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ELECTROPHYSIOLOGICAL STUDIES ON INITIATION AND REVERSAL OF THE HEART BEAT IN *CIONA INTESTINALIS**

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INTRODUCTION

The heart of the tunicate *Ciona intestinalis* is a simple tube composed of epithelial muscle cells which—though isolated from any neural connexions—is capable of producing repeated contractions at reasonably constant frequency. It is thus a myogenic pacemaker system in which spontaneous rhythmic activity is derived from some intrinsic source. The simplicity of this system makes it a useful preparation for the study of pacemaker activity. The cells appear morphologically homogeneous; they are arranged in a single layer, and consequently activity initiates in and is propagated along the surface of a cylinder. The tubular nature of the heart makes analysis of the directionality of contractile events convenient, and it permits morphological and physiological characterization of the two ends.

The tunicate heart exhibits a reversal phenomenon in which a series of peristaltic contractile waves initiated at one end and conducted to the other is followed by a new series of contractions initiated at the previously passive end and conducted in the opposite direction. This phenomenon, which occurs intermittently and apparently without pattern, was first noted by van Hasselt (1824) and has puzzled workers for over 100 years. Several hypotheses have been developed to explain it (back pressure: Haywood & Moon (1950, 1953), Kriebel (1964); pacemaker fatigue: Krijgsman (1956), Krijgsman & Krijgsman (1957); extrasystole: Mislin (1964, 1965), Mislin & Krause (1964); CO_2 concentration: Brocas *et al.* (1966*a*, *b*); these will be considered later in light of data obtained in the present study. Millar (1953*a*) and Krijgsman (1956) have reviewed the observations made by early workers.

In an effort to elucidate their organization and functional characteristics, cellular elements of the heart of *Ciona intestinalis* have been studied by extracellular and intracellular recording techniques. The results, together with light-microscopic and electron-microscopic observations, are used to construct a model of the interactions between cells and groups of cells that explains the mechanical phenomena observed.

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MATERIALS AND METHODS

Live material

Specimens of *Ciona intestinalis* were obtained from the underside of floats in the yacht harbour in Monterey, California. They were kept at approximately 13° C. in laboratory aquaria for periods ranging from 3 to 6 weeks.

The anatomy of *Ciona* has been described in detail by Brien (1948) and Millar (1953*a*). The body is cylindrical, remains attached at its posterior end to the substratum, and has two siphons at its anterior end: an incurrent (antero-ventral) siphon and an excurrent (antero-dorsal) siphon. The heart is an inverted V-shaped tube lying

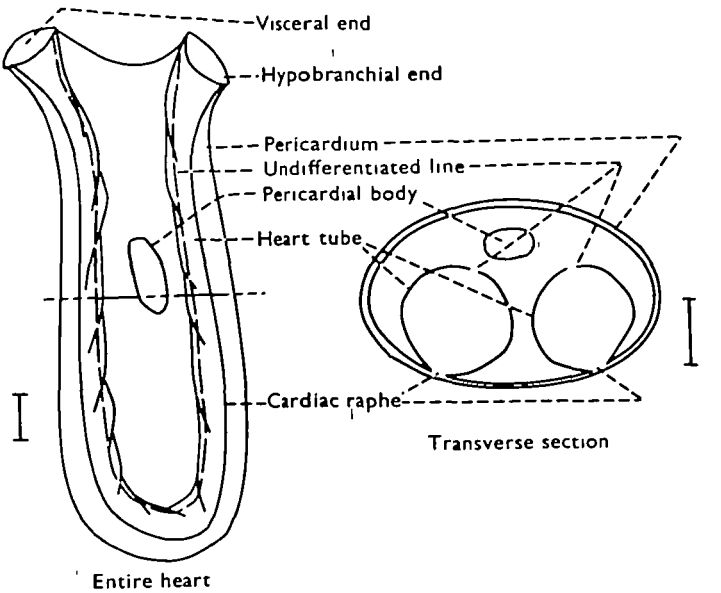

Fig. 1. Left, diagram of the entire heart. Right, cross-section of the heart taken at the level of the horizontal line shown on the entire heart. Calibrations: 1 mm.

under the branchial basket; the anterior limb of the V ends near the endostylar appendix, and the posterior limb ends on the stomach. The antero-ventral end of the heart, from which the subendostylar sinus or ventral vessel arises, will be referred to as the hypobranchial (H) end; the posterior end of the heart, from which the cardio-stomachic vessel or visceral sinus arises, will be referred to as the visceral (V) end.

The heart, enclosed in the pericardium, develops as an invagination of the pericardial wall and remains attached to the pericardium along its length by means of the cardiac raphe. The cardiac raphe passes along the right-dorsal side of the heart. The undifferentiated line (Fernandez, 1904) lies opposite the raphe and passes along the length of the left, ventral side of the heart. The pericardial body, a conspicuous ball within the pericardial cavity, has been described by Millar (1953*a*) as an aggregate of degenerating blood cells and cardiac fibres which have sloughed off the heart (Fig. 1).

Dissection was accomplished by the following procedure: the tunic was cut away and the body wall musculature was cut longitudinally, allowing the viscera to float in

the sea-water bath in which the animal was immersed. The subendostylar sinus and the cardio-stomachic vessel were cut transversely and the heart, with the pericardium intact, was lifted free of the animal and transferred to the experimental chamber. The pericardium was then trimmed away to the raphe, leaving the heart tube exposed.

Extracellular recording and stimulation

Excised hearts were placed in the recess (1·5 cm. × 0·5 cm. × 0·5 cm.) of a small wax dish filled with sea water, and transverse partitions of petroleum jelly were used to isolate regions of the heart.

Recordings were made using extracellular glass capillary suction electrodes with a tip diameter of approximately 100 μ connected to a syringe by polyethylene tubing. Platinum or silver wire was inserted into the glass capillary through a small hole in the polyethylene tube at its base. All junction points of the electrode components were sealed with dental wax. The shielded leads of the platinum or silver wires were connected through a.c.-coupled amplifiers to a multiple-beam cathode ray oscilloscope. Sea water was drawn up into the suction capillary to make contact with the wire; the heart tissue was attached to the tip of the capillary and held in place by a vacuum exerted by the syringe. Potential changes were measured by recording differentially between the suction electrode and a nearby platinum or silver wire in the medium bathing the preparation; a third electrode connected the bathing medium to ground.

Stimulating electrodes were either suction electrodes constructed with silver wire in the same manner as recording electrodes, using silver wire in the bath, or a pair of silver wire electrodes placed in the bathing solution. Brief pulses from an electronic stimulator were applied between the inside and the outside of the suction capillary, or across the two silver wires.

Intracellular recording

Intracellular recordings were made from pinned-out, excised hearts using glass 3 M-KCl-filled microelectrodes of 15–30 MΩ resistance (measured in 3 M-KCl) suspended in a 'floating' arrangement from fine silver wires. Potential differences were recorded between the micro-electrode and an indifferent electrode in the sea-water bath. The shielded silver wire leads were connected through a neutralized capacity input amplifier (Bioelectric Instruments Co.) to one of the oscilloscope beams.

