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Cardioregulatory Role of the Abdominal Ganglion of APLYSIA CALIFORNICA

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CARDIOREGULATORY ROLE OF THE ABDOMINAL GANGLION OF *Aplysia californica*

by

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Each person whose signature appears below certifies that he has read this thesis and that in his opinion it is adequate, in scope and quality, as a thesis for the degree of Master of Science.

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INTRODUCTION

Myogenicity, extrinsic nervous regulation, response to neurohormones, and inflow-outflow pattern are some of the features of the molluscan heart in general found to be in common with the vertebrate heart. The simpler organization of the nervous system as well as the abundance of animals and the low cost of procurance and maintenance make the mollusc Aplysia desirable for a comparative physiological study of a myogenic heart.

Literature Review

The heart of the mollusc Aplysia has been investigated for many years. Automaticity, nervous regulation, mechanics of contraction, and response to chemicals are some of the physiological characteristics that have been studied.

Myogenicity

Among the results of several lines of investigation indicating the myogenic nature of the Aplysia heart are the automaticity of beats in isolated hearts and fragments, the shape of the electrocardiogram, and the effect of neurohormones.

Straub (1901), Schoenlein (1894), and Carlson (1905) reported that the isolated, unstretched heart of Aplysia continues to beat, while Ransom (1883) and Jullien (1937) indicated that even unstretched ventricular fragments beat. Contrarily, L. Fredericq (1922) and Heymans (1924) reported the failure of empty hearts to beat. In 1960, Wright found that isolated trabeculae attached to the ventricular node had a

spontaneous frequency of 28 to 31 beats/min. in A. vaccaria. Stretching the trabecula to 1.2 times resting length increased the rate to 50 beats/min. Atrial trabeculae rarely had spontaneous beats.

Hoffmann (1910) used a string galvanometer with two electrodes placed lengthwise across the heart and obtained a smooth electrocardiogram (ECG) devoid of the rapid impulses found in ECG's from neurogenic hearts. Wright (1960) obtained a smooth electrogram with a suction electrode on the ventricular node. (McCann, 1965, gives a comparison of microelectrode recordings from a neurogenic heart and a myogenic heart.)

10^{-10} M acetylcholine (ACh) applied to nodal tissue resulted in complete cardiac arrest in A. vaccaria (Wright, 1960) while 10^{-11} M ACh on A. limacina heart (Reiter, 1957) resulted in 40% reduction in beat amplitude with no chronotropic effect. Heymans (1924) on A. limacina and Jullien (1937) on A. fasciata obtained negative tonotropic and chronotropic effects. Heymans (1924) found epinephrine to activate the Aplysia heart.

Two other chemicals tested on the Aplysia heart are 5-Hydroxytryptamine (5-HT) and lysergic acid diethylamide (LSD). Loveland (1963) found evidence favoring 5-HT as a probable mediator for excitation in the heart of Venus, a marine gastropod. However he failed to identify 5-HT in the perfusate of the stimulated heart-nerve preparation. Welsh and Moorehead (1959), using fluorescence spectroscopy, found 5-HT in the Venus ganglia. Wright (1960) found that 10^{-10} M 5-HT applied to the normally beating A. vaccaria heart results in immediate, long-lasting excitatory effects which are blocked by high intensity

stimulation of the left connective. LSD-25 inhibited both normally beating and 5-HT excited hearts. Gaddum and Paasonen (1955) found LSD to be an unsatisfactory 5-HT antagonist in some molluscan hearts.

Aplysia have not been studied histologically to determine the presence of nerve cells or ganglia in the heart. The general picture varies considerably for gastropods. There is evidence for nerve cells in some gastropod hearts but their function is unknown (Bullock and Horridge, 1965).

Extrinsic Nervous Regulation

The nature of extrinsic cardioregulation by the nervous system has been studied by many investigators in various species of Aplysia. These studies involved electrical stimulation of nerves related to cardiac function and direct stimulation of the heart itself. In 1901 (see note, Table 1), Bottazzi and Enriques, reporting on the effects of nicotine on the visceral ganglion, concluded that the cell bodies of the cardiac nerve fibers are located in this ganglion. The axons in the pleuro-visceral connectives synapse with these cells which give rise to the cardioregulatory nerves. Carlson (1904) reports that nerve fibers from the visceral ganglion innervate the heart at both the aortic and atrial ends. Dogiel mentioned in 1877 that nerve fibers from the branchial ganglion enter the base of the gill near the atrium. Stimulation of the ganglion resulted in acceleration of the heart. Bottazzi and Enriques (1900) reported cardiac acceleratory fibers arising from the left visceral ganglion.

The results of stimulation studies on Aplysia before Wright's work in 1960 are tabulated in Table 1.

Table 1. Results of early studies on *Aplysia* heart and abdominal ganglion nerve stimulation.

Investigator(s)	Date	Species	Stimulation Site	Result(s)	Citation
Dogiel	1877		Branchial ganglion	Acceleration	Wright (1960)
Dogiel	1877		Pleurovisceral connective	Acceleration	Carlson (1905)
Ransom	1884		Pleurovisceral connective	Acceleration	Carlson (1905)
Yung	1887		Heart	Inhibition	Krijgsman and Divaris (1955)
Schoenlein	1894	*limacina*	Heart	Inhibition	Krijgsman and Divaris (1955)
Schoenlein	1894		Pleurovisceral connectives	Acceleration	Carlson (1905)
Schoenlein	1894	*limacina*	Pleurovisceral connectives	No visible effect	Wright (1960)
Schoenlein	1894	*limacina*	Ctenidial nerve	Contraction of gill and ventricle	Wright (1960)
Bottazzi and Enriques	1900*	*limacina* and *depilans*	Visceral nerve	Acceleration	Krijgsman and Divaris (1955) Hill and Welsh (1966)
Bottazzi and Enriques	1901*		Pleurovisceral connectives	Acceleration	Carlson (1905)

Investigator(s)	Date	Species	Stimulation Site	Result(s)	Citation
Carlson	1905	californica	Pleural ganglion, visceral nerves, and connectives	Acceleration	Carlson (1905)
Heymans	1924	limacina	Visceral nerve	Acceleration	Krijgsman and Divaris (1955)
Fredericq	1939	limacina	Visceral nerve	Acceleration	Krijgsman and Divaris (1955)

*Both dates were cited for the same article. I was unable to trace exact date of the original publication.

Carlson (1905) found that the exposed but intact heart of A. californica continued beating for an hour after surgery. Electrical stimulation of the pleural ganglion or visceral nerve in such a heart resulted in augmentation of the rhythm. In a resting heart stimulation of the nerve initiated a series of beats in both the ventricle and atrium. Then Carlson ran a series of experiments to determine areas where the cardiac nerve fibers enter the heart. Stimulation of the visceral nerve of a heart severed at the aortic sinus resulted in augmentation of rate of beat in both heart chambers, with the greatest effect on the atrium. Equal results were obtained in both chambers of a heart severed at the atrioventricular junction. Hearts severed at the base of the atrium remained contracted and could not be used. His conclusion was that accelerator nerve fibers enter the ventricle at the aortic sinus and enter the atrium at its base.

Wright's (1960) dissertation on the heart of Aplysia vaccaria is the most recent work on extrinsic neural regulation. His results were significant in that stimulation of the left pleuro-visceral connective at high intensities produced inhibition, a finding not previously reported except in the case of direct stimulation of the heart. Pulses of 10 milliamperes (ma) intensity and 10 milliseconds (ms) duration at a frequency of 50 Hertz (hz) were required to obtain inhibition of the heart. During the early post-inhibition period, positive chronotropic and inotropic effects were recorded. Low intensity pulses on the order of 5 ma resulted in positive chronotropic and inotropic effects. Stimulation of the right connective resulted in acceleration at all intensities. Wright showed that inhibition is mediated through the branchial

nerve and ganglion while acceleratory fibers are contained in the pericardial nerve from the visceral ganglion to the heart.

Visceral Ganglion

The visceral (abdominal) ganglion of Aplysia has been widely utilized for studies in cellular electrophysiology, neuronal control and response in behavior, photochemical responses, and circadian rhythm of single cells. The primary advantages are the relatively small total number of neurons in the ganglion, large identifiable monopolar neurons and their close proximity to the surface. In a 20 cm A. californica (relaxed length) the largest (giant) cell is on the order of 400 microns in diameter while in a 25 cm A. vaccaria it is approximately 800 microns (Bullock, 1961). In larger animals the giant cell is about 1 mm. Coggeshall (1967), Frazier et al. (1967), on A. californica and Kandel et al. (1967) in their series of three papers facilitated use of this ganglion as an experimental preparation by classifying 30 large neurons according to 1) anatomical position, 2) morphology, 3) spontaneous firing pattern, 4) response to iontophoretic injection of ACh, 5) connections to the nerves, 6) response to nerve stimulation, and 7) interconnection with other neurons within the ganglion.

The ganglion is enclosed within a tough translucent sheath containing nerves, muscle cells and blood vessels (Coggeshall, 1967). The blood vessels travel throughout the sheath but do not enter the nervous system within.

Frazier et al. (1967) devised a simple, flexible system for numbering the cells and determining location within the ganglion. The ganglion was arbitrarily divided into quadrants with left and right determined from the dorsal view while facing rostrally. The quadrants

were called left rostral quarter ganglion (LRQG), left caudal quarter ganglion (LCQG), right rostral quarter ganglion (RRQG), and right caudal quarter ganglion (RCQG). The cells were numbered according to right and left sides, e.g., R1 through R16 and L1 through L14. Some of the cells were labeled as parts of clusters with properties similar to an identifiable cell within the area since these cells were not individually identifiable in themselves.

Two distinct colorations are present in the cells, some cells being whitish and others having an orange pigment. The white cells, thought to be neurosecretory, are characterized by the presence of large numbers of granules, paucity of synaptic connections, and regular firing.

Iontophoresis is a technique for injecting controlled amounts of ions from a microelectrode into a conductive medium such as intracellular fluid. An electrical current is used to regulate the quantity and flow rate of ions into a highly localized area around the microelectrode tip (Curtis, 1964). Five categories of response of the abdominal ganglion cells to iontophoretic injection of acetylcholine (ACh) were listed by Frazier et al. (1967) according to the classification by Gerschenfeld and Tauc (1964).

Cell Type	Response to Acetylcholine
D	Depolarizing
H	Hyperpolarizing
D-H	Double response (cell L7)
DINI	D cell with noncholinergic spontaneous inhibitory postsynaptic potentials (IPSP's)
DILDA	D cell with inhibition of long duration following excitation or inhibition but does not have spontaneous IPSP's

Cells L1, R1, and R2 are the only cells (of the 30 identified cells of Frazier et al., 1967) which send their axons to the circumesophageal ganglia through the pleurovisceral connectives. Oddly, these are all classified as silent cells. In general, identified cells send their axons into the ipsilateral peripheral nerves. All the R cells except R16 send their axons into the branchial nerve while nearly all L cells have their axons in the genital-pericardial nerve. The axons of cells R15 and R16 cross over into the genital-pericardial nerve while L7 and L14 send axons into the branchial nerve. Some of the cells send axons into more than one nerve and the description given provides only a general idea of the organization of the abdominal ganglion.

Using simultaneous recordings from two and three cells, Kandel et al. (1967) have identified some of the synaptic connections between identified cells and some inferred interneurons. Anatomical evidence from study of serial sections by light microscopy was obtained in some instances to verify monosynaptic connections. The effect of interneuron I (L10) was found to be inhibitory on cells L2, L3, L4, and L6 and excitatory on R15 and R16. (This is of particular interest since these cells have their axons in the genital-pericardial nerve.) Confirmation that the opposite synaptic effects were mediated by a common transmitter was obtained by the effects of iontophoretic injection of ACh and curare on the cells. Kandel et al. (1969) and Waziri and Kandel (1969) have expanded on the outline of interconnections within the ganglion showing the effects of interneuron I and II (unidentified cell) on their follower cells.

Behavior, Learning and Single Cells

Bruner and Tauc (1964, 1966a, b) showed that habituation of the cephalic tentacle contractile response of A. depilans and A. punctata to drops of sea water is at the central neuronal level and not a peripheral sensory adaptation. In whole animal experiments, they observed that contraction of the tentacle would diminish as a function of repeated stimuli (habituation). Recovery to the initial amplitude of contraction was brought about by a rest period of several minutes or by application of a stronger stimulus (dishabituation) such as scratching the skin. A compound EPSP in the left giant cell was seen to habituate and dishabituate under similar circumstances. Cerebral nerve stimulation of a preparation of isolated ganglia resulted in habituatory responses in the whole animal experiments. Results from the isolated ganglia preparation indicated that habituation occurred at a central location since no sensory receptors were involved.

Kandel (1970) described the results of a series of experiments on the gill withdrawal reflex of A. californica (Kupfermann and Kandel, 1969; Pinsker et al., 1970; Kupfermann et al., 1970; Castellucci et al., 1970). Whole animal experiments showed quantifiable habituation of a defensive gill withdrawal response to stimulation of the siphon and mantle area. There is also a spontaneous gill movement different from the evoked withdrawal response (Peretz, 1969; Kupfermann and Kandel, 1969). The spontaneous movements remained constant before, during and after habituation, indicating that gill fatigue is not the cause of habituation. In addition, strong stimuli produced less habituation than weak, contrary to what one would expect if muscle fatigue were the mechanism for habituation.

Kupfermann et al. (1969, 1970) and Castellucci et al. (1970) worked out a "wiring diagram" for the motoneurons involved in both the defensive gill withdrawal reflex and spontaneous gill movements, partly by direct data and partly by inference. Both intact animal and abdominal ganglion-siphon nerve-skin preparations were used to obtain the following experimental results.

1. Habituation occurs at the motoneuron EPSP level and not at the gill muscle or sensory receptors.
2. Dishabituation also occurs at the central motoneuron synapse.
3. Mechanoreceptor neurons are located within the abdominal ganglion. Baylor and Nicholls (1968) classified certain identifiable neurons in the leech central ganglia as being sensory.

Some general conclusions that were drawn are: 1) habituation and dishabituation are plastic changes that occur in a "hard wired" system as opposed to new growth, 2) direct inhibition is not involved, and 3) both phenomena act independently at a common synapse.

Summary of Particularly Relevant Literature

1. Studies on the heart have shown that there is an extrinsic regulatory mechanism present in Aplysia (Carlson, 1904, 1905; Krijgsman and Divaris, 1955; Wright, 1960).
2. The large identifiable cells in the abdominal ganglion have been mapped as to location, ACh response, connections to nerves, and spontaneous firing rhythm (Frazier et al., 1967).
3. Interconnection and interaction between some cells have been studied (some by inference) and "wiring diagrams" worked out

(Kandel et al., 1967; Kandel et al., 1969; Waziri and Kandel, 1969).

4. Behavioral studies have expanded knowledge and understanding of cellular events and interconnections and produced further anatomical information of possible relevance to the heart (Bruner and Tauc, 1966a, b; Kupfermann and Kandel, 1969; Pinsker et al., 1970; Kupfermann et al., 1970; Castellucci et al., 1970; Kandel, 1970; Peretz, 1969).

In the literature to date, investigations on neuro-regulation of the heart have been based on whole nerve stimulation and anatomical studies. No attempt has been made to determine neuronal correlates of cardiac control as has been reported for other organ systems (Bruner and Tauc, 1966a, b; Peretz, 1969; Kupfermann and Kandel, 1969). Also heart contractions in the whole nerve studies have utilized isotonic levers but not blood pressure measurements as the indicator for activity.

Objectives

The primary purpose of this study was to investigate the correlation of neuronal activity with the regulation of the heart. The detailed configuration of identified cells in the abdominal ganglion provided a clue as to which cells to try first. The existence of extrinsic nervous regulation of the heart suggested the feasibility of locating at least one neuron that would modulate or be modulated by heart activity. A secondary goal was to determine the cardiac response to whole nerve stimulation at various intensities. The investigation was conducted in four major steps as outlined below.

1. Use an isolated ganglion preparation to learn microelectrode techniques, obtain familiarity with the anatomy of the ganglion, and determine response of the identified cells to nerve stimulation.
2. Correlate spontaneous single cell activity with action potentials on the cardiac nerves.
3. Determine the cardiac response to stimulation of the nerves using an abdominal ganglion-intact heart preparation measuring blood pressure as an index of heart activity.
4. Correlate single cell spikes from neurons in the abdominal ganglion with heart activity.

MATERIALS AND METHODS

Animal Procurement and Care

Aplysia californica Cooper (identified by Dr. L. R. Winkler) ranging in weight from 80 to 520 grams were purchased from Pacific Biomarine Corporation or collected along the southern California coast. These were kept in a 70 gallon artificial sea water (Instant Ocean) aquarium at 16° C until used. Four 40 watt fluorescent tubes controlled by a timer provided illumination on a 6 a.m. to 6 p.m. daily cycle. Wakame (dried seaweed purchased at a Japanese market) was fed to the animals about once a week. They were also occasionally fed the kelp *Egregia* obtained from Pacific Biomarine Corporation.

Basic Surgical Preparation

Hypothermia, induced by keeping the animal at 4.5° C for about one hour, aided in reducing secretions during the removal of the purple and opaline glands. The animal was first allowed to extend itself in a dissection tray and placed in the cold room. Forty-five minutes later it was pinned out by the parapodia and foot (Fig. 1). Ten more minutes in the cold room usually sufficed to relax the animal following the pinning procedure. The opaline gland duct was then clamped off, the purple gland, shell, and shell covering removed and the animal opened by incisions along the base of the parapodia from the caudal end forward to the rostral edge. A dorsal midline incision from the rostral end of the parapodium to the level of the rhinophores completed exposure of the viscera, circumesophageal ring, visceral ganglion and pleuro-visceral connectives. Removal of the opaline gland and ligation and cutting of

the connectives from the pleural ganglia completed the basic surgery. Nerves were ligated to reduce leakage of ions from the cut ends.

Isolated Ganglion Preparation

Following the basic procedure outlined, the peripheral nerves from the abdominal ganglion were ligated and transected, leaving the maximum length possible attached to the ganglion. Careful dissection of the branchial and genital-pericardial nerves was required because of their attachment to the connective tissue close to the ganglion. The isolated ganglion was pinned (Minutien 0.15 mm diam. insect pins) by the connective tissue to a rubber ring on the light pedestal in the perfusion chamber (Fig. 4). The nerves were placed on pairs of Ag-AgCl electrodes mounted on a special nerve ring to simplify the procedure of recording and stimulating. A General Electric 253-X prefocused bulb energized at 3 volts, direct current, for low intensity and 4.5 volts for high intensity was used to trans-illuminate the ganglion. Perfusion with artificial sea water at room temperature maintained the ganglion. The cells were observed under a Zeiss stereo zoom microscope at magnifications ranging from 25x to 100x (Fig. 2).

Intact Heart - Abdominal Ganglion Preparation

The anterior and posterior aorta and gastroesophageal artery (Hyman, 1967) were ligated and cut as far distal to the heart as possible. Removal of the seminal vesicle, and digestive tract from esophagus to gizzard, followed this step. The digestive gland was removed from the rest of the visceral mass by cutting the connective tissue along the mantle edge. The siphon musculature and rectum were surgically removed along with the digestive gland to reduce artifacts from muscle contraction other than the heart itself.

Pliable tubing (2mm I.D.) was used to cannulate the atrium for perfusion with artificial sea water containing 100 mg% glucose to help maintain the heart over a period of 8 to 14 hours. A 5 mm incision slightly caudal and to the left of the rostral attachment of the gill provided the opening for the atrial cannula. Pressure was measured through a cannula inserted into the previously ligated gastroesophageal artery. The gill was ligated in sections and removed to reduce pressure changes due to contraction of muscles extraneous to the heart.

The reproductive organs were also removed, with extra care being taken in removing the hermaphroditic duct since the genital nerve lies within the connective tissue of this duct. Extreme care had to be taken not to puncture the pericardium.

The abdominal ganglion was pinned to the light pedestal. Some of the muscular tissue was also pinned to reduce stretching of the branchial and genital-pericardial nerves and to keep the heart in a relatively fixed position.

Intact Animal Heart Rate

In three experiments the heart beat was externally observed (on the left body wall over the heart) and heart rates were counted on the resting animal. In three experiments a needle connected to a Statham pressure transducer was inserted into the hemocoel and relative hemocoel pressure changes were measured. The heart contractions, reflected as fluctuations in the hemocoel pressure, were recorded on a Grass Polygraph.

Recording Instrumentation and Methods

Electrodes. Glass micropipettes were pulled from Pyrex tubing (2 mm O.D., 0.5 mm wall thickness) on a horizontal two stage puller

patterned after the Chowdhury Pipette-Puller. (See Appendix for details on microelectrode puller.) The microelectrodes, filled with a 0.2 M $CaCl_2$, 0.8 M KCl solution, ranged from 10 to 50 megohms impedance with tip sizes of approximately 0.5 micron diameter. Electrode impedance was measured using the square wave method provided with the BAK Electrometer Amplifier. Advancement of the electrode was controlled in micron steps by a Trent Wells hydraulic micromanipulator.

Isolated Ganglion Preparation. (See Figure 5)

Intact Heart-Abdominal Ganglion Preparation. (See Figure 6)

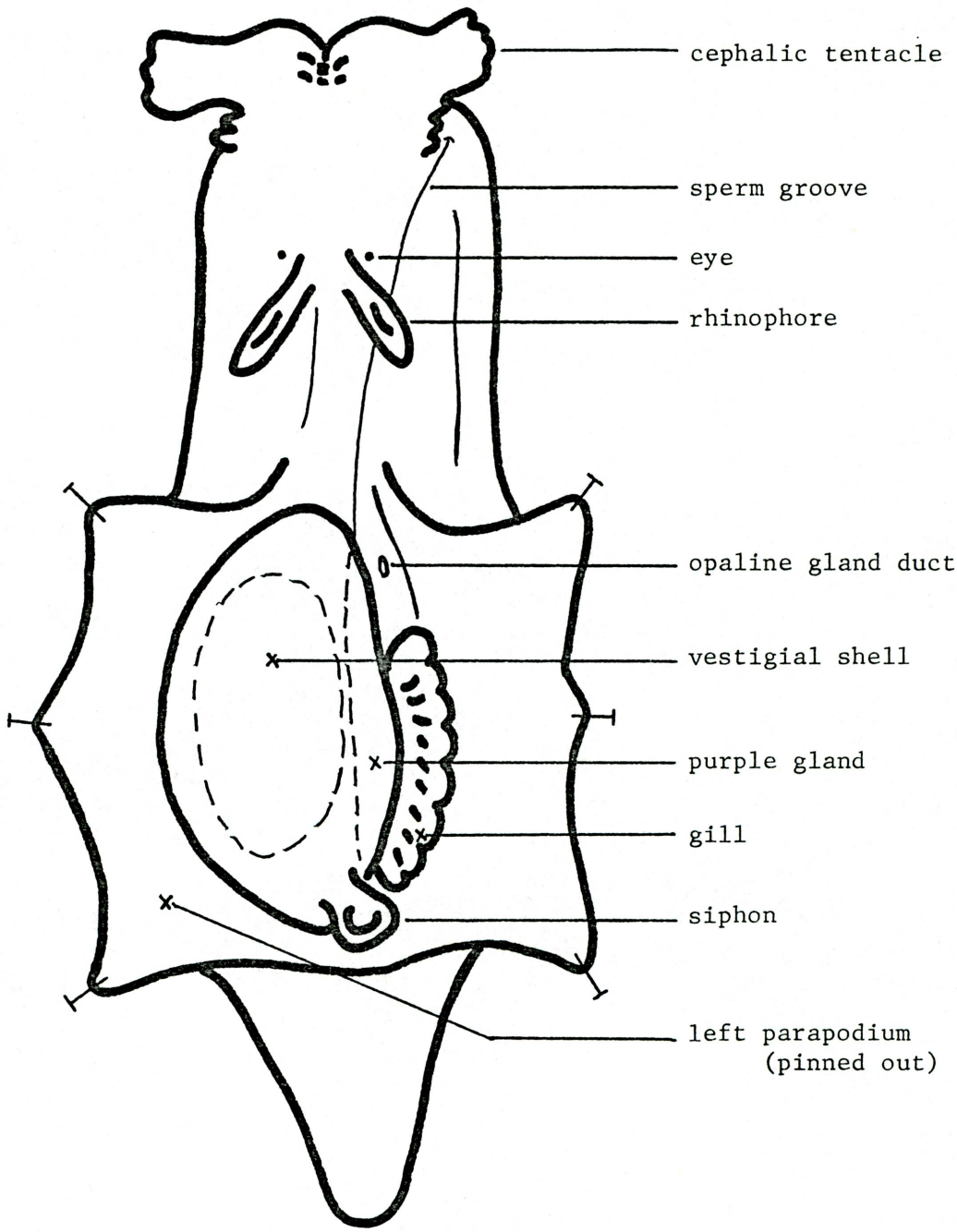
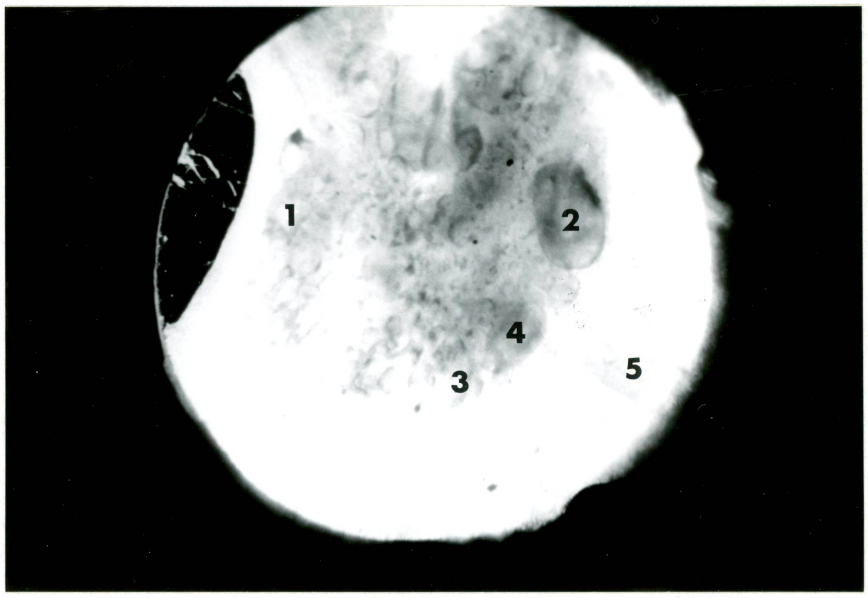


FIGURE 1. EXTERNAL FEATURES OF Aplysia californica

FIGURE 2. DORSAL VIEW OF ABDOMINAL GANGLION

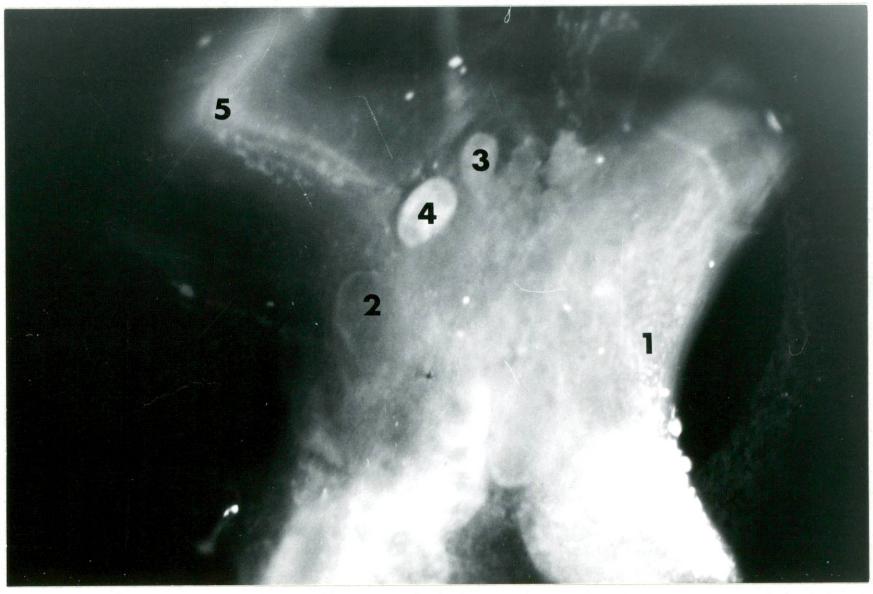


A. Approximate magnification: 25X (transmitted light)

1. Region of L7; cell is not clearly outlined in these photographs
2. R2, the giant cell of the abdominal ganglion
3. R15, a white cell with a bursting rhythm
4. R14, a neurosecretory cell
5. Branchial nerve

