data are available (Zwicky and Hodgson, 1963; Sherman and Pax, 1968; Sherman, et al., 1969). The heart of limulus has become the classic example of the neurogenic heart, but little has been published about the anatomical organization of its cardiac ganglion. Most of what is known about the morphology of this ganglion is based upon highly selective methylene blue stain or has been obtained as a secondary objective of some specific physiological study.

Milne-Edwards (1873) noted nerve elements associated with the Limulus polyphemus heart, but Patten and Redenbaugh (1900) were the first to describe the gross anatomy of the heart and the cardiac ganglion. Nukada (1925), using methylene blue stains, described three nerve cell types within the ganglion. Nukada considered the largest of the cell types to be autorhythmic since their distribution correlated with the distribution of pacemaker regions reported earlier by Carlson (1904c). From a more physiological standpoint, Heinbecker (1936) attempted to correlate cell types with electrical activity in the ganglion. By progressively sectioning the ganglion, he was able to show that spontaneously active sections always contained at least one large cell. Fedele (1942) described five nerve cell types in the ganglion and attempted to describe cellular interaction on a morphological basis.

The physiology of the cardioregulatory nerves has been most studied in the Crustacea. The distribution, ramification, and function of these nerves is known for a number of species (Carlson, 1905a; Smith, 1947; Maynard, 1953, 1961a; Florey, 1960). The presence of pericardial organs and the influence of these organs on heart rate has been discussed by several authors (Carlisle and

Knowles, 1959; Belamarich, 1963; Cooke, 1966). In the arachnids, the cardioregulatory nerves of scorpions have been found to originate from the cerebral ganglion (Police, 1902, 1903), and Zwicky (1968) has been able to trace part of the pathway of these nerves by transection. On the basis of electrical stimulation experiments of the brain of spiders, Carlson (1905a, 1909) suggested that cardioregulatory fibers are present. Wilson (1967) and Legendre (1968) show that nerves enter the cardiac ganglion, but it is yet to be determined whether these nerves are in fact cardioregulatory in nature. Patten and Redenbaugh (1900) were the first to describe the presence of segmental cardiac nerves in limulus, but Carlson (1905b) described the system in its entirety and studied by electrophysiological methods the relationship of this system to the heart. Heinbecker (1933) described the effects of electrical stimulation of both cardioinhibitory and cardioacceleratory nerves upon the activity of the cardiac ganglion. Pax and Sanborn (1964, 1967a, 1967b) and Pax (1969) have studied the various components of the cardioregulatory system in limulus. No pericardial ramifications of the cardioregulatory nerves have been described in spiders, scorpions or limulus.

Since the heart of the decapod crustaceans has been so well studied and since limulus has been shown to have a number of characteristics in common with the Crustacea, a comparison of these two hearts seems in order. The specific number, type, and arrangement of cells within the limulus ganglion has not yet been reported nor has the structure of the ganglion itself been examined. These features are well known in the Crustacea. The cardioacceleratory nerves in limulus have not been traced farther than the pericardial area. In order to analyze ganglionic aspects of cardioregulation, it is necessary to know the relationship of the cardioacceleratory nerves to the cardiac ganglion. Do these nerves enter the ganglion proper as in the case of the Crustacea or do they form a neuro-hemal structure which is in turn responsible for cardioacceleration?

The question, stated simply, is whether or not the limulus cardiac ganglion is similar in structure and function to the cardiac ganglion of the Crustacea. This study was undertaken to provide some of this information.

Anatomical Background

The anatomical studies of Patten and Redenbaugh (1900) are the best account of the general anatomy of the *L. polyphemus* heart and its innervation. The heart is located directly beneath the carapace just dorsal to the intestine. It extends from a point midway between the lateral eyes to the seventh pair of entapophyses in the opisthosoma. It has the general appearance of a segmented tube and attains a length of about 15 cm in an adult, fully one-half the length of the body. There are eight pairs of ostia on the dorso-lateral surfaces of the heart. These ostia divide the heart into nine unequal segments.

Eleven arteries are given off from the heart, three from the anterior end, and one pair from each of the four anterior segments. Blood is carried from each gill in a large branchio-cardiac canal to a pericardial sinus. From this sinus blood enters the heart through the ostia.

In the opisthosoma the large pericardial sinus around the heart is enclosed by a membranous connective tissue pericardium. Ventral to the heart, it is a well-defined layered membrane which attaches to the bases of the branchial cartilages, but lateral to the heart it becomes thinner and joins the connective tissue lining of the branchio-cardiac canals. Dorsally the pericardium is attached to the carapace at successive entapophyses. In the prosoma the ventral pericardium is similar to that in the opisthosoma and is continuous with it. Dorsal to the heart the opisthosomal extensor muscles lie immediately above the heart and there is no pericardium interposed between these muscles and the heart.

The heart is held in position in the pericardial sinus by suspensory connective tissue fibers. Laterally, eight pairs of connective tissue supports, the alary muscles of Van der Hoeven, arise from the lateral edge of the heart at the level of each ostium. Each of these supports fuses distally with the pericardium. The ventral surface of the heart is attached to the pericardium throughout its entire length by numerous connective tissue fibers, but dorsally the heart is suspended only at certain points. In the opisthosoma there is a band of connective tissue fibers opposite each of the ostia while in the prosoma two masses of connective tissue provide support: one between the second and third pair of ostia, and the other between the first and second pair of ostia.

The heart itself is composed of three layers: an inner muscle layer, a median basement membrane, and an outer layer of thick connective tissue fibers. This outer layer of fibers anastomoses with the connective tissue fibers suspending the heart in the pericardial sinus. These connective tissue fibers also surround the cardiac ganglion.

The cardiac ganglion extends almost the entire length of the heart and occupies the outer mid-dorsal aspect. The ganglion has its greatest width in the fourth, fifth and sixth segments and it becomes progressively narrower both anteriorly and posteriorly. The ganglion forms the center of the intrinsic cardiac nervous complex which is confined mainly to the dorsal and lateral sides of the heart. Numerous anastomosing and branching nerve bundles are given off by the ganglion to the myocardium. These branches come to form more or less definite tracts at the lateral edges of the heart, which are known as the lateral cardiac nerves. These lateral nerves are much smaller than the ganglion and they contain no nerve cell bodies.

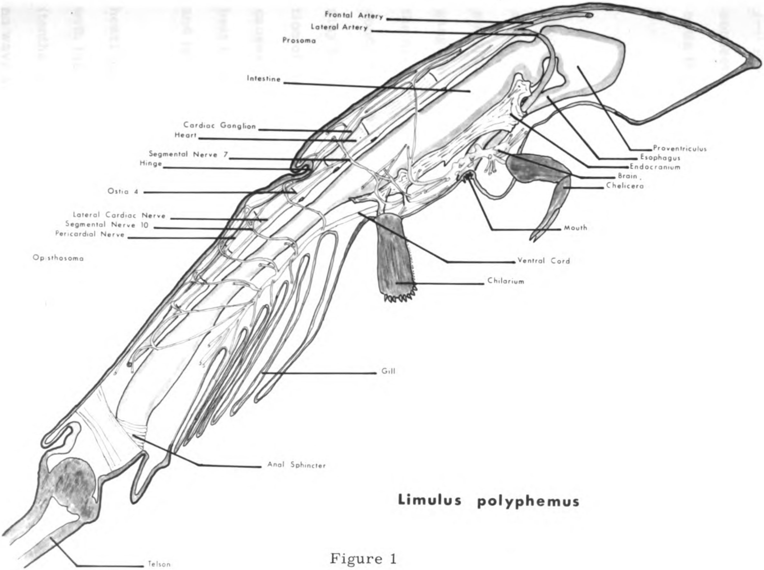
The intrinsic nervous complex is connected with the central nervous system by an extensive system of nerves. A pair of nerves is given off from the dorsal surface of the brain just above each pair of nerves to the walking appendages. These nerves go dorsally to innervate the integument, the viscera, and the dorsal musculature with the posterior three pairs of nerves sending fibers to the cardiac ganglion. The anterior pair of nerves from each of the abdominal ganglia takes a similar course and makes connections similar to those made by nerves from the dorsal side of the brain. Since these nerves innervate structures dorsal to the level of the brain and ventral cord, Patten and Redenbaugh called them "hemal" nerves to distinguish them from the ventral nerves of the walking appendages and gills, which they designate the "neural" nerves.

Of the dorsal nerves arising from the brain only the posterior three pairs connect with the cardiac ganglion (hemal nerves 6-8 of Patten and Redenbaugh). The cardiac branches of the seventh and eighth dorsal nerves fuse on each side to form a large nerve which passes to the dorsal hypodermis. Branches of this nerve join the cardiac ganglion between ostia III and IV. The main body of this nerve goes to supply the large opisthosomal extensor muscles and to make up the pericardial nerve which is a relatively large nerve trunk that runs parallel to the heart on each side just outside the lateral pericardium (see Figure 1). The sixth dorsal nerve sends branches to the opisthosomal extensor muscles and the cardiac ganglion. These branches join the cardiac ganglion near ostia II.

The dorsal segmental nerves from the abdominal ganglia (hemal nerves 9-13 of Patten and Redenbaugh) send branches to the digestive tract and integument. These nerves continue dorsally and before penetrating the dorsal pericardium to connect with the cardiac ganglion, each nerve sends a communicating branch to the pericardial nerve.

There is considerable individual variation in the exact position of entrance of the segmental cardiac nerves into the cardiac ganglion. Generally the cardiac nerves from the five abdominal ganglia enter the cardiac ganglion opposite the five pairs of posterior ostia (Carlson, 1905b). The fused seventh and eighth nerve enters the cardiac ganglion sometimes at the level of the second pair of ostia, sometimes in the middle of the third segment and sometimes

Figure 1. -- A semi-diagrammatical section of L. polyphemus showing the general placement of organs and the system of nerves connecting the cardiac ganglion with the ventral nervous system (Modified from Patten and Redenbaugh, 1900).



Limulus polyphemus

Figure 1

just posterior to the third pair of ostia (Carlson, 1905b). The connections of the sixth nerve appear consistently at the level of ostia II (Pax, 1964).

Physiological Background

Carlson, (1904a, 1904b) found that the limulus heart when exposed from the dorsal side *in situ* or when removed from the animal continues to beat for hours. The heart appears to contract simultaneously throughout its entire length. Section of the cardiac ganglion results in incoordination of the beating in the two ends of the heart. The lateral nerves are not essential to coordination or conduction (Carlson, 1904a). The whole heart and the lateral nerves may be sectioned, leaving only the ganglion intact, and the coordination of both ends will be maintained. Removal of the cardiac ganglion causes immediate and permanent cessation of beating. The heart beat in limulus, therefore, has its genesis in the cardiac ganglion, and is thus neurogenic.

Carlson's indirect evidence of the neurogenic origin of the heart beat was supported by Hoffman (1911), who was able to obtain with the use of a string galvanometer a continuous series of rapidly (tenths of a millisecond) oscillating waves separated by periods with no wave activity. Garrey (1912) verified Hoffman's findings of the oscillatory character of the electrocardiogram of limulus and studied the modification of normal activity under the influence of changes in temperature and of drugs. However, Nukada (1918), Hoshino (1925), Dubussion (1930) and Monnier and Dubussion (1931) could not find oscillatory discharges from the cardiac ganglion and they described a series of slow waves similar to that of the vertebrate heart. It remained for Rijlant (1931) to resolve these disagreements. By using an improved cathode ray oscilloscope, he was able to record the oscillatory character of the electrical activity from the isolated cardiac ganglion. This electrical activity consists of periodic changes in potential lasting 1.2 seconds. These bursts of activity are separated by periods of quiescence of 2 seconds. Each of these potential changes is composed of a series of waves. Three different types of activity were obtained: 100 to 200 fast waves; 10 to 30 slow waves; or simultaneously fast and slow waves. The maximum action potential obtained was 150 to 200 uV while the normal value was 100 uV.

Under normal conditions the activity starts in the region corresponding to the fifth segment and is conducted up and down the ganglion at an average speed of 75 cm/sec (Edwards, 1920; Pond, 1921; Rijlant, 1931). The first sign of the beginning of a burst is a sharp initial potential change which is immediately followed by a series of slow waves each lasting about 100 msec and repeating about ten times during each burst (Rijlant, 1932). The first of the slow waves has the largest amplitude and succeeding waves are progressively damped until undetectable. Superimposed on the slow waves is a series of fast waves (spikes) appearing initially at a high frequency but rapidly declining in parallel with the decline in amplitude of the slow waves (Heinbecker, 1936). Armstrong, et al. (1939) and Prosser (1943a) showed that the slow potentials are largest in segment five and spread more slowly than the spikes.