Light microscopy

Tissues were fixed in sea-water Bouin's solution, dehydrated in ethyl alcohol, embedded in paraffin, and stained with Masson's trichrome (Humason, 1967) or silver (Rowell, 1963). Tissues for whole mounts were hydrated, stained with a mixture of methylene blue and azure II, and dehydrated. All tissues were mounted on slides in Permount.

Dilute methylene blue in sea water was used as a selective supra-vital stain to examine living preparations for the presence of nerve fibres.

RESULTS

Spontaneous activity

Activity was recorded with one or more suction electrodes placed at intervals along the isolated heart, and long series of multiple recordings were made from a number of preparations in patterns of normal activity. Several variations in this pattern were noted. The frequency of beat initiation could be regular or irregular over a given period; any single heart could change from one of these modes to the other intermittently. Reversals sometimes occurred immediately, i.e. the length of time between

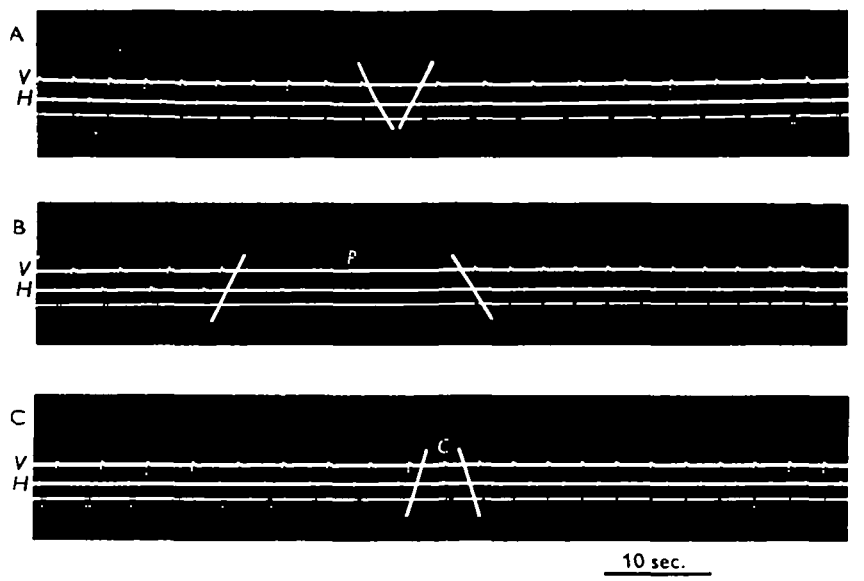

Fig. 2. Examples of reversals in beat direction. A, immediate reversal (*V*-to-*H* to *H*-to-*V*); B, reversal preceded by a pause, *P* (i.e. a period greater than the normal interbeat interval) (*H*-to-*V* to *V*-to-*H*); C, reversal preceded by a collision, *C* (i.e. beats began at approximately the same time at each end and cancelled along the length of the heart) (*H*-to-*V* to *V*-to-*H*). Slash lines indicate reversed directions of beating. *H* indicates the electrode placed near the hypobranchial end of the heart; *V* indicates the electrode placed near the visceral end. The direction of propagation is determined by the temporal relations of the events recorded by the two electrodes. When the beat direction is *H*-to-*V*, a contractile event is first recorded at the *H* electrode and then at the *V* electrode; when the beat is *V*-to-*H* an event is first recorded at the *V* electrode and then at the *H* electrode. When collisions occur, events are recorded nearly simultaneously by both electrodes. Bottom trace: key. Calibration: 10 sec.

the end of a series of beats in one direction and the beginning of a series of beats in the opposite direction was approximately that of a single interbeat interval (Fig. 2A). On other occasions, reversals were preceded by a pause, the duration of which was greater than a normal interbeat interval (Fig. 2B); and they were sometimes accompanied by collisions, i.e. beats began at approximately the same time at each end and cancelled by meeting along the length of the heart (Fig. 2C). Pauses and collisions also occurred in the absence of reversals. Single beats sometimes escaped from one end of the heart in the midst of a beat series initiated at the opposite end. Various combinations of collisions, pauses, and escape beats were frequently observed prior to reversal,

or interrupted a series of beats that were propagating primarily in one direction. No particular type of spontaneous activity characterized either heart end, and no specific events were consistently associated with reversals.

The examination of quiescent preparations and of pieces of hearts revealed other characteristics of the system that generates spontaneous activity. Contractions could be evoked from quiescent hearts by gently probing the tissue with a small glass rod. Touching the middle of the heart initiated a contractile wave which spread in both directions toward the ends of the heart. Occasionally, in failing preparations, spontaneous beats did not originate at the ends of the heart but instead began at various points along its length. Stretch applied from both ends of the spontaneously active, intact heart produced an increase in frequency.

Excised hearts were cut transversely into pieces as small as 3–4 mm. wide, or longitudinally into strips 1 mm. wide. All such pieces exhibited contractile activity. The frequency of beating was variable in different pieces; slower frequencies were probably caused by injury. Cutting the heart into longitudinal strips lacking the raphe showed that it is not the pathway for conduction, as was suggested by Ebara (see Krijgsman & Krijgsman, 1957). All pieces could show repetitive propagated activity. Some pieces exhibited reversal of direction of the propagated contractions, though such pieces were not consistently obtained from one region in different preparations.

The cutting experiments clearly show that all parts of the heart are capable of initiating contractions in either direction. No hierarchy of frequency was detected among the pieces of the heart; thus, it appears that there is no longitudinal gradient of pacemaker potentiality. The results, in agreement with those of Mislin (1964, 1965) and Mislin & Krause (1964) on transverse cutting, strongly suggest that the heart comprises multiple pacemakers.

An attempt was made to measure the relative conduction velocities for beats conducted *V*-to-*H* and *H*-to-*V* by using two suction recording electrodes placed a constant distance apart in any given experiment. Early experiments (Anderson, 1965) indicated that the time required for a *H*-to-*V* beat to traverse the distance between the two electrodes was always less than that required for a *V*-to-*H* beat. Later experiments, in which care was taken to place both electrodes on the undifferentiated line at the extreme ends of the heart, demonstrated that the relative conduction velocities were not consistently greater in the *H*-to-*V* direction, but instead were variable.

This variation may occur because contractile activity is not always initiated at exactly the same site, and the same conduction pathway is not always active (see below). Recently, Kriebel (1967) has demonstrated that conduction velocities are greater in the mid-regions of the arms of the heart than in the apex. His results indicate that the average conduction velocity is 13 mm./sec.

Response to electrical stimulation

Trains of brief, constant-frequency, electrical stimuli of systematically varied voltage were applied to the ends of the heart. The latency between the stimulus and the resulting beat varied from 500 msec. at low stimulus frequencies to 650 msec. at high stimulus frequencies. Stimulus frequencies ranged from one per second, a frequency somewhat greater than the rate observed in spontaneously active hearts, to

two per second. Stimulus trains equal to or less than the spontaneous beat in frequency were not effective. The heart could be driven at a maximal frequency, above which increases in stimulus amplitude or frequency had no effect. Absolute thresholds varied from heart to heart, and the highest driven frequency ever observed was 2·6 beats per second (Fig. 3B).