Legend applies to both photographs

B. Approximate magnification: 25X (incident light)



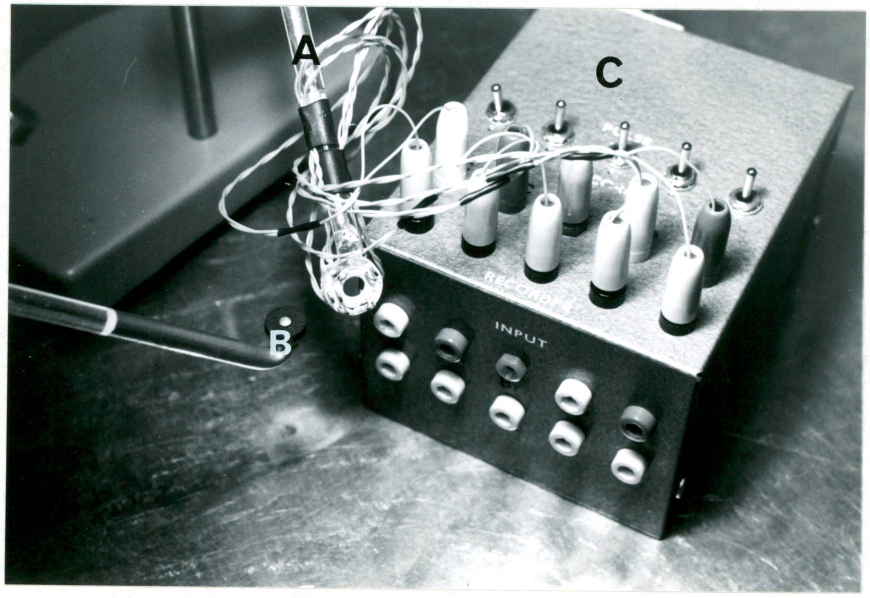


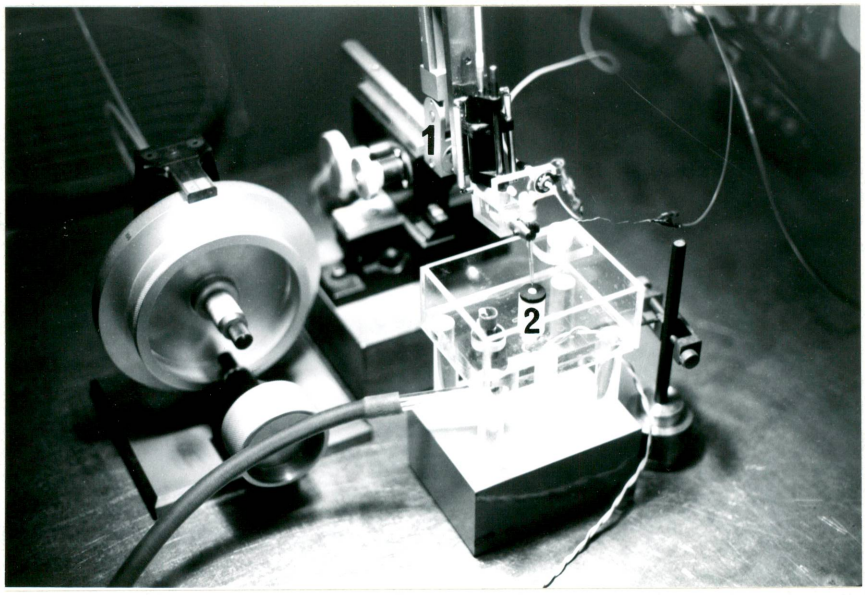
FIGURE 3. NERVE HOOK, LIGHT FOOT, AND R-S BOX

A. Nerve hook with 5 pairs of electrodes

B. Light foot for pinning and transillumination of ganglion in whole animal

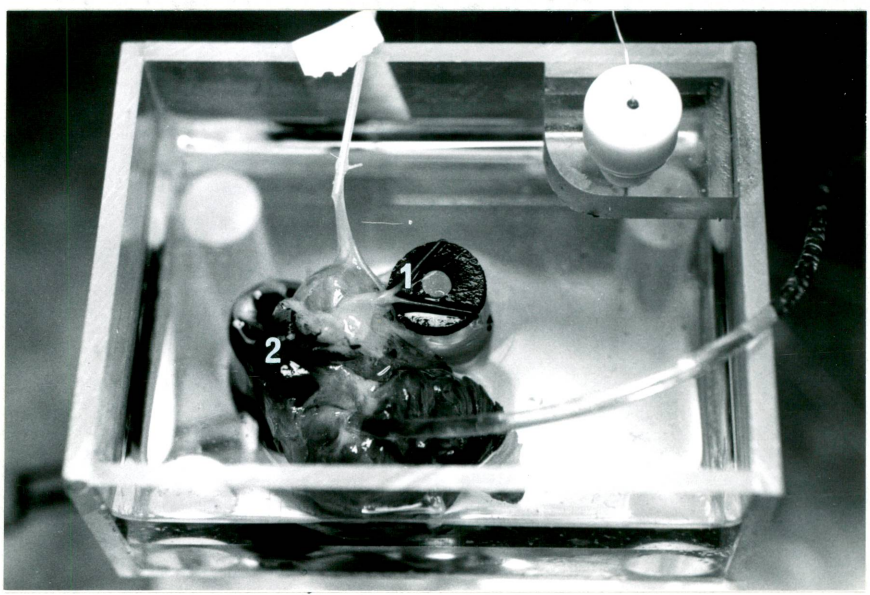
C. Record-Stimulate (R-S) Box for recording from or stimulating the ganglion nerves

FIGURE 4. HEART-GANGLION PREPARATION AND CHAMBER



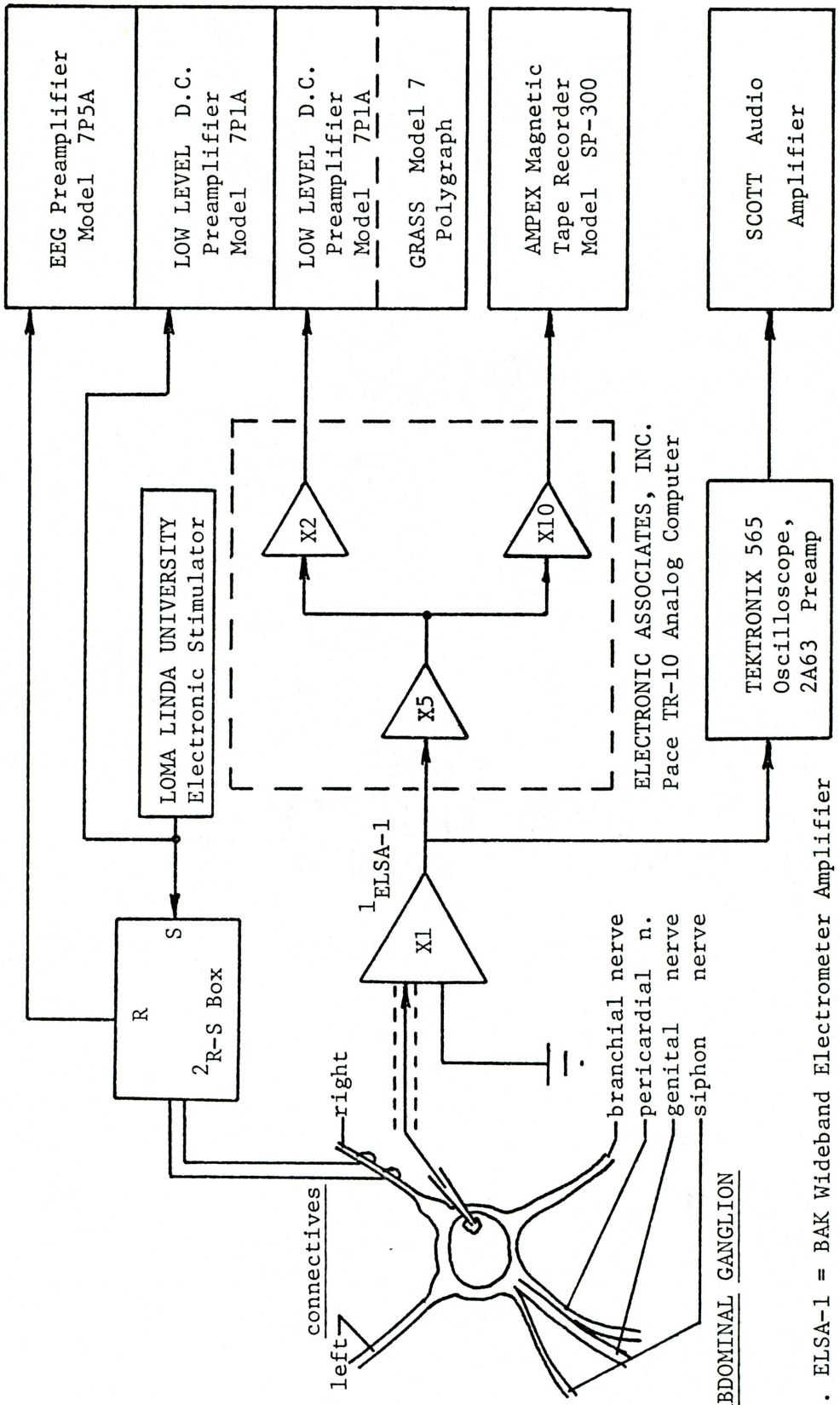
A. Chamber with associated recording apparatus

1. Trent Wells Hydraulic Micromanipulator
2. Light pedestal for pinning and illuminating ganglion



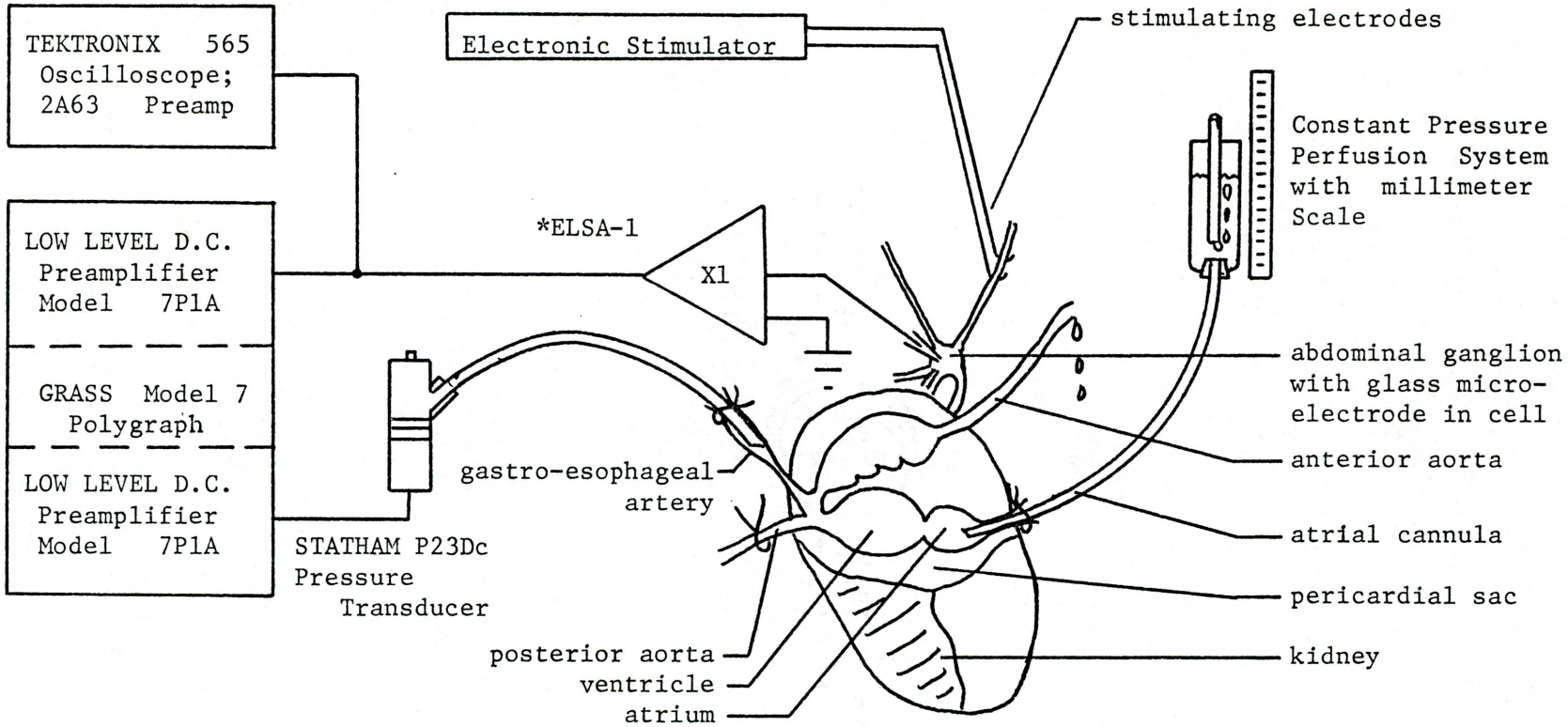
B. Heart-ganglion preparation in the chamber

1. Ganglion on light pedestal but not pinned
2. Perfused heart



1. ELSA-1 = BAK Wideband Electrometer Amplifier
2. R-S Box = Record-Stimulate switching box

FIGURE 5. ISOLATED GANGLION PREPARATION AND RECORDING APPARATUS



*ELSA-1 = BAK Wideband Negative Capacitance Electrometer Amplifier

FIGURE 6. INTACT HEART-ABDOMINAL GANGLION PRAPARATION

RESULTS

Intact Animal Heart Rate

Table 2 is a tabulation of the mean heart rate of quiescent animals in artificial sea water at room temperature. Heart rate was determined by measurement of hemocoel pressure or visual observation. The pulsations in hemocoel pressure (Fig. 8A, B) correlated well with rhythmic physical pulsations on the dorso-lateral surface of the animal over the area of the heart.

Table 2. Mean resting heart rate of intact animals

Body Weight (grams)	Heart Rate (beats/min)	Measurement	Water Temperature (°C)
370	25.7	hemocoel pressure	23.2
380	21.0	hemocoel pressure	-
300	22.7	hemocoel pressure	-
410	22.0	visual count	-
> 520	22.0	visual count	-
370	19.5	visual count	21.6

Results from Whole Nerve Stimulation

Genital-Pericardial Nerve. Preliminary evidence from two of three animals indicates the presence of inhibitory axons in the genital-pericardial nerve complex (Table 3). In animal 37 (Fig. 10-13) the stimulation responses were purely genital-pericardial nerve, since the nerve was cut at the ganglion. In the other 2 subjects (53 and 54), the branchial and genital-pericardial nerves were intact but the ganglion of animal 54 had been pre-treated with pronase (see comments on possible

damage from pronase). In the pronase treated preparation only acceleratory responses were obtained.

Siphon Nerve. A response to siphon nerve stimulation was present in all of the 5 animals tested (Table 7). Both excitatory and inhibitory results were obtained from 2 of the 5 animals tested. The sampling was inadequate to show the overall effect of the siphon nerve in the 3 remaining animals but clearly inhibitory responses were obtained in 2 of them. Cardiac arrest was obtained in 4 of the 5 animals and the effectiveness of the siphon nerve in stopping the heart is demonstrated in Fig. 26, 27C, and 27D.

Right Connective. Predominantly inhibitory and some excitatory responses resulted from right connective stimulation, with dual effects seen in 4 of 11 animals (Table 4a). Of the 11 tested, 2 animals had exclusively acceleratory responses while 3 had only inhibitory results. The sum of the normalized heart rate change (NHRC) figures in Table 4b shows that negative chronotropic changes (slowing of heart rate) were more readily obtained than acceleratory results. Normalization was necessary because the total number of stimulations was not the same in each subject, e.g., without normalization animals 44 and 51 would have accounted for 61.3% of the negative chronotropic results, thus introducing a large weighting factor.

Some of the general results from right connective stimulation are shown in Fig. 14-18. Important is the occurrence of cardiac arrest in 6 of the 11 animals (Table 4). Correcting for the total number of stimulations to account for the subthreshold values, calculation of the relative frequency of arrest is:

$$\% \text{ Arrest} = 15/(101-21) \times 100 = 18.75\%.$$

This adds to the evidence for the inhibitory control exerted by the right connective. The arrests occurred only in animals with negative chronotropism or no rate change. Figure 16 illustrates the cardiac arrest from right connective stimulation in one of the animals and also demonstrates the consistency and low threshold in this particular case (16A, B, & C). In two of the animals tested (44 & 51) no cardiac arrest occurred after the branchial nerve was cut but inhibitory results were still obtained (Fig. 17).

The dual response to right connective stimulation (Fig. 24B) during a given stimulation period was not seen very often but this further illustrates the presence of dual innervation within the nerve. This type of effect is difficult to demonstrate if one factor overrides the other.

Left Connective. Left connective stimulation resulted primarily in inhibitory responses, as indicated by the occurrence of negative chronotropism in 5 animals out of 10 tested and cardiac arrest in 5, as compared to cardioacceleration in only 3. Table 5a gives the results for the 10 animals including the total number of stimulations, threshold intensity and number of subthreshold stimulations. There were only 8 cases of cardioacceleration, all from only 3 of the 10 animals with the results heavily weighted from animal 34. The normalized heart rate change ratio (NHRC ratio = - NHRC/+NHRC, Table 5b) of 2.35 further indicates the dominance of the inhibitory over the excitatory axons in the left connective.

Cardiac arrest in response to left connective stimulation resulted for intensities ranging from 0.3 volts (10 ms, 5 hz stimuli, Fig. 19C) to 5.0 volts (Fig. 20C) in one of the animals with a range of values in

between among the various animals. The relative frequency of cardiac arrest after correction for subthreshold stimulations was:

% Arrest = 10/(80-11) x 100 = 14.5%, a value slightly lower than for right connective stimulation (18.75%).

Branchial Nerve. Preliminary evidence from 2 of 2 animals (44 and 51) indicates that the branchial nerve has a significant role in the cardiac arrest resulting from stimulation of the connectives. In both animals cardiac arrest resulted from connective stimulation with the branchial nerve intact but once the nerve was cut, only bradycardic responses were obtained (Fig. 17 & 21). The branchial nerve was not directly stimulated in either of these animals. Results from 4 other animals (Table 8) show that branchial nerve stimulation did not evoke arrest except in one instance (Fig. 29C). Thus results of direct stimulation do not directly agree with the circumstantial evidence obtained by cutting the branchial nerve. It may be that some synergistic inhibitory pathways are removed when the branchial nerve is cut and that arrest results only when all inhibitory axons are intact.

There are both acceleratory and inhibitory fibers in the branchial nerve of A. vaccaria (Wright, 1960) but my results from A. californica were not conclusive. Only 1 of 4 animals showed significant results (Table 8). In animal 54, direct stimulation of the branchial nerve produced both excitatory and inhibitory responses with clear instances of cardioacceleration shown in Fig. 31. It was difficult to obtain consistent results for the following reasons. The exposed portion of the branchial nerve is very short and it goes through a "tunnel" to the branchial ganglion in the area of the gill artery. Dissection of the nerve to obtain additional exposure for stimulation possibly damages

some of the short nerves from the branchial ganglion. Also the close quarters cause stretching as well as shunting of the stimulating electrode with sea water and muscle tissue, the degree of shunting changing from one preparation to the next.

Pericardial Nerve. The predominant response to pericardial nerve stimulation was cardioacceleration, as partially illustrated in Fig. 28. Results from the 4 animals tested are given in Table 6. There were 26 acceleratory responses to 33 stimulations of the pericardial nerve with only 3 cases of slowing, which all occurred in one animal (No. 42). Of these 3, 2 were dual responses, initial acceleration followed by a slight slowing concomitant with decreased pulse pressure.

General Observations. The increased responsiveness of both the left and right connective with increased stimulus frequency is demonstrated in Fig. 25 where pulse intensity and duration were kept constant. No responses were obtained at 5 hz but at 50 hz, inhibitory results were recorded. The lower (5.0 hz) frequency was normally used because it was considered to be more in the physiologic range for Aplysia.

Figures 23 and 31B show the effectiveness of single pulses, in a few cases where it was tried, in evoking cardiac responses. In 5 of 11 trials some response was obtained ranging from positive tonotropic effects to cardiac arrest (one case). This result is not altogether surprising in light of the burst response evoked in some neurons by single pulse stimulation of the nerves. The burst of response in L14 (Fig. 38A) from left connective stimulation was also obtained with single pulses.

Cardiac arrhythmia resulted from right (Fig. 18A, B, & 24B, C) and left (Fig. 18C, 22, & 24A) connective stimulation for intensities

ranging from 0.2 to 2 volts in one of the animals (51). This was not a general phenomenon among the animals tested for response to connective stimulation. There was no definite pattern in duration of arrhythmia.

In some instances the pericardium was cut to determine whether the positive tonotropic effects observed in intact hearts were from the contraction of the pericardium and not the heart. Figure 9 shows that even with the pericardium cut, positive tonotropic responses could be obtained. In 3 of the 5 animals with the pericardium opened, left connective stimulation evoked positive effects and right connective stimulation did the same in 4 of 7 animals. Carlson (1905) also showed positive tonotropism in records taken with isotonic levers attached to completely exposed unperfused hearts.

Intracellular Data Related to Heart Rhythm Changes

Cell L7. Figures 32-35 show the relationship of high frequency spike bursts in L7 to cardioinhibition in one isolated, perfused, heart-abdominal ganglion preparation. Cell L7 was identified by its location and orange pigmentation (not peculiar to L7). (It was not possible to use the added criteria of relatively equal size and close proximity to the readily identifiable L11 since L7 was larger than usual and L11 was smaller than in previous preparations. Generally L11 is a large cell located close to the emergence of the genital-pericardial nerve from the ganglion.)

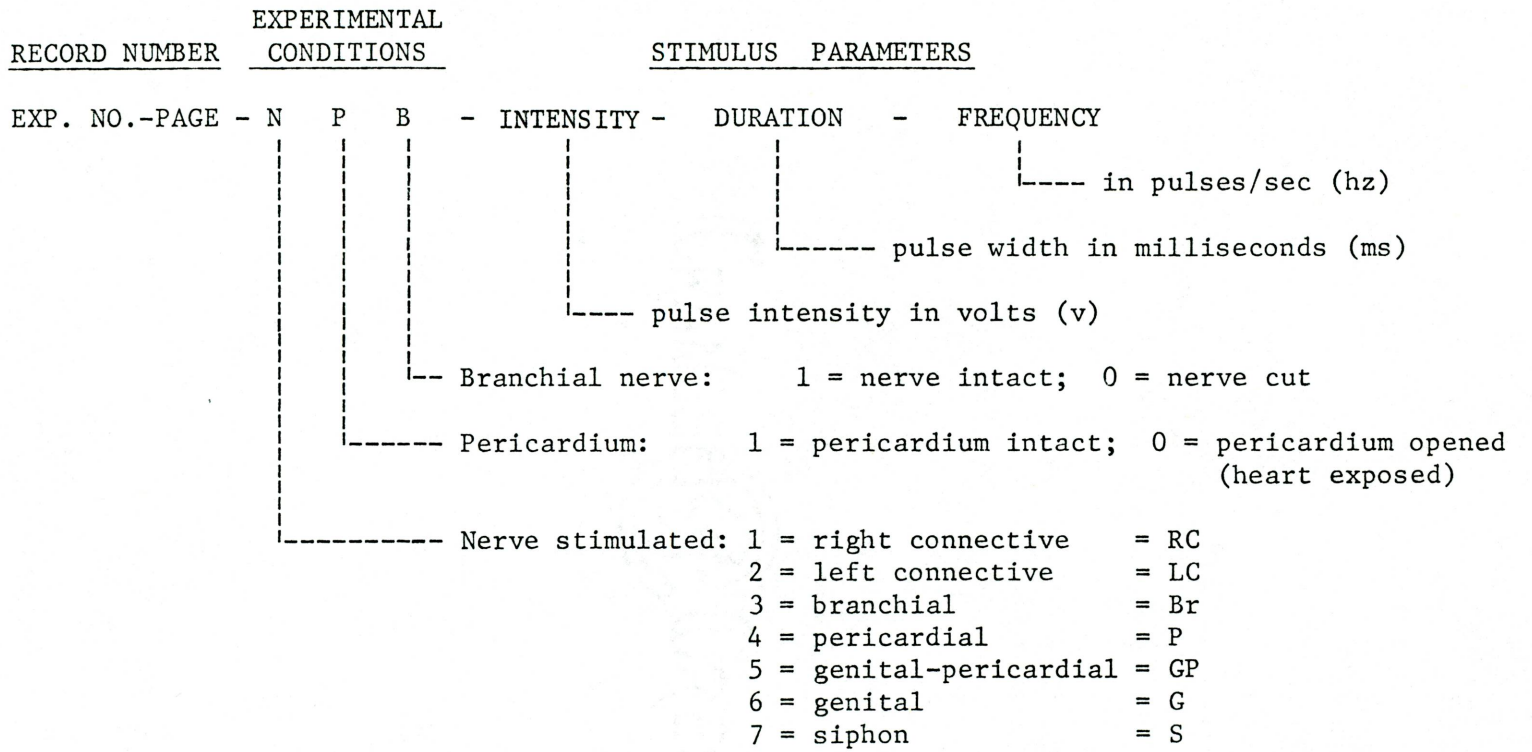
Abrupt, high frequency spike bursts in cell L7 occurred 7 times while recording from it in animal 49 and in 6 of these there was an accompanying bradycardia following the burst. This phenomenon was seen only in this subject. Four of these results are shown in Fig. 32, 33A,

34B, and 35A, while the single case of spike burst following bradycardia is shown in Fig. 33B. Milder, more equivocal increases in spike bursts in L7 associated with bradycardia were seen several times and were detected in the audio system during the course of the recording session. A rather subjective evaluation of the results, including the cases of relatively mild increases in L7 spike frequency, is tabulated in Table 9.

Cell L10. In one (No. 52) of 3 animals where penetration into cell L10 was successful, there was some evidence of an associative relationship between inhibition of long duration in the cell and slowing of the heart (Fig. 37). There was no obvious correlation between the two events in the other two animals but in one case the spike activity did not have the bursting pattern seen in Fig. 36 and 37. Figure 36 illustrates the types of diverse relationships (in animal 52) which made interpretation difficult even at the subjective level. Combinations of inhibition of long duration and bradycardia from one animal (No. 52) were categorized into 5 groups with the number of occurrences of each (Table 10).

Other cells tested for correlation of spike activity with heart rhythm were L14 (Fig. 38), L2 (Fig. 35B), L9, L12, L13, R2, R12, and R15. There were no obvious correlative activities in these cells.

Figure 5. Key for the notation used to identify the blood pressure recordings.



EXAMPLE: 51-684-211-.2-10-5

Experiment 51, page 684, stimulated left connective, pericardium intact, branchial nerve intact, LC stimulated with 0.2 volt, 10 milliseconds duration, 5 hz pulses.