The cardiac ganglion is connected with the ventral ganglion at the level of each of the five posterior pairs of ostia and to the brain at the level of the second and third pairs of ostia. Cardio-inhibitory nerves are carried in the last two pairs of dorsal nerves arising from the brain; stimulation of either of these two nerves at points where they emerge from the brain results in a decrease in heart rate and strength of beating (Carlson, 1905b; Pax and Sanborn, 1964). Cardioacceleratory nerves are carried in the segmental cardiac nerves that arise from each of the five abdominal ganglia. Stimulation of the ventral cord or any of the abdominal ganglia results in an increased rate of heart beat (Carlson, 1905b; Heinbecker, 1933). However, the abdominal nerves have been shown to also carry inhibitory nerves (Pax, 1969). The characteristics of inhibition found in the abdominal cardiac nerves are similar to those for the cardioinhibitory nerves arising from the brain.

Heinbecker (1933) recorded the electrical activity in the cardiac ganglion during stimulation of the inhibitory nerves. Such stimulation lowered the amplitude of the slow potentials, decreased the rate of the fast discharge and shortened the total duration of each burst. Stimulation of the accelerators increased the amplitude of the slow waves, increased the frequency of the fast discharge and lengthened the burst duration (Heinbecker, 1933).

MATERIALS AND METHODS

Mature male and female Limulus polyphemus with body lengths between 25 and 30 cm were used for these studies. They were shipped by air express from Gulf Specimen Company, Panacea, Florida, and maintained at first in a cold box in moist excelsior at 5° C. Pax and Sanborn (1967a) report that responses of animals so maintained did not vary for at least six weeks. Later, the animals were maintained in running artificial sea water at 15° C. These animals remained active for long periods of time and on several occasions trilobite larval stages were found.

Histological Studies

Preparation of the cardiac ganglion for histological section requires isolation of the heart. The method for heart isolation has been previously described (Pax and Sanborn, 1967a). After the heart was isolated, a glass tube (6 mm OD) was inserted into the lumen of the heart to prevent distortion during fixation. At this time most of the loose connective tissue around the ganglion was dissected away.

A heart supported by the glass tube was then placed in Gilson's fixative for 12 hours (Gray, 1954). Other fixatives, notably formalin, Bouin's and Zenker's, were used; however, none were satisfactory since most of the cytoplasmic detail was lost.

Each heart was washed in tap water for 30 minutes after fixation. The glass tube was then removed and the heart was cut into pieces approximately 2 cm in length. These were dehydrated in graded alcohols, cleared in xylene and embedded in paraffin by usual methods. Serial frontal sections and serial cross sections were cut at 7 u and stained by Bretschneider's method (Gray, 1954). Bretschneider's method was modified by the addition of 0.2% Chromotrope 2R (Allied Chemical) to the counterstain in order to enhance nucleolar staining. Ganglia wholemounts stained by the reduced methylene blue method (Pantin, 1946) were also examined. Fresh frozen 10 u sections were used to monitor the amount of shrinkage and distortion caused by fixation.

The serial frontal sections were used in a reconstruction of the cardiac ganglion. A section was projected by a microslide projector utilizing a 10X objective and traced on paper. Each subsequent section was then superimposed on the previous tracing until the entire ganglion was reconstructed. By this method the types of cells present and their distribution along the length of the ganglion was determined for seven hearts.

Some of the cells in the cardiac ganglion contain a yellow pigment which is strongly autofluorescent. The total number of these cells can be easily counted under a fluorescent microscope. For this procedure, ganglia were removed from isolated hearts, placed on a glass slide, dried in a desiccator jar under vacuum for 24 hours and then examined under a Leitz fluorescent microscope utilizing a mercury vapor lamp with 400 mu filters. The pigment was also observed in situ with a Leitz microspectrometer and a Photovolt microphotometer and the resultant absorbance spectrum plotted.

Physiological Studies

Stimulation of segmental nerves 6-11 was performed at three different levels: 1) ventrally as the nerves leave the central nervous system; 2) pericardially, both above and below the pericardial nerve; and 3) dorsally as the nerves enter the cardiac ganglion. Two different animal preparations were used: 1) hearts isolated from the animals and 2) intact hearts within partially dissected animals.

The preparation used for ventral stimulation of cardioregulatory nerves has been previously described (Pax, 1969) and was followed in part. The gills, genital opercula and legs were removed. A longitudinal dorso-ventral cut through the entire thickness of the animal was made about 4 cm on either side of the midline for a length approximating the underlying heart. Transverse cuts were made through the animal at the anterior and posterior boundaries of the heart. This leaves a rectangular block of tissue which includes most of the musculature, the digestive tract and the ventral nervous system; and preserves the heart and pericardium as well as the cardioregulatory nerves. This rectangular block was pinned ventral side up in a shallow tray. The abdominal nerve cord and the brain were exposed along their entire length and nerves 6-11 were cut free at their point of emergence and dissected free of underlying tissue laterally to the point where they turn dorsally. The ventral cord and brain were then removed. A portion of the ventral abdominal muscles and the intestine were removed to expose the heart from ostia VIII to the posterior terminus. The mechanical activity of the heart was monitored at the eighth segment. Stimulation was accomplished by lifting the nerve onto a pair of platinum hook electrodes.

Pericardial stimulation was accomplished by continued dissection of the above preparation. The ventral abdominal muscles and the digestive tract were removed so that the ventral pericardium was exposed in its entirety. The pericardium was slit unilaterally at the level of the entrance of the branchio-cardiac canals into the pericardium and the pericardial nerve from the hinge of the carapace to the pericardial branch of the eleventh segmental nerve was exposed. By following the pericardial branch of the segmental nerve to the main nerve tract, the segmental nerves were easily located. Stimulation was accomplished by lifting the nerve onto the hook electrodes. The mechanical activity was monitored at the eighth segment.

The preparation used for dorsal stimulation of the cardioregulatory nerves was prepared as follows: The dorsal exoskeleton was sawed through just lateral to the underlying heart and these cuts were joined by transverse anterior and posterior cuts. The rectangular piece of isolated exoskeleton overlying the heart was then removed by lifting and scraping it free of the underlying tissues. The exoskeleton must be separated carefully from the underlying tissue so that the segmental nerves to the heart are not destroyed. The entire heart and the overlying tissue were removed and placed in a tray dorsal side up. The heart was stretched to its original length and pinned in place. Segmental nerves 9-11 were dissected from the tissue overlying the heart. These nerves are most easily found by first locating the pericardial nerve and following it to the area in which the cardiac branch crosses the pericardial nerve. The fused nerve 7 and 8 is found anterior to the overlying tissue and does not require dissection. Nerve 6 lies in a mass of connective tissue at ostia II and must be dissected free. These nerves were lifted onto the hook electrodes for stimulation. Mechanical activity was monitored at the second heart segment.

Carlson (1907a) reported that heart wall tension and intra-lumenal pressure are factors that influence both the rate and the strength of beating of the heart. For the isolated heart, longitudinal tension approximating that of the intact heart was obtained by stretching the heart to a length equal to that present before removal from the animal. In those preparations used for ventral and pericardial stimulations, the pericardium and the suspensory ligaments remain intact and thus the longitudinal tension is not disturbed. In order to approximate the normal intra-lumenal pressure, a gravity-fed reservoir of Chao's saline solution (Chao, 1933) was connected by cannula to the posterior end of the heart. Later this system was replaced by a peristaltic perfusion pump and artificial sea water. The hearts were perfused at a rate of 15 ml/min, the route of perfusion fluid being that of normally expelled blood. Perfusion was begun as soon as the preparation was pinned to the tray. All experiments were conducted at room temperature (20-26° C).

Records of the mechanical activity of the hearts were obtained by the use of an E & M myograph "A" transducer. Since the main component of tension development in the heart is radial due to the circularly directed muscle fibers, the transducer was attached laterally to the heart muscle. The transducer output was recorded on an E & M Physiograph.

Stimulation was accomplished by a Grass S 4 stimulator and isolation unit. Pax (1969) reported a mean rheobase of 2.5 V with a chronaxie of 0.5 msec for the abdominal segmental nerves. Because of the relatively high threshold and long chronaxie, he routinely used a stimulus strength of 5 V with a duration of 3 msec and found that at all stimulus frequencies maximum increase in rate occurred only after 30-40 seconds of stimulation and the maximum rate change during this time was produced by stimulus frequencies between 5 and 10/sec. Thus one and two minute stimulations of all nerves in all areas studied were carried out at 5/sec at 5 V (duration 3 msec). Pax and Sanborn (1964) note that maximum slowing of the heart due to stimulation of nerves 7 and 8 occurs with stimulation frequencies between 10 and 80/sec at 40 V and 1 msec duration. Thus nerves 7 and 8 were also stimulated in this study at stimulus strengths of 6 V for 5 msec duration and 20/sec. Increase in either voltage or duration during a stimulation period did not change the on-going pattern, showing that all stimulations were above threshold.

Since normal heart rates in limulus vary greatly from animal to animal (Pax and Sanborn, 1964), each animal is used as its own control. All rates in this study are expressed as change in heart rate. The change in rate is determined by the difference between the experimentally altered heart rate and the control rate, which is that rate during the minute prior to stimulation. Thus positive values are indicative of excitation and negative values are indicative of inhibition. The averaged rate for each ten second period is plotted.

Extract Studies

Since the effects of secretions from the pericardial organs upon the heart beat of crustaceans is well known (Belamarich, 1963; Belamarich and Terwilliger, 1966; Cooke, 1966), various tissues of *L. polyphemus* (muscle, ventral cord, brain, heart, hypodermis, pericardial tissue) were tested to determine what effect they might have on heart rate. These tissues were macerated in Chao's saline by mortar and pestle (10 mg tissue per 1 ml saline). The filtrate from the various tissues was tested on the isolated heart, which, in this case, was dissected free of all extraneous tissues. Aliquots of approximately 20 cc were used to bathe the heart or were perfused through the heart. The mechanical activity of the heart was monitored at segment two as previously described.

RESULTS

Gross Morphology of the Ganglion

Under low magnification (30X), the unfixed cardiac ganglion appears as a large bundle of intertwining nerve fibers with intermingled masses of cells. A few individual large cell bodies are discernible by the presence of pigment within them and at various levels along the ganglion some of these cell bodies bulge from the surface of the ganglion.

In cross sections from the middle segments of the heart, it is apparent that the ganglion consists of two morphologically distinct areas: a ventral region containing most of the ganglionic cell bodies, and a dorsal region composed primarily of nerve fibers (Figures 2c, d, e). The ventral region I have designated the cellular portion of the ganglion; the dorsal region the fiber tract.

The cellular portion makes up the bulk of the ganglion. It is continuous from ostia III to the posterior terminus of the heart. This cellular portion contains most of the cell bodies of the ganglion as well as a number of connective tissue elements. Some nerve

Figure 2. -- Representative sections of the cardiac ganglion of L. polyphemus.

A. Sagittal section from the fifth heart segment.

B. Cross-section through second segment.

C. Cross-section through fourth segment.

D. Cross-section through seventh segment.

E. Cross-section through eighth segment.

In A dorsal is to the left. A large unipolar cell is visible. The area to the left of this cell is the fiber tract; the area to the right the cellular portion. In B the entire cross-section is fiber tract but in C, D, and E the cellular portion can be seen underlying the fiber tract. Calibration is 200u.

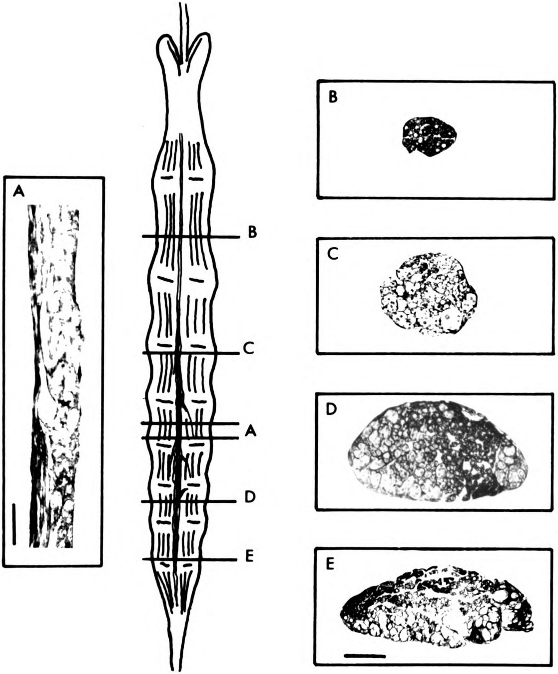


Figure 2

fibers of various diameters are also found throughout this region of the ganglion.