One-to-one following of stimuli by contractile events developed gradually, as shown in Fig. 3A, several stimuli (beginning at upward arrow) were usually required before the driven periodicity was established. Upon cessation of stimulation (downward arrow) the driven rhythm sometimes persisted for one to three beats before the heart slowed to its natural frequency. In a few cases the heart continued in the imposed rhythm for more than an hour after stimulation was stopped (Fig. 3B).

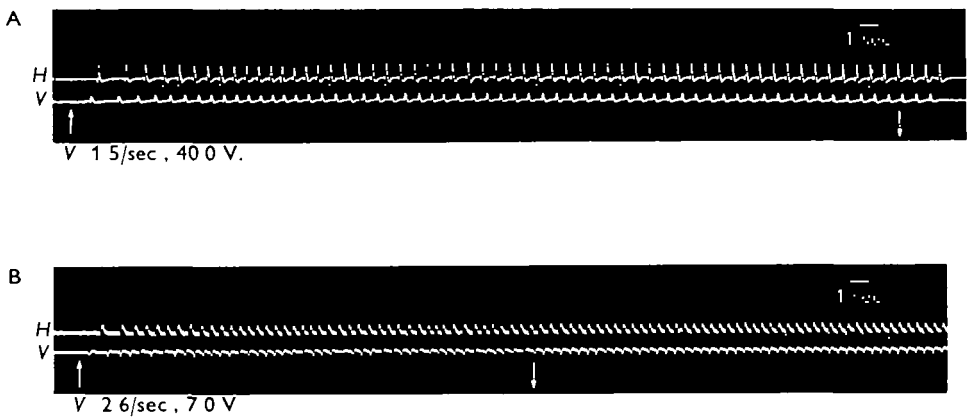

Fig. 3. Trains of brief pulse electrical stimuli applied to the visceral end of the heart. The beginning of stimulation is indicated by an upward arrow, its cessation by a downward arrow. Several stimuli were required before one-to-one following was established in both A and B. In A, two beats continued in the imposed rhythm before the heart slowed to its natural frequency. In B, the heart continued to beat in the imposed rhythm for more than an hour after stimulation was stopped. Calibrations: 1 sec.

Trains of stimuli at a constant frequency, but below the strength necessary for one-to-one driving, could evoke beats at a constant, higher-than-normal rate; these accelerated beats had no fixed phase relation to the stimuli. When such a train was applied at constant voltage, increases in its frequency caused increases in the frequency of beat initiation; the resulting frequency of beating was constant for each stimulus train, but the duration of the inter-beat intervals decreased as the frequency of the stimulus train increased. Increasing the amplitude of a subthreshold stimulus train kept at a constant frequency also yielded an increase in the frequency of beat initiation (Fig. 4A, B).

Fig. 4C shows that when activity was initiated spontaneously at one end, reversals could be produced by stimulating the opposite end at a voltage sufficient to yield one-to-one driving, as long as the stimulus frequency was higher than the opposing spontaneous frequency.

By simultaneously delivering pulse trains through two stimulating electrodes, one placed at each end of the heart, it was possible to control the direction and frequency of contractions. At voltages and frequencies within a range in which one-to-one following

was achieved, either end could be established as the dominant end. Its frequency of beating could be set and, by changing the stimulating conditions, reversal could be effected. The continuous record in Fig. 5 illustrates such an experiment. The original beat direction was *V*-to-*H* (slash lines indicate beat direction); stimulation was initiated at *b* at a frequency and amplitude appropriate to cause a reversal to the *H*-to-*V* direction (A); stimulation was then begun at *a* and the intensity was increased to a value great enough to yield reversal of the beat direction to *V*-to-*H* (B); stimulation was stopped at *a*, and the beat direction, influenced by the continued stimulation at *b*, reversed to the *H*-to-*V* direction (C). Several seconds were required for the heart to recover (not shown in the figure) after stimulation at *b* was stopped.

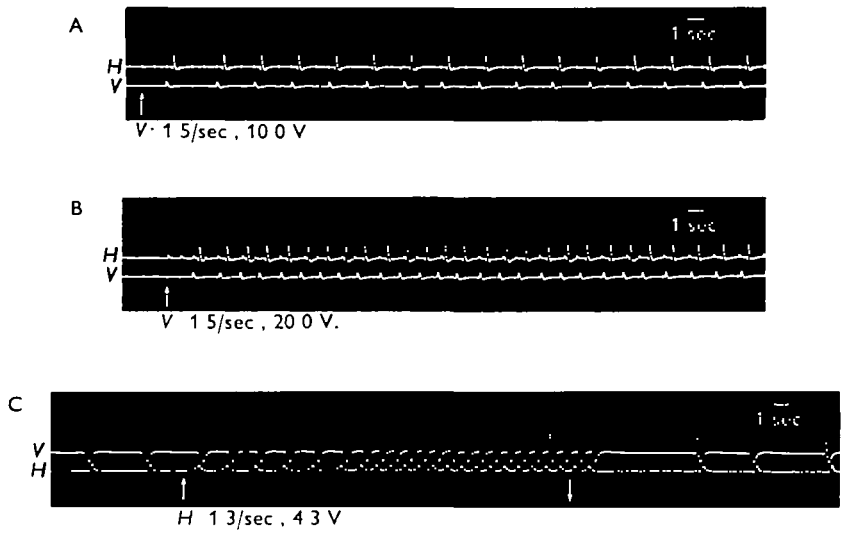

Fig. 4. A and B, subthreshold stimulation applied to the driving end (*V*) of the heart at a constant frequency with increasing voltage. The frequency of beating increased as the intensity of the stimulus train increased. In C, reversal and one-to-one following were produced by applying a train of stimuli of threshold intensity (upward arrow) at the driven end (H) of the heart. Upon cessation of stimulation (downward arrow) the natural spontaneous frequency and direction of beating were resumed. Calibrations: 1 sec.

Attempts were made to determine the differences between hypobranchial and visceral ends on the basis of the responses of each to electrical stimulation, and to determine whether the differences observed depend upon the morphological identity of the respective ends or upon their momentary status as driver (the end initiating beats) or driven (the inactive end). A stimulating electrode was placed at each end of the heart and recordings were made with suction electrodes from two points in the central region. The threshold voltage for one-to-one driving was determined for trains of stimuli that were increased in frequency by tenths of stimuli per second from one to 1·9 (where possible). To ensure that recording conditions were as similar as possible, threshold values for driving at a given frequency were determined for one end and immediately thereafter for the opposite end.