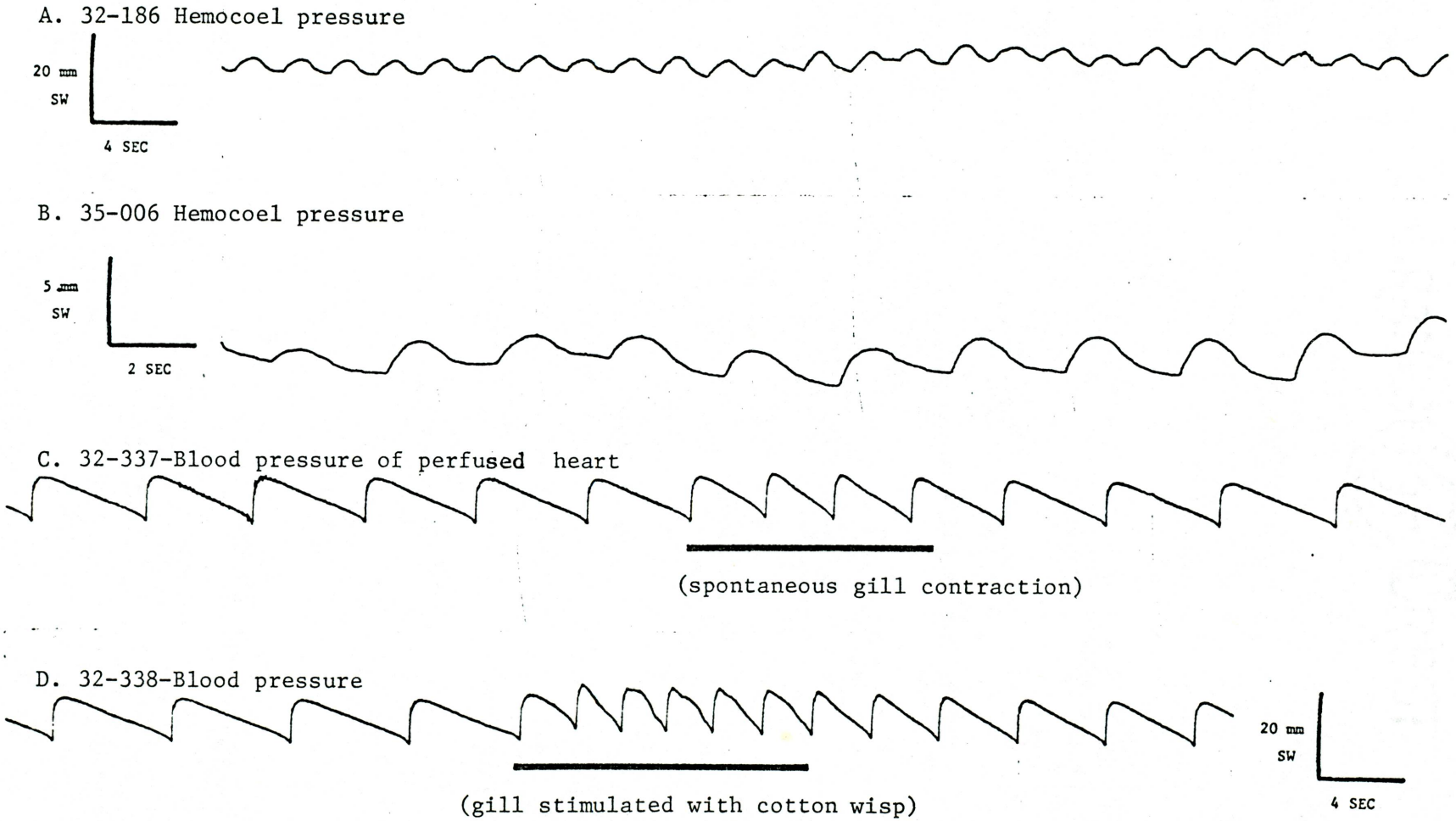


Fig. 8. A: Hemocoel pressure from animal 32. Mean heart rate (HR) = 25.7 beats per minute (bpm). B: Hemocoel pressure, animal 35. HR = 22.7 bpm. C: Cardioacceleration resulting from spontaneous gill contraction. Control HR (CHR) = 11.5 bpm, HR during gill contraction = 17.3 bpm. D: Cardioacceleration resulting from gill stimulation. CHR = 10.8 bpm, experimental heart rate (EHR) = 21.6 bpm.

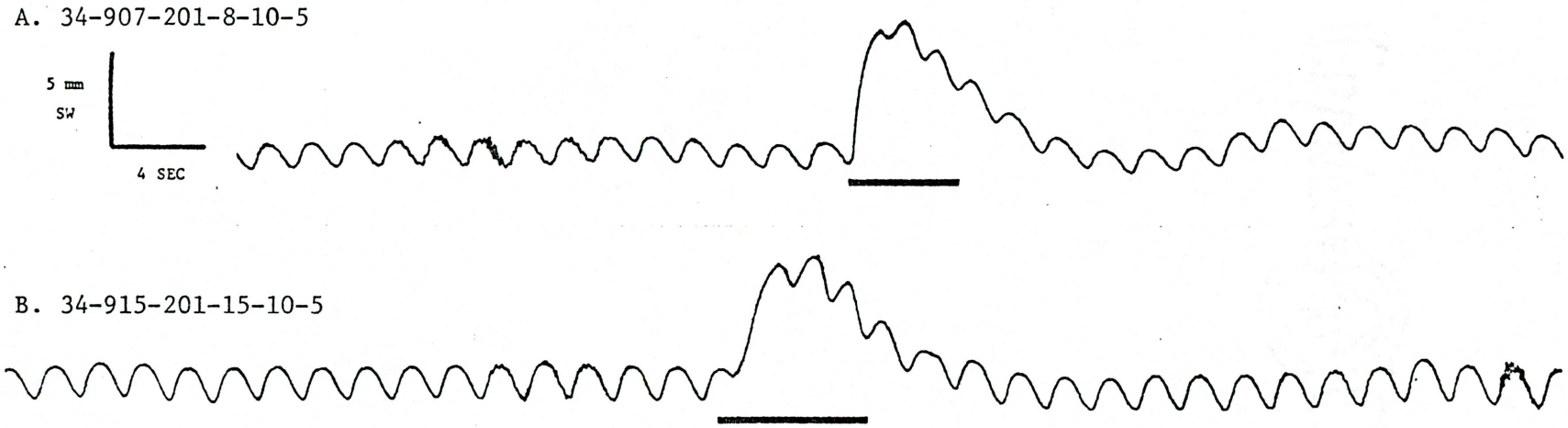


Fig. 9. Positive tono- and chronotropic results from LC stimulation with pericardium cut open. Note the low pulse pressure. A and B form a continuous record. P_i = 25 mmSW. A: CHR = 32.4 bpm, BP = 1.7 mmSW. EHR = 37.6 bpm. B: CHR = 30.6 bpm, BP = 1.8 mmSW. EHR = 34.6 bpm.

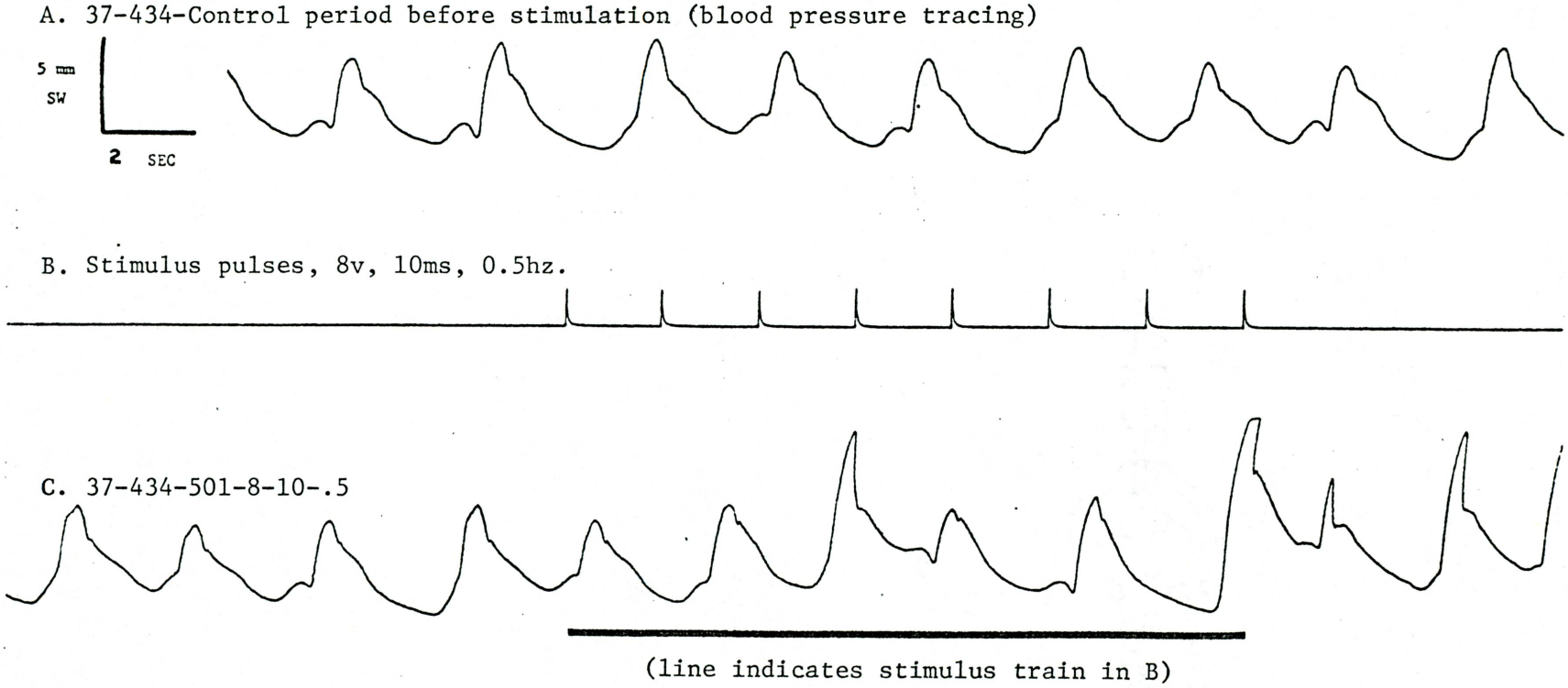


Fig. 10. Cardioacceleration from low frequency (0.5 hz) stimulation of the genital-pericardial nerve, continuous pressure recording (A-C). P_i = 20 mmSW. A: Pre-stimulus control period. HR = 20.5 bpm, BP = 4.3 mmSW. B: Individual stimulus pulses (also indicated by dark line in C). C: Positive tono-, ino-, and chronotropic results. EHR = 22 bpm, BP = 10 mmSW (peak value).

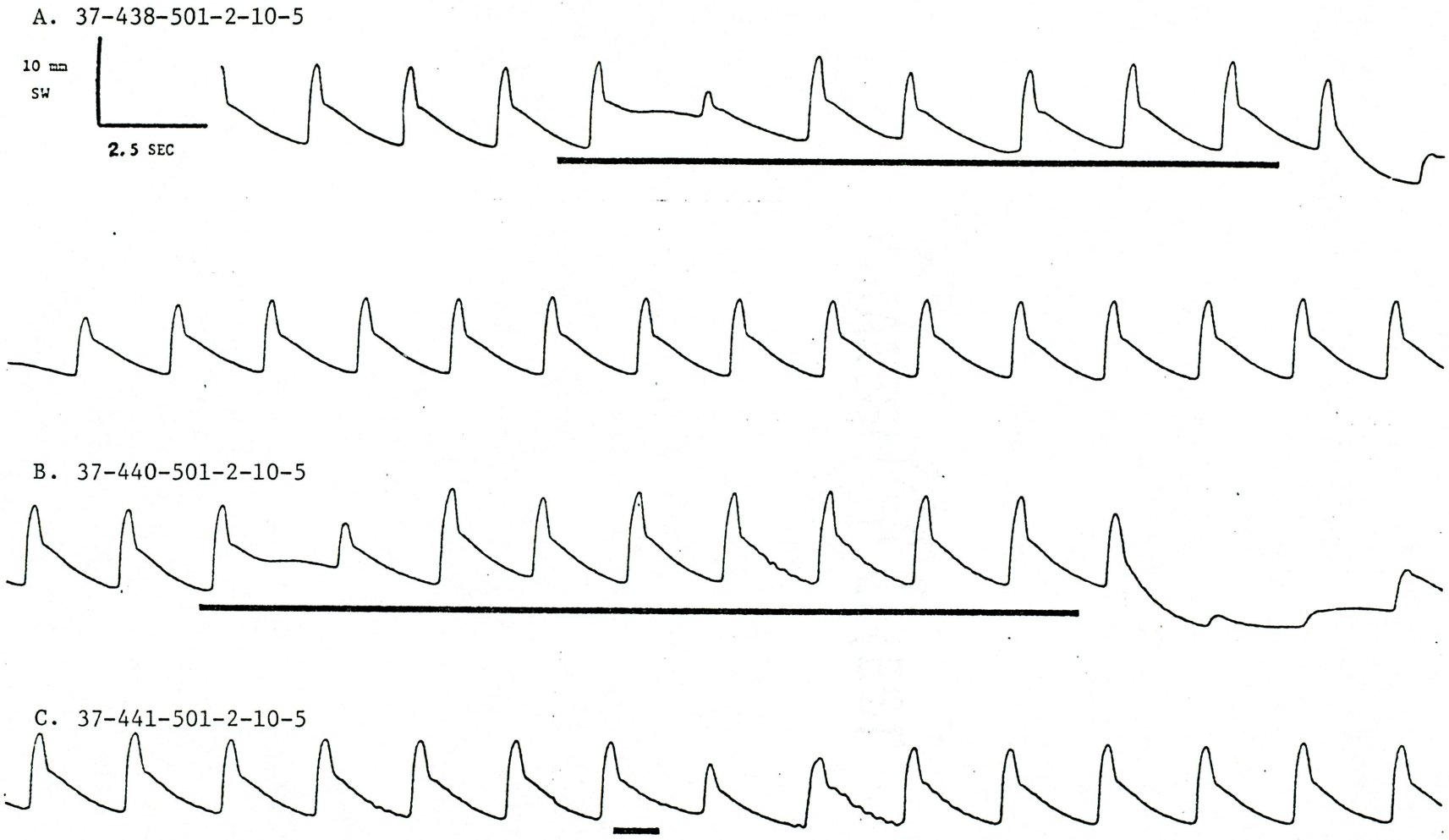


Fig. 11. Bradycardia resulting from GP stimulation at higher stimulus frequency (5 hz) and intensity (2 v). P_i = 20 mmSW. Note the inhibitory post-stimulus response. A: CHR = 26.7 bpm, BP = 9 mmSW; EHR = 24.6 bpm, BP = 9.5 mmSW. B: CHR = 27 bpm, BP = 9 mmSW; EHR = 25.2 bpm. C: Short burst of 6 stimulus pulses applied to GP resulted in bradycardia from 26.6 bpm to 25 bpm.

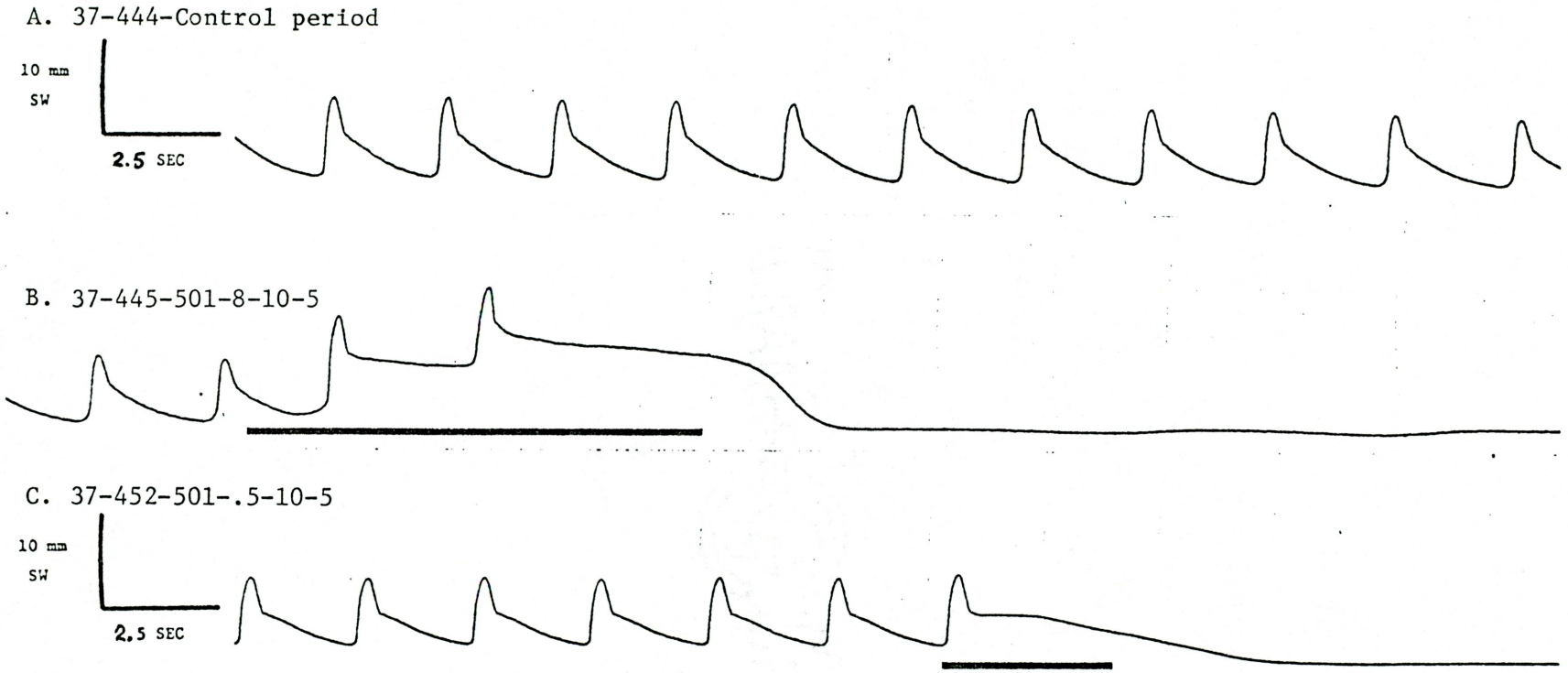


Fig. 12. Cardiac arrest resulting from GP stimulation. P_i = 20 mmSW. A: Control period, A and B are continuous. HR = 22 bpm, BP = 8 mmSW. B: Positive tonotropic effect preceding slowing and eventual cardiac arrest. Heart resuscitated by hydraulic massage (oscillate perfusion pressure by squeezing the plastic sea water reservoir bottle). C: Cardiac arrest from 0.5 volt, 5 hz stimulus. HR = 23 bpm, BP = 7 mmSW.

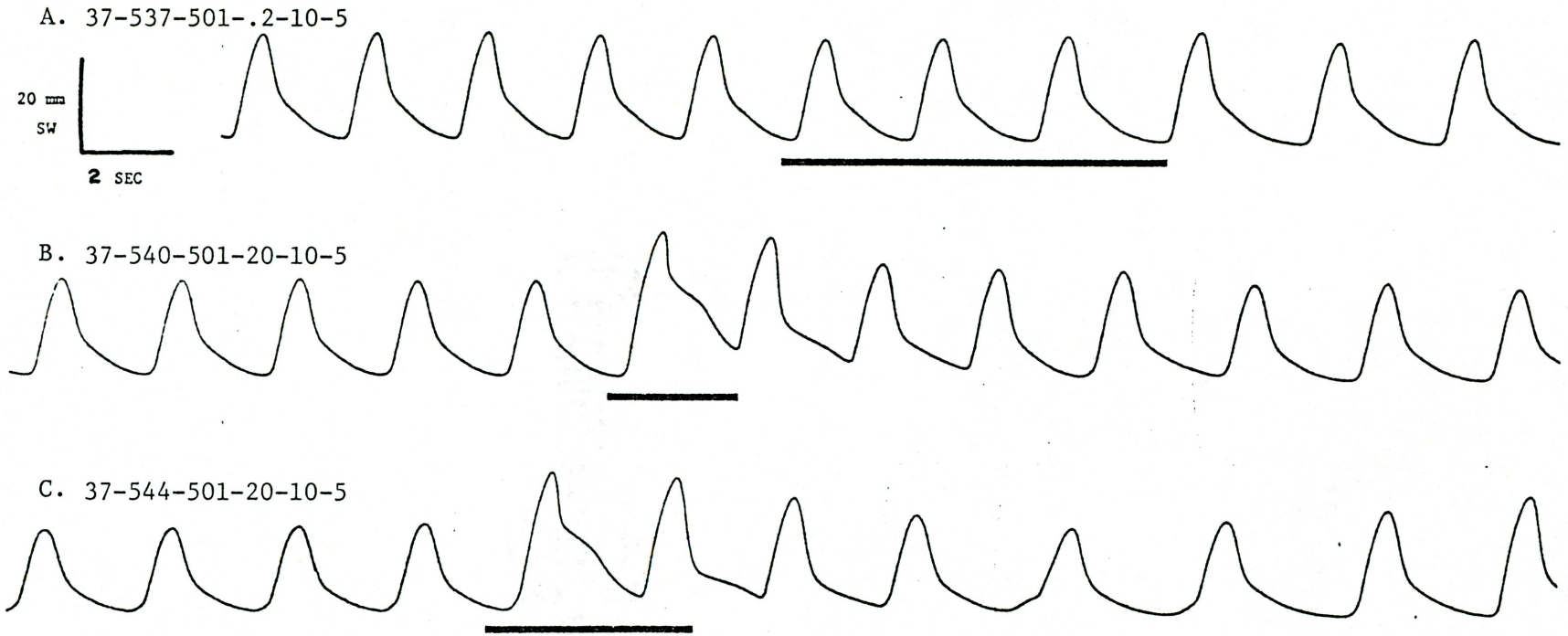


Fig. 13. Opposite results from high and low intensity stimulation of the genital-pericardial nerve. P_i = 15 mmSW. Note the increased blood pressure in contrast to Figures 11 & 12. A: Low intensity stimulation (0.2 v). CHR = 24.1 bpm, BP = 22 mmSW; EHR = 22.1 bpm, BP = 21 mmSW. B: High intensity stimulation (20 v). CHR = 23 bpm, BP = 20 mmSW; EHR = 25.2 bpm, BP = 31 mmSW (peak for one heart beat). C: High intensity (repeat 20 v) stimulation. CHR = 21.6 bpm, BP = 18.5 mmSW. No change in heart rate; however, note the inotropic effect as in B and the post-stimulus bradycardia.

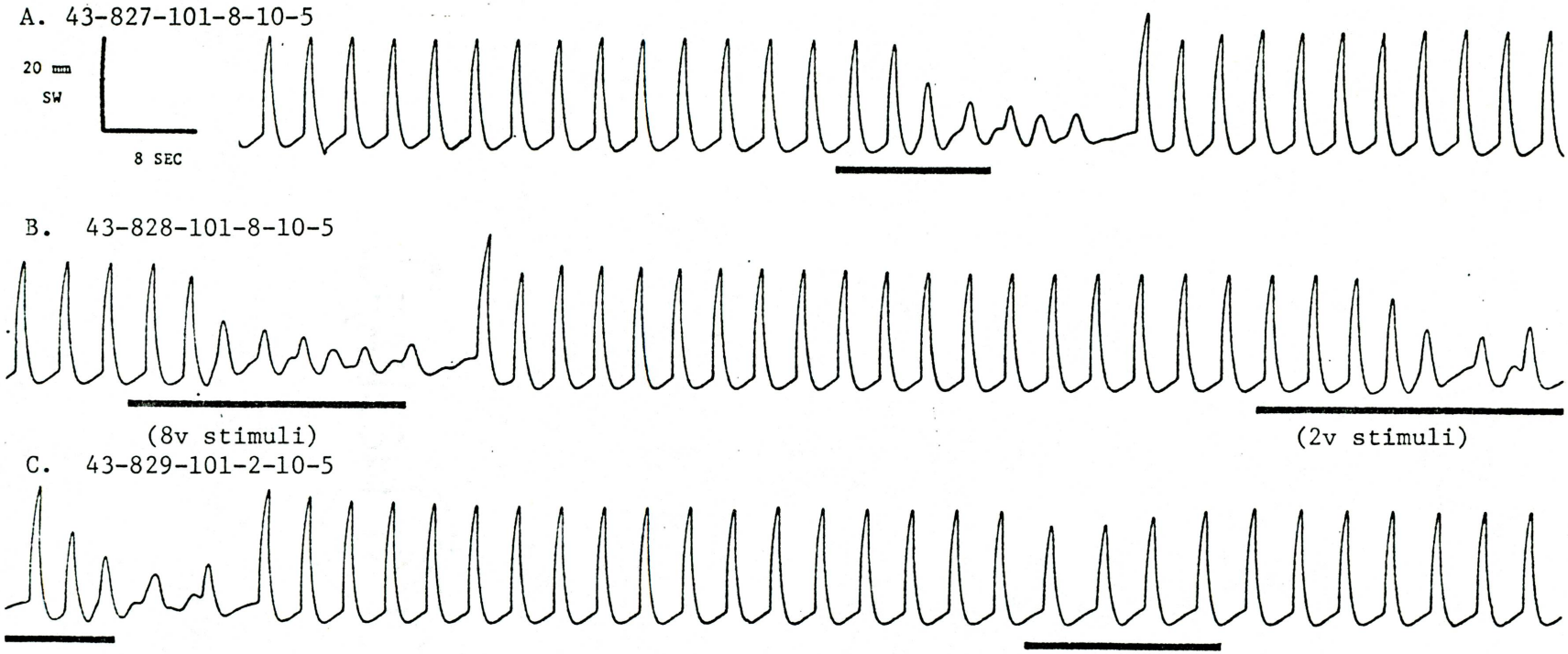


Fig. 14. Opposite results from RC stimulation at differing stimulus intensities. P_i = 58 mmSW, continuous recording. (Pericardium cut open.) A.: Cardioacceleration at 8 v. CHR = 16.1 bpm, BP = 24 mmSW; EHR = 18.3 bpm, BP = 9 mmSW minimum. B.: Cardioacceleration at 8 v. CHR = 16 bpm, BP = 26 mmSW; EHR = 19.2 bpm, BP = 5.5 mmSW. Mixed result with 2 v stimuli; initial speeding followed by slowing and "escape" during stimulus period. CHR = 16 bpm, BP = 24.5 mmSw. Maximum HR = 19.4 bpm, minimum HR = 12.6 bpm. C: Slowing with 2 v stimulation. CHR = 15.3 bpm, BP = 24.7 mmSW; EHR = 13.5 bpm, BP = 21 mmSW. Note the decrease in both pressure and rate in contrast with results from 8 volt stimulation.