The fiber tract extends the entire length of the heart and has approximately the same cross sectional area throughout segments three through eight. It consists mainly of nerve processes and connective tissue elements. The number of fibers within a segment varies greatly. This variation is due to fibers arriving from the cellular portion and to branches leaving the fiber tract. Generally, 30-40 fibers are found in segment one and 80-120 fibers are found in segments two and three. Posterior to ostia III, the number of fibers increases to 200-300, with the largest number of fibers occurring in segment five. The number of fibers decreases posterior to segment five and in the posterior half of segment eight and in segment nine the number of fibers again approaches 30-40.

The oval nuclei of fibroblasts are easily recognized in the fiber tract and hemocytes which tend to aggregate in intra-ganglionic spaces are often seen. Anterior to ostia III isolated groups of small cells are scattered among the fibers, while posterior to ostia III the fiber tract is free of these cell groups. A few individual small bipolar cells are also found in the fiber tract. A connective tissue layer surrounds the cellular portion and in sagittal sections it can be seen that processes from the cell bodies in the cellular portion pass through this layer to enter the fiber tract (Figure 2a).

The nerve processes leave the fiber tract in bundles of various sizes and spread over the myocardium. There is an almost segmental arrangement of these bundles as they leave the ganglion. The largest bundle is usually found in the fifth segment and contains 80 to 100 fibers. It may run anteriorly or posteriorly and may occupy either the left or right side. The contralateral bundle is smaller in size, but there are usually more bundles on that side. These tracts may bifurcate immediately or they may travel several millimeters before branching. Segments three through eight give rise to at least one bundle of large size containing 40-80 fibers, one or two bundles of medium size containing 20-40 fibers and several smaller bundles of 5-20 fibers. All bundles branch randomly and small bundles of one to five fibers are found throughout the cardiac muscle mass. Individual fibers have not yet been traced to individual muscle cells (see Abbott, et al., 1969). The bundles that remain on the surface of the heart come to form more or less definite tracts at the lateral edges of the heart. These lateral nerves contain 80-100 fibers when counted at their greatest diameter, which is in the fifth segment.

Cells of the Cardiac Ganglion

In addition to the fibroblasts of the connective tissue elements, six types of cells are clearly recognizable by morphological characteristics. A description of each of these cell types follows. Cell sizes are expressed for a heart of 15 cm length.

1) Pigmented unipolar cells. -- The pigmented unipolar cells have cell bodies approximately 120 u in diameter which may be either round or oval (Figures 3, 4, 5). They give rise to a single large process approximately 40 u in diameter. This process often bifurcates soon after leaving the cell (Figure 3). Processes from different unipolar cells often cross one another with little if any connective tissue between them (Figure 6). These fibers can be traced for some distance and pass eventually into the fiber tract.

The unipolar cell is invested by a connective tissue capsule. This capsule may be thick (up to 10 u) as when the cell body bulges from the ganglion or it may be thin (2 u) as when the cell body lies within the ganglion. In either case the capsule thins as it approaches the process and does not appear to continue along the process. Connective tissue fibers of the capsule anastomose with other connective tissue fibers to form the fibrous network seen throughout the cellular portion of the ganglion.

Around the cell body and within the capsules of the unipolar cells, there are numerous small cells, approximately 7-10 u in diameter. These small cells bear a constant relationship to the process of the unipolar cell. In cells that bulge from the ganglion,

Figure 3. -- A pigmented unipolar cell from the fifth heart segment. The large axon can be seen to bifurcate a short distance from the cell body. Small non-pigmented cells (arrows) are always present within the capsule of the unipolar cell. Calibration is 30 u.

Figure 4. -- A pigmented unipolar cell bulging from the ganglion. Here the small non-pigmented cells surround the process. Calibration is 40 u.

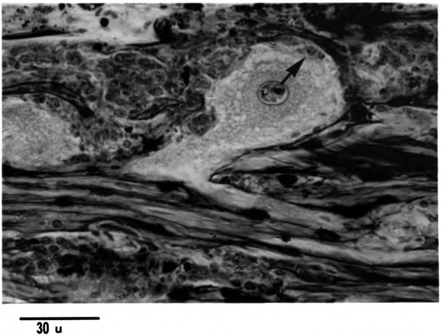


Figure 3

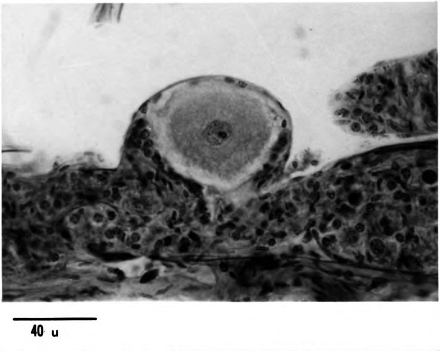


Figure 4

Figure 5.--A pigmented unipolar cell lying within the ganglion. Here the small non-pigmented cells occur at the side opposite the process. Pigment granules are visible (arrow). Calibration is 50 u.

Figure 6.--Frontal section of the ganglion in heart segment 5. Three unipolar cells are visible. Note the manner in which the axons of two of the unipolar cells come to lie adjacent to each other. Calibration is 100 u.

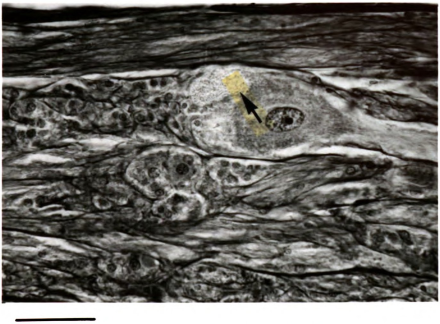


Figure 5

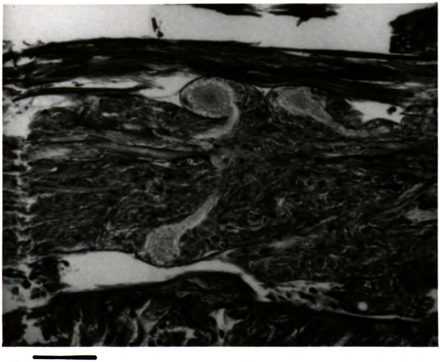


Figure 6

they aggregate around the process (Figure 5); while in those cells which lie within the cellular portion, the small cells lie at the end opposite the process (Figure 4).

The nucleus of the unipolar cell is round or oval and occupies the center of the cell; it contains scattered chromatin and one nucleolus. The cytoplasm is finely granular becoming reticular near the cell membrane. No cytoplasmic inclusions are visible. However, there are pigment granules at the periphery just below the cell membrane. These granules are approximately 1 u in diameter and in the unipolar cells they are usually concentrated in one large group. These granules are responsible for the fluorescence seen in all pigmented cells (Figure 7). At least four and perhaps five bands of absorbance (Figure 8) are seen in in vivo determinations: 590, 570, 510, 490 and 470 mu.

The unipolar cells occur in all segments of the heart except anterior to ostia III (Figure 9c). They are most numerous in segments five and six. The size of the cells varies with position, becoming smaller in segments seven and eight. The mean number of unipolar cells was 124 with a range of 93 to 163 in the seven hearts studied.

2) Pigmented large bipolar cells. -- The pigmented large bipolar cells are approximately 120 u in length and 90 u in width

Figure 7. -- A section of the unfixed cardiac ganglion viewed through a fluorescent microscope (excitation wave length 400 mu). The fluorescence of a large pigmented bipolar cell (arrow) and several unipolar cells can be seen. Calibration is 100 u.

Figure 8. -- The absorbancy spectrum of the pigment when measured *in vivo*. There are two major absorbancy bands at 510 and 490 mu. Three other bands possibly exist at 590, 570 and 470 mu. The arrows mark these bands.

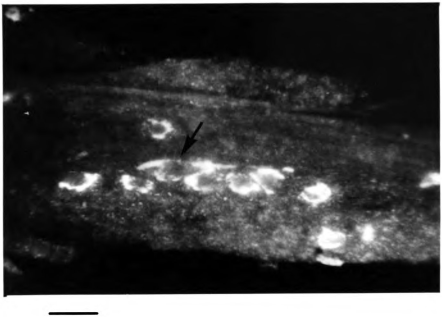


Figure 7

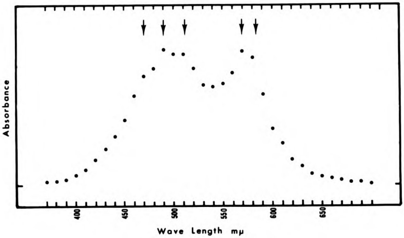


Figure 8

Figure 9. -- The distribution of the various pigmented cell types along the length of the ganglion. Each bar represents the mean determined from seven different hearts. The vertical lines give the range.

A. Total number of pigmented cells.

B. The multipolar cells.

C. The unipolar cells.

D. The large bipolar cells.

E. The small bipolar cells.

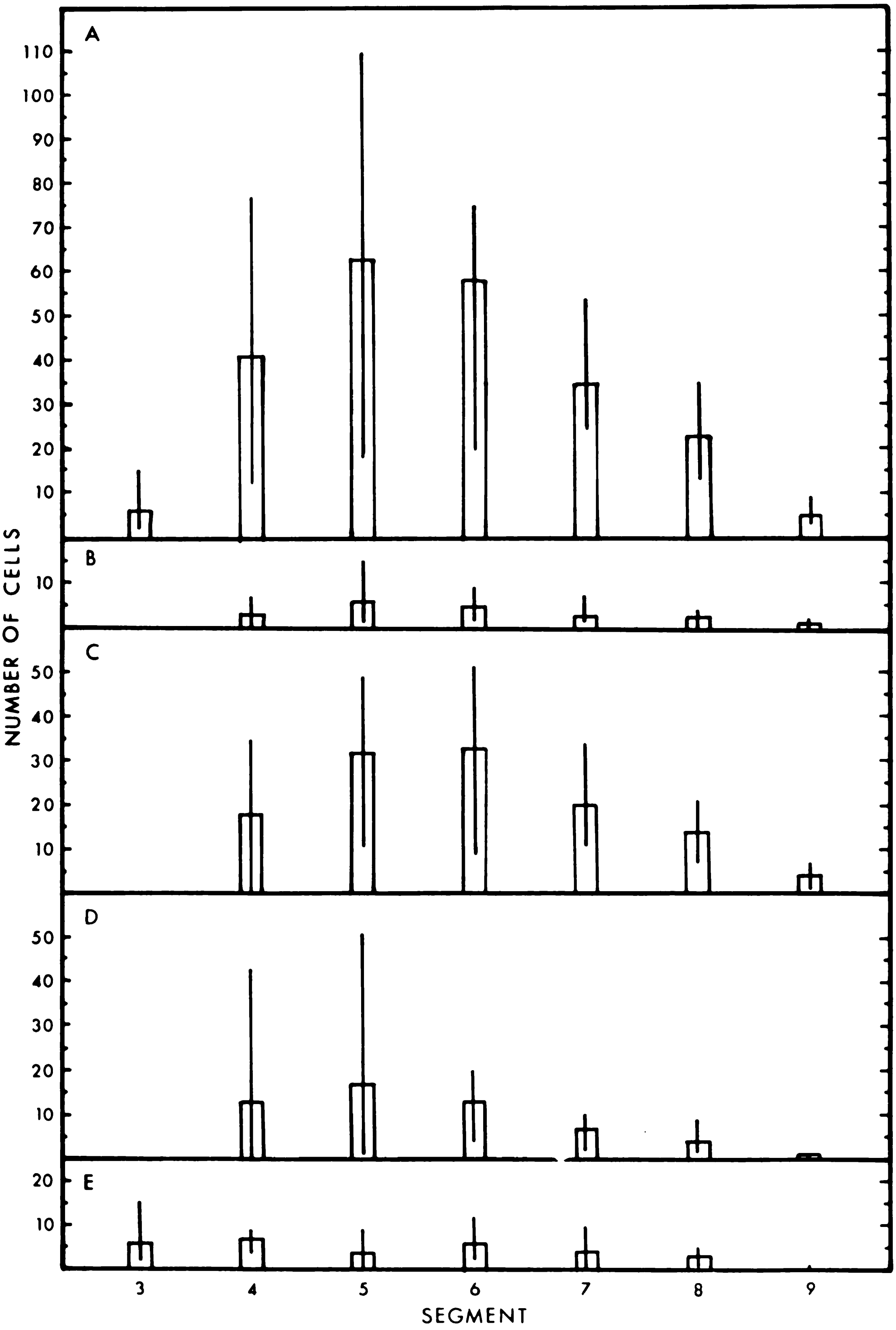

A
B
C
D
E
NUMBER OF CELLS
110
100
90
80
70
60
50
40
30
20
10
3
4
5
6
7
8
9
SEGMENT

Figure 9

(Figure 10). They have two large processes of equal size. These processes are sometimes seen to bifurcate, although bifurcation has not been found within 200 u of the cell body. The long axes of the bipolar cells are parallel to the length of the ganglion. The bipolar cells exhibit varying degrees of connective tissue investment. When lying adjacent to the fiber tract or at the surface of the cellular portion, they have a heavy capsule (10-15 u thick) and are almost always fusiform. When lying within the cellular portion, they have less connective tissue investment (up to 5 u) and may assume a more contorted form. The capsule is formed from several layers of connective tissue. It maintains the same thickness over the cell body and continues at this thickness along the processes for some distance.