The current required to yield one-to-one driving for a train of stimuli at a given frequency varied markedly from heart to heart; however, the thresholds for one-to-

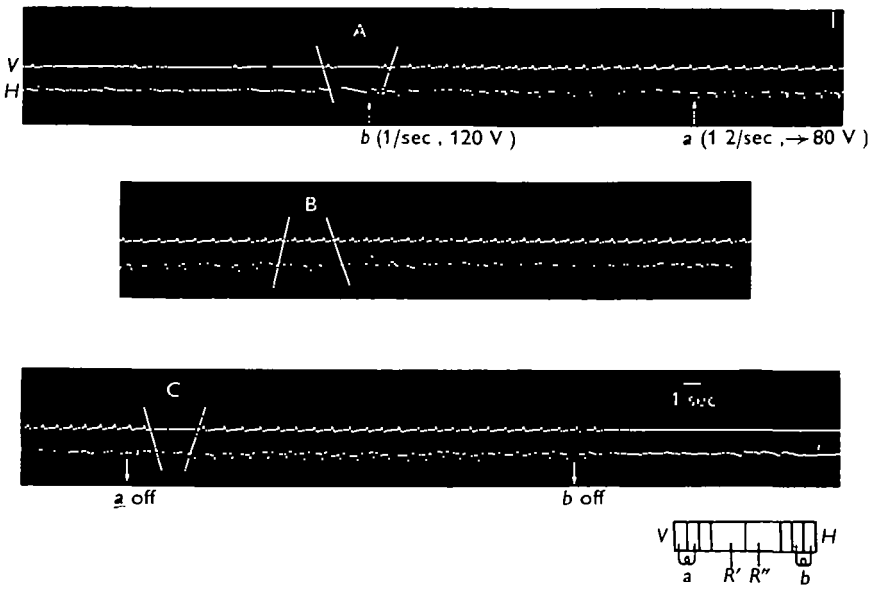

Fig. 5. Simultaneous stimulation of the two ends of the *Ciona* heart. The direction of spontaneous beat was *V*-to-*H* (slash lines indicate beat direction); stimulation was initiated at *b* at a frequency and voltage appropriate to cause a reversal to the *H*-to-*V* direction (A); stimulation was then begun at *a* and the intensity was increased in order to produce a reversal to the *V*-to-*H* direction (B); stimulation was stopped at *a*, and the direction reversed to the *H*-to-*V* direction (C). Recovery after the stimulation was stopped at *b* is not shown in the record. Calibration: 1 sec.

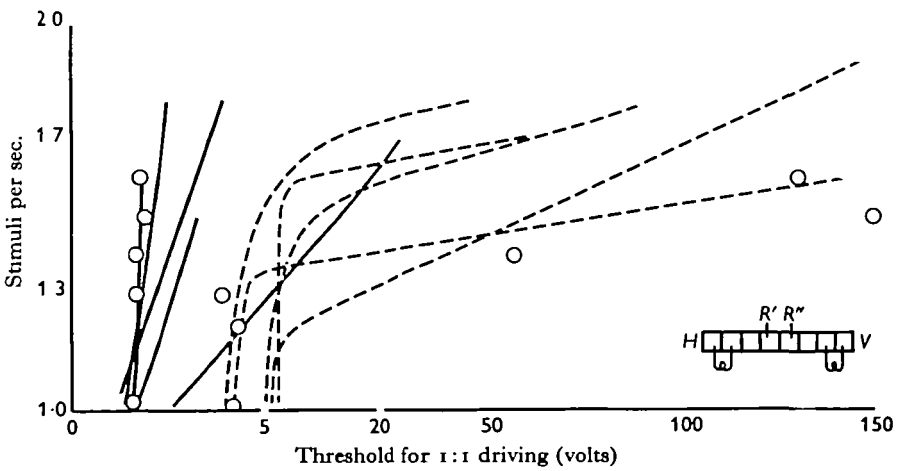

Fig. 6. Frequency-threshold graph. Points are shown for one experiment only; the other curves represent best fits for similar but unplotted data. The threshold voltage for one-to-one driving was determined for trains of stimuli per sec. from 1 to 1·9 (where possible); trains of stimuli were applied to each end of the heart when it was driving and also when it was being driven. When the beat was *H*-to-*V* and when it was *V*-to-*H* the *H* end (solid lines) showed a linear response pattern while the *V* end (broken lines) showed a non-linear one. —, Hypobranchial; – – –, visceral.

one driving were consistently higher at high frequency than at low frequency. The frequency/threshold graph of Fig. 6 indicates this relationship for each end. Whether the original spontaneous beat direction was *V*-to-*H* or *H*-to-*V*, the hypobranchial end exhibited a much more linear response pattern than the visceral end.

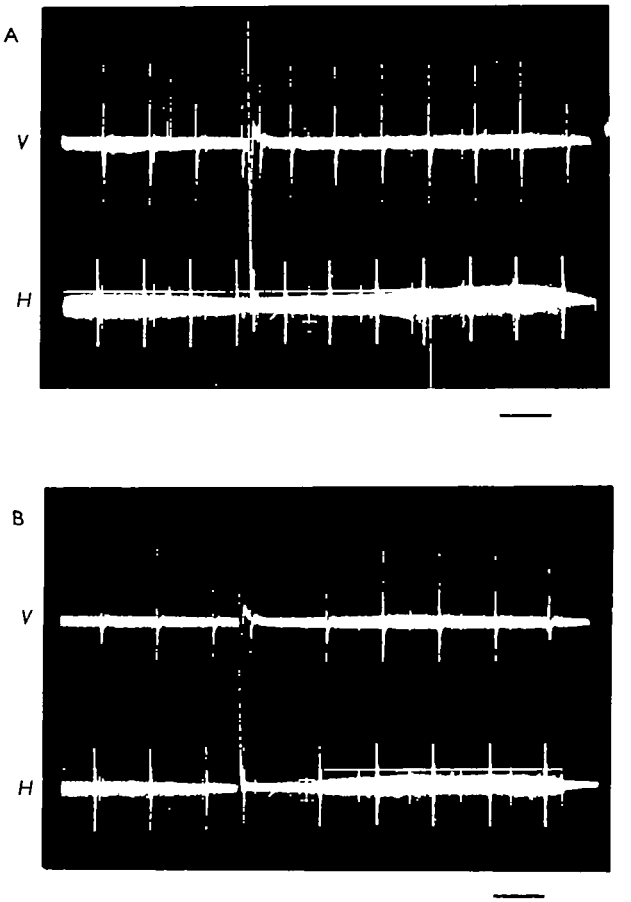

Fig. 7. Single stimuli applied to the heart (*H* end in A and B) at an intensity great enough to produce a conducted contractile wave. In A the sum of the interval preceding the interpolated event and the one following it was approximately equal to the average of the preceding interbeat intervals. In B, the sum of the interval preceding the interpolated event and the one following it was approximately equal to twice the average of the preceding interbeat intervals. In both A and B the interpolated beat failed to alter the phase of the rhythm permanently. Calibrations: 5 sec.

Single stimuli strong enough to produce a conducted contractile wave were applied to an end of the heart during periods of relatively constant frequency at various times in the normal beat cycle. In most cases the interpolated event did not affect the pacemaker system; that is, the sum of the interval preceding the interpolated event and the one following it was approximately equal to the average of the preceding interbeat intervals (Fig. 7A). In other instances the interpolated event was incorporated into the rhythm; although it occurred earlier than the predicted spontaneously initiated beat, the spontaneous beat following it occurred at approximately the same time as would

have been expected if no stimulated event had been interpolated (Fig. 7B). In effect, the rhythm behaved as though a spontaneous impulse were lost following the evoked beat. In both A and B, the interpolated beat failed to alter permanently the phase of the rhythm.