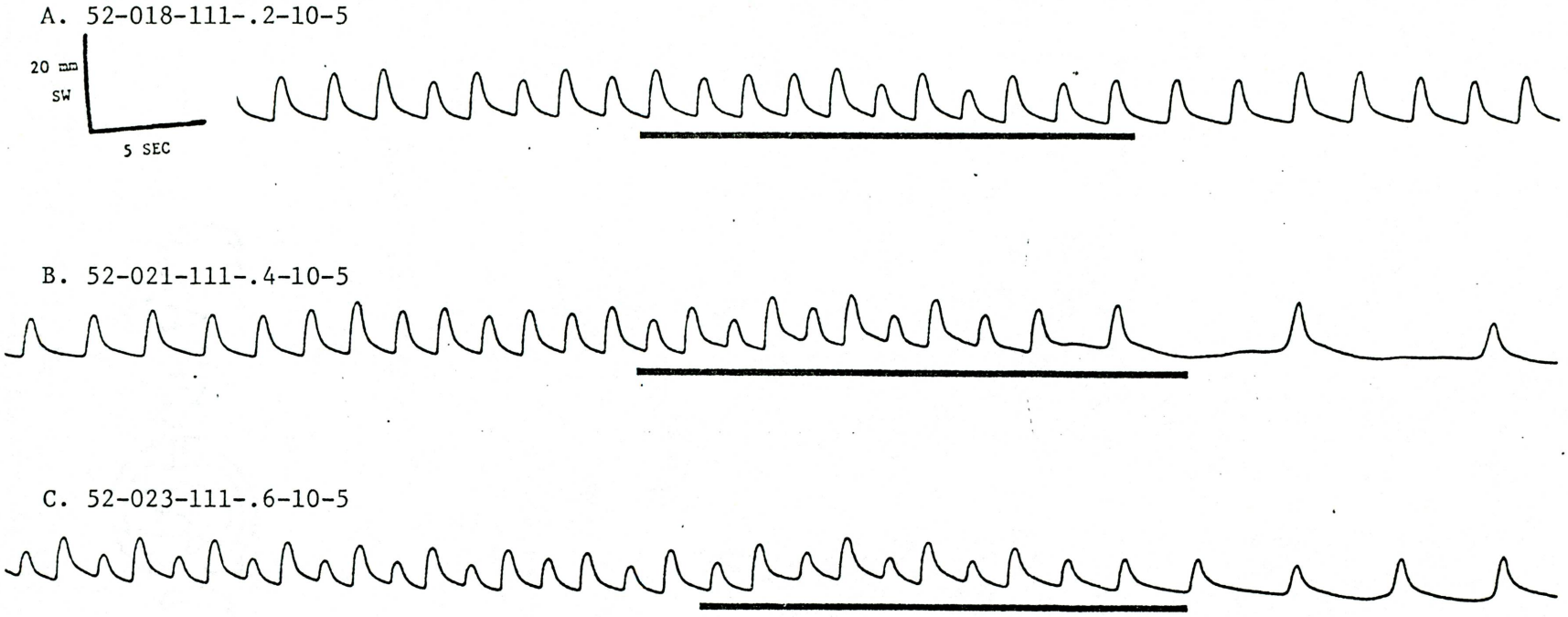


Fig. 15. Post-stimulus bradycardia resulting from RC stimulation in an intact heart preparation. P_i = 30 mmSW. A: slowing from low intensity stimulation (0.2 v, 5 hz). CHR = 29.3 bpm, BP = 10 mmSW; EHR = 22.2 bpm. B: bradycardia resulting from 0.4 v, 5 hz stimulation. CHR = 31.9 bpm, BP = 10 mmSW; EHR = 7.2 bpm min. C: bradycardia resulting from 0.6 v stimulation. CHR = 34.3 bpm, BP = 9 mmSW; EHR = 13.6 bpm min. The values given for experimental rates are from the post-stimulation period. In B and C, there was a slightly increased rate and positive tonotropic effect during the stimulus period.

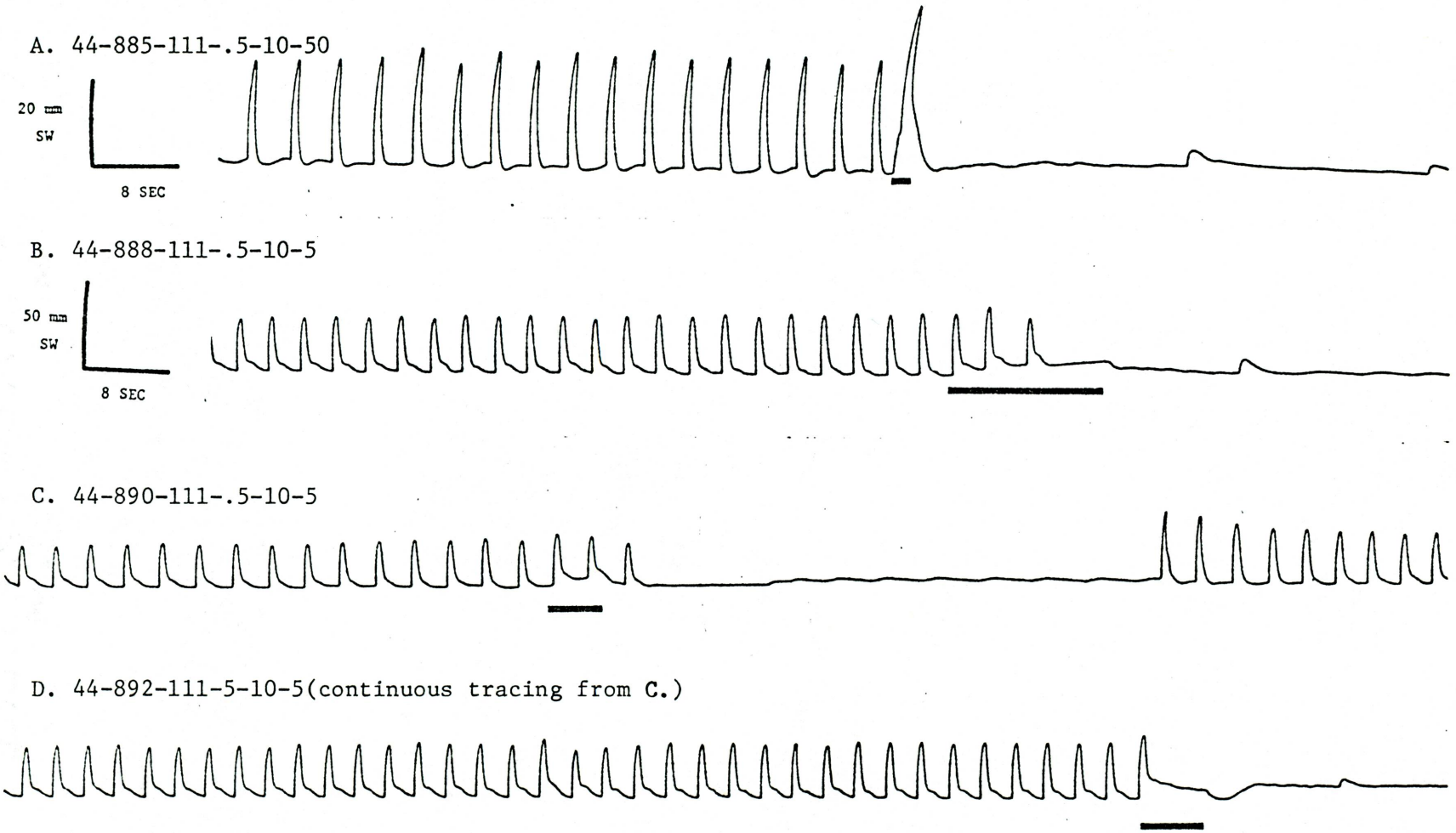


Fig. 16. Cardiac arrest resulting from RC stimulation of an intact heart preparation. A: High frequency stimulation (50 hz) at 0.5 v. HR = 17.2 bpm, BP = 27 mmSW. Resuscitation by hydraulic massage. B: Low frequency (5 hz), low intensity (.5 v) stimulation. HR = 19.5 bpm, BP = 35 mmSW. Resuscitation by massage. C: Arrest with spontaneous recovery in 50 sec. resulting from 0.5 v, 5 hz stimulation. D: Arrest with recovery in 3.7 minutes resulting from 5.0 v, 5 hz stimulation.

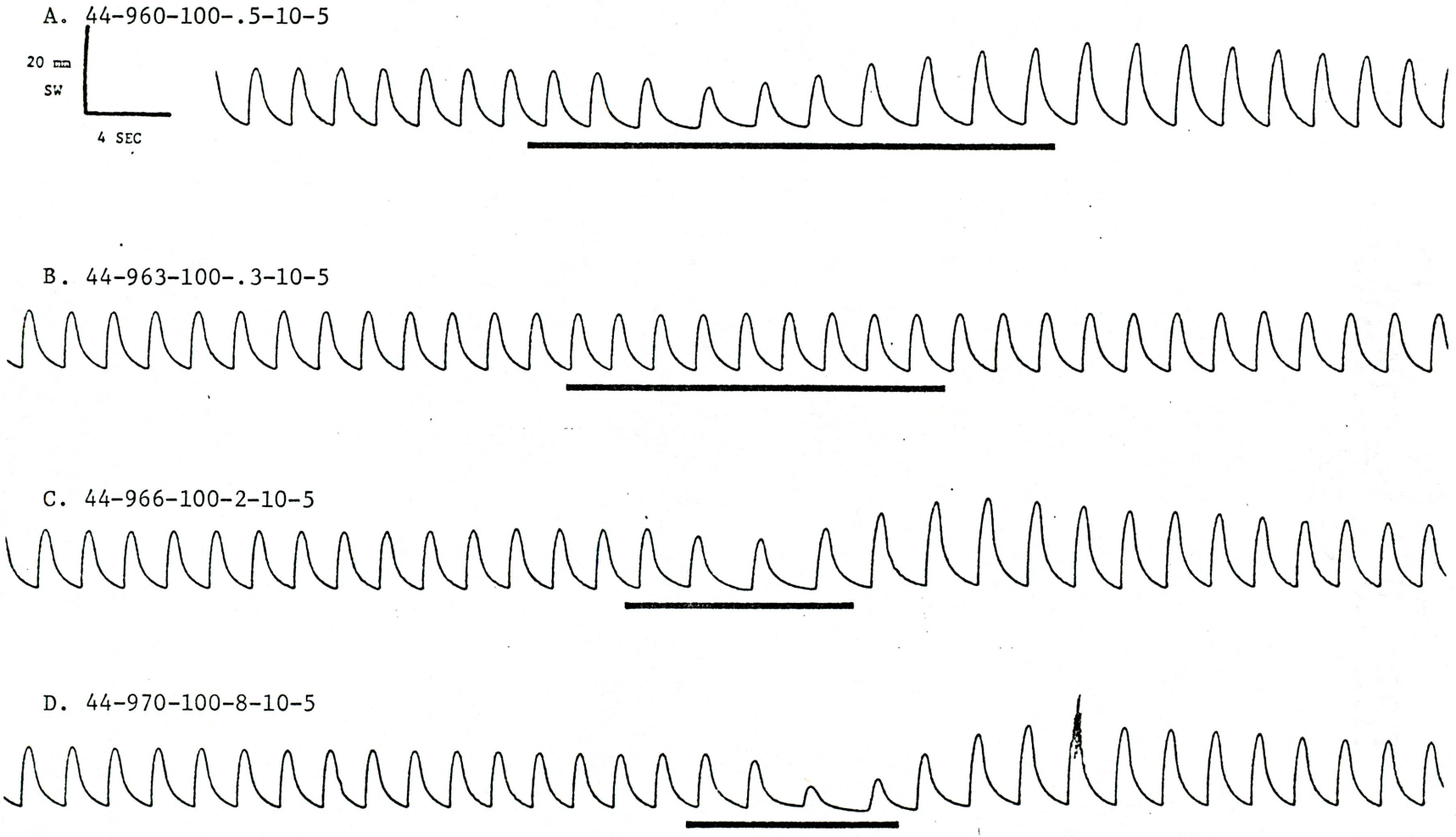


Fig. 17. Reduced inhibitory response to RC stimulation with cutting of Br nerve and pericardium (compare with Figure 16). A: Inhibitory response from 0.5 v, 5 hz stimulation. CHR = 30 bpm, BP = 13 mmSW; EHR = 23.4 bpm, BP = 9 mmSW minimum. B: No effect at 0.3 v stimulation. C: Cardioinhibition from 2 v stimulation. CHR = 29.2 bpm, BP = 13.5 mmSW; EHR = 22.2 bpm, BP = 12 mmSW min. D: Greater inhibition from 8 v stimulation. CHR = 30.9 bpm, BP = 13.3 mmSW; EHR = 23.4 bpm, BP = 4.5 mmSW min.

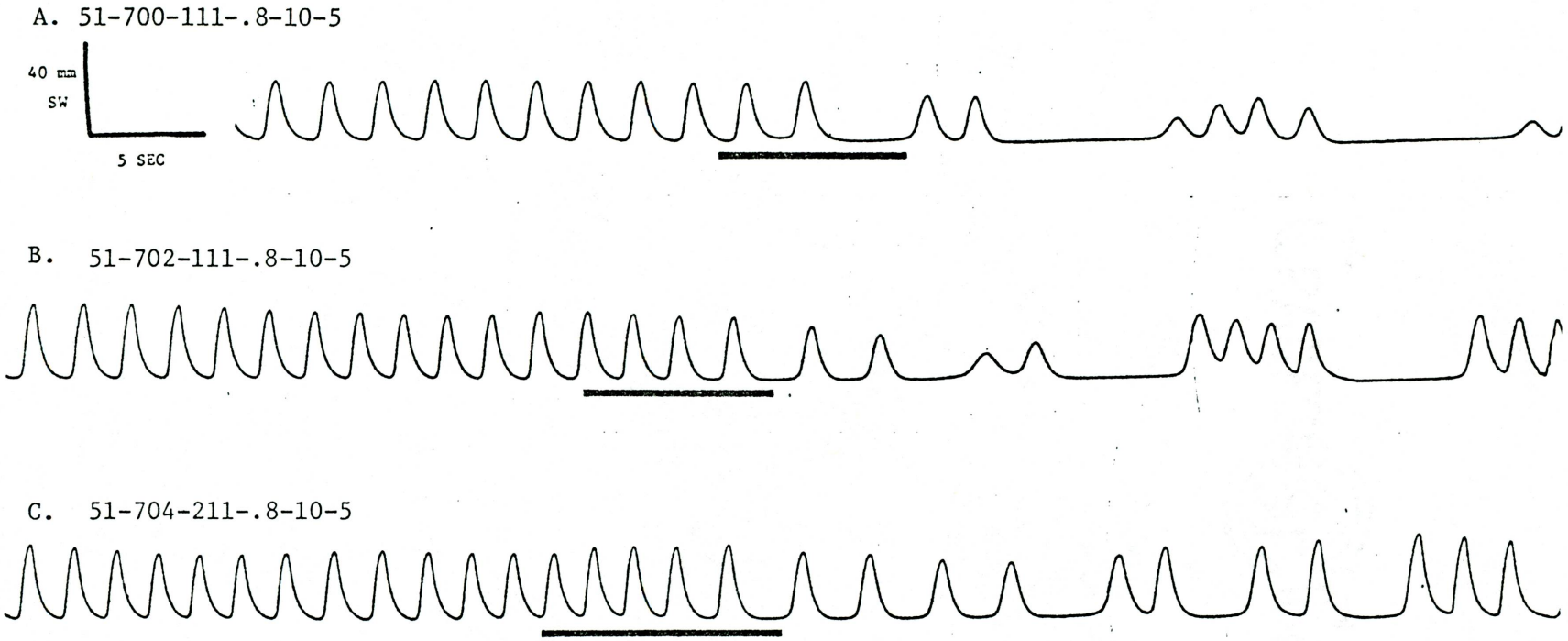


Fig. 18. Cardiac arrhythmia resulting from RC stimulation (LC in 18C) at 0.8 v, 5 hz. P_i = 50 mmSW. A: CHR = 26.3 bpm, BP = 24 mmSW, Recovery Time (RT) = 85 sec. RC stimulation at 0.6 v failed to elicit any change in cardiac activity. B: RC stimulation as in A. CHR = 30.4 bpm, BP = 28 mmSW, RT = 73 sec. Note that recovery from threshold RC stimulation takes longer than for LC stimulation. C: LC stimulation at 0.8 v intensity. CHR = 30 bpm, BP = 28 mmSW, RT = 37 sec. The response to LC stimulation has decreased since the previous stimulation (see Fig. 22C).

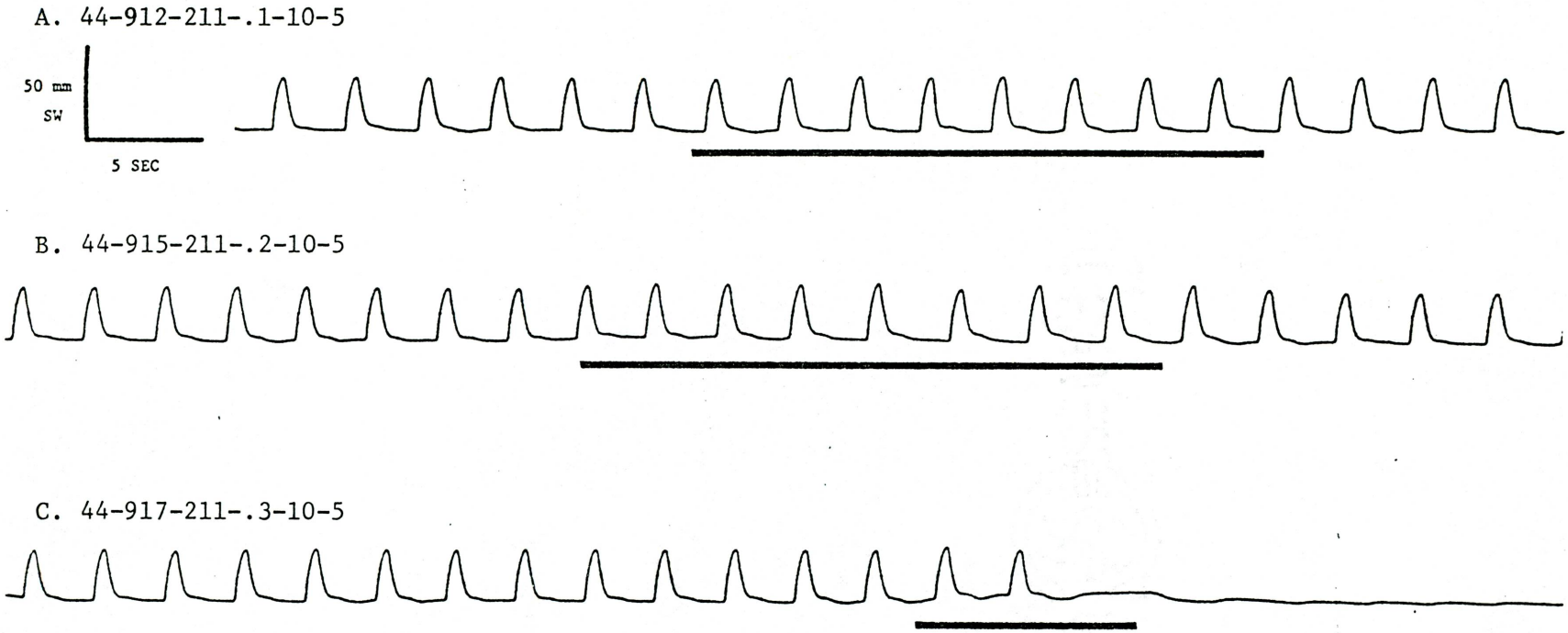


Fig. 19. Threshold response of intact heart to LC stimulation. A: No response to 0.1 v, 5 hz stimulation. HR = 18.8 bpm, BP = 27.5 mmSW. B: Slowing from 0.2 v stimulation. CHR = 19.4 bpm, BP = 27.5 mmSW; EHR = 17.7 bpm, BP = 30 mmSW. Note the small positive tono- and inotropic responses. C: Cardiac arrest from 0.3 v stimulation with spontaneous recovery in 45 sec. HR = 19.5 bpm. BP = 27.5 mmSW.

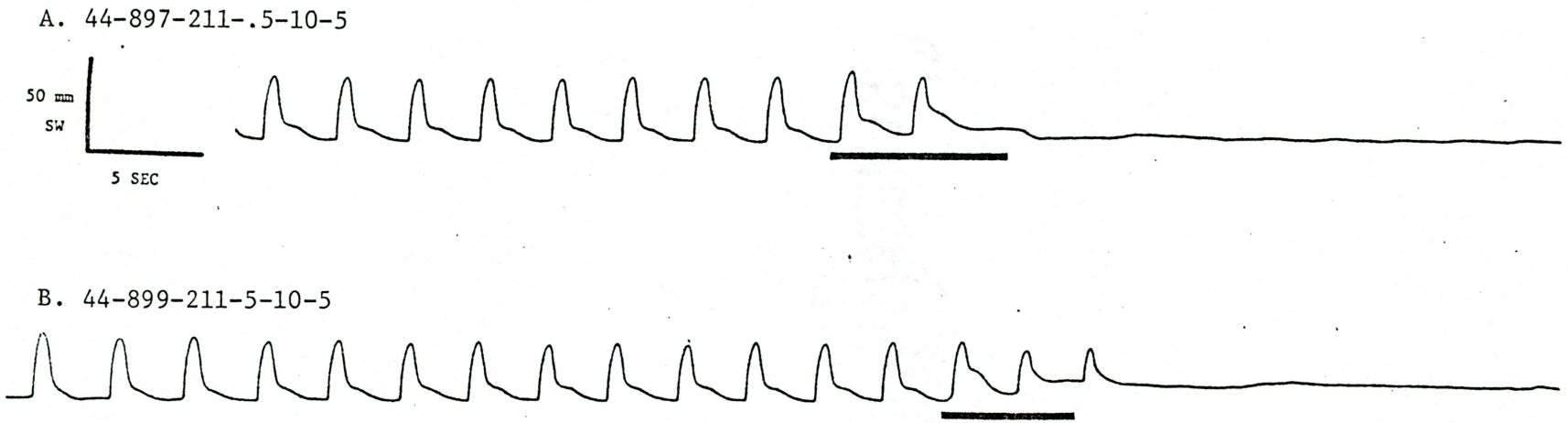


Fig. 20. Cardiac arrest resulting from LC stimulation. A: Arrest from low intensity stimulation (0.5 v, 5 hz) with spontaneous recovery in 55 seconds. HR = 19 bpm, BP = 33.7 mmSW. B: Arrest from higher intensity (5.0 v) stimulation with recovery in 80 seconds. There was a slight increase in rate prior to arrest. Note that the tonotropic effect is larger from the higher intensity stimulus. HR = 19.9 bpm, BP = 31.2 mmSW.

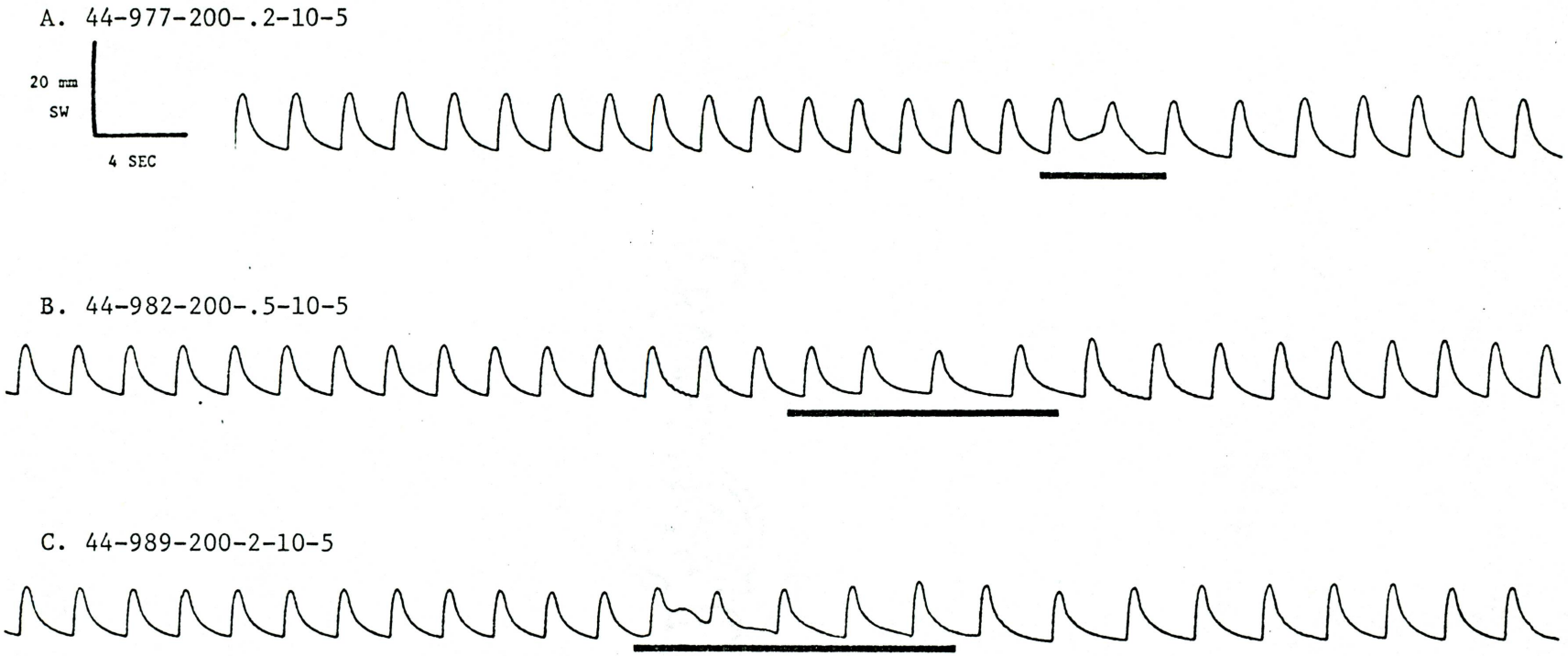


Fig. 21. Reduction in response to LC stimulation with cutting of the pericardium and the branchial nerve as evidenced by absence of cardiac arrest at equivalent stimulus intensities (compare with Figure 20). A: Slowing with positive tonotropism from low intensity stimulation (0.2 v, 5 hz). CHR = 27.5 pbm, BP = 11.5 mmSW; EHR = 24 bpm. B: Cardioinhibition from 0.5 v stimulation. CHR = 25.9 bpm, BP = 10.3 mmSW; EHR = 19.7 bpm, BP = 9 mmSW min. C: Slowing and positive tonotropic effect with 2 v stimulation. CHR = 26.2 bpm, BP = 10 mmSW; EHR = 21 bpm.