The round or oval nucleus contains scattered chromatin and at least one nucleolus. The cytoplasm is finely granular becoming reticulated toward the cell membrane. No cytopolasmic inclusions were noted. Aggregations of pigment granules are found at both poles of the cell.

While this cell and the previously described unipolar cell are of the same size, the bipolar cell is easily distinguished from the unipolar cell. Grossly the pyriform appearance of the unipolar cell contrasts with the fusiform appearance of the bipolar cell. No small cells of the type seen within the capsules of the unipolar cells

Figure 10. --A pigmented large bipolar cell lying adjacent to the fiber tract in the fifth heart segment. Calibration is 40 u.

Figure 11. --A pigmented multipolar cell lying within the cellular portion of the ganglion. Two processes are visible in the photograph: a large process at center right; a smaller process at upper left. Non-pigmented cells and processes of other pigmented cells appear in the photograph also. Calibration is 20 u.

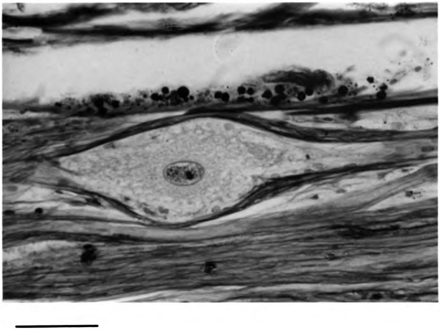


Figure 10

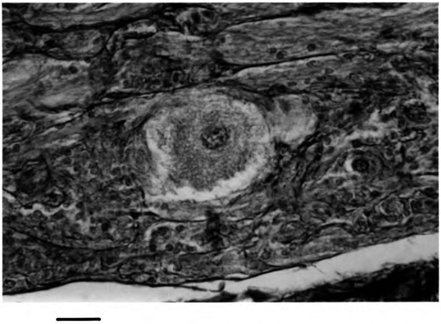


Figure 11

are found within the capsules of the bipolar cells. And the capsule of the unipolar cell does not continue along the process as it does on the processes of the bipolar cell.

The pigmented large bipolar cells are present in all segments posterior to ostia III (Figure 9d). They are by far the most variable in number among the various cell types. The mean total number of these cells in the seven hearts studied was 55 with a range from 18 to 132.

3) Pigmented multipolar cells. -- The pigmented multipolar cells are approximately 60-80 u in diameter (Figure 11). These cells occur in the cellular portion and are located well away from the fiber tract. A few connective tissue fibers are seen surrounding these cells, but there is no distinct capsule. Small non-pigmented cells often lie in close proximity and are occasionally seen to abut this cell.

The nucleus of the multipolar cell is round and exhibits scattered globular chromatin and at least one nucleolus. The cytoplasm is finely granular with no distinct inclusions. The pigment granules are concentrated in several groups close to the cell membrane and near the processes.

These cells are distributed evenly along the ganglion posterior to ostia III and are the most constant in number from one heart to the next (Figure 9b). The mean number of these cells was 21 in the seven hearts studied with a range of 13 to 31.

In my examination of methylene blue stained ganglia, I have been unable to count more than 30 large stainable cells in any one ganglion, although numerous small cells were visible. This number corresponds most closely with the number of pigmented multipolar cells counted by other means. Moreover the cells with connective tissue capsules, namely, the pigmented unipolar and bipolar cells, do not appear to be stained by methylene blue since in several instances these cells, though unstained, were visible due to their pigment inclusions. Thus it seems that only the unencapsulated cells are stained by methylene blue.

4) Pigmented small bipolar cells. -- The pigmented small bipolar cells are approximately 40 u in length and are always fusiform (Figure 12). They lie exclusively within the fiber tract and like the large bipolar cells, they are invested by a heavy capsule. Their long axes are parallel to the long axis of the ganglion.

The round nucleus usually has two large nucleoli and small inclusions which have a staining affinity similar to that of the nucleoli. Scattered chromatin is also present in the nucleus. The cytoplasm is finely granular and more deeply staining than that of

Figure 12. --A pigmented small bipolar cell lying within the fiber tract. Calibration is 20 u.

Figure 13. --Non-pigmented multipolar cells (arrows) are found within the cellular portion of the ganglion and adjacent to pigmented cells. Here two of these cells lie adjacent to a unipolar cell. Small non-pigmented cells are also present. Calibration is 30 u.

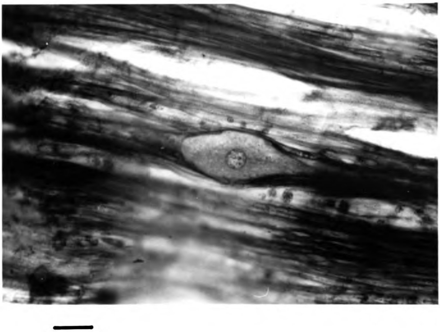


Figure 12

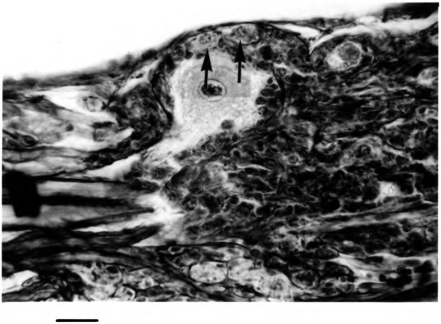


Figure 13

the large bipolar cell. No cytoplasmic inclusions are found. Pigment granules are limited to the polar regions.

These cells may be found in all segments of the heart (Figure 9e). They averaged 31 in the seven hearts studied. The range was 22 to 47.

5) Non-pigmented multipolar cells. -- The non-pigmented multipolar cells are approximately 20-30 u in diameter (Figure 13). They have a rounded or oval appearance with their processes pushing out between the small non-pigmented cells which always surround the multipolar cell body. These multipolar cells are found throughout the cellular portion of the ganglion and are often found in close association with the pigmented multipolar cells. They also often occur in isolated groups anterior to ostia III in the fiber tract.

The nucleus is round with extensive chromatin and in most cases there are two or more nucleoli. The cytoplasm stains more darkly than that of the pigmented cells and it has a heterogeneous granular appearance with distinct granules forming at the periphery. Darkly staining cytoplasmic inclusions are often seen.

No attempt was made to count these cells, but the number of cells of this type greatly exceeds that of any of the pigmented cell types severalfold.

6) Small non-pigmented cells. -- The small non-pigmented cells are approximately 7-10 u in diameter and are by far the most numerous of cells within the ganglion (Figure 13). They occur within the capsules of the pigmented unipolar cells and are seen filling the interstices around the non-pigmented multipolar cells in the cellular portion of the ganglion. Anterior to ostia III, they can be found in the fiber tract where they surround the non-pigmented multipolar cells.

These cells have large nuclei which may assume various shapes from round to kidney-shaped. No distinct nucleolus can be seen and the sparse granular chromatin is scattered throughout the nucleus. Occasionally enlarged nuclei with chromatin in strands are seen. The cytoplasm of this cell is extremely pale staining in comparison to that of the other cells and darkly staining inclusions are often present in the cytoplasm. Frequently only the nucleus is conspicuous. The cell form is highly irregular, but no processes can be seen.

It was impossible to count these cells.

Variation in Pigmented Cells

The numbers and types of pigmented cells were determined on a segment to segment basis by reconstructive drawings. The mean total number of pigmented cells for the seven hearts studied was 231 with a range of 171 to 305. This mean total cell number agrees well with that obtained from the five hearts studied by the fluorescent microscope method: 229 cells with a range of 190 to 265.

The total number of pigmented cells within the ganglion is not related to the length of the ganglion (Table 1). Of the seven ganglia I studied, the shortest had the largest number of pigmented cells, while the longest was third in number. There is also no apparent relationship between the numbers of individual cell types and the length of the heart. The small bipolar cells and the multipolar cells are most constant in numbers from one ganglion to the next. The large bipolar cells are the most variable in number.

Table 1 also shows the segment by segment cell totals in the seven hearts studied. There is no consistent pattern of cell distribution within the ganglion. The greatest concentration of cells occurs in the middle segments, but the segment containing the most cells in any one heart may be either segment four, five or six.

Figure 14 presents cell placement for unipolar and bipolar cells in the seven hearts studied. Each vertical bar represents the number of cells within 5% of the total length of a segment. As can be seen from this figure, there is no relationship between the cell placement in the ganglion and external heart features such as ostia.

Table 1. --Distribution of total cells by segment and cell types for seven hearts.

HEART NUMBER	1	2	3	4	5	6	7	MEAN
HEART LENGTH (cm)	8	10	10	10	14	14	18	
TOTAL CELLS	305	171	183	277	195	252	236	231
CELLS/SEG								
3	5	3	6	15	7	2	7	6
4	77	53	15	42	12	50	41	41
5	110	37	18	89	69	82	34	63
6	65	20	75	72	54	62	56	58
7	25	31	38	29	34	31	54	35
8	18	21	27	26	12	22	35	23
9	5	6	4	4	7	3	9	5
CELL TYPES								
UNIPOLAR	117	97	119	148	93	163	133	124
LARGE BIPOLAR	132	34	18	52	51	40	60	55
MULTIPOLAR	20	18	22	31	13	23	17	21
SMALL BIPOLAR	36	22	24	46	38	26	26	31

Figure 14. -- The distribution of unipolar and bipolar cells along the ganglion. Each square represents one cell.

A. Unipolar cells.

B. Bipolar cells.

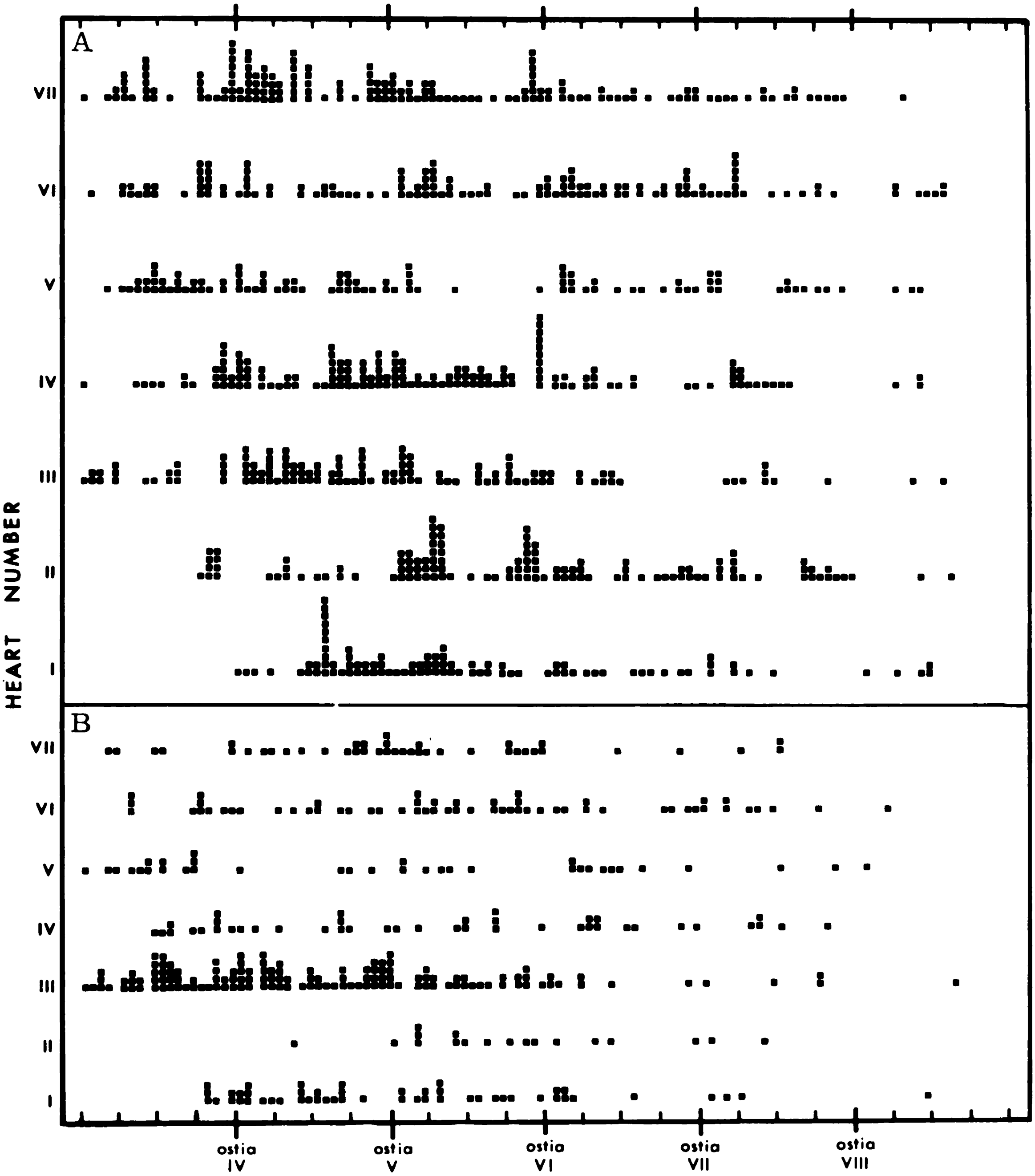

A
B
HEART NUMBER
VII
VI
V
IV
III
II
I
ostia IV
ostia V
ostia VI
ostia VII
ostia VIII

Figure 14

Nor does the cell pattern appear to be consistent from ganglion to ganglion. Rather the cells appear to be random in placement but most numerous in segments four, five and six (see Figure 9a also).