These data imply that the tunicate heart does not respond in the usual way to attempts to reset its pacemaker rhythm, and therefore does not possess the diagnostic characteristic of the relaxation-oscillation type of pacemaker (Bullock & Horridge, 1965). However, the heart shows such extreme variability in frequency of spontaneous activity that the experimental recognition of reset is difficult; furthermore, the heart is a population of many cells and, most probably, of many pacemaker units. Applied stimuli presumably evoke responses because those cells having pacemaker capabilities are poised near their threshold. Because different pacemaker units may have different frequencies and phases at a given time, the reset of any single pacemaker unit will not be apparent when the entire population of units in a given region is stimulated. Since the individual units are in different states of activity at any given point in time, they are differentially accessible to the stimulus—i.e. enough units are affected by the stimulus to yield a contractile response, but a significant number of pacemaker elements remain unaffected. An extra event in a series of spontaneous events occurring at low frequency would not necessarily shift the phase of the overall rhythm, because the redundancy of the entire system ensures production of the latter. Since all of the pacemaker elements in a region need not be mobilized in order to produce a response to the interpolated stimulus, a single event will not necessarily affect the synchrony of the entire system of pacemaker elements that acts to produce contractions. Indeed, it has been shown previously that a number of stimuli in a train are needed before a heart will respond in a one-to-one fashion to each stimulus of the train. It is likely that several stimuli are required to synchronize all of the active pacemaker sites in the end of the heart at the imposed frequency.

Other workers (Quincke & Stein, 1932; Mislin & Krause, 1964) have shown that extracellularly applied electrical stimuli affect the tunicate heart. Recently, Sugi, Ochi & Udo (1965) have investigated the effects of single stimuli and stimulus trains on hearts of *Ciona intestinalis*. It was shown that the strength of the contractile responses increased as a function of increasing stimulus intensity. The threshold for production of a propagated contraction was lower at an active pacemaker region than at the inactive end. Trains of stimuli caused the hearts to beat at an increased rate; however, stimulus trains having a frequency low enough to produce one-to-one following were not used.

Isolated hypobranchial and visceral ends

Experiments were performed in which conduction was blocked by tying a ligature around the centre of the heart. Studies by earlier workers have shown that in ligated hearts each end is unaffected by activity originating at the opposite end; thus each end beats toward the centre independently of the other (Mislin & Krause, 1964; Krijgsman & Krijgsman, 1959; von Skramlik, 1926*a*). These experiments were carried out on isolated hearts as well as on hearts within the intact circulatory systems of minimally dissected animals. After the ligature was tied, activity was observed for 30–45 min. to make certain that the contractions were consistently initiated at the ends of the

heart and were conducted toward the centre. Contractions sometimes began at the centre, immediately after the ligature was tied. In about 10% of the cases in which hearts were ligated within intact circulatory systems, beats intermittently began at the ligature and were conducted toward one of the ends of the heart. This type of activity continued for periods of 3 to 4 hr. In the majority of cases, contractions initiated at the ligature consisted of one or two beats, and were followed by a long series of contractions initiated at the end, lasting several minutes.

The activity of the two ends was simultaneously recorded for periods ranging from 20 to 45 min.; the inter-beat intervals were measured and plotted against time in order to describe the variations in frequency over long periods. Figure 8 illustrates data from a typical experiment treated in this way. The visceral end was characterized by approximately regular variations between high and low frequency. In different preparations the duration of these cycles usually ranged from less than two minutes to three minutes, peak-to-peak. Frequency ranges for the hypobranchial end were variable from heart to heart; in some cases a constant frequency was maintained, while in others there were irregular variations.

Some hearts did not exhibit the reversal phenomenon during the 1 hr. observation period preceding ligation. In most of the cases, when conduction was blocked at the centre of the heart, the end previously shown to be inactive never attained a frequency level greater than that of the dominant end.

When trains of brief pulse (10–50 msec. duration) stimuli of a strength adequate for 1:1 driving were applied to the visceral end of the isolated ligated heart for periods of one, two, and three times the observed length of the period of high-frequency beating, there was no marked change in the length of this period. On the basis of these data it appears that this longer-period rhythm of the visceral end cannot be reset. Further experiments, using temperature changes as well as trains of electrical stimuli in an attempt to affect the periodic variations in frequency, must be carried out before the nature of this rhythmic process can be elucidated.

Intracellular recording of spontaneous activity

While activity of the heart was being monitored with an extracellular suction electrode, single cells were penetrated with glass KCl-filled micropipettes. Because the tissue moved during contractions and because the diameter of the cells was small (6 μ), it was often difficult to keep an electrode inside a cell. Resting potentials of successfully impaled cells were approximately -50 mV. Intracellular recordings which did not show a resting potential in the range of -50 mV. for at least 1 min. were discarded.

Depolarizing potential changes were usually associated with contractile events in a fixed time relation. Figure 9 illustrates some simple monophasic action potentials. These typically showed a rise time of approximately 120 msec. and a decay time of approximately 580 msec., and usually displaced the membrane potential to zero at their peaks without overshooting. Miller & McCann (1962) have also recorded intracellular potential changes from *Ciona* hearts; their recordings show (see Hecht, 1965) a resting potential of -48 mV. Some of the potential changes they recorded showed an overshoot of 2 mV. or less. Kriebel (1967) has recently reported resting potentials of -71 mV. and potential changes of 75 mV. in hearts of *Ciona*.