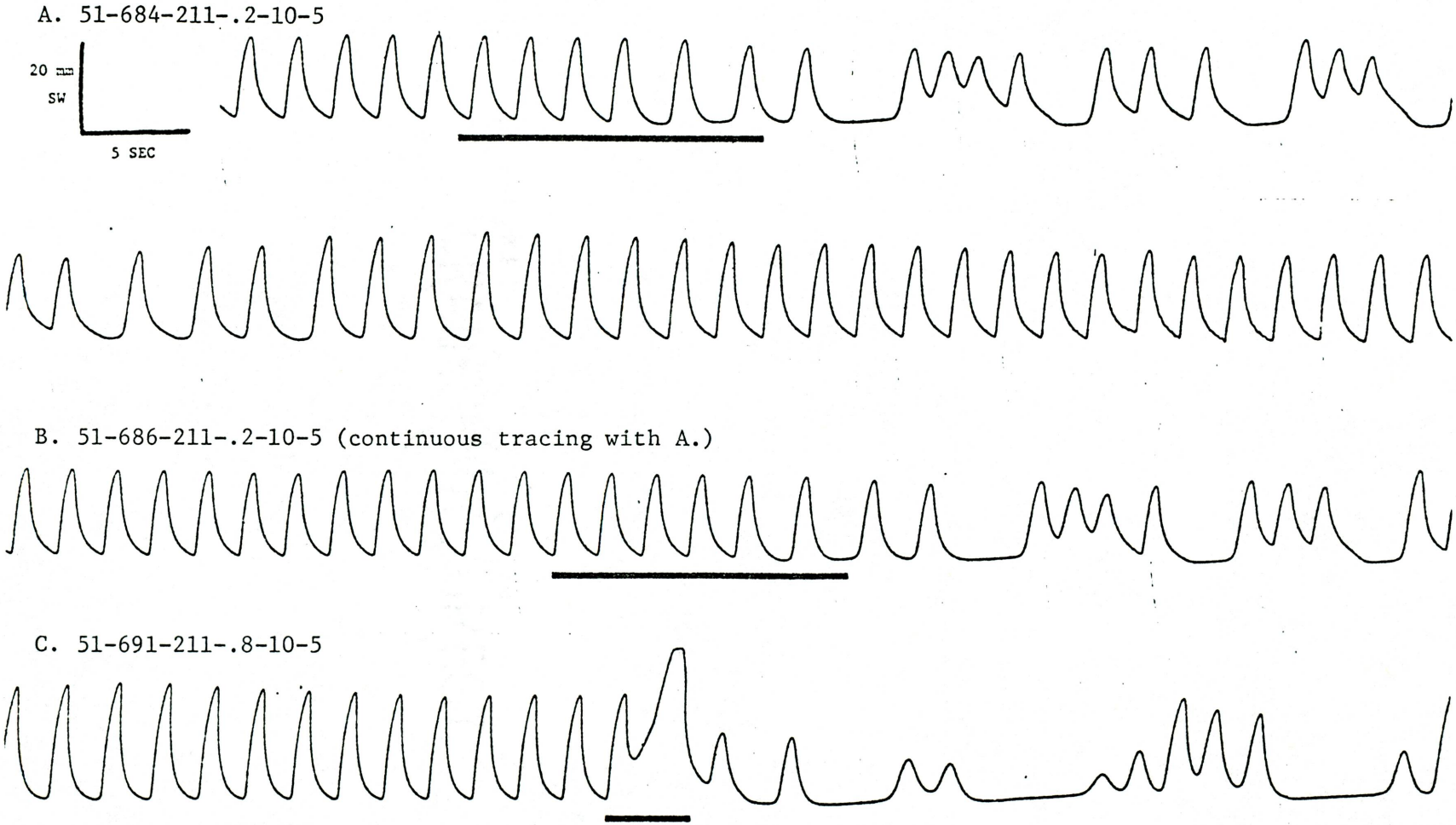
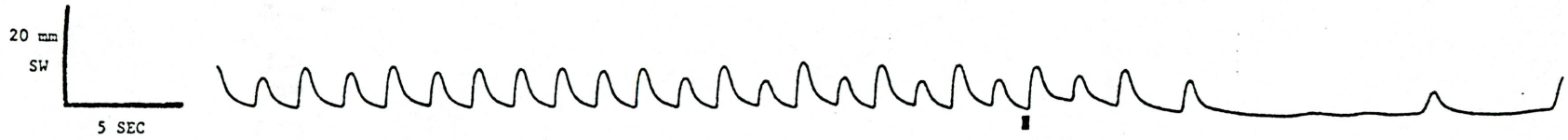


Fig. 22. Cardiac arrhythmia resulting from LC stimulation. P_i = 50 mmSW. A: arrhythmia from 0.2 v, 5 hz stimulation. CHR = 27 bpm, BP = 18 mmSW. Recovery time (RT) from arrhythmia = 35 sec. B: same stimuli as A. CHR = 28.1 bpm, BP = 20 mmSW, RT = 37 sec. (RT for 0.4 v stimulation was 45 sec.) C: large positive tono- and inotropism in addition to arrhythmia from 0.8 v stimulation. CHR = 27.9 bpm, BP = 24 mmSW, RT = 50 sec.

A. 52-028-211-8-10-0



B. 52-029-211-8-10-0 (A & B are continuous)

Fig. 23. Response of intact heart to single pulse stimulation (8 v, 10 ms) of left connective. P_i = 30 mmSW. A: bradycardia resulting from single pulse. CHR = 37.1 bpm, BP = 16 mmSW; EHR = 5.8 bpm (minimum). B: same as A. CHR = 33.4 bpm, BP = 14 mmSW; EHR = 7.2 bpm min.

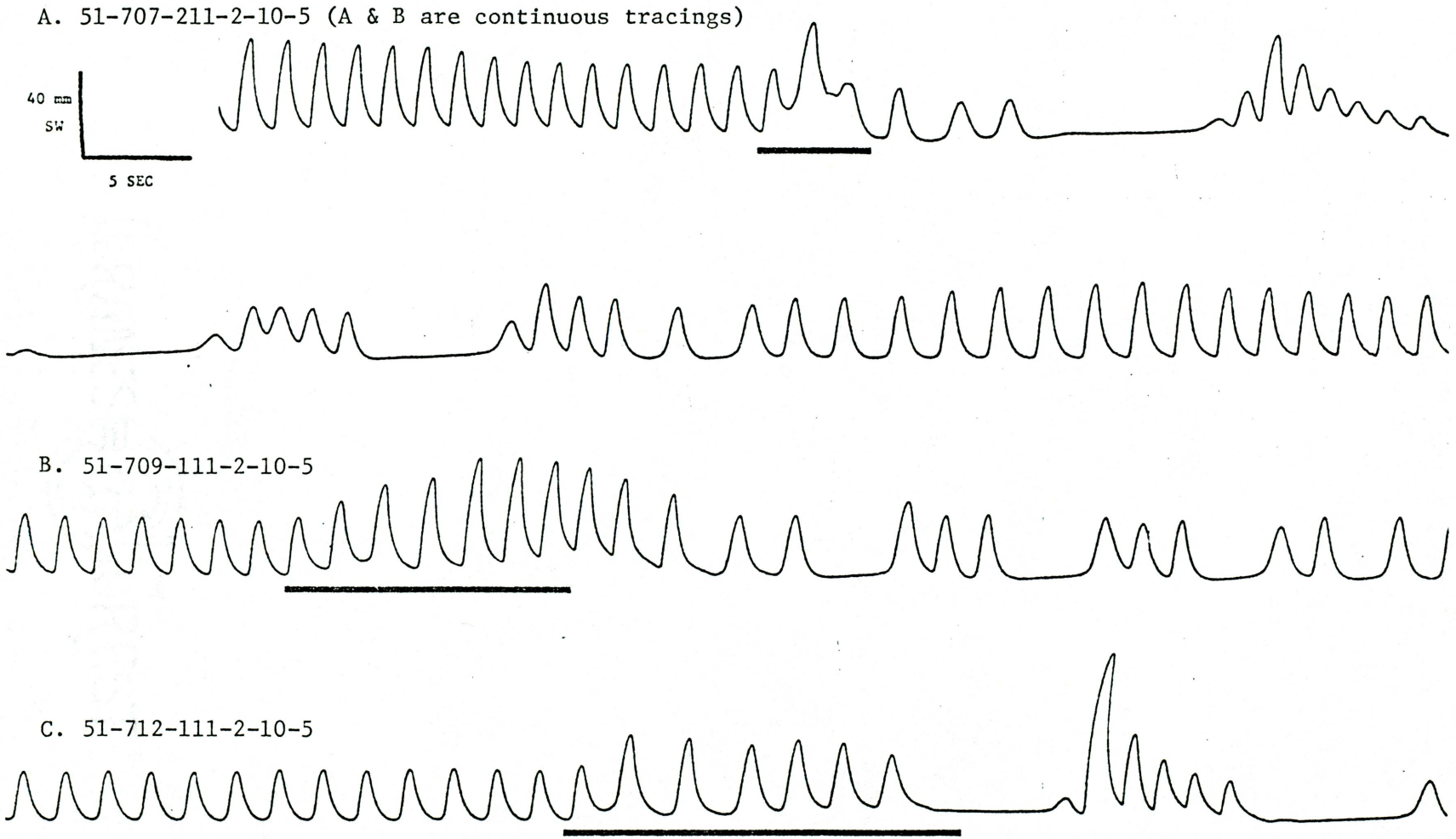


Fig. 24. Mixed response to RC and LC stimulation at higher intensities in comparison with results in Figures 18 & 22. P_i = 50 mmSW. A: positive tono- and inotropism followed by arrhythmia from LC stimulation at 2 v. CHR = 35.7 bpm, BP = 31 mmSW, RT = 65 sec. B: slowing followed by acceleration with RC stimulation at 2 v. CHR = 32.8 bpm, BP = 30 mmSW, RT = 35 sec. C: same as B. CHR = 29.4 bpm, RT = 60 sec.

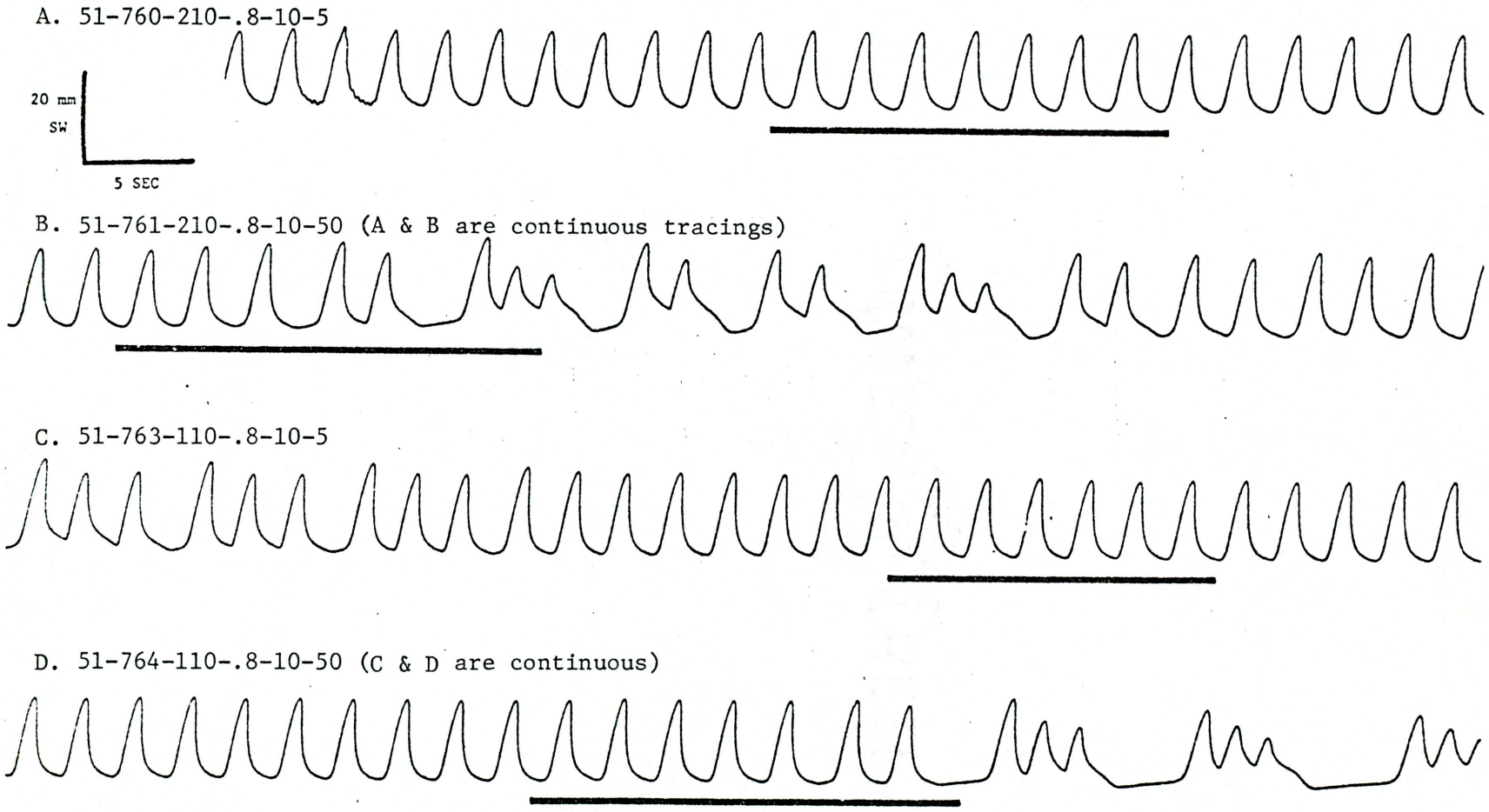


Fig. 25. Effectiveness of 50 hz stimuli (in contrast with 5 hz; Br cut). A: no heart rhythm change from LC stimulation at 0.8 v, 5 hz. HR = 25 bpm, BP = 17.5 mmSW. B: arrhythmia and slowing with LC stimulation at 0.8 v, 50 hz. C: no change as in A for RC stimulation. HR = 25.2 bpm, BP = 18 mmSW. D: slowing followed by arrhythmia from RC stimulation at 0.8 v, 50 hz.

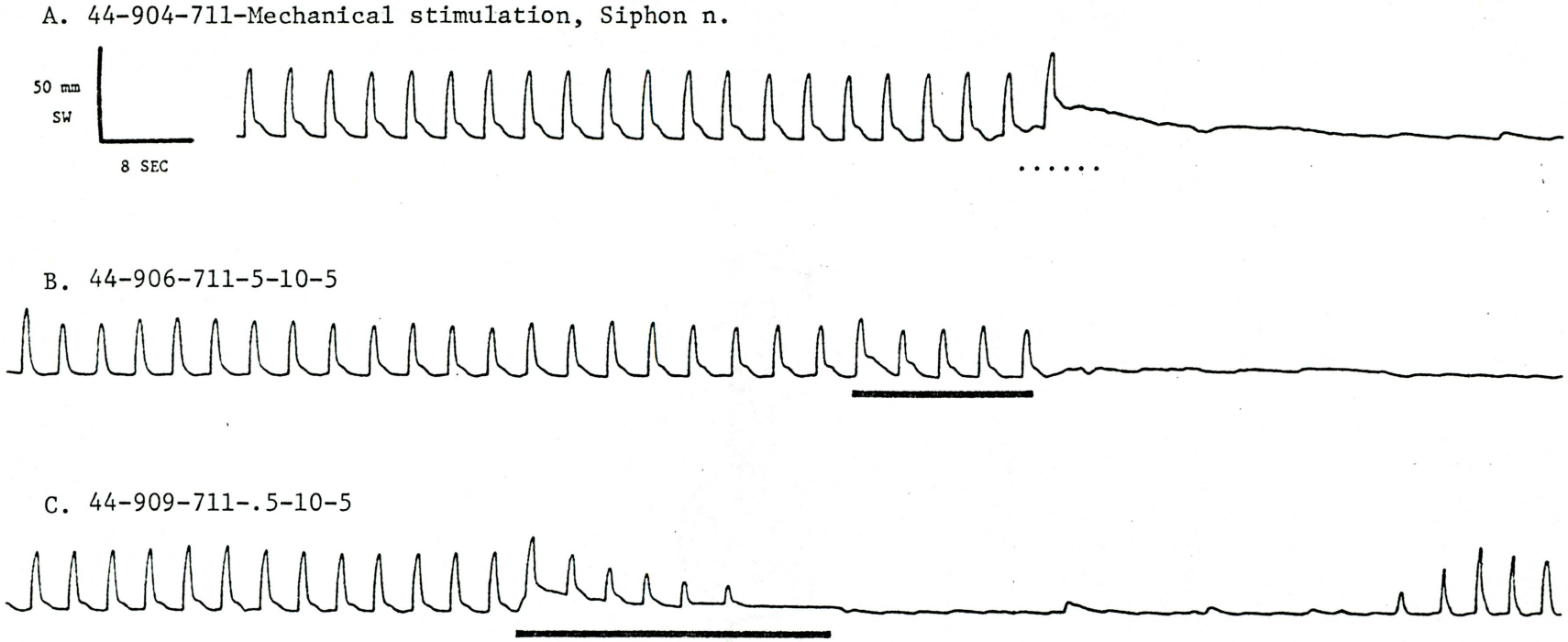


Fig. 26. Cardiac arrest in intact heart with siphon nerve stimulation (siphon nerve is cut peripheral to the electrode). A: Arrest resulting from mechanical stimulation in placing the siphon nerve on the stimulating electrode. HR = 17 bpm, BP = 35 mmSW. Spontaneous recovery in 90 sec. (Contraction of the pericardial musculature and ejection of purple secretion was observed at this time.) B: Arrest following stimulation at 5 v, 5 hz with spontaneous recovery in 72 sec. HR = 16.5 bpm, BP = 25 mmSW. C: Arrest from low intensity stimulation (0.5 v) with recovery in 58 sec. HR = 17.8 bpm, BP = 31.2 mmSW.

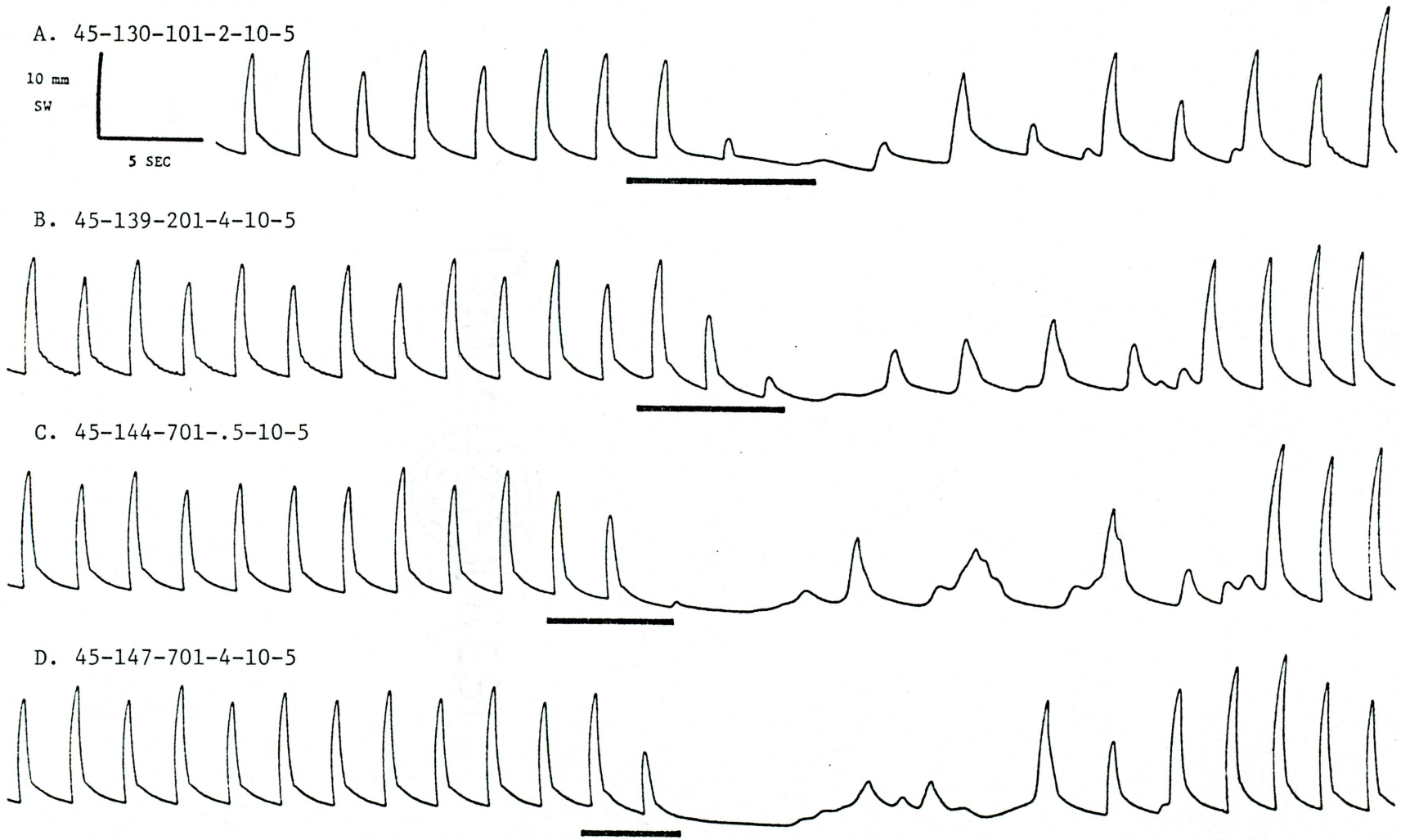


Fig. 27. Inhibitory responses of exposed heart, Br intact. P_i = 50 mmSW. A: RC stimulation at 2 v, 5 hz. HR = 20.4 bpm, BP = 15.5 mmSW. B: LC stimulation, 4 v, 5 hz. HR = 23.2 bpm, BP = 14.5 mmSW. (Results at 2 v were equivocal.) C: S nerve stimulation, 0.5 v, 5 hz. HR = 22.9 bpm, BP = 15 mmSW. D: S nerve stimulation, 4 v. HR = 23.4 bpm, BP = 14.2 mmSW. Note the arrhythmia during recovery.

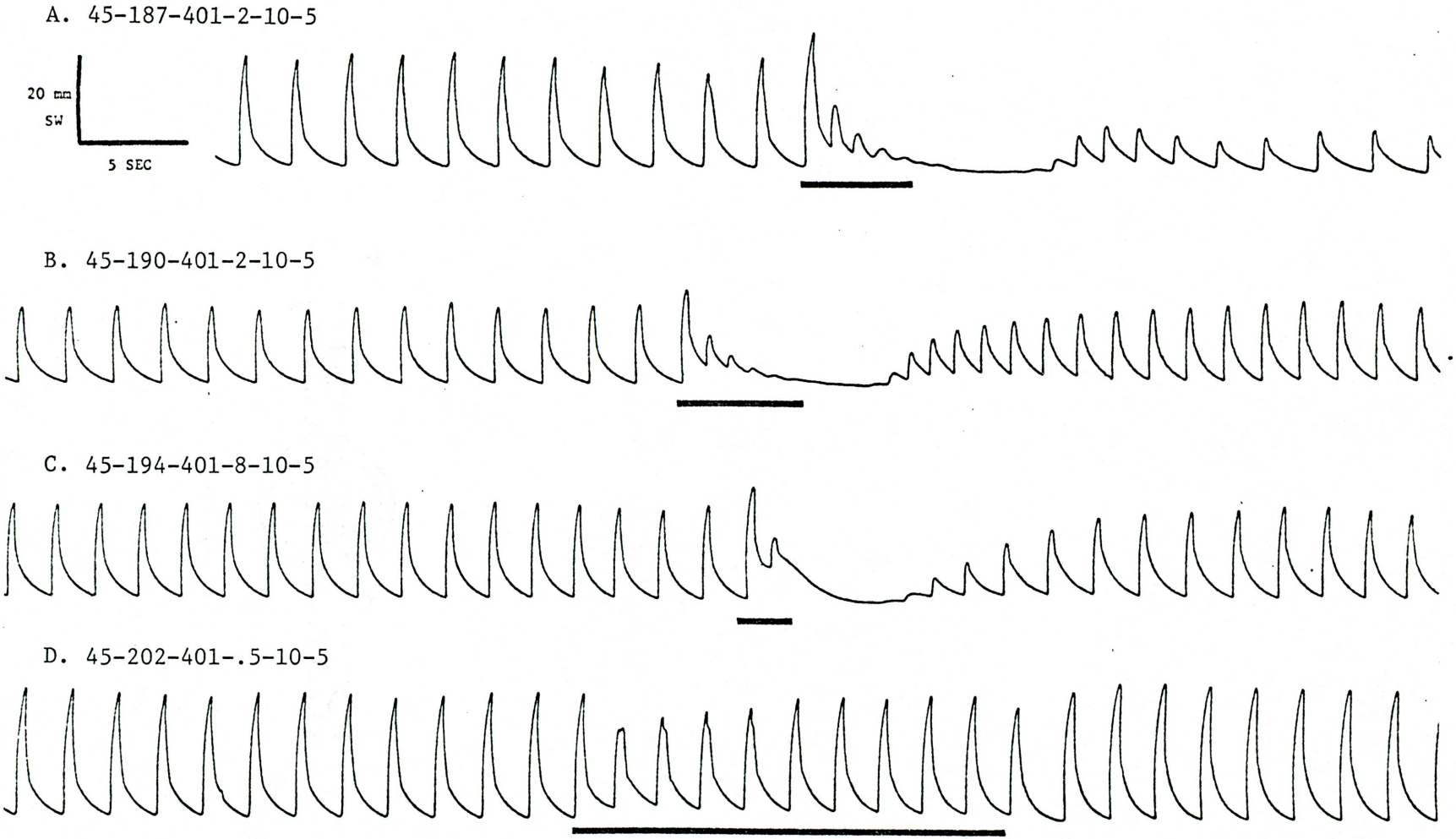


Fig. 28. Cardioacceleration from P nerve stimulation (compare with Fig. 23 & 24). P_i = 50 mmSW. A, B, & C show acceleration with positive tono- and inotropic effects. A: = 24.4 bpm, BP = 26 mmSW; EHR = 57.1 bpm. B: CHR = 26.8 bpm, BP = 19 mmSW; EHR = 60 bpm. C: CHR = 29.2 bpm, BP = 21 mmSW; EHR = 50 bpm (single beat). Note the sharp rate increase in each case with decreased pressure following. D: CHR = 27 bpm, BP = 29.7 mmSW; EHR = 30.6 bpm max. Speeding was slight with the lower voltage (0.5 v).

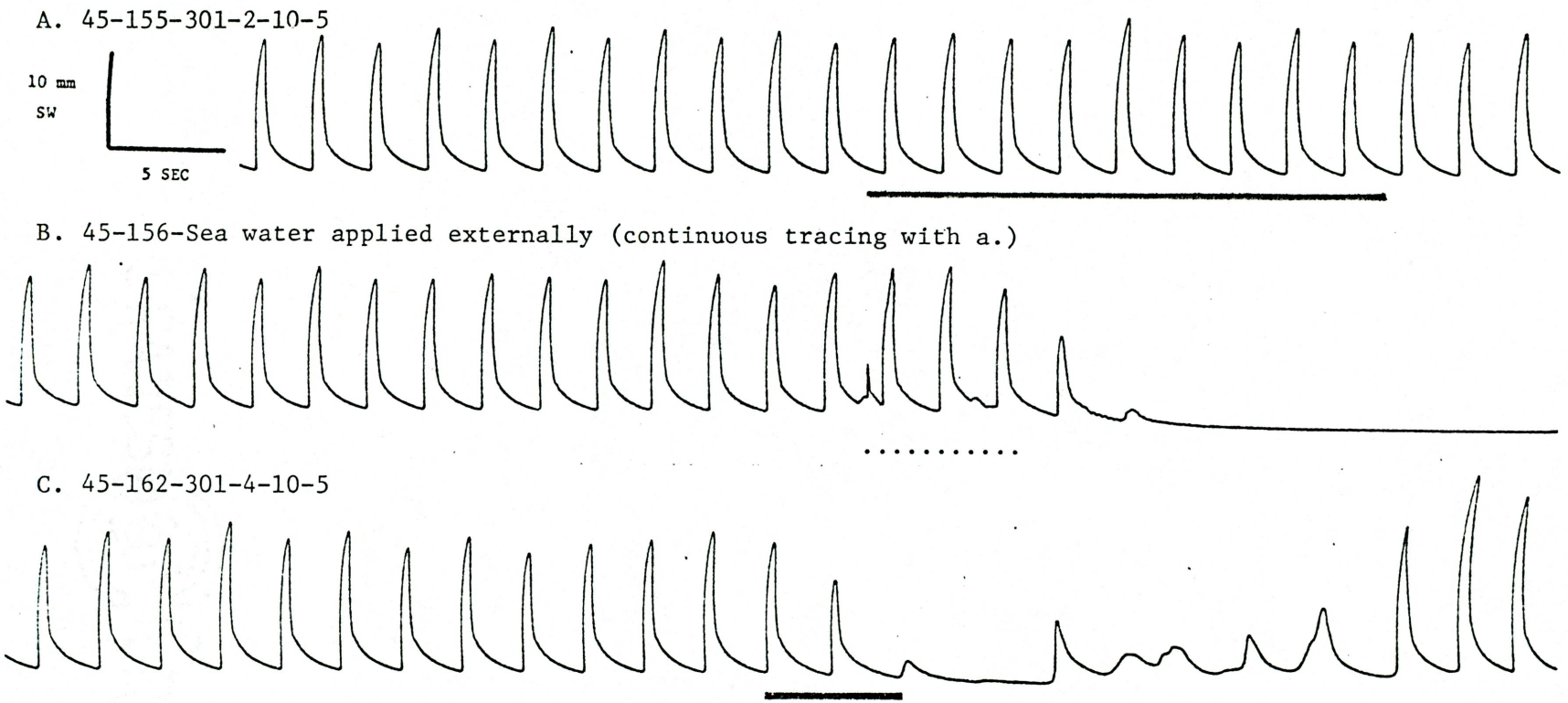
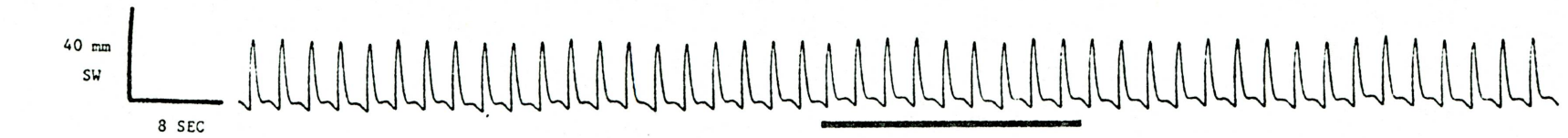


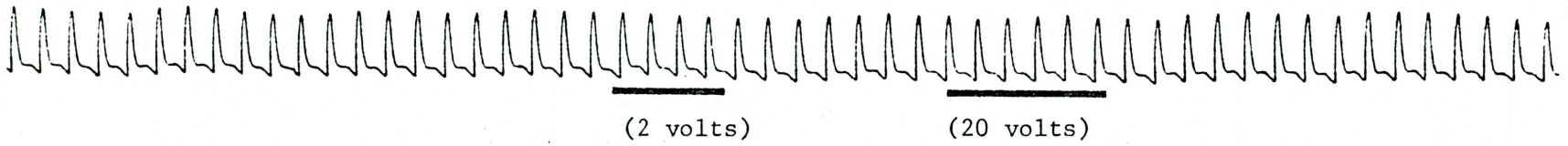
Fig. 29. Cardioinhibition resulting from Br nerve stimulation. P_i = 50 mmSW. A: No change from 2 v, 5 hz stimulation. HR = 23.8 bpm, BP = 14 mmSW. B: Cardiac arrest from drops of SW applied to the musculature in the area of the perfusing catheter (approximate time denoted by dotted line). A and B form a continuous recording. C: Cardioinhibition from Br stimulation at 4 v, 5 hz. HR = 22.4 bpm, BP = 15 mmSW.