The cells of the cardiac ganglion also exhibit much individual variation in size within a single cell type. In a single heart of 18 cm length, the unipolar cells varied from an average diameter of 115 u to 155 u within the middle segments (Figure 15). However, cell size within a ganglion is influenced by position since those cells in segments four, five and six are on the average larger than those of seven, eight and nine. Cell size is also influenced by heart length. Figure 15 presents data on the relationship between heart length and the size of the unipolar cells in segment five for ten hearts. As can be seen from this figure, the cell size increases as the length of the heart increases.

It is also to be noted from Figure 15 that cell size is consistent whether measured from fresh frozen sections or fixed stained sections, showing that measurable shrinkage did not occur during processing.

Role of the Cardioregulatory Nerves

The segmental nerves have been shown to connect the brain and ventral cord with the cardiac ganglion (Carlson, 1905b). Stimulation of these nerves near their origins in the brain or ventral cord

Figure 15.--The relationship between heart length and diameter of the unipolar cells. Each vertical line represents the range in cell diameters measured for ten different cells chosen at random from segment five.

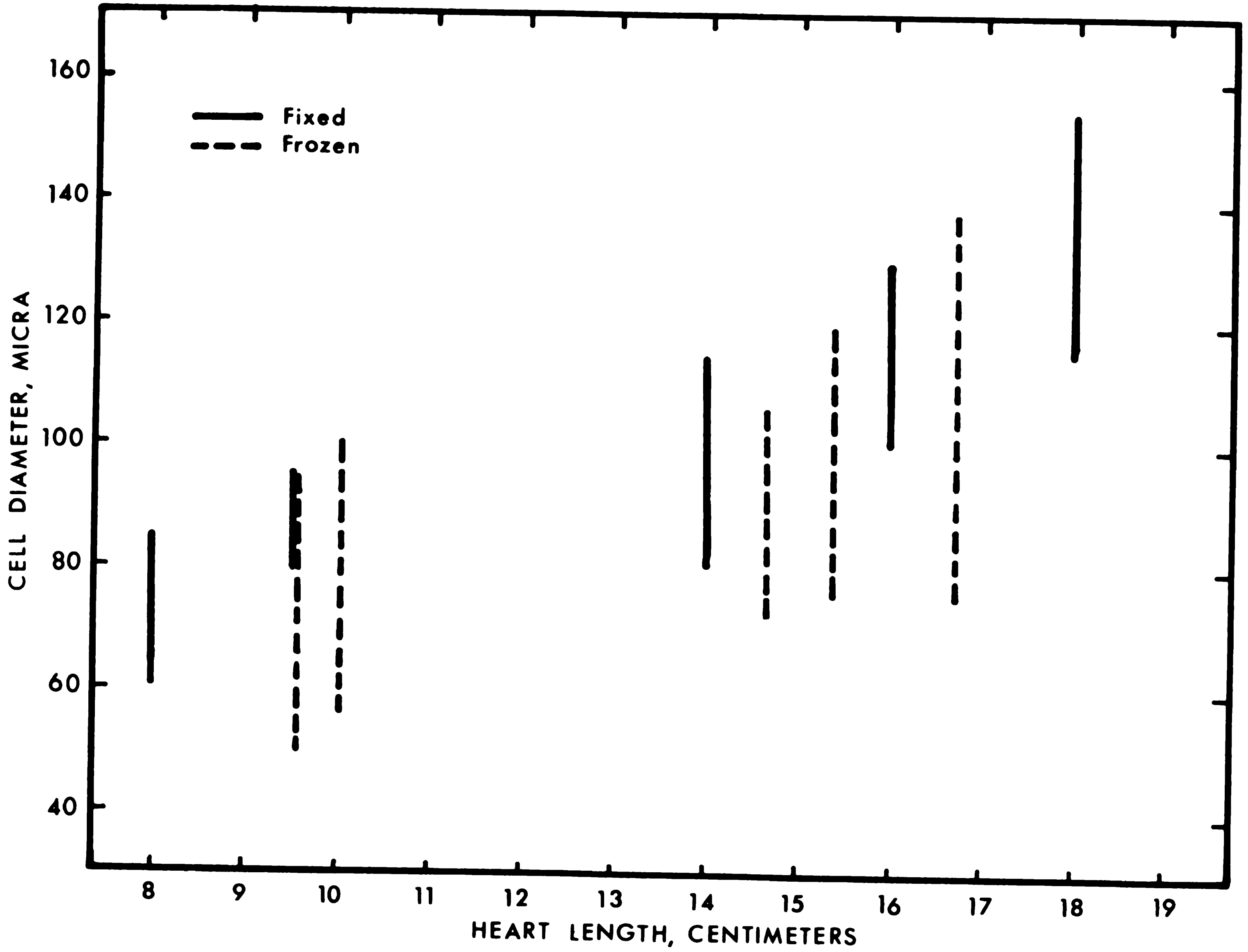

Fixed
Frozen
CELL DIAMETER, MICRA
160
140
120
100
80
60
40
8
9
10
11
12
13
14
15
16
17
18
19
HEART LENGTH, CENTIMETERS

Figure 15

causes a change in heart rate; nerves 7 and 8 are inhibitory (Carlson, 1905b; Pax and Sanborn, 1964) while nerves 9-13 are predominantly acceleratory (Carlson, 1905b; Pax, 1969). Several questions arise from these observations: Do the cardioregulatory fibers have direct connections with the ganglion? Is a neurohormonal system involved? Is there a combination of direct connections and neurohormonal systems? Do the segmental nerves carry both cardioinhibitory and cardioacceleratory fibers? What is the arrangement of the cardioregulatory fibers? Are the pericardial nerves involved in cardioregulation?

Partial solution of these questions can be carried out by an examination of the segmental cardiac nerves and an isolated heart preparation. Since stimulation of the segmental nerves at their origins produces a change in heart rate, what change occurs when these nerves are stimulated at various locations along their tracts and when various branches are stimulated? If the response pattern in the heart is observed during these stimulations, the fibers which are responsible for cardioregulation can be traced. If stimulation of the segmental nerves to an isolated heart preparation in which extraneous tissues have been removed produces the same response pattern as before, then the segmental nerves carry fibers which enter the cardiac ganglion and which are responsible for the inhibition and acceleration seen when the ventral nervous system is stimulated.

In this study nerves 7 and 8 were stimulated ventrally near their origins in the brain and dorsally as they enter the cardiac ganglion. Likewise, the segmental nerves 9-11 were stimulated ventrally near their origins in the ventral cord, pericardially as they cross the pericardial nerve and dorsally as they enter the cardiac ganglion. The results of such stimulation were compared with one another.

Stimulation of the segmental cardiac nerves 7 and 8 at their origins (5 V, 3 msec, 5/sec) resulted in a slowing of the heart rate (Figure 16). At least three phases of response can be seen: 1) time for maximum inhibition, 2) minimum rate and 3) recovery time. The most rapid change in heart rate occurs before the first 30 seconds of stimulation.

The mean response to stimulation of nerve 7 is an inhibited rate throughout the stimulation period. In one individual the response did not occur until after 30 seconds of stimulation. In all other animals, inhibition begins immediately and establishes the new rate within the first 30 seconds of stimulation. The response does not end with cessation of stimulation since the heart rate requires several seconds to return to the original rate. In one

Figure 16. -- Change in heart rate as a result of stimulation of nerves 7-11 at their origins (stimulus strength: 5 V, 3 msec, 5/sec). The arrows mark the duration of stimulation. The vertical lines give the range.

A. Nerve 7 (N = 6)

B. Nerve 8 (N = 7)

C. Nerve 9 (N = 6)

D. Nerve 10 (N = 10)

E. Nerve 11 (N = 7)

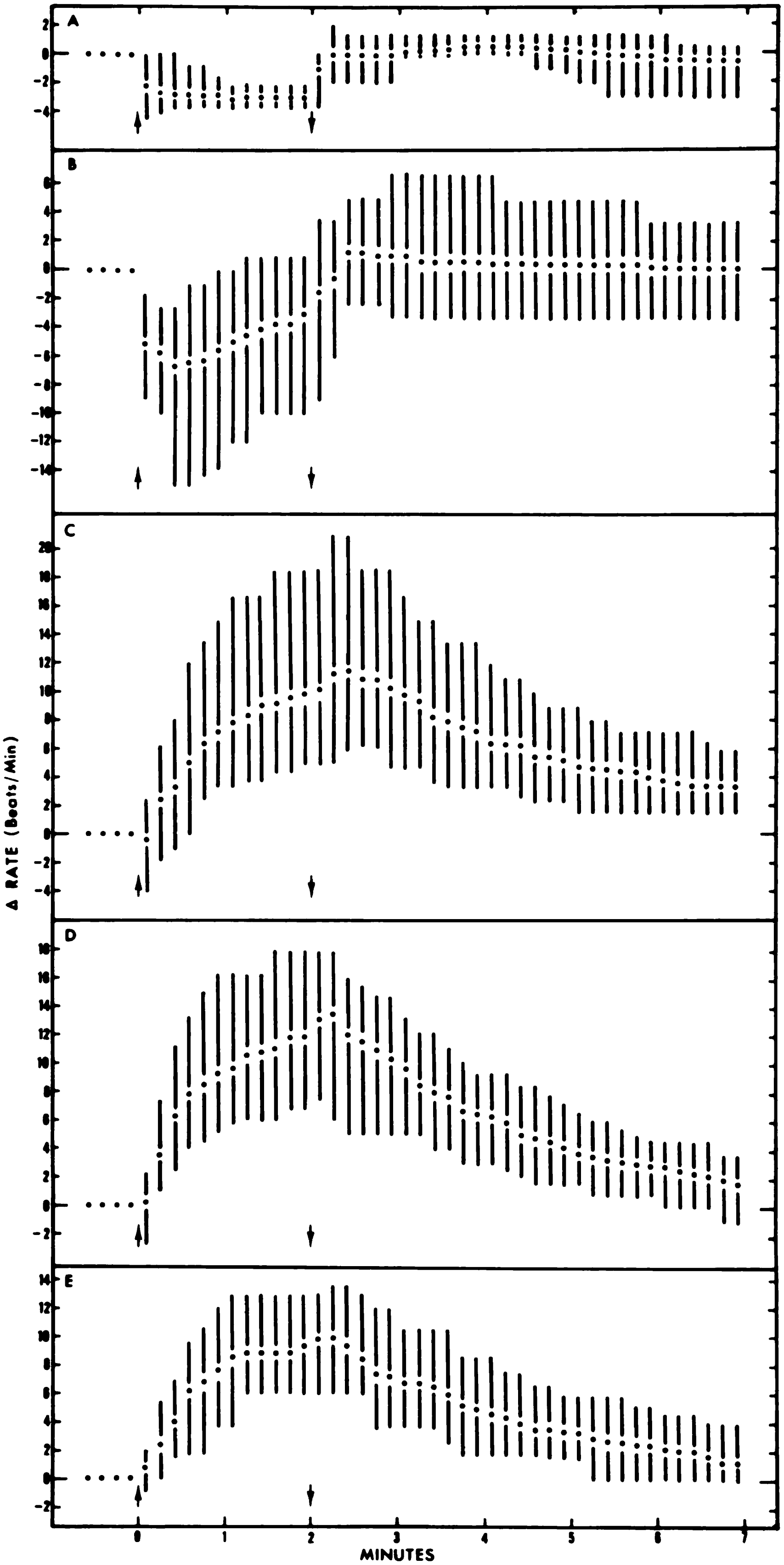


Figure 16

individual the heart established a new rate one beat per minute faster than the original rate. In other animals the heart rate returned to the original level and maintained that rate or after several minutes began a slower rate. The mean deviation from the original rate is less than one beat per minute for the entire five minutes following stimulation.