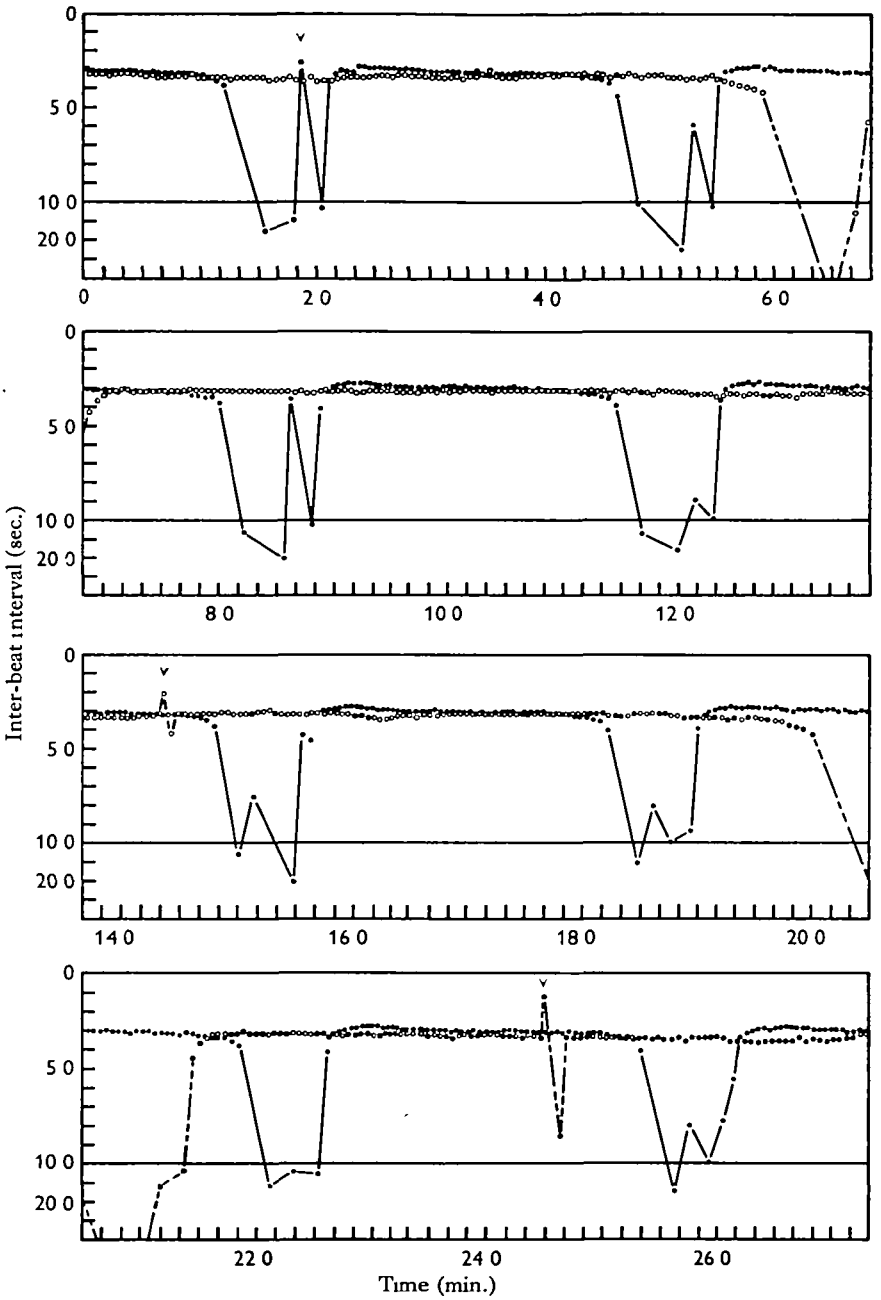

Fig. 8. Spontaneous activities recorded simultaneously from the ends of a heart ligated in the middle. Interbeat intervals (ordinate) were plotted against time (abscissa). The visceral end (closed circles) was characterized by regularly varying levels of high and low frequency; in this preparation, the hypobranchial end (open circles) varied more or less regularly between levels of high and low frequency, but with longer periods of high frequency than those of the visceral end. Arrows point to events which may have been escape beats (i.e. single beats which sometimes escaped from one end of the heart in the midst of a beat series initiated at the opposite end) in a non-ligated preparation. Collisions in non-ligated hearts are thought to result from interactions between similar frequency levels at the two ends. These results are considered in detail in the discussion section of this paper.

Complex action potentials which exhibited pre- and post-potentials were also observed; their amplitudes were often less than those of the simple potentials. In some cases, as shown in Fig. 10, depolarizing potentials of low amplitude (5–10 mV.) occurred at a constant frequency but showed varying phase relations with the main electrical events and therefore were not directly associated with them. Figure 11 illustrates a slow depolarizing event produced in the absence of both mechanical

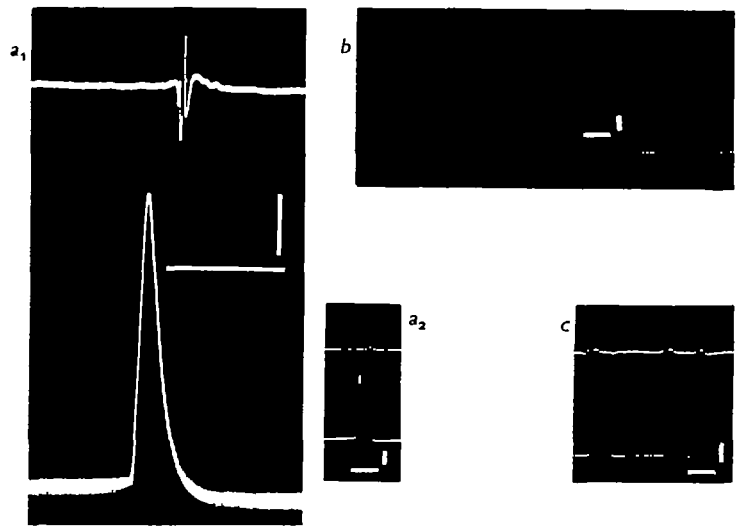

Fig. 9. Simple monophasic action potentials. Top trace: extracellular recording of contractile activity. Bottom trace: intracellular recording. a_1 is an enlargement of a_2; *b* shows a potential change recorded at a faster sweep speed than in *a* and *c*; *c* illustrates the relationships of the resting potential, the peak of the action potential and the zero line, as it was recorded when the electrode was taken out of the cell. Amplitude calibrations: 10 mV.; the calibrations in *a* and *c*, 1 sec.; in *b*, 0·1 sec.

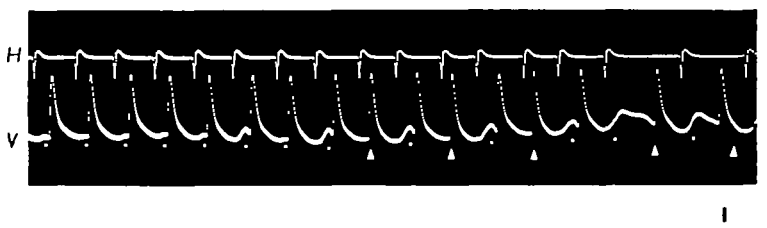

Fig. 10. Complex action potentials. Top trace: extracellular recording. Bottom trace: intracellular recording. The large amplitude potential changes in the bottom trace are associated with the contractile activity of the heart. The low-amplitude (5–10 mV.) potential changes, which are marked by the closed circles, occurred at a constant frequency. The arrows indicate where the low-amplitude events are presumed to have occurred, if they had not been obscured by the main electrical events. Amplitude calibration, 10 mV.; time calibration: one sec.

activity and the electrical activity that usually accompanies contraction. It is not clear whether this slow depolarizing event was generated in the cell from which the recording was made, or whether it originated in a nearby cell which was electrotonically coupled with the recorded cell.

During collisions, action potentials with double peaks were frequently recorded from cells in the central region of the heart. Figure 12 shows examples of the series of

changes during collisions prior to a reversal. Cells did not always exhibit changes in the shapes of action potentials that could be associated with the direction of peristaltic contractions or with the various types of reversals observed. Figure 13 is an example of an intracellular record in which potential changes were similar during *V*-to-*H* beats, collisions, and *H*-to-*V* beats.

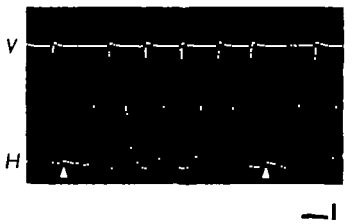

Fig. 11. Small depolarizing potential changes (indicated by arrows) occurring in the absence of mechanical and electrical activity. Top trace: extracellular recording. Bottom trace: intracellular recording. Amplitude calibration, 10 mV.; time calibration, one sec.