A. 54-822-211-2-10-5

B. 54-824-211-20-10-5

C. 54-825-111-2-10-5, 20-10-5



D. 54-834-511-.4-10-5

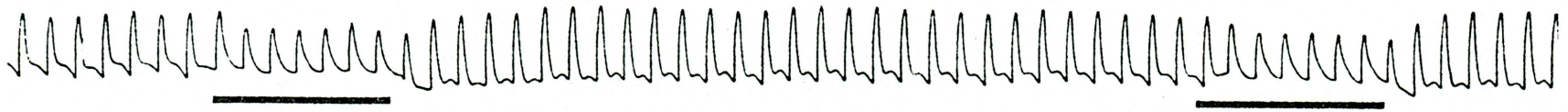


Fig. 30. Possible damage from pronase (20 mg/ml; 20 minutes on dorsal surface of ganglion sheath). No changes were observed with stimulation of the left (A,B) and right (C) connectives at 2 and 20 volts, 5 hz. Direct GP stimulation (D) at a lower intensity than the above (0.4 v, 5 hz) resulted in cardio-acceleration, decreased pulse pressure, and increased tone, implying damage somewhere between the input and output nerves.

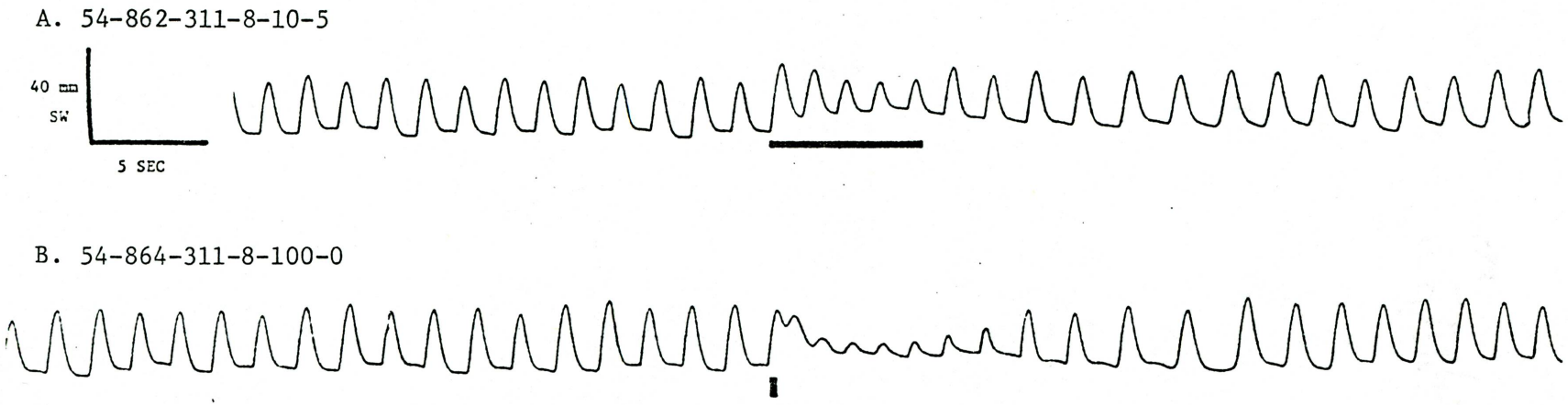


Fig. 31. Cardioacceleration from branchial nerve stimulation of pronase treated ganglion (compare with Fig. 30). A: stimulation a 8 v, 5 hz resulted in acceleration with a long term positive tonotropic effect and single beat inotropism. This is the only preparation that showed acceleration with Br stimulation (with exception of animal 32 which was perfused through the gill). CHR = 34.7 bpm, BP = 25 mmSW; EHR = 40 bpm max., BP = 12 mmSW min. (Stimulation at 2 v was ineffective.) B: cardioacceleration with positive tonotropism from single 8 v, 100 ms pulse. CHR = 31.9 bpm, BP = 30 mmSW; EHR = 54.5 bpm max., BP = 4 mmSW min.

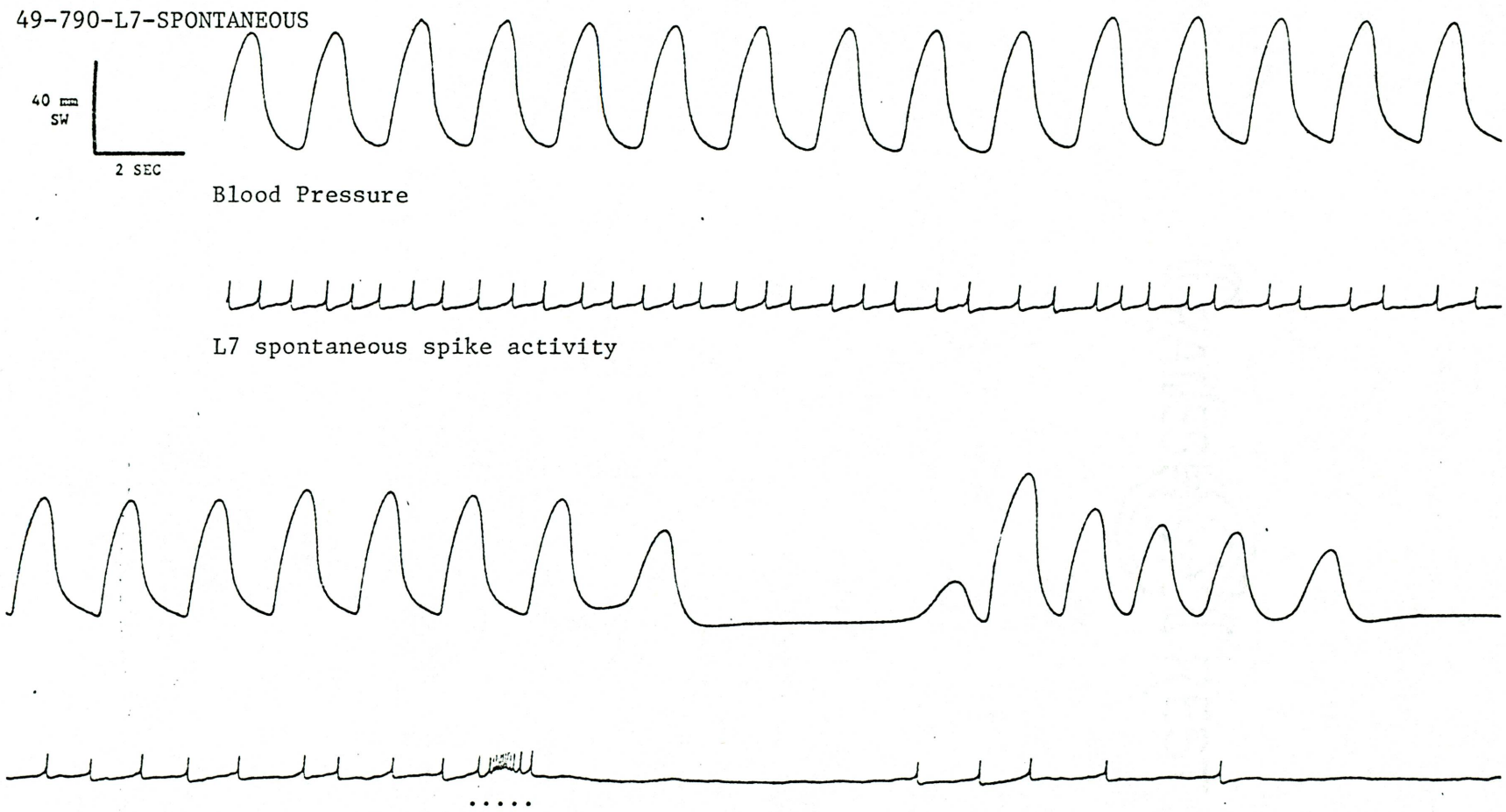


Fig. 32. Spontaneous high frequency burst in L7 followed by cardioinhibition. P_i = 35 mmSW. Control period: HR = 30.5 bpm, BP = 53 mmSW, maximum spike frequency = 2 hz. Spike burst frequency: 20 hz.

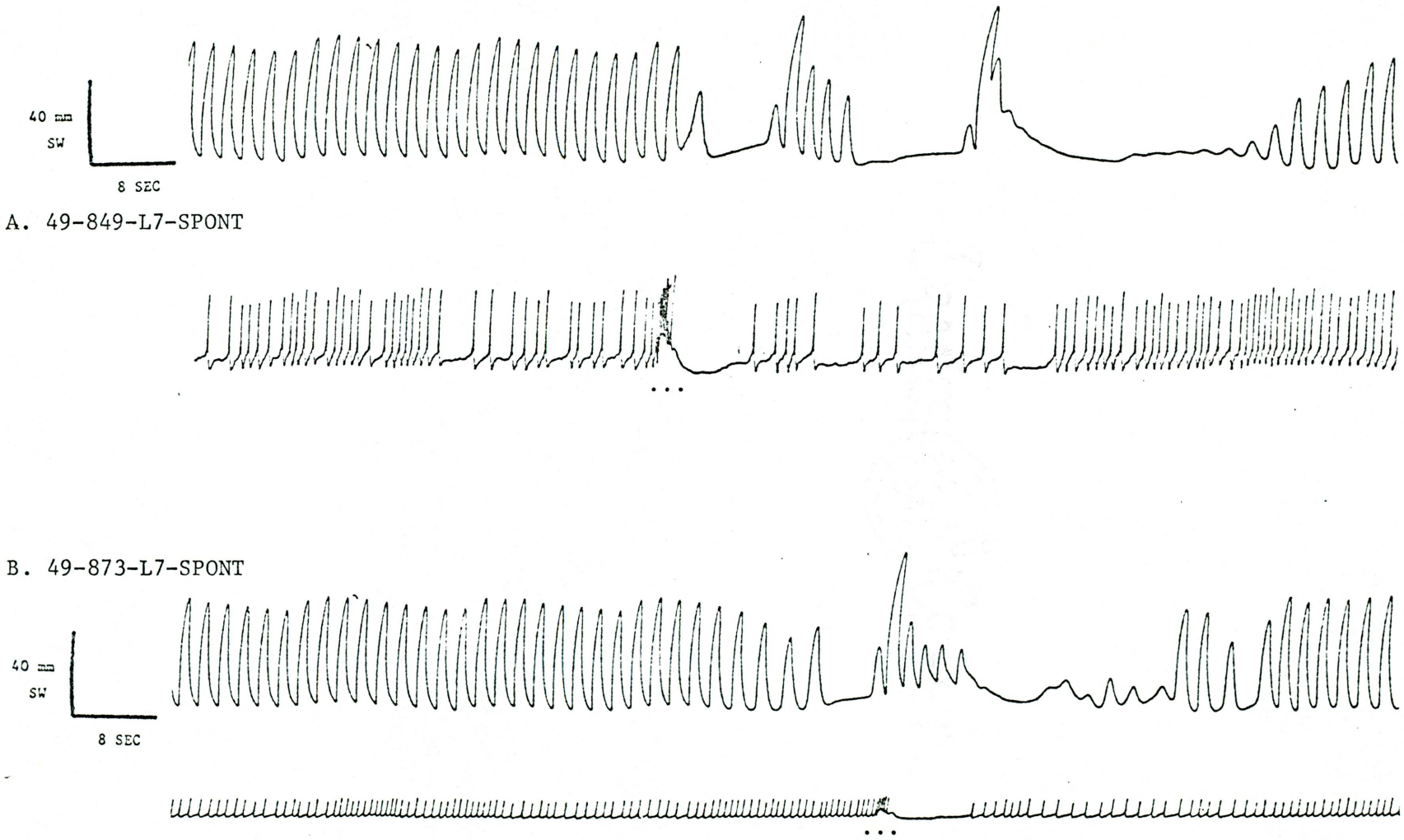


Fig. 33. Mixed results with spike burst in L7. A: Spike burst precedes bradycardia, arrhythmia. HR = 30 bpm, BP = 54 mmSW. B: Onset of bradycardia precedes spike burst. HR = 31.8 bpm, BP = 51 mmSW.

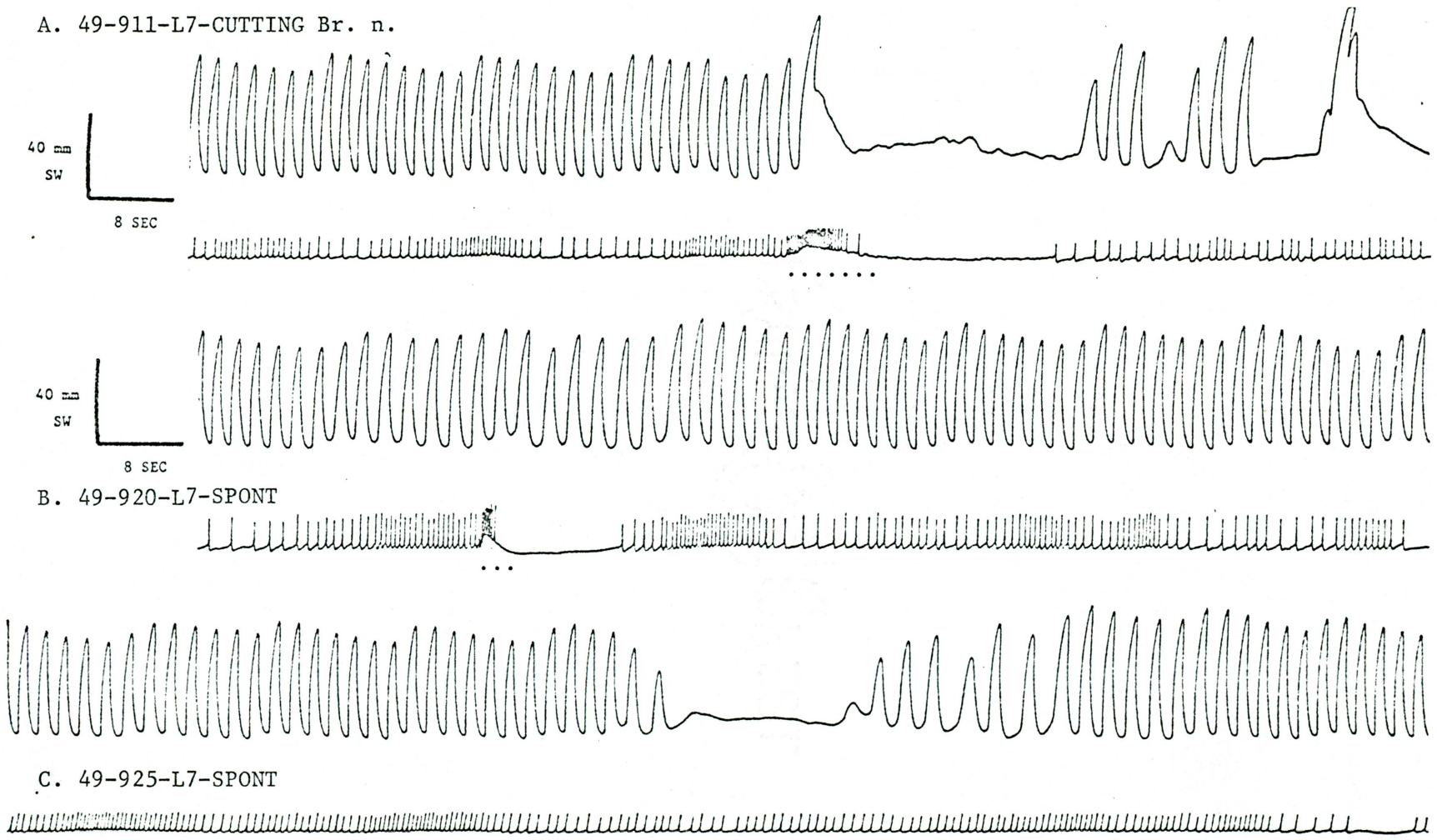


Fig. 34. Branchial nerve cut. A: Spike burst in L7 with cardiac arrest in cutting Br. Inotropic and tonotropic effects present. B: Spike burst followed by moderate bradycardia with Br cut. HR = 32 bpm, BP = 51 mmSW. C: Cardiac arrest without apparent change in L7 spike rhythm. HR = 31.6 bpm, BP = 48 mmSW.

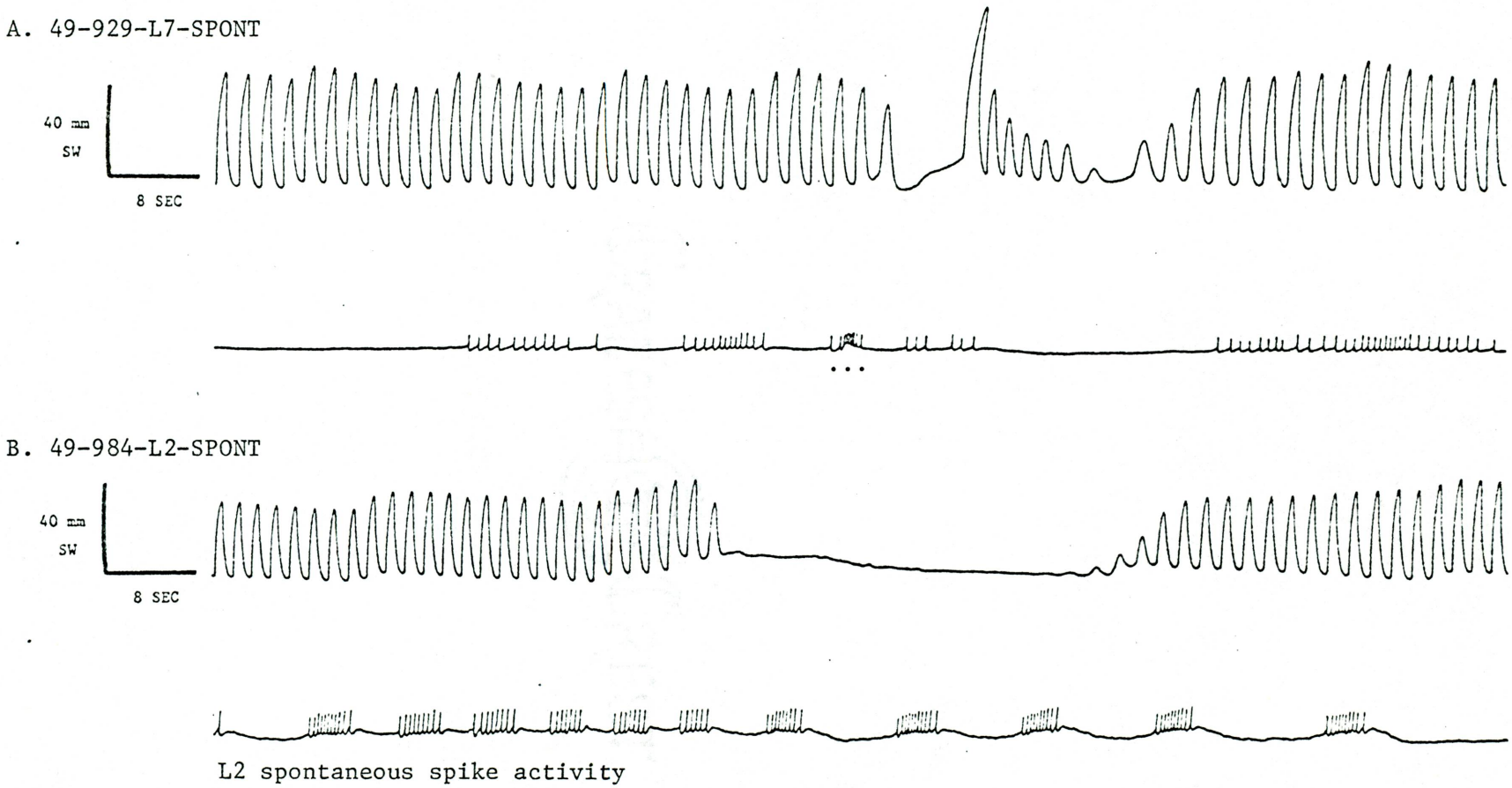


Fig. 35. A: L7 spike burst followed by bradycardia and arrhythmia. HR = 31.3 bpm, BP = 47 mmSW. B: No apparent change in L2 burst rhythm with cardiac arrest.

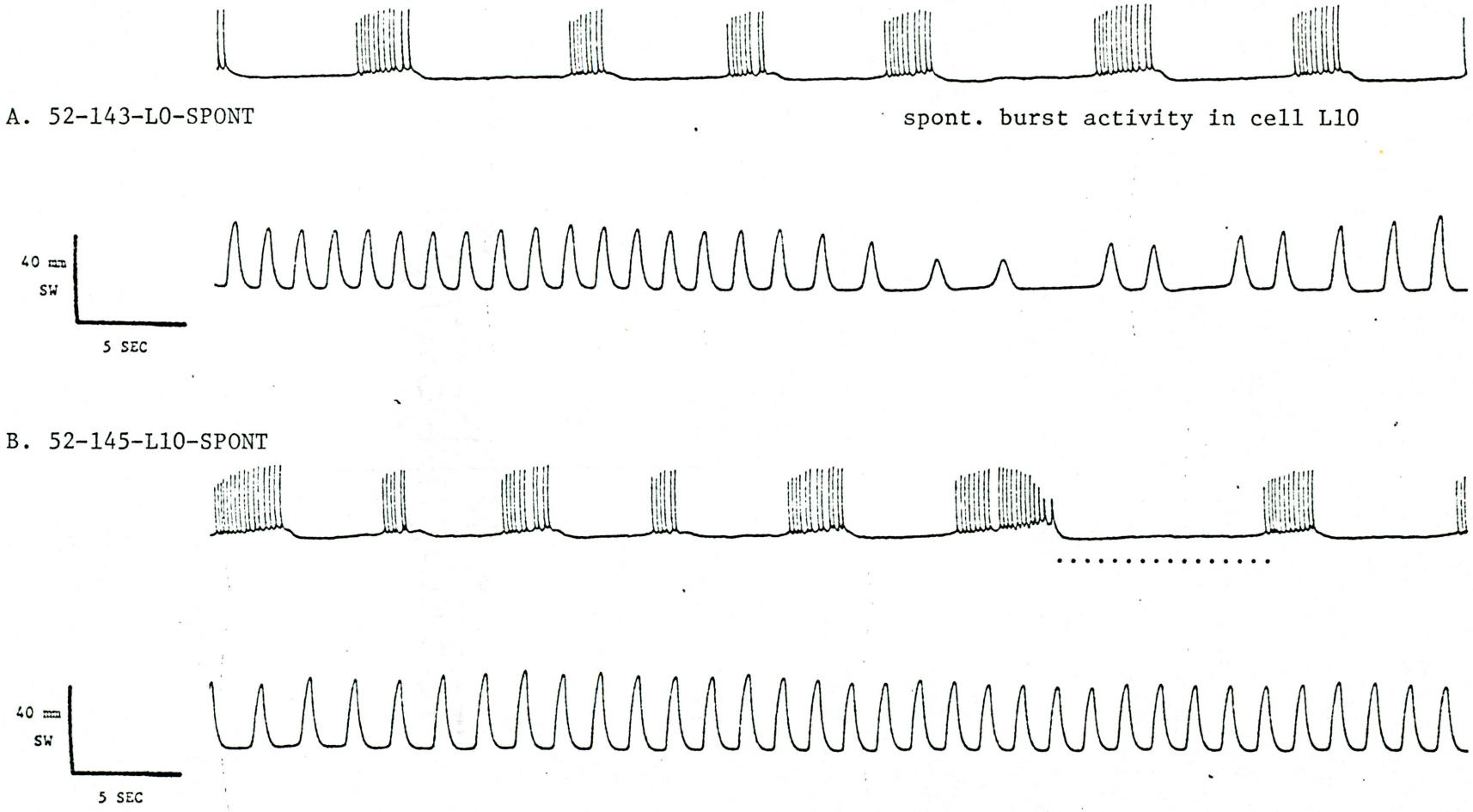


Fig. 36. Variations in relationship between L10 burst rhythm and heart activity. P_i = 30 mm glucose-sea water (G-SW). A: Bradycardia with no apparent change in L10. HR = 38.1 bpm, BP = 27 mmSW. B: Inhibition of long duration (ILD) in L10 with no apparent change in heart rhythm. HR = 37.5 bpm, BP = 32 mmSW.