The mean response to stimulation of nerve 8 differs markedly from that of nerve 7. Inhibition begins immediately at the onset of stimulation and reaches maximum inhibition at 30 seconds. However, from this time on the mean heart rate returns toward the original rate. At the end of the stimulation period inhibition is one-half the value of maximum inhibition. The mean heart rate here as in stimulation of nerve 7 requires several seconds to recover. Not all individuals return to the original rate. The extremes in the responses to stimulation of nerve 8 during the recovery phase are an increase of seven beats per minute before returning toward the original rate and a maintained depression of three beats per minute. This variation suggests that the response to stimulation of nerve 8 is more or less an individual pattern. However, there is a characteristic feature in all response patterns except one. The rate after cessation of stimulation increases and then decreases. Indeed, the mean response shows an increase of one beat per minute before returning to the original rate. These differences in response patterns to stimulation of nerves 7 and 8 suggest that there is a basic difference in the fibers that make up these nerves.

Since Pax and Sanborn (1964) observed that stimulus frequencies of 10-80/sec produced maximum response of the inhibitory nerves, nerves 7 and 8 were also stimulated at 20/sec. The response is similar to that seen when these nerves are stimulated at 5/sec except that there is greater inhibition of the heart rate. In both cases the inhibition is closely coupled to stimulation with maximum inhibition occurring within a few seconds after stimulation begins and a return toward original rates immediately after release from stimulation. The response to stimulation of nerve 8 again shows a post-stimulation increase but it is not detectably different from that obtained by stimulation at 5/sec.

Stimulation of the segmental cardiac nerves 9-11 at their origins increased the heart rate (Figure 16). The response patterns to stimulation of nerves 9-11 resemble one another. Three phases of response can be seen: 1) rise time, 2) maximum rate and 3) recovery time. The most rapid change in mean rate occurs during the first 30-40 seconds of stimulation with a lessened increase in rate during the remainder of stimulation. Maximum mean rate is not reached until 10-20 seconds following cessation of stimulation.

Individually, however, the maximum rate may be reached during the last few seconds of stimulation and this rate is then maintained for several seconds after cessation of stimulation. It is also to be noted that an initial inhibition is present in some individuals. In these individuals the maximum rate always occurs after cessation of stimulation. But these two response characteristics do not always occur together since maximum rate may occur after cessation of stimulation in an individual in which initial inhibition does not occur. Recovery requires several minutes, usually between four and ten, and it occurs in a very nearly exponential pattern. Recovery also shows much variation; some hearts return to the original level quickly and establish a new level of beating at a slower rate; while other hearts may establish a slightly faster rate and not return to the original rate at all. The response pattern is the same whether the stimulation lasts for one or two minutes (Figure 17).

Individual nerves cause relatively consistent responses with similar stimulations over long periods of time. Figure 18 presents the results of repeated ventral stimulation of nerve 9 over a two hour period. The initial rate and the maximum rate varied over the range of four beats per minute during the various stimulations; however, the recovery phase shows less variation.

Figure 19 displays the mean response to stimulation of nerve 9 when stimulated in three different locations: 1) at its

Figure 17. -- Change in heart rate as a result of stimulation of nerve 10 at its origin (stimulus strength: 5 V, 3 msec, 5/sec) for one and two minute stimulation periods. The vertical lines give the range.

A. Nerve 10 stimulated for 2 minutes (N = 10)

B. Nerve 10 stimulated for 1 minute (N = 5)

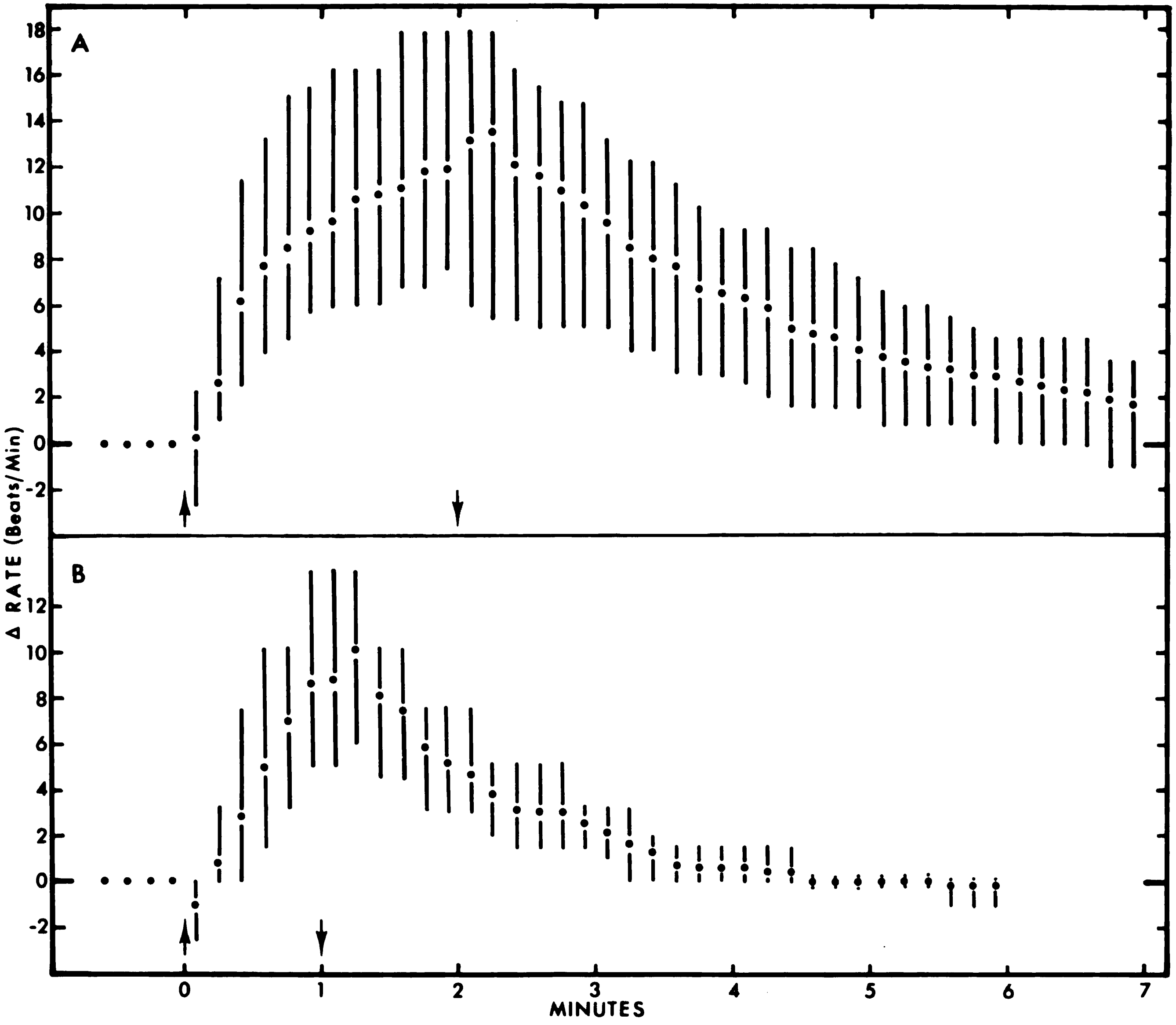

A
B
Δ RATE (Beats/Min)
18
16
14
12
10
8
6
4
2
0
-2
12
10
8
6
4
2
0
-2
0
1
2
3
4
5
6
7
MINUTES

Figure 17

Figure 18. -- Change in heart rate as a result of stimulation of nerve 9 at its origin (stimulus strength: 5 V, 3 msec, 5/sec). Stimulation was for one minute every twenty-five minutes (N = 5). The arrows mark the duration of stimulation. The vertical lines give the range.

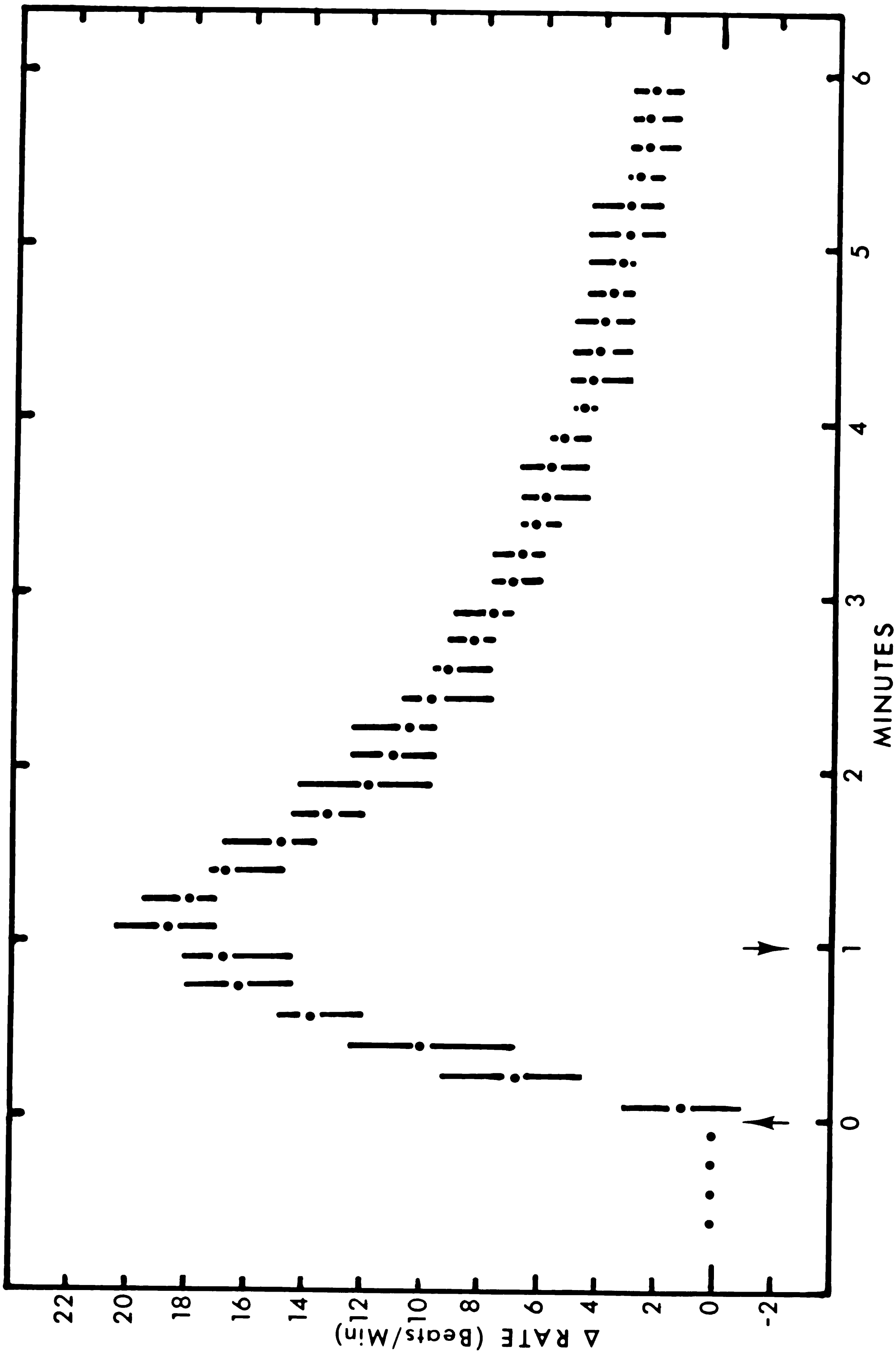


Figure 18

origin, 2) at the level of the pericardial nerve and 3) at the level of the cardiac ganglion. There are no detectable differences in the responses. Nerves 10 and 11 give similar results when stimulated in the corresponding locations.

Figure 20 displays the responses to stimulation of fused nerve 7 and 8 and nerves 9-11 when stimulated as they enter the cardiac ganglion. There is little difference in the mean responses to stimulation of these nerves dorsally and the responses when they are stimulated ventrally (see Figure 16). The ranges of responses overlap to the extent that the responses in both instances are essentially the same.

My observations essentially confirm Carlson's (1905b) observation that nerves 7 and 8 carry inhibitory fibers and Pax and Sanborn's (1964) observation that fused nerve 7 and 8 enters the ganglion. Also Pax's (1969) observation that nerves 9-13 carry excitatory fibers is confirmed. These experiments show, however, that the rise time, maximum rate and recovery phase of the response to stimulation of nerves 9-11 are essentially the same no matter where stimulation occurs; thus cardioacceleratory fibers like cardioinhibitory fibers enter the ganglion and their effect appears to be directly on the ganglion and not through some intermediary neurohormonal system.