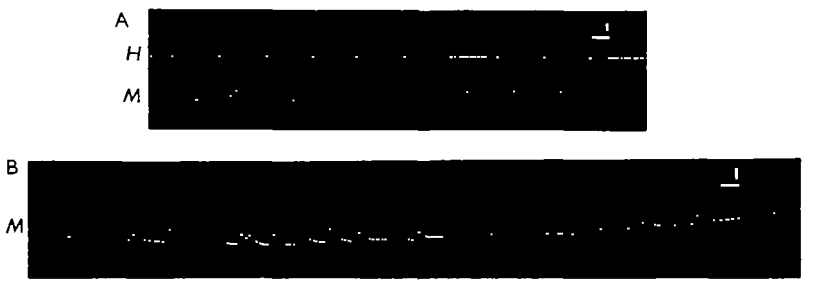

Fig. 12. Complex depolarizations associated with collisions prior to reversals. A and B are two recordings taken from separate experiments in which collisions were observed while the activity was simultaneously recorded. In A, the top trace is an extracellular recording; the bottom trace is an intracellular recording. In B, only the intracellular recording is shown. In both cases the intracellular potential changes were recorded from the middle (*M*) region of the heart. Amplitude calibration, 10 mV.; time calibration: 1 sec.

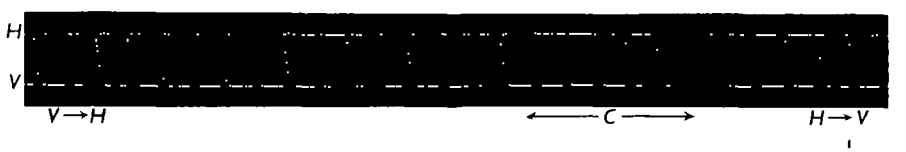

Fig. 13. Potential changes recorded when the beat was *V*-to-*H*, during collisions, and when the beat was *H*-to-*V*. Top trace: extracellular recording. Bottom trace: intracellular recording. The shape of the intracellular action potentials did not change during collisions nor when the heart reversed its direction of beat. Amplitude calibration: 10 mV.; time calibration, one sec.

Although intracellularly recorded electrical events usually accompanied each contractile wave, there were three occasions in this series of experiments in which a cell was electrically quiescent while beats occurred in the rest of the heart. In Fig. 14 the top trace (extracellular record) shows a series of contractions initiated and conducted along the heart; the bottom trace (intracellular record) illustrates the activity of a single cell.

In hearts which beat for a time at a frequency greater than that usually observed (e.g. 0·8–1·0 beats/sec.) some of the intracellularly recorded potential changes in the

series were decreased in amplitude; a few also showed double peaks. Examples of this phenomenon are given in the intracellular record of Fig. 15. The decreased amplitude recorded from cells involved in high-frequency activity suggests that they may have been in a state of relative refractoriness.

Sometimes, usually in old and failing preparations, contractions initiated at one end

Fig. 14. Example of the absence of intracellular potential changes during contractile activity. The top trace (extracellular record) records a series of contractions initiated and conducted along the heart; the bottom trace (intracellular record) illustrates the activity of a single cell which, for a period of time, did not follow the events taking place in the rest of the heart. Amplitude calibration, 10 mV.; time calibration, 1 sec.

Fig. 15. Activity recorded from a heart which beat at a frequency greater than that usually observed. Top trace: extracellular recording. During high-frequency activity the amplitude of some of the intracellular potential changes (bottom trace) was reduced; a few of these events also had double peaks. Amplitude calibration, 10 mV.; time calibration, 1 sec.

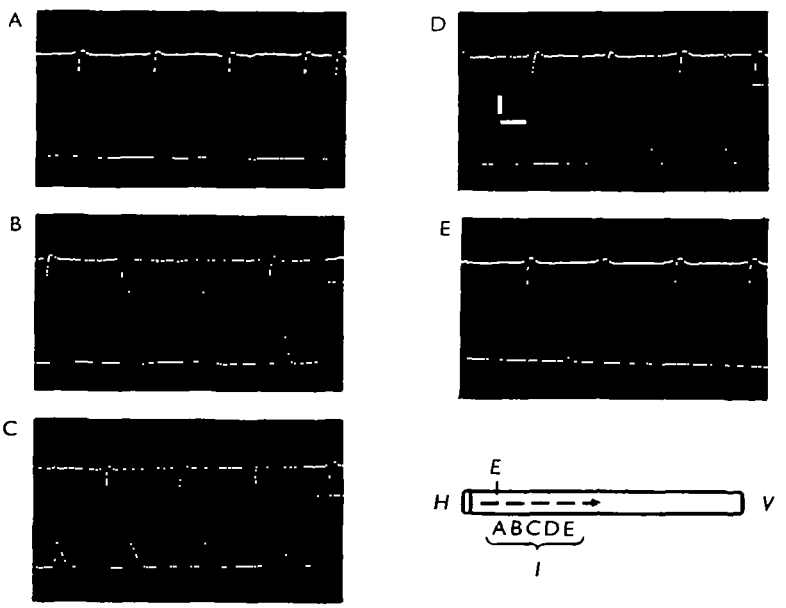

Fig. 16. Graded electrical activity recorded when a beat did not propagate the full length of the heart. A–E show intracellular recordings (bottom traces, *I*) made at loci of increasing distances from the extracellular suction electrode (top traces, *E*). All the records show normal resting potentials; electrical events near the point at which contractile activity was initiated were of maximum amplitude while electrical events near the point at which conduction stopped were of reduced amplitude. Amplitude calibration, for all records, 10 mV.; time calibration, one sec.

of the heart were not conducted the full length of the heart. Fig. 16A–E shows intracellular records taken at different loci between the end (A) where beats were initiated, and the centre of the heart (E), where beats stopped. All records showed normal resting potentials; electrical events recorded near the point at which beats were initiated were of maximum amplitude, while those recorded near the point at which conduction ceased were of reduced amplitude. It seems most reasonable to suppose that electrical changes of high amplitude are associated with fully developed contractile events, while those of lower amplitude are associated with less vigorous or failing contractions. The potential changes remain coupled to the contractile mechanism, but conduction from the active area is decremental.

It could be argued that some of the intracellular potential changes recorded in these experiments are movement artifacts, since they are of long duration and their amplitude is directly related to the strength of mechanical activity. Data have been presented previously, however, showing that more than one type of activity may be found within a given cell: small depolarizing potential changes can occur at a constant frequency that is unrelated in phase to the potential changes associated with contractions (Fig. 10); small depolarizations can occur in the absence of mechanical activity (Fig. 11); and occasional potential changes with double peaks occur during series of high-frequency contractions (Fig. 15). In these examples, beats proceeded in only one direction; therefore, no complex mechanical changes took place. If such potential changes were artifacts of co-ordinated mechanical phenomena, all intracellular changes would have shown constant phase relations and no potential changes would have occurred in the absence of mechanical activity.