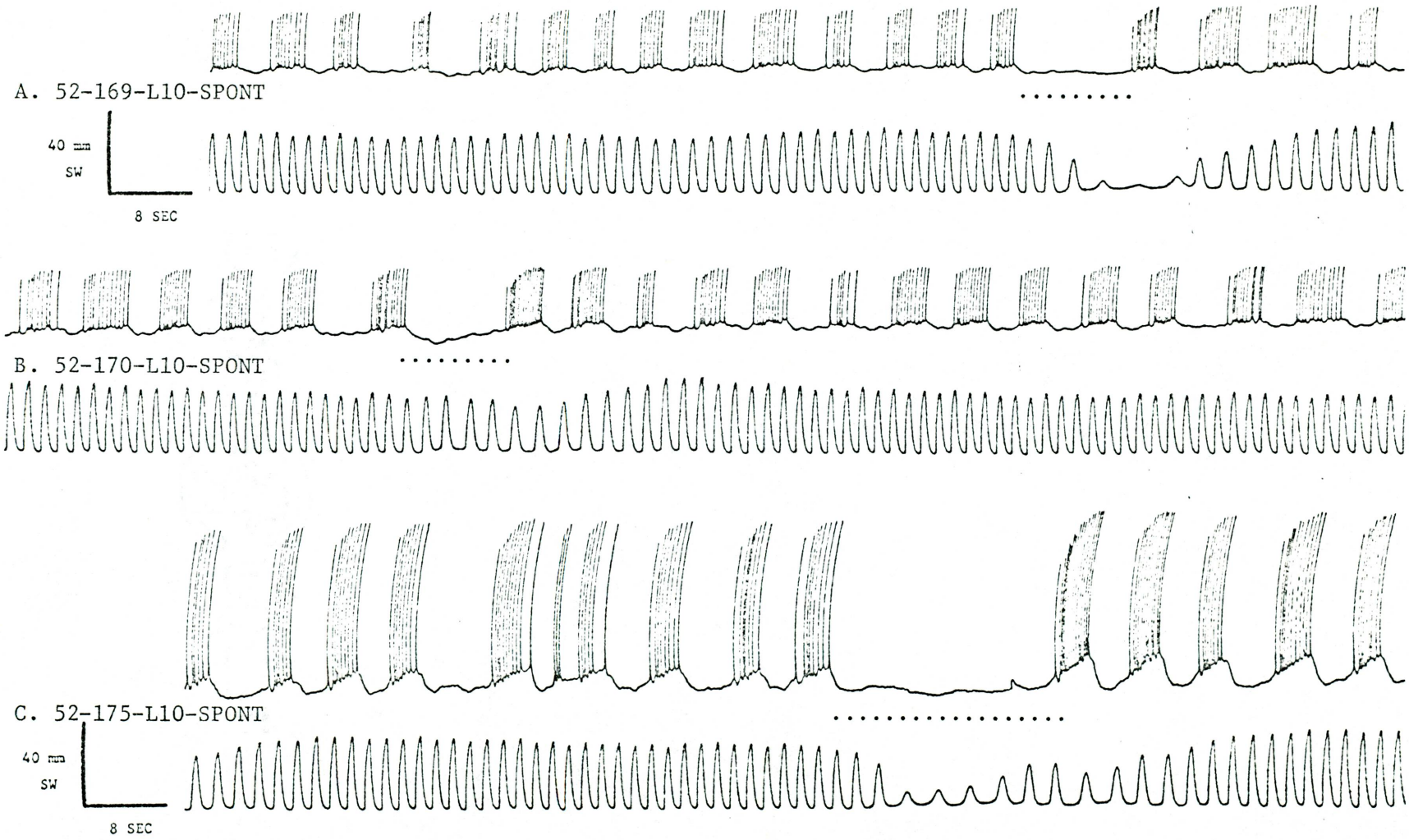


Fig. 37. Cardioinhibition associated with ILD's (indicated by dotted line) in L10. P_i = 30 mmG-SW. A: HR = 37.5 bpm, BP = 30 mmSW. B: HR = 40 bpm, BP = 29 mmSW. C: HR = 37 bpm, BP = 31 mmSW.

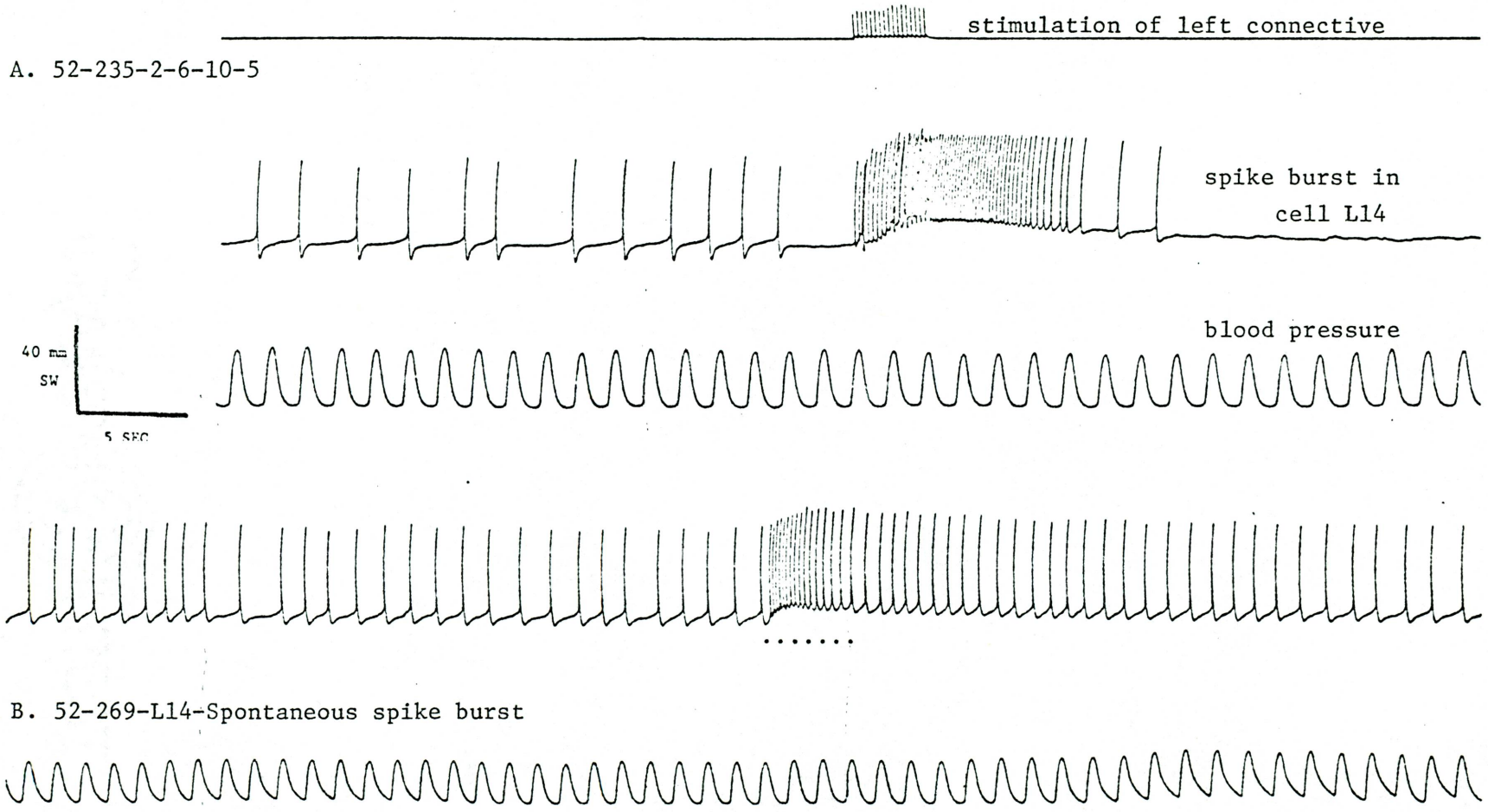


Fig. 38. Relation of L14 high frequency spike burst to cardiac activity. P_i = 30 mmSW. A: burst response in L14 triggered by LC stimulation at 6 v, 5 hz. There was no change in heart rhythm. B: spontaneous burst in L14 with no apparent change in heart rhythm.

Table 3a. Results from genital-pericardial nerve stimulation

Animal 37[1]

Control		Stim.[2]	TONO	INO	HRC	A	FIG.
(HR)	(BP)	(Volts)					
19.8	5	8[3]	+	+	+	0	10C
26.7	9	2	+	-	-	0	11A
27	9	2	+	-	-	0	11B
26.6	9	2[4]	+	-	-	0	11C
22	8	8	+	0	-	+	12B
23	14	0.5	+	0	-	+	12C
23.7	14	0.5	+	0	-	+	
24.2	35	0.1	0	0	0	0	
24.0	35.5	0.2	0	-	-	0	
24.1	22	0.2	0	0	-	0	13A
25.2	20	20	+	+	+	0	13B
21.6	18.5	20	+	+	0	0	13C
23.2	28	8	+	+	$\pm$	0	
20.4	24	4	+	+	$\pm$	0	
20.3	23	2	+	0	$\pm$	+	

1. Animal 37. Genital-pericardial nerve was cut at the ganglion and stimulated toward the heart. Perfusion pressure ranged from 15 to 20 mm sea water. Pericardium was opened.

2. Stimulus pulses were of 10 ms duration at 5 hertz unless otherwise specified. Pulse trains were of various lengths.

3. 0.5 hertz stimulus

4. Short duration stimulus pulse train

5. HR = heart rate in beats/min, BP = blood pressure in mm sea water, TONO = tonotropic effect, INO = inotropic effect, HRC = heart rate change, A = cardiac arrest, + = positive or increase, - = negative or decrease, 0 = no change.

Table 3b. Results from genital-pericardial nerve stimulation

Control (HR)	Control (BP)	Stim. (Volts)	TONO	INO	HRC	A
Animal 53[1]						
26.2	10	8	-	0	0	+
24.8	31	.8	+	0	±	0
26.1	34	2[2]	+	0	0	0
25.2	35	2	+	0	±	0
26.1	40	8	0	0	-	+
23	40	8[3]	0	0	-	0
28.7	35	8	0	0	-	+
Animal 54[4]						
26.8	24	.8	+	+	+	+
25.1	23	.2	0	0	0	0
24.6	28	.4	+	0	+	0
24.9	32	.4	+	0	+	0
26	28	.4	+	0	+	0
29.2	26	.3	0	0	0	0
27.6	29	.3	0	0	0	0
26.1	29	.6	0	0	0	0
25.4	30	2	0	0	+	0

1. Animal 53. Intact pericardium and branchial nerve.

2. Short stimulus train, applied too soon after previous stimulus.

3. Stimulated too soon after previous stimulus.

4. Animal 54. Pronase treated ganglion. Intact pericardium and branchial nerve.

Table 4a. Chronotropic effects from right connective stimulation

Animal	Total Stim.	No. Sub-threshold	Threshold (Volts)	+ HRC	- HRC	0 HRC	A	NIL
34	5	0	<2	3	0	2	0	0
37	2	0	>2	0	0	2	0	2
38	6	0	0.5	0	2	4	3	1
39	9	4	>1	2	0	7	0	4
42	10	0	<0.5	0	4	6	0	0
43	9	2	>0.5	5*	2*	3	2	3
44	15	2	0.3	1	7	7	5	4
45	1	0	<2	0	1	0	1	0
51	30	6	<0.8	1*	12*	18	2	14
52	9	4**	0.4	1	3	5**	0	5
53	5	3	>0.4	0	0	5	2	3
11	101	21		13	31	59	15	36
						-21		-21
					Corrected totals#	38		15

* One of these had a dual response (±).

** Three of these were from single pulse stimulation.

Totals corrected for subthreshold stimuli.

1. HRC = heart rate change, + = increase, - = decrease, 0 = no rate change, A = cardiac arrest, NIL = no contractility or rate changes evoked by stimulation.

2. Threshold was taken as the lowest intensity that evoked a response. Anything above 1 volt was regarded as suprathreshold even if no response resulted.

Table 4b. Normalized results from right connective stimulation

Animal	Number Stimulations	+HRC	-HRC	+NHRC[1]	-NHRC
34	5	3	0	1	0
37	2	0	0	0	0
38	6	0	2	0	1
39	9	2	0	1	0
42	10	0	4	0	1
43	9	5	2	0.71	0.29
44	15	1	7	0.12	0.88
45	1	0	1	0	1
51	30	1	12	0.08	0.92
52	9	1	3	0.25	0.75
53	5	0	0	0	0
	Totals	13	31	3.16	5.84

1. NHRC = normalized heart rate change

a. $+NHRC = \frac{+HRC}{(+HRC) + (-HRC)}$

b. $-NHRC = \frac{-HRC}{(+HRC) + (-HRC)}$

c. $\text{HRC ratio} = \frac{-HRC}{+HRC} = \frac{31}{13} = 2.38$

d. $\text{NHRC ratio} = \frac{-NHRC}{+NHRC} = \frac{5.84}{3.16} = 1.85$

Table 5a. Chronotropic effects from left connective stimulation

Animal	Total Stim.	No. Sub-threshold	Threshold (Volts)	+ HRC	- HRC	0 HRC	A	NIL
34	7	1	> 1	6	0	1	0	1
37	3	0	> 8	0	0	0	0	3
38	2	0	< 0.5	0	2	0	1	0
39	3	1	> 0.5	1	0	2	0	1
43	2	0	>20	0	0	2	0	2
44	15	2	0.2	1	10	2	4	2
45	10	5	2	0	4	5	1	6
51	19	0	< 0.2	0	12	7	1	5
52	14	0	0.2	0	6	7	0	7
53	5	2	> 0.2	0	0	5	3	2
10	80	11		8	34	31	10	29
						-11		-11
			Corrected totals*			20		18

* Totals corrected for subthreshold stimuli.

1. HRC = heart rate change, + = increase, - = decrease, 0 = no rate change, A = cardiac arrest, NIL = no contractility or rate changes evoked by stimulation.

2. Threshold was taken as the lowest intensity that evoked a response. Anything above 1 volt was regarded as suprathreshold even if no response resulted (e.g., Animals 37 & 43).

Table 5b. Normalized results from left connective stimulation

Animal	Number Stimulations	+HRC	-HRC	+NHRC[1]	-NHRC
34	7	6	0	1	0
37	3	0	0	0	0
38	2	0	2	0	1
39	3	1	0	1	0
43	2	0	0	0	0
44	15	1	10	0.09	0.91
45	10	0	4	0	1
51	19	0	12	0	1
52	14	0	6	0	1
53	5	0	0	0	0
	Totals	8	34	2.09	4.91

1. NHRC = normalized heart rate change

a. $+NHRC = \frac{+HRC}{(+HRC) + (-HRC)}$

b. $-NHRC = \frac{-HRC}{(+HRC) + (-HRC)}$

c. $\text{HRC ratio} = \frac{-HRC}{+HRC} = \frac{34}{8} = 4.25$

d. $\text{NHRC ratio} = \frac{-NHRC}{+NHRC} = \frac{4.91}{2.09} = 2.35$

Table 6. Results from pericardial nerve stimulation

Animal	Stimulations	Heart Rate Changes			No Effect[1]
		Increase	Decrease	No Change	
42	9	7*	3*	1	0
43	5	4	0	1	1
45	9	6	0	3**	2
52	10	9	0	1	1***

1. No effect signifies no contractility, as well as no rate changes.

* Two of these were dual results, speeding followed by slowing.

** Two of these were subthreshold stimuli.

*** This was a result from a cut pericardial nerve stimulated towards the ganglion to look for a possible feedback pathway.

Table 7. Results from siphon nerve stimulation

Animal No.	Control (HR)	Control (BP)	Stimulus (Volts)	TONO	INO	HRC	ARREST	
42	17.6	24	20	0	0	±	0	
	17.5	24	0.5	0	0	±	0	arrest later
	20.2	20.5	2	0	+	-	0	
	22	21	2	0	0	-	+	
	22.9	23	8	0	-	±	0	
44	16.5	25	5	0	0	0	+	
	17.8	31.2	0.5	+	0	0	+	
*	25.6	9.5	0.5	0	0	0	0	
*	24.6	9.8	2	0	0	+	0	
*	25.8	9	8	0	0	+	0	
45	22.9	15	0.5	0	0	0	+	
	23.4	14.2	4	0	0	0	+	
51	24.5	8	2	+	0	0	+	
52	38.8	10	0.8	+	0	+	0	
	38.8	11	8.0	+	0	+	0	

1. Animal 42. Pericardium and branchial nerve intact.

2. Animal 44. * = both the pericardium and the branchial nerve were cut (previously intact).

3. Animal 45. Pericardium cut, branchial nerve intact.

4. Animals 51 and 52. Both pericardium and branchial nerve intact.

5. Stimulus pulses were of 10 ms duration at 5 hertz unless otherwise specified. Pulse trains were of various lengths.

6. HR = heart rate in beats/min, BP = blood (pulse) pressure in millimeters sea water, TONO = tonotropic effect, INO = inotropic effect, HRC = heart rate change, Arrest = cardiac arrest, + = positive or increase, - = negative or decrease, 0 = no change.

Table 8. Summary of chronotropic effects of nerve stimulation

Animal No.	RC			LC			Br			P			G-P			S		
	+HRC	-HRC	A	+HRC	-HRC	A	+HRC	-HRC	A	+HRC	-HRC	A	+HRC	-HRC	A	+HRC	-HRC	A
34	3	0	0	6	0	0												
37	0	0	0	0	0	0							5	11	4			
38	0	2	3	0	2	1												
39	2	0	0	1	0	0												
42	0	4	0							7	3	6				3	5	1
43	5	2	2	0	0	0				4	0	0						
44	1	7	5	1	10	4										2	0	2
45	0	1	1	0	4	1	0	1	1	6	0	3				0	0	2
51	1	12	2	0	12	1										0	0	1
52	1	3	0	0	6	0	0	0	0	9	0	0				2	0	0
53	0	0	2	0	0	3	1	2	0				2	5	3			
54	0	0	0	0	0	0	5	6	0	—	—	—	5	0	1	—	—	—
Totals	13	31	15	8	34	10	6	9	1	26	3	9	12	16	8	7	5	6

1. HRC = Heart Rate Change, + = increase, - = decrease, A = cardiac arrest, 0 = no stimulus response.

2. Blank spaces indicate no stimulation for that particular nerve.

Table 9. Occurrence of high frequency spike burst in cell L7 relative to bradycardia in animal 49

Event	Number	Percentage
Burst initiated prior to bradycardia	26*	45.6
Burst initiated after bradycardia	5**	8.8
Burst without bradycardia	1	1.7
Bradycardia without burst	25	43.9
Total	57	

* Six of these were of the obvious type seen in Fig. 32. Many of the others were subjectively interpreted.

** One of these was of the abrupt type, high frequency spike bursts (Fig. 33B).

Table 10. Occurrence of ILD in cell L10 relative to bradycardia in animal 52

Event	Number	Percentage
ILD initiated before bradycardia	11	34.4
ILD simultaneous with bradycardia	3	9.4
Bradycardia initiated before ILD	5	15.6
ILD without bradycardia	3	9.4
Bradycardia without ILD	10	31.2
Total	32	

ILD = inhibition of long duration

DISCUSSION

Viability of Heart-Abdominal Ganglion Preparation

The heart-ganglion preparation described in the methods section of this paper remains viable over long periods. In one preparation (expt. 49, the one from which L7 data was taken), the heart continued beating vigorously for 17.5 hours from the time of initial cannulation. The pulse pressure was 46 mmSW and heart rate 25 beats/min after this long period. There was a great variability in performance from preparation to preparation and even during a given experiment, with pulse pressures ranging from 0.9 to 42 mmSW and heart rates from 4.3 to 39.2 beats/min under control conditions. Probable factors influencing the performance were faulty atrial cannulation, damage during surgical isolation of the genital and pericardial nerves, and pericardial puncture in the separation of the heart from the digestive gland. At times the pericardium and/or heart were accidentally punctured, allowing the perfusate to flow through the leaks. Generally a leaky heart did not beat very well. The cannulation procedure was complicated by 1) removal of the gill and 2) presence of the branchial ganglion close by.

Gill removal was necessary even when cannulation was instituted through the gill artery, because contraction of the gill muscles caused an increase in intrapericardial pressure with a concomitant change in blood pressure. The need for gill removal made atrial cannulation more difficult since the catheter, which was ligatured at the base of the gill, tended to be more leaky. The catheter was not tied directly to the atrium to reduce the hazard of cutting any nerves at the base of the atrium.

Relation of Single Neuron Activity to Heart Rhythm

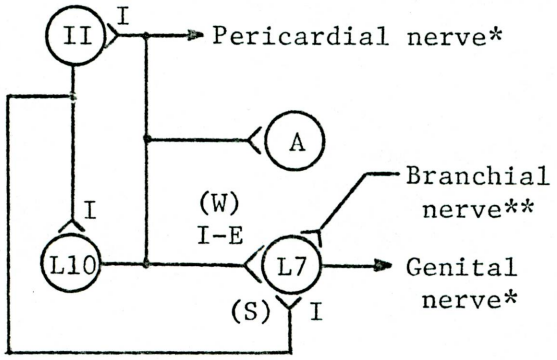
Cell L7. The distinctive high frequency spike burst in cell L7 preceding the onset of bradycardia (Fig. 32, 33A, 34B, & 35A) is a correlated but not causative activity. The basis of this conclusion is that induced bursts in L7 upon release from hyperpolarization, and steady high frequency spike activity following cell penetration, elicited no changes in cardiac rhythmicity. This behavior is similar to that of the type iv cells described by Peretz (1969) in his study of spontaneous gill movements in Aplysia. Type iv cells were those which evoked no gill movements when activated by hypopolarization, failed to reduce movements when hyperpolarized, and yet had increased spike activity during spontaneous gill movements. L7 was mentioned by Peretz as being in a class of cells having axons in both the ctenidio-genital (genital) and branchial nerves. This is a probable explanation for the spike burst seen when the branchial nerve was being cut (Fig. 34A) and adds weight to the identification of this cell as being L7 instead of L11 (see Results, page 29).

Cell L10. There is a parallel but not causative relationship between the inhibition of long duration (ILD) in L10 and slowing of the heart. This conclusion is based on: 1) somewhat subjective evidence from one of 3 animals tested, 2) the intercellular connections between L10, L7, and interneuron II (an unidentified neuron in the abdominal ganglion) described by Waziri and Kandel (1969), and 3) the studies on defensive and spontaneous gill withdrawal (Kupfermann and Kandel, 1969) and spontaneous gill movements (Peretz, 1969).

Nineteen of the 32 events tabulated in Table 10 show that an ILD in L10 occurred together with bradycardia. In 11 of these, the onset of

ILD preceded the bradycardia. If L10 were causing bradycardia to occur indirectly, say by disinhibiting an inhibitory neuron, I would expect to see a greater percentage of the ILDs initiated prior to the bradycardic response. Hyperpolarization of L10 for a period comparable to an ILD would have helped in clarifying the question of causation. Were the ILD a causative factor, one would expect a high correlation between the hyperpolarization period and both the timing and occurrence of bradycardia. There would not necessarily be a 1:1 correlation since there probably are several cells involved in evoking cardioinhibition, as with the gill withdrawal (Kupfermann and Kandel, 1969, and Peretz, 1969).

Waziri and Kandel (1960) showed some of the synapses between cells L10, L7, and interneuron II according to the diagram modified below.



A = to other follower cells
II = interneuron II
E = excitatory synapse
I = inhibitory synapse
I-E = rate dependent dual synapse
(W) = weak effect on follower cell
(S) = strong effect on follower cell

*Peretz (1969)

**Kupfermann and Kandel (1969)

Interneuron II is involved in the spontaneous gill movement (Peretz, 1969, and Kupfermann et al., 1969). Peretz also reported that L10 was inhibited during, but did not contribute to, the gill movement. L10 could have a similar correlated activity associated with the heart, due to interneuron II activity. A unifying concept would be to suggest that the activity I saw in both L7 and L10 are a reflection of the gill movements. The disturbing factor is that from the evidence seen in Fig. 8C

and 8D, one would expect cardioacceleration associated with gill contraction rather than bradycardia. However, the gill was removed in both preparations so a direct comparison is not valid. The idea could be tested by recording gill movement, blood pressure, and L10 and L7 activity simultaneously.

Regulatory Function of the Abdominal Ganglion Nerves

The results of whole nerve electrical stimulation in my study gives strong evidence for the presence of cardioinhibitory nerves in the perfused, intact heart-abdominal ganglion preparation. Primarily inhibitory responses were obtained from both the right and left connectives and the siphon nerve. Carlson (1905) found no evidence of cardioinhibition in the unperfused heart of A. californica with atrium removed. In A. vaccaria, Wright (1960) obtained both inhibitory and excitatory responses from stimulation of the left connective and the branchial nerve but got only acceleratory responses from the right connective. He perfused the intact heart through an atrial cannula and recorded contractions with an isotonic lever. The lever was attached to the heart by a small hook passed through a small slit in the pericardium.

I was also able to obtain evidence, corroborating Carlson (1905) and Wright (1960), indicating the cardioacceleratory effect of pericardial nerve stimulation. It is not surprising that Carlson got only acceleratory responses in that he had cut the siphon, gill, and osphradial nerves (presumably the siphon, genital, and branchial nerves) and was using a preparation that maintained rhythmic contractions for not more than 20 minutes. This left only the pericardial nerve connected to

the heart. Wright reported that the pericardial nerve contained only acceleratory fibers.

The results from siphon nerve stimulation (Fig. 26, 27C, D) indicate the presence of a feedback pathway. Jahan-Parwar et al. (1969), Peretz (1969), and Kupfermann and Kandel (1969) have shown the existence of sensory feedback into the ganglion from the peripheral nerves. A possible function of this pathway is the coordination of the respiratory and cardiovascular systems mentioned by Peretz (1969). Possibly contraction of the siphon initiates propulsion of the blood into the gill vein and sensory feedback to the abdominal ganglion inhibits or excites the heart, depending on the timing, to coordinate circulation. For example, inhibition during siphon contraction would relax the heart to enhance filling and a vigorous contraction could follow. Simultaneous recordings of hemocoel pressure, gill, and siphon contractions may be useful in showing the sequence of events in coordination of the respiratory and cardiovascular systems as postulated by Peretz.

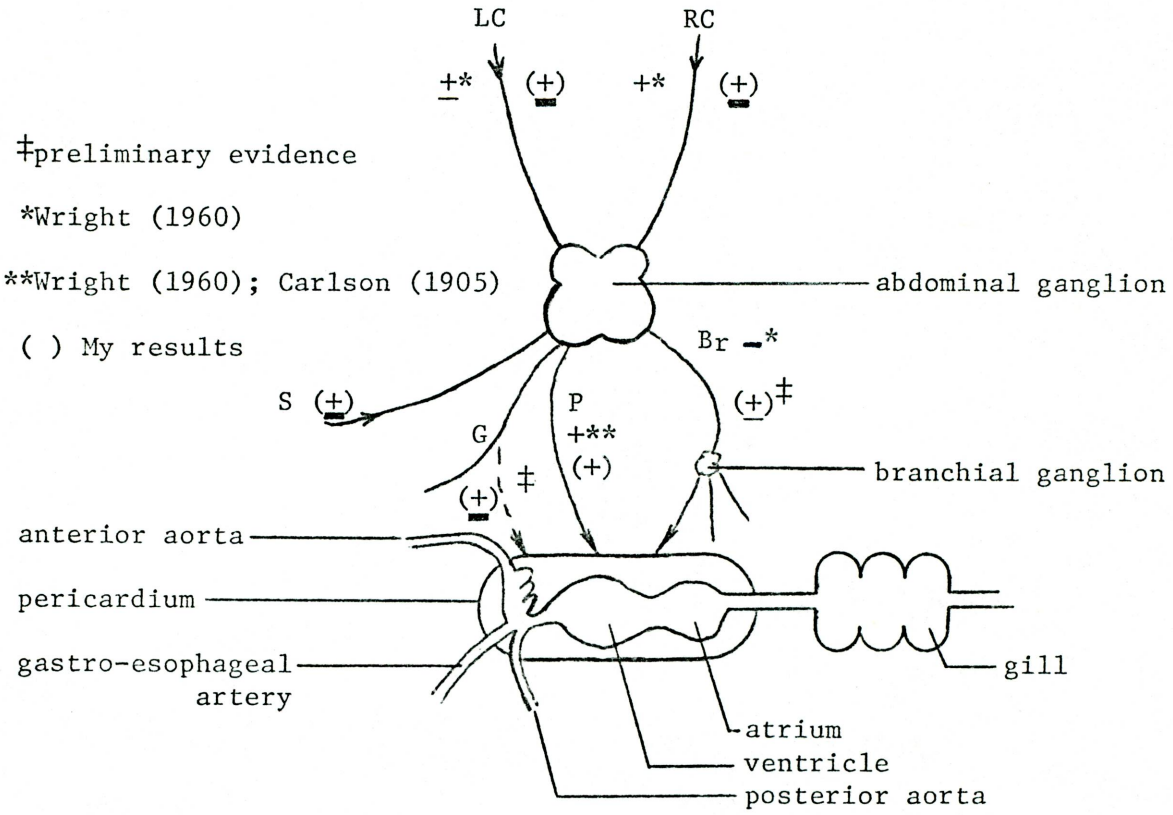
Three factors combine to give strong preliminary indications that there are cardioinhibitory axons in the genital nerve. The evidence is as follows:

1. Preliminary evidence for inhibitory responses to genital-pericardial nerve complex stimulation,
2. Nearly exclusively acceleratory reponses from pericardial nerve stimulation, and
3. Inhibitory responses to left (Fig. 21) and right (Fig. 17) connective stimulation with the branchial nerve cut, leaving only the genital-pericardial nerve connected to the heart in both cases. A second animal gave similar results.