Figure 19. -- Change in heart rate as a result of stimulation of nerve 9 in three different locations (stimulus strength: 5 V, 3 msec, 5/sec). Arrows mark the duration of stimulation. The vertical lines give the range.

A.	Ventral stimulation	(N = 5)
B.	Pericardial stimulation	(N = 8)
C.	Dorsal stimulation	(N = 6)

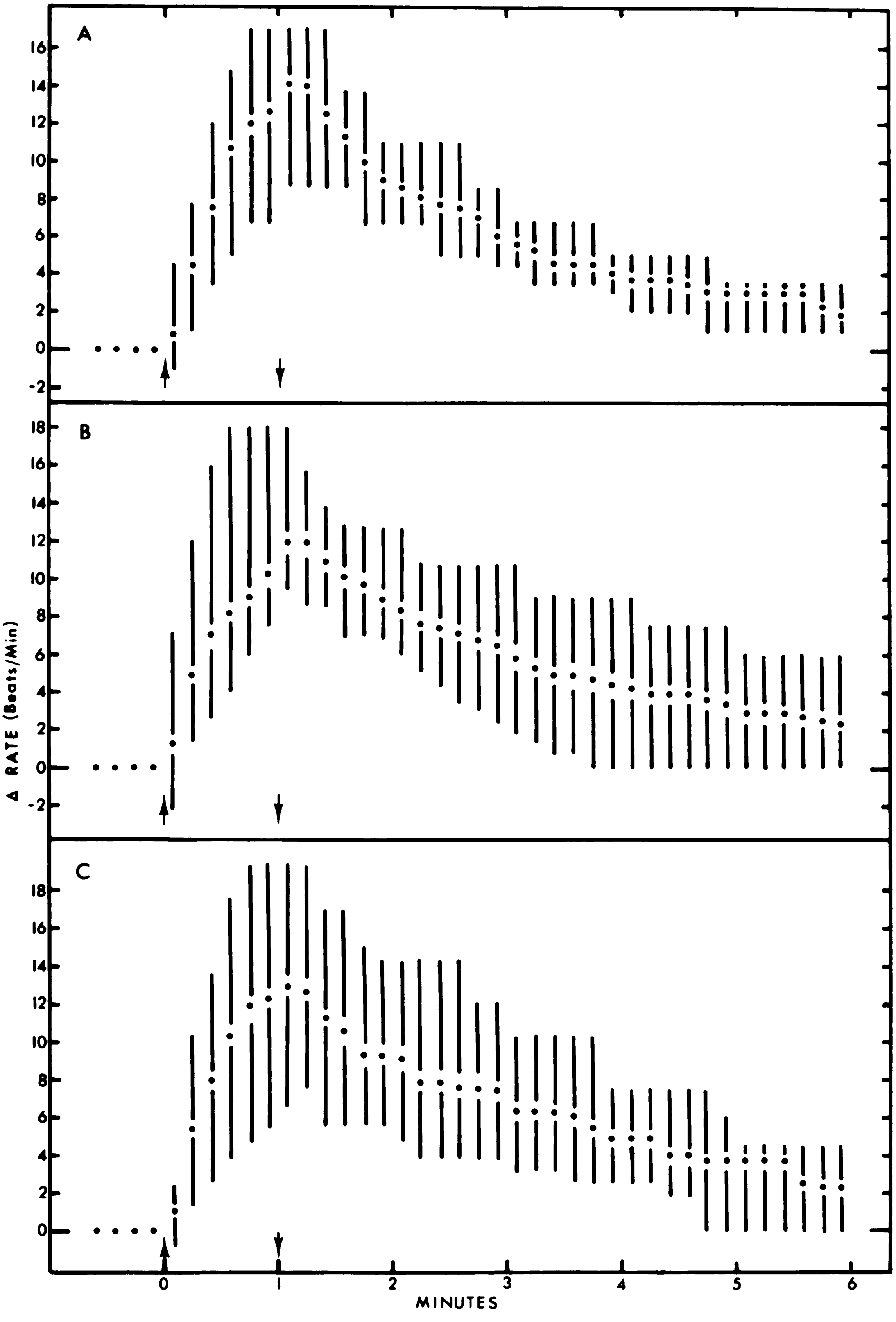

A
B
C
Δ RATE (Beats/Min)
16
14
12
10
8
6
4
2
0
-2
18
16
14
12
10
8
6
4
2
0
-2
18
16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
6
MINUTES

Figure 19

Figure 20. -- Change in heart rate as a result of stimulation of fused nerve 7 and 8 and nerves 9-11 as they enter the cardiac ganglion (stimulus strength: 5 V, 3 msec, 5/sec). The duration of stimulation is marked by arrows. The vertical lines give the range.

A. Fused nerve 7 and 8 (N = 9)

B. Nerve 9 (N = 6)

C. Nerve 10 (N = 8)

D. Nerve 11 (N = 5)

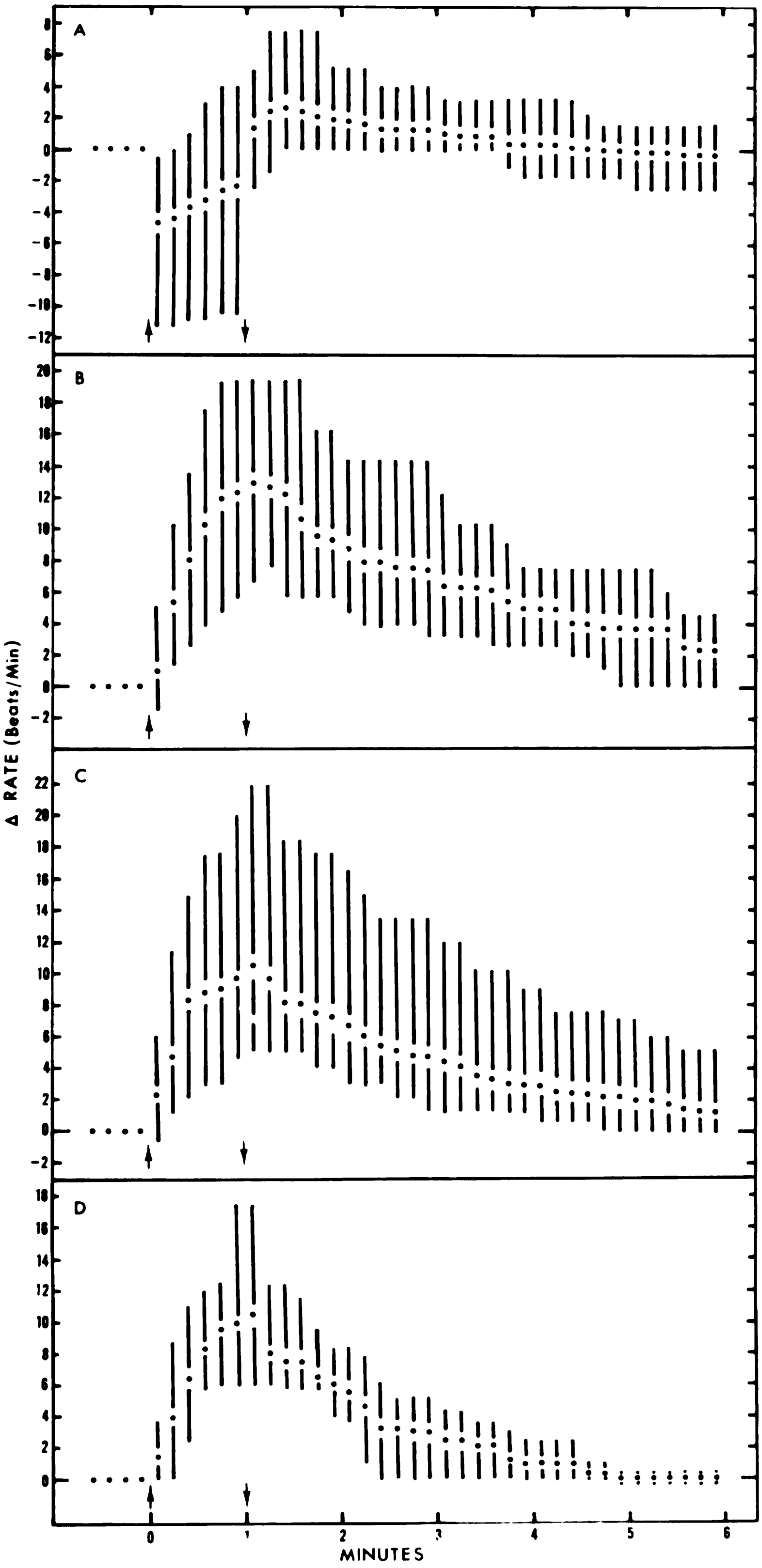

A
8
6
4
2
0
-2
-4
-6
-8
-10
-12
B
20
18
16
14
12
10
8
6
4
2
0
-2
C
22
20
18
16
14
12
10
8
6
4
2
0
-2
D
18
16
14
12
10
8
6
4
2
0
Δ RATE (Beats/Min)
0
1
2
3
4
5
6
MINUTES

Figure 20

Two other nerves were also stimulated: segmental nerve 6 and the pericardial nerve. Segmental nerve 6 was non-responsive when stimulated both at its origin and where it joins the ganglion. This confirms Carlson's (1905b) observation that nerve 6 is non-responsive when stimulated at its origin; but not Pax's (1966) observation that nerve 6 was inhibitory when stimulated near its point of entry into the ganglion. This difference can probably be explained since fused nerve 7 and 8 enters the ganglion at various locations. The number of branches of this nerve entering the ganglion also varies; on occasion two and sometimes three branches instead of the usual one are found. It is possible that one of these branches entered the ganglion near nerve 6 and Pax stimulated this combination. While nerve 6 does not apparently function in cardioregulation, its function is still unclear.

Stimulation of the pericardial nerve and the pericardial branches of the segmental nerves does not affect heart rate. It does, however, cause contractions of the opisthosomal extensor muscles.

Tissue Extracts

There have been no reports of pericardial organs in *L. polyphemus*; and in my observations of the pericardial region, I could find no nerve trunks or endings such as those described in the Crustacea. The pericardial area is richly supplied with nerves of one to five fibers, many of which branch from the segmental nerves. Other small nerves branch from the pericardial nerve. Since stimulation of nerves in this area was previously shown to be ineffective in altering heart rate, tissue extracts were utilized to determine if some tissue might contain a substance capable of affecting heart rate.

Of the experiments utilizing tissue extracts, only two tissues show consistent effects upon the heart. Tissue extracts (filtrate of 10 mg tissue per 1 ml saline) of ventral cord and brain depress heart rate by four to ten beats per minute while extracts of the pericardial tissues increase heart rate by eight to twelve beats per minute. Boiling the extract and applying the recovered filtrate produced the same results as fresh materials. These results suggest that some substances are present which have effects upon the heart, but further work is necessary to determine if they are part of an organized system affecting heart rate.

DISCUSSION

Morpohology of the Cardiac Ganglion

The organization of the cardiac ganglion of L. polyphemus differs markedly in many respects from that of other arthropods for which comparative information is available. In the crustaceans, arachnids, and limulus the heart is tubular and a cardiac ganglion lies on the dorsal midline. In isopods and decapods this ganglion lies on the inner surface of the heart; but in the amphipods, stomatopods, scorpions, spiders and limulus, it lies on the outer surface of the heart.

No further comparisons can be made with the arachnids, since quantitative data have not been reported. In the malacostracans which have been studied, the total number of cells is usually small. In amphipods and isopods there are only six neurons in the ganglion (Alexandrowicz, 1954); in stomatopods the number of neurons is 14 (Irisawa and Irisawa, 1957); and six neurons are present in the mysidicean Praunus flexuosus (Alexandrowicz, 1955). The decapod cardiac ganglion also contains a limited number of cells; five large and four small cells have been found in lobsters and crabs (Alexandrowicz, 1932), but eight large and eight small cells have been found in the crayfish *Astacus potamobius* (Alexandrowicz, 1929) and only five cells in the shrimp *Alpheus spp* (Suzuki, 1935). The cardiac ganglion of *L. polyphemus* differs markedly from that of the malacostracans with respect to both the total number of cells as well as the number of cell types. The total number of cells in the ganglia I studied averaged 231 and there were six cell types.

Not only is the total cell number in malacostracans small, it is also remarkably constant for any particular species. In the lobster *Homarus americanus*, for example, in only three of about 150 cardiac ganglia the total number of cells was 10 rather than nine (Maynard, 1961). The total number of cells in the cardiac ganglion of limulus ranged from 171 to 305 in the twelve hearts studied.

In the malacostracan cardiac ganglion the large cells are usually anterior and small cells posterior. The large cells range up to 200 u in large decapods and are generally multipolar; the small cells are about 100 u and are always multipolar, although they often have one large process (Bullock and Horridge, 1965). The arrangement in *L. polyphemus* is different. Six morphological cell types are distinguishable and, unlike the situation in the malacostracan cardiac ganglion, unipolar and bipolar cells are the dominant cell types. Moreover, the cell types are not segregated on any morphological basis, but lie randomly along the length of the ganglion.