If there were no inhibitory axons from the genital nerve, one would have to postulate the presence of inhibitory axons in the pericardial nerve when the branchial is cut and cardioinhibition persists. The results of pericardial nerve stimulation strongly dictate otherwise. The genital nerve was generally cut at the peripheral end at the genital ganglion but the small branches from the main trunk along the distance between the abdominal and genital ganglia may not have all been cut. If so, there could have been small branches from the genital nerve entering into the pericardium and to the heart.

A proposed diagram of the contribution of the abdominal ganglion nerves in cardioregulation is shown in Fig. 39.

FIGURE 39. Cardioregulatory role of the abdominal ganglion nerves.



Possible Damage to Ganglionic Function by Pronase

My single attempt at using pronase (20 mg/ml for 20 min.) to soften the connective tissue sheath around the abdominal ganglion of a 470 gram A. californica showed signs of possible damage to synaptic activity. Hafemann and Miller (1967) used pronase at 20 mg/ml for 30 minutes on the A. vaccaria abdominal ganglion with no loss in synaptic activity but A. californica does not have the extra individualized neuronal layer found in vaccaria. Stimulation of the connectives at 20 volts (Fig. 30A, B, C) was ineffective, whereas acceleratory responses were obtained from genital-pericardial nerve stimulation at 0.4 volt (Fig. 30D) and branchial nerve stimulation at 8 volts (Fig. 31). Another disturbing factor is that the sheath was still difficult to penetrate after the enzyme softening.

Procedural Changes for Future Experiments

Some procedural improvements which would enhance consistency in the results and their interpretation are discussed below.

1. Use of suction stimulating electrodes would give a more uniform stimulus field around the nerve and also keep the nerve from drying out on the electrodes. During the later stages of a given series of stimulations, the nerve being stimulated tended to dry out and this changed the resistance in the stimulating circuit.
2. Systematic rotation of nerves being stimulated would avoid the problem of habituation.
3. A well-planned routine for nerve stimulation to enhance statistical interpretation of the data. This was not done

since the primary goal had been to obtain single neuronal correlates to cardiac activity and only corroborative information was sought for whole nerve data.

4. Simultaneous intracellular recordings from two or three neurons along with blood pressure and gill movement recordings would aid in determining correlation between spike and cardiac activity as well as interplay between cells.
5. Determine the particular behavioral role of a cell by electrically or chemically controlling the membrane potential, hence the spike activity.
6. Use acetylcholine antagonists on the heart to demonstrate the excitatory response to nerve stimulation more clearly.
7. Demonstrate the direct role of nerve pathways to the heart by reversible block, such as sucrose gap or cooling, instead of cutting.

SUMMARY

An isolated heart-abdominal ganglion preparation of the mollusc Aplysia californica was developed and used to study the cardioregulatory function of the ganglion and nerves. The heart was perfused with artificial sea water through an atrial cannula and the gastro-esophageal artery was catheterized for blood pressure monitoring. The presence and cardioregulatory effects of axons in the nerves was investigated by electrical stimulation. Fine-tipped glass microelectrodes inserted into ganglionic neurons were used to determine the relationship between the neuronal and cardiac activities.

The principal results are listed below.

1. Primarily inhibitory responses are obtained from both right and left connective stimulation, with some evidence for excitatory axons.
2. The siphon nerve exerts a strong inhibitory influence on the heart, probably acting as part of a feedback pathway.
3. Predominantly acceleratory responses are elicited from pericardial nerve stimulation (confirming work of Wright, 1960, and Carlson, 1905).
4. A high frequency spike burst in L7 precedes bradycardia, a coincidental but not deterministic relationship obtained from one subject.
5. Preliminary evidence indicates the presence of a non-deterministic inhibition of long duration in cell L10 associated with bradycardia.
6. There is speculative evidence for inhibitory axons in the genital nerve.

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APPENDIX

I. General Information on *Aplysia californica*

A. Taxonomy (Hyman, 1967)

1. Phylum: Mollusca
2. Class: Gastropoda
   a. Subclass: Opisthobranchia
3. Order: Anaspidea or Aplysiacea
4. Family: Aplysiidae
5. Genus: *Aplysia* (also *Tethys*, MacGinitie, 1949)
6. Species: *californica*
7. Common name: Sea hare

B. Habitat

1. Limited to tropical and subtropical waters (Hyman, 1967)
2. On the Pacific coast of North America, ranges from Monterey Bay on the Central California coast to the Gulf of California (Winkler, 1959).
3. *A. californica* numerous at depths of 30-40 feet, as deep as 100 feet (Limbaugh; cited by Winkler and Dawson, 1963). Usually caught in tide pools where there is abundance of algae.

C. Size

1. *A. californica*: Grow up to 16 pounds (MacGinitie, 1949).
2. *A. vaccaria*: Approximately 35 pounds, 30 inches long (Limbaugh; cited by Winkler and Dawson, 1963).

D. Food

1. Herbivorous

2. Red, green, and brown marine algae (Winkler, 1959)

II. Electrode Puller

Previous knowledge of the terminology and parameters involved in microelectrode pulling is assumed in this section. (See Frank and Becker, 1964, for an excellent treatise on the subject of microelectrodes.)

Strong, sharp pointed glass electrodes were needed to penetrate the tough tissue surrounding the abdominal ganglion of Aplysia. We (see asknowledgement) designed and built a microelectrode puller described below as the PDC puller, for making electrodes of this type following many of the basic ideas of the Chowdhury (1969) machine now commercially available. The Chowdhury Pipette-Puller has a horizontal two-stage pull with air jet cooling for obtaining relatively large cone angles. The pipette is chucked between two carriages which are pulled in opposite directions by a spring and solenoid to provide tension on the glass.

The photodetection-digital controller (PDC) puller main frame is constructed with DOW magnesium tooling plate. The two carriages are made of the same material because of its light weight and ease of matching. (Fig. A-1 shows various front panel components described in the text.) Teflon tracks guide the carriages providing a grease-free, smooth bearing surface. Both carriages and tracks need to be cleaned occasionally due to the accumulation of dust particles and fingerprints.

Tension in the first stage is produced by the weight of the solenoid plunger and an adjustable coil current. The current range is adjustable from 0.0 ampere (A) to 1.0 A with a typical value of 0.4 A.

The second pull solenoid current is not adjustable and is dependent on the setting for the first pull, ranging from 1.25 A to 1.5 A with a typical value of 1.4 A. Estimates of pulling force taken from STROKE-PULL curves for the Guardian 4HD-DC solenoid are as follows:

1st pull = 8 oz.

2nd pull = 80 oz.

Adding the plunger weight of 2.3 oz. to each of these values results in a starting pull ratio of each stage as 82.3 oz./10.3 oz. = 8/1. A peak value of 14 pounds pull is achieved by the solenoid with the plunger at the end of its travel but the glass separates before this force is exerted.

Timing of the second stage pull events is controlled by a photocell-program card-digital logic arrangement. The photocell detector sends a trigger pulse to the logic elements each time the program card lines up in the proper position to let light shine through. Location of the two holes in the program card determines the start and end points of the second stage. A photocell detector was used instead of a microswitch to reduce switch malfunction problems and to facilitate future changes in programming capability.

A front panel RESET pushbutton switch initiates action by turning on the heater and first stage solenoid current. Duration of the first stage pull is determined by the 1) heater current, 2) type of glass, 3) initial pull force, and 4) initial clamping position. The INITIAL POSITION is a relative measure of how much the glass elongates before the strong second pull starts. The logic circuit disables the heater, pull solenoid, and air valve once a pull cycle is completed, thus allowing the operator to

remove the electrodes and reload the machine without accidentally turning on any component that could be injurious (Fig. A-2, A-3, & A-4).

The MANUAL pushbutton switch enables the operator to cycle the puller through each stage manually without any glass being pulled. The object of having this capability is to facilitate troubleshooting the electronics.

The objective of a strong second pull with rapid cooling is to obtain a large cone angle at the tip. The large cone angle with very small tip results in a sharp, strong electrode without excessively high resistance (Chowdhury, 1969). Argon was used for cooling the glass because of its inertness and the availability of a low pressure regulator, although compressed air would probably serve the purpose. The gas is directed onto the glass through a pair of counter-opposed jets during the second stage at pressures ranging from 0.5 to 30 pounds/square inch. Pressures between 0.5 to 2 pounds/square inch gave the best results. The greatest problem in producing straight electrodes was getting uniform cooling of the glass. Closing one jet resulted in curvature toward the side with gas blowing on the electrode indicating that uneven cooling and not the force of the blast was responsible for the bending (at low gas pressures).

Electrodes of approximately 0.5 micron tip diameter with 10 to 50 megohms resistance (filled with 0.2 M $CaCl_2$ and 0.8 M KCl) were obtained at 50 to 65% yield once the desired settings were determined for a given shape. The major problem was bending at the tip of the electrode. Phase contrast microscopy and an ocular micrometer were used in the early stages of development to determine constancy of the tip diameter. Subsequently, gross inspection of the electrodes at 10 to 30 X was used

for quality control purposes. Occasionally a microscope with a 45X objective and 10X ocular micrometer (0.25 micron/division) was used for checking the tip size.

"Automatic" is an inappropriate word to describe electrode pulling with the PDC puller. The operator has a few parameters to work with to produce electrodes of a desired length, tip diameter, and cone angle. Even with a given group of settings, the vagaries of glass dynamics, gas flow, and timing make electrode pulling a real challenge. One empirically arrives at a satisfactory combination of parameter settings to produce the desired results.

A few of the parameters and their resultant effect are given below.

1. Heater temperature

   Increased heat results in longer, thinner electrodes.

2. Gas pressure

   Gas pressure regulates cone angle and tip length. Increased pressure results in larger cone angles and shorter tip length.

3. First stage pulling force

   Electrode length is an inverse function of initial pull, probably because the glass starts to elongate at a lower temperature.

4. Initial position

   The INITIAL POSITION is a measurement reflecting the position of the solenoid plunger in the coil or the distance between the chuck and heater coil. This is the most difficult parameter to adjust consistently due to the small range involved (approximately 4 mm). For a given coil current, the initial pulling force is greater if more of the solenoid plunger is in the coil.

The distance of chuck movement before onset of the second pull is reduced with a larger value for INITIAL POSITION. Both effects result in shorter electrodes.

Sequence of Events in Pulling Glass Microelectrodes

Stage I. Initial Pull

A. Glass tubing chucked into place noting INITIAL POSITION

B. RESET switch turns on the following:

1. Heater (current adjustable)

2. Pull solenoid (on low current to provide some tension in addition to weight of solenoid armature; adjustable)

Stage II. Second Pull

A. Heater turned off

B. Pull solenoid turned on high current for maximum acceleration of glass

C. Air valve turned on for rapid cooling

Stage III. End of Pull

A. Heater remains off

B. Solenoid turned off

C. Air valve turned off

Truth Table

Stages	Elements			
	$\overline{A}$	$\overline{B}$	C	
I	1	1	0	$\overline{A}$ controls heater relay
II	0	1	1	$\overline{B}$ plus C controls pull solenoid
III	0	0	0	C controls air valve solenoid

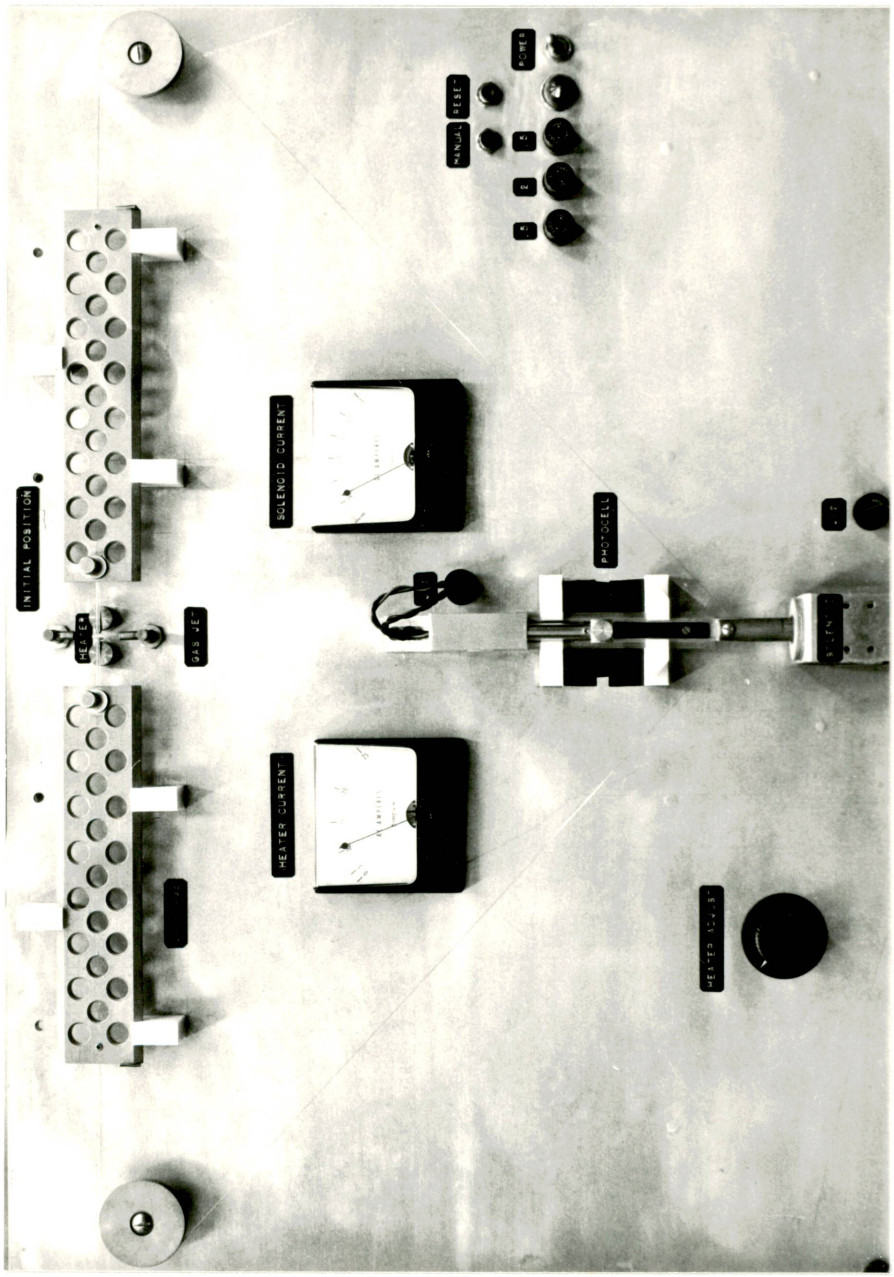


FIGURE A-1. PDC MICROELECTRODE PULLER FRONT PANEL

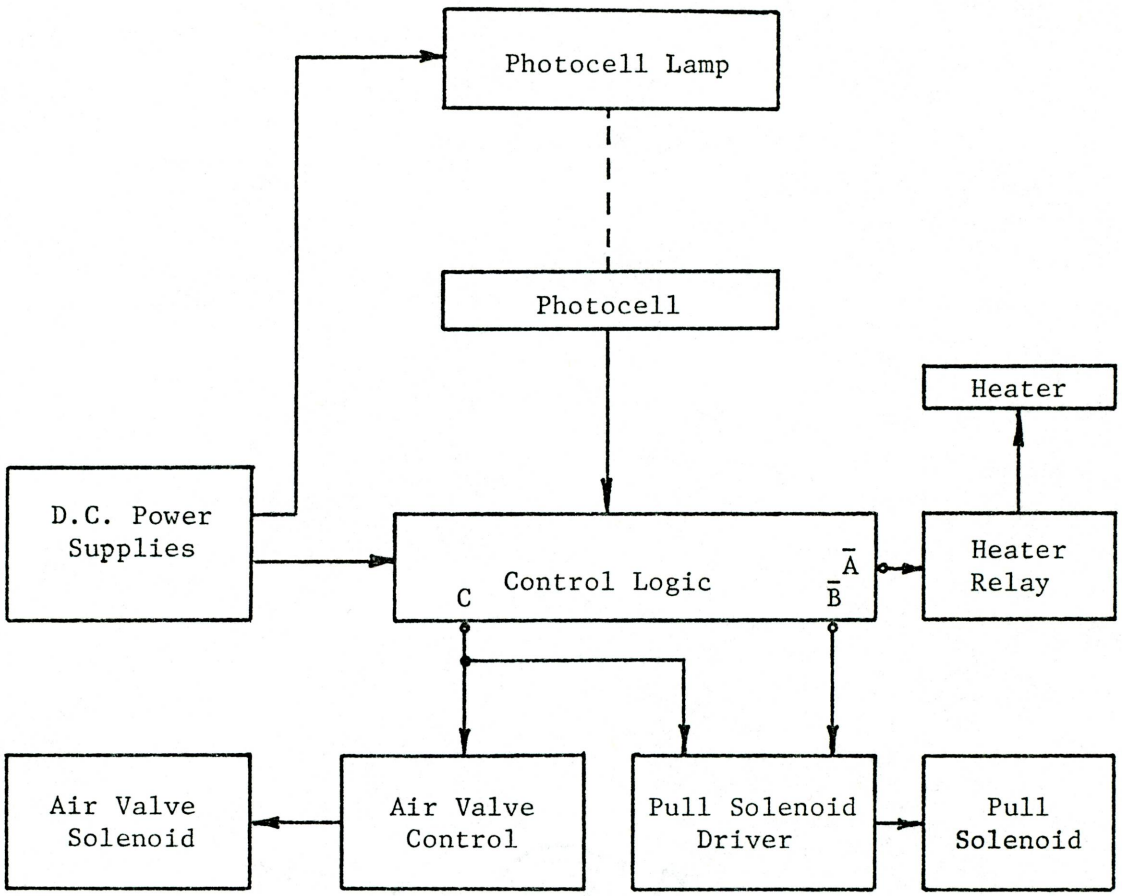


FIGURE A-2. MICROELECTRODE PULLER BLOCK DIAGRAM

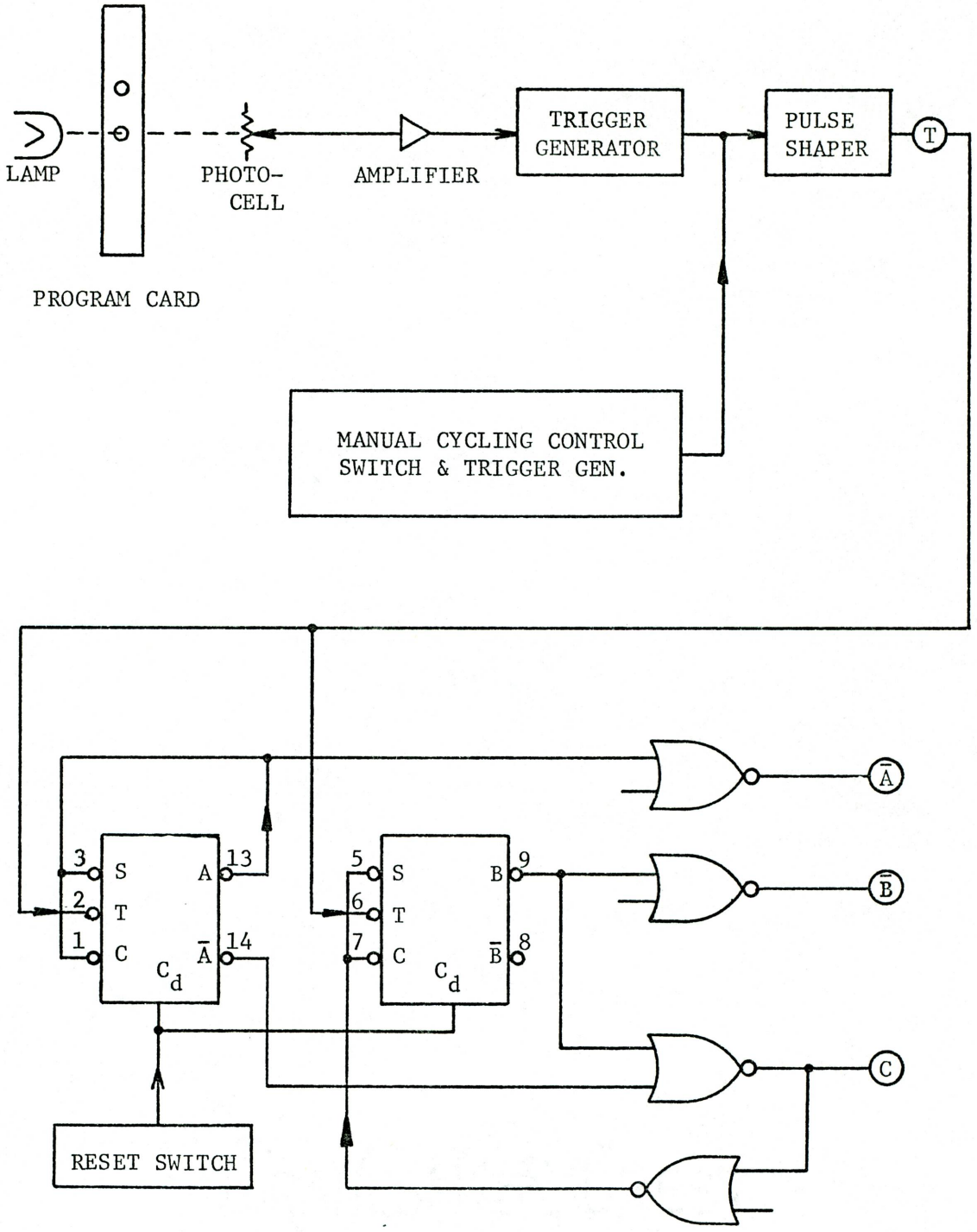
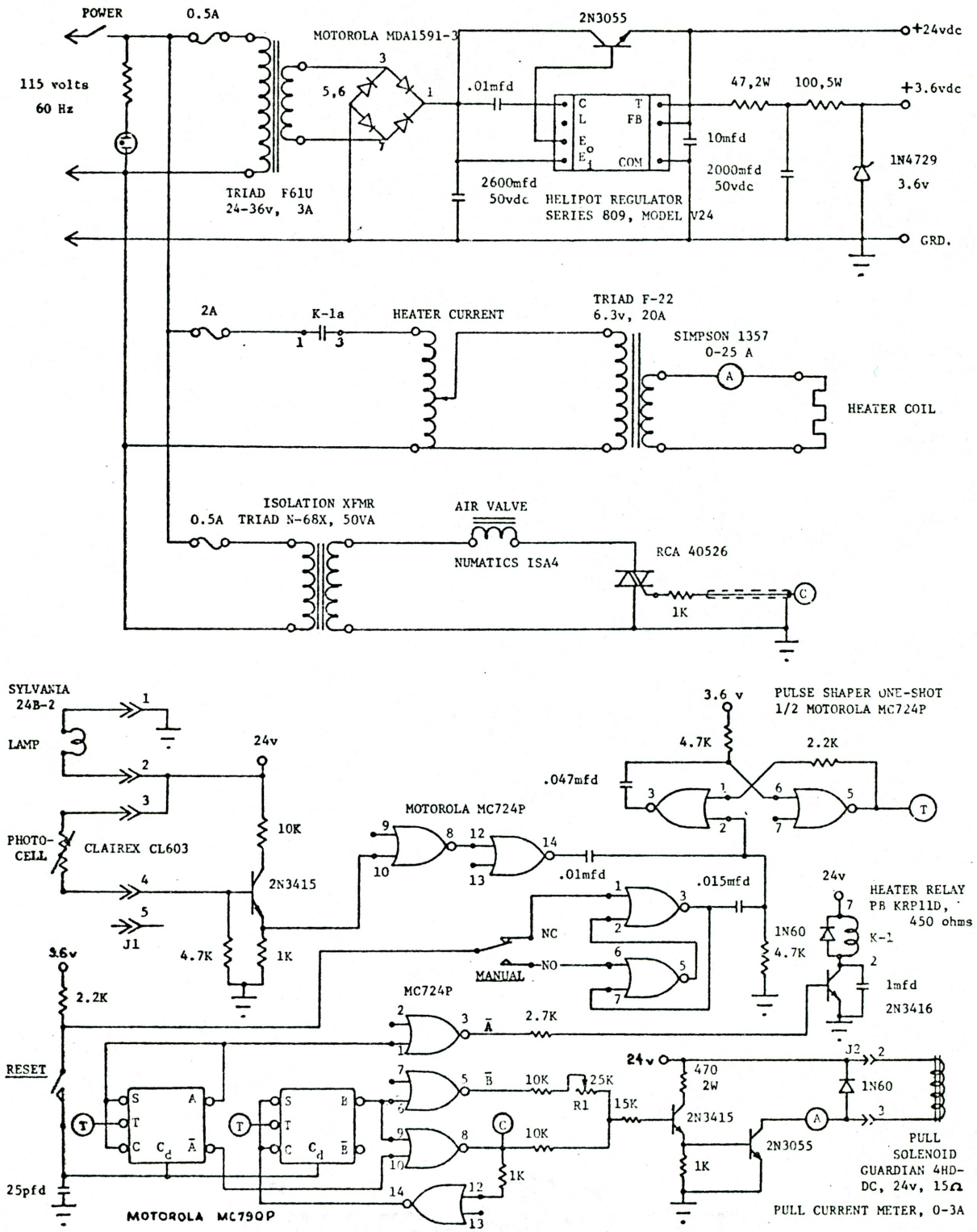


FIGURE A-3. PULL CYCLE CONTROLLER BLOCK DIAGRAM

FIGURE A-4. PDC MICROELECTRODE PULLER SCHEMATIC DIAGRAM



LOMA LINDA UNIVERSITY
Graduate School

CARDIOREGULATORY ROLE OF THE ABDOMINAL GANGLION OF *Aplysia californica*

by

George Maeda

An Abstract of a Thesis

in Partial Fulfillment of the Requirements for the Degree

Master of Science in the Field of Physiology and Biophysics

June 1971

ABSTRACT

An isolated heart-abdominal ganglion preparation of this mollusc was developed and used to study the cardioregulatory function of the ganglion and nerves. The heart was perfused with artificial sea water through an atrial cannula and the gastro-esophageal artery was catheterized for blood pressure monitoring. The presence and cardioregulatory effects of axons in the nerves was investigated by electrical stimulation. Fine-tipped glass microelectrodes inserted into ganglionic neurons were used to determine the relationship between the neuronal and cardiac activities.

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1. Primarily inhibitory responses are obtained from both right and left connective stimulation, with some evidence for excitatory axons.
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4. A high frequency spike burst in L7 precedes bradycardia, a coincidental but not deterministic relationship obtained from one subject.
5. Preliminary evidence indicates the presence of a non-deterministic inhibition of long duration in cell L10 associated with bradycardia.
6. There is speculative evidence for inhibitory axons in the genital nerve.