In the crustaceans the cardiac ganglion consists mainly of a limited number of cell bodies, their branched axons, and neuropile. The neuropile of the ganglion is regionally clumped and surrounded by glial cells (Alexandrowicz, 1932). In limulus there is a separation of the cardiac ganglion into only two recognizable regions; the cellular portion and the fiber tract. Unlike the crustacean ganglion, neuropile and cell body regions are not clearly segregated.

Thus in all aspects of the cardiac ganglion discussed above, namely, the total cell number, variability in cell numbers, distribution of cell types along the ganglion, and arrangement of the components of the ganglion, limulus differs markedly from that of the malacostracans. In light of such differences, the question may be raised as to whether there is a functional difference between these cardiac ganglia. The answer to such a question must await a physiological analysis of the various cell types.

Also from a broader viewpoint, the limulus cardiac ganglion appears to be different from many other arthropod nervous structures. In those systems thoroughly analyzed on a morphological basis, there appears to be a constancy in cell number as well as cell arrangement, so that specific neurons can be identified from animal to animal and the three dimensional distribution of cell bodies and connectives can be mapped. This has been possible in such structures as the lobster cardiac ganglion (Maynard, 1953; Hagiwara and Bullock, 1957; Hartline, 1967), the crayfish ventral nerve cord (Kendig, 1967), the locust thoracic ganglion (Wilson, 1961) and the cockroach metathoracic ganglion (Cohen, 1967). Such a specific arrangement of elements apparently does not occur in the limulus cardiac ganglion. There is a large variation in total cell numbers as well as a large variation in the total number of each cell type from one ganglion to the next.

Regulation of the Neurogenic Heart

Numerous factors are capable of altering the activity of arthropod hearts (see Krijgsman, 1952, for review). The usual endpoint used in evaluation of activity is either a change in rate or in amplitude of contraction. Interpretation of the results of such changes is complicated since there may be many sites of action: pacemaker cells, motor cells, coordinating system or regulation system of the ganglion or neuromuscular junctions or muscle itself.

The similarity between the activity of the crustacean heart and that of limulus is amplified by the similarity of factors modulating the activity of the heart. There is positive correlation between temperature and the rate of beating (Carlson, 1906b; Garrey, 1920, 1932a; Seiwell, 1930). Distension of the heart increases the heart rate (Carlson, 1904c, 1907a; Garrey, 1930; Dubisson, 1931; Isquierdo, 1932). The effects of electrical stimulation have been reported (Samojloff, 1930; Garrey, 1932b; Garrey and Knowlton, 1934; Bullock, et al., 1943), a variety of ions have been tested for their effects (Carlson, 1906c, 1906d, 1906e; Prosser, 1943b; Prosser and Brown, 1961) and numerous pharmacological agents which cause a change in both rate and strength of beating have been reported (Carlson, 1904a, 1906f, 1907b; Welsh, 1939, 1942; Prosser, 1942; Smith, 1947; Pax and Sanborn, 1967a, 1967b; Abbott, et al., 1969). While these studies have described many factors modulating heart action, no study has yet been made concerning the problems of intraganglionic relationships.

Anatomy of cardioregulatory nerves. -- The similarity of the anatomy of the cardioregulatory nerves of the Crustacea and limulus is also strikingly similar. Cardioregulatory nerves in decapods were indicated early by several investigators (Dogiel, 1876; Plateau, 1880; Conant and Clark, 1896). Carlson (1905a, 1906a) studied the nerves of *Panulirus sp.* and came to the conclusion that two nerve pairs from the subesophageal ganglion innervate the heart, the anterior pair inhibitory and the posterior pair acceleratory. The entrance of the inhibitory nerves into the hearts of various crabs was observed by Alexandrowicz (1932), Heath (1941), Wiersma and Novitski (1942) and Smith (1947). Maynard

(1953) observed in Panulirus sp. that the extrinsic cardioregulatory nerves consist of two pairs of cardioacceleratory nerves (posterior) and one pair of cardioinhibitory nerves (anterior) which arise in the anterior portion of the thoracic ganglion. These nerves run dorsally and laterally to join the pericardial plexus where fibers from each come together to form the dorsal nerve which runs to the cardiac ganglion. This anterior-posterior arrangement of cardioregulatory nerves is the same as found in limulus. However, the cardioregulatory nerves of limulus enter the ganglion individually rather than forming a single nerve.

Function of cardioregulatory nerves. -- Stimulation of inhibitory nerves in both decapod Crustacea and limulus produces similar effects. Heart rates in both groups are decreased within a few seconds of application of above threshold stimuli and return to the original rate a few seconds after cessation of the stimulus. In the case of the crustacean, Florey (1960) reported that frequencies of stimulation less than 15/sec were ineffective and frequencies above 35/sec result in heart standstill. In the range of frequencies between 15-35/sec the decrease in heart rate varies almost exponentially as the frequency of stimulation is increased. Pax and Sanborn (1964) report that frequencies as low as 2.5/sec cause slowing of the heart of limulus and that as frequency is increased the heart rate reaches a maximum depression at a stimulation frequency of 15/sec and remains at that level up to a stimulation frequency of 90/sec. Frequencies above 90/sec were found to be less effective in rate depression and even greater stimulation frequencies accelerate the heart. Both Smith (1947) and Florey (1960) report heart stoppage in the Crustacea and Carlson (1905b) reports similar occurrences in limulus, but Pax and Sanborn (1964) were unable to produce heart standstill.

When stimulating nerves 7 and 8 with frequencies of 80-100/sec, Pax and Sanborn (1964) report an acceleration of heart rate and suggest that some excitatory fibers are contained in the inhibitory nerves of limulus. Both Terzuolo and Bullock (1958) and Florey (1960) describe post-inhibitory excitation; but they do not discuss it. Later Florey (1968) showed that in the crayfish *Pacifiastacus leniusculus* some individuals may have both inhibitory and acceleratory fibers in a single nerve rather than the usual separate nerves. Thus the presence of a post-stimulatory increase may indicate that acceleratory fibers are present in the predominantly inhibitory nerves of both limulus and the Crustacea. The range of responses in nerve 7 as reported here indicates that inclusion of acceleratory fibers probably occurs only rarely. In nerve 8, however, the slope of the mean response and the post-stimulatory increase indicates that probably most animals have acceleratory fibers in this nerve.

Excitation is similar in limulus and the Crustacea. It does not start abruptly as does inhibition, but develops gradually and reaches a maximum only after some seconds of stimulation. The heart rate remains elevated after cessation of stimulation and returns slowly to the original rate. Both the acceleration and the after-effects are much more pronounced in limulus than in the Crustacea.

Limulus also shows two other differences in response to stimulation of the acceleratory nerves; often there is an initial inhibition and a post-stimulatory increase. The initial inhibition seen in nerves 9-11 is similar to that seen in nerves 7 and 8. In both cases the inhibition is closely coupled to stimulation with inhibition occurring immediately. Pax (1969) examined the frequency-response characteristics of both sets of nerves and found that the acceleratory nerves like the inhibitory nerves give marked inhibitory effects only with stimulus frequencies in excess of 10/sec. Thus the inhibition reported here is below the maximum that might be expected. The post-stimulatory increase occurs during the 10-20 seconds after stimulation which is the main period of recovery from the influence of inhibition. Thus this increase suggests that the ganglion has been released from inhibition and the full effect of stimulation of the acceleratory fibers is then seen.

Initial inhibition and the post-stimulatory increase are evidence that inhibitory fibers are present in the abdominal nerves. Since the inhibitory effects are more marked in nerve 9, it appears that more fibers are present in this nerve or lower thresholds are present. The number of inhibitory fibers is not known but must vary from individual to individual since the range of responses is large.

Neurohormonal regulation. --In recent years a great deal of attention has been focused on the brachyuran pericardial organs discovered and described by Alexandrowicz (1953). Because of the location of the pericardial organs in relation to the heart, Alexandrowicz and Carlisle (1953) believed that secretions of the pericardial organs could be involved with regulation of the heart. Maynard (1961a, 1961b) demonstrated that cell groups from the ventral ganglia send axons to the pericardial organs by way of the first seven segmental nerves and suggested on morphological evidence that at least three types of fibers are present. Maynard and Maynard (1962) described a number of bipolar cells within these organs. Various substances have been proposed as the active principle (Belamarich, 1963; Belamarich and Terwilliger, 1966) and Cooke (1966) has reported the sites of action of pericardial organ extract on the lobster heart. All this work has not yet demonstrated that the heart is the primary target of pericardial organ substances. Indeed, Cottrell and Osborne (1969) suggest that placement of neurosecretory systems near the heart is ideally situated for the release of a substance causing the general stimulation of an animal. Release of such a substance would first accelerate the heart and thus promote its own distribution throughout the rest of the animal; whereas, release of the substance elsewhere would take longer for distribution and initiation of stimulation would be delayed. Thus the primary target of the pericardial organs of Crustacea may not be the heart and secretions of these organs may not function in normal cardiac regulation.

No such organs have yet been described for limulus, but extracts of tissues from the pericardial area consistently caused an increase in heart rate. It cannot yet be stated whether this increase is due to the activity of a neurohormonal system or due to a general cytoplasmic substance of the pericardial tissues.

Function of the Cells of the Cardiac Ganglion

Morphology of a structure is at best only a small part of an overall understanding of a structure. An equally important aspect of the structure is function. The function of the cardiac ganglion is initiation of the heart rhythm. However, since the ganglion is composed of many smaller components, it is necessary to examine each of these with regard to function. Function for the cells of the cardiac ganglion can be suggested if the data from the distribution of the pigmented cells (Figure 9) and some data from previous physiological studies are used.

With the establishment of the neurogenicity of the limulus heart (Carlson, 1905b), early workers began looking for the pacemaker and its constituents. Garrey (1930) was the first to examine the ganglion systematically for its pacemaker. He noticed that with section of the heart, each piece maintains the same rate as the original but the pieces no longer beat synchronously. Continued cutting of the ganglion produces contractile segments except anterior to ostia III and posterior to ostia VIII. Prosser (1941) was able to record slow waves externally from the ganglion. These slow potentials are largest in segments four, five and six, less in segments three, seven and eight and not present in segments one, two or nine.

Since the limulus ganglion shows a morphological change at ostia III, two aspects of cardiac ganglion organization are suggested by the work of Carlson, Garrey and Prosser. First, pacemaker cells probably lie within the cellular portion of the ganglion.

Secondly, since small bipolar cells and non-pigmented cells are found anterior to ostia III, they probably do not participate in ganglion activity initiation.

Carlson (1905b) demonstrated that when the intra-cardiac pressure is increased there is an increase in heart rate. Garrey (1930) noted that when the anterior end of the animal was raised, the increased intra-cardiac pressure altered the character of the contraction. These observations suggest the probability of a stretch receptor-pacemaker interaction. Bullock and Horridge (1965) indicate that most of the stretch receptor cells studied thus far are bipolar cells. There are two types of bipolar cells in limulus cardiac ganglia; whether one or both are stretch receptors is an open question.

This leaves only two cell types, the pigmented unipolar and the pigmented multipolar, which may be involved in the production of the heartbeat. Heinbecker (1936) found that when he cut the ganglion into 3-6 mm pieces, only some of the pieces were spontaneously active. By histological examination of these pieces he found that pieces showing spontaneous activity always contained at least one large unipolar cell as well as several multipolar cells. Pieces of ganglion containing only multipolar cells were not spontaneously active. On this basis Heinbecker concluded that the large unipolar cells are responsible for the initiation of the heartbeat.

Heinbecker, however, did not obtain any sections in which only the unipolar cell was present; in all cases there were at least a few multipolar cells. Since the sample size in his experiments was quite small and since he did not distinguish between pigmented and non-pigmented cells, it is possible that a pigmented multipolar cell was always present when the piece showed spontaneity and absent when it did not. For this reason the evidence presented by Heinbecker does not appear to clearly demonstrate that the unipolar cell is necessarily responsible for the initiation of the heartbeat. Obviously further physiological experimentation is needed before specific functions can be assigned to each of the cell types found within the cardiac ganglion of _L. polyphemus_.

CONCLUSIONS

This study provides the basis for future physiological examination of the L. polyphemus cardiac ganglion, especially electrophysiological studies of individual ganglionic cells. Since the cell types, cell numbers and distribution along the ganglion are now known, it will be possible to make comparisons of physiological data from one heart to the next. Likewise, since the cardioregulatory nerves enter the ganglion directly, ventral nervous system influence on the cardiac ganglion can now be examined by electrophysiological methods.

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