Anatomy

The observations concerning the general organization of the heart made by earlier investigators, summarized and augmented by Millar (1953*a*), have been confirmed in the present study. The myocardium consists of single-layered, transversely striated, epithelial muscle cells. The cells are tapered at their ends, have diameters of approximately 6·0 μ range in length from 60 to 80 μ, and lie parallel to one another at an angle of approximately seventy degrees with respect to the raphe and the undifferentiated line. Fernandez (1904) described the undifferentiated line as being composed of a single layer of epithelial cells from two to five cells wide. He suggested that the undifferentiated line provides a mechanical support for the insertion of muscle fibres. The cells do not contain myofibrillar elements. Ichikawa (1966) observed tight junctions at the interfaces of cells in the undifferentiated line, and at the interfaces between cardiac muscle cells and the cells in the undifferentiated line.

Earlier workers (Alexandrowicz, 1913; Florey, 1951) suggested that the heart of *Ciona intestinalis* was innervated by fibres from the main ganglion, which is located in the inter-siphonal region. Millar (1952, 1953*a*) found no evidence for the existence of neural tissue in the heart of *Ciona*. He did see, however, a ring of connective tissue cells at each end of the heart which line the large vessels for a short distance from the ends of the heart; he suggested that these cells might have been mistaken for neural tissue. The results of Ichikawa (1966) agree with those of Millar. No evidence for innervation of the heart was found by the methods used in the present experiments.

Attempts to determine by pharmacological methods whether the heart is myogenic

or neurogenic have produced confusing results. Bacq (1935) and Krijgsman & Krijgsman (1959) reported that acetylcholine and adrenalin had no effect on the heart of *Ciona*. Scudder, Akers & Karczmar (1963) and Sugi & Matsunami (1966) stated that adrenalin increased the frequency of both ends of the heart and that acetylcholine showed no effect. Sugi and Matsunami also demonstrated that serotonin increases the frequency of the visceral end and reduces that of the hypobranchial end.

Although the myogenic hearts of clam and vertebrate respond to acetylcholine and to adrenalin in a way opposite to that of the neurogenic lobster heart, it is not reasonable to conclude that all other cardiac systems can be categorized on the basis of the same tests. Furthermore, the interpretation of results obtained from pharmacological studies of the *Ciona* heart is made difficult in view of its broad variability in spontaneous activity. On the other hand, substantial morphological and physiological data obtained by other workers support the position that the myocardium is not innervated.

DISCUSSION

The results described above suggest that the activity of the heart of *Ciona intestinalis* is determined by its intrinsic pacemaker capabilities; that is, the repetitive initiation of contractile waves results from periodic changes within the cells of the heart, without necessary extrinsic stimuli. The possibility that environmental changes may modify the overall frequency of beat initiation and the pattern of reversal is not excluded.

Schulze (1964), who investigated the ultrastructure of the *Ciona* heart, reported that there are no desmosomal structures connecting the cells. Ichikawa (1966), however, describes (also in *Ciona*) a number of tight junctions at the interfaces of adjoining myocardial cells. Such junctions probably provide low-resistance pathways for the spread of excitation. Kriebel (1966) measured the attenuation of rectangular current pulses applied across the myocardium and found that the transverse electrical resistance was 230 Ω/cm.^{2}. These findings indicate that current flow perpendicular to the plane of the heart tissue is virtually non-existent, and thus spread of excitation is by local current flow along the longitudinal axis of the heart. In addition, on the basis of his studies of the conduction velocities of contractile waves in the *Ciona* heart, Kriebel (1967) demonstrated that a wave-front must traverse one cell in 0·3 msec. and that this period of time is insufficient for the activation of a transmitter mechanism.

Intracellular recording of spontaneous activity revealed that any one cell may communicate with neighbouring cells by means of electrotonic coupling. This was clearly shown in the experiment of Fig. 10, in which low-amplitude depolarizing potential changes occurred at a regular frequency, but not in phase with the main electrical events associated with the contractions. It must be presumed that the cell from which the recording was made was in the vicinity of a cell whose output frequency was different from the contraction frequency. During collisions, certain cells (Fig. 12) responded electrically to two sources of input, thereby producing doublet potentials of reduced amplitude. In Fig. 15, during activity at especially high frequency, some potentials showed double peaks as well as reduced amplitude. Here the recorded cell was presumed to be in communication with other rhythmic sources with which it usually appeared to be phase-locked; because of the relative refractoriness imposed by the high frequency, a lag was produced which resulted in a slight phase

separation of the inputs. Figure 14 illustrates one of the rare instances in which a cell produced no activity while the rest of the heart maintained contractile activity. These results all provide strong evidence for the presence of foci of activity (or inactivity) which are not directly associated with the main contractile event.

The tunicate heart may initiate contractile waves at constant or irregular frequency in either direction, and can intermittently change from one pattern or direction to another. Low frequencies of spontaneous beat initiation can be greatly altered by a slight change in the rise time of the potentials, or by minor shifts in threshold (Bullock & Horridge, 1965). Variation in the rhythm may result from the homogeneous cellular construction of the heart; several pacemaker loci may exist, each having a different inherent frequency at a given time. The pacemaker unit exhibiting the greatest frequency of autogenic depolarization and recovery synchronizes and drives the contractile system. In *Aurelia* (Horridge, 1959), the leading pacemaker role is rotated among several equivalent pacemakers. Horridge postulated that in that system the redundancy of pacemakers produces, in addition to a high overall frequency, greater regularity.

The frequency of any given unit may vary periodically. At times it may lead, and at other times it slows so that a higher-frequency unit takes over as driver. Such periodic variations presumably depend upon variations in membrane conductance, which in turn may be based on metabolic processes within the cell. Strumwasser (1963, 1965, 1966) has postulated the production of a depolarizing substance by an enzyme system in the cells of the parietovisceral ganglion in *Aplysia*; variations in the level of this depolarizing substance are thought to result in variations in the excitability, and hence in the activity, of the cell.

As long as the inherent frequencies of the active pacemaker units are clearly different, the fastest one will control the contractile activity of the heart. However, if two or more pacemaker units have only slightly differing frequencies and if their capacities for producing autogenic activity are approximately equal at a given time, the rhythm might be a result of interactions between both centres. In this way, various patterns of beat initiation and deviations from constant frequencies would be produced. As in the outputs of coupled oscillators (Solberger, 1965; Mercer, 1965), interacting pacemaker sites (i.e. two mechanisms acting on one another, each changing the other) may undergo resonance, interference and frequency demultiplication.

The electrical changes recorded from cells in the tunicate heart quite probably result from complex interactions between cells within a large population. The cellular components may possess similar structural and physiological characteristics; by virtue of their associations, they produce variable intracellular voltage changes and variable rhythms of spontaneous events.

The ligation experiments demonstrate that the system of beat initiation at one end of the heart often changes rhythmically between periods of high and low frequency of initiation (see Fig. 8). It is possible to use this behaviour in proposing a new reversal mechanism for the intact heart, based on interactions between changing frequency levels at the two ends. As long as the frequency of beat initiation is greater at one end, that end maintains a dominance by blocking potential beats initiated from the opposite end, presumably by resetting the individual pacemaker elements.

The level of excitability of any single pacemaker unit in the driven end is not great enough to synchronize that region into a rhythm of greater frequency than that of the

driving end. Therefore, the driven end contracts in the rhythm of the driving end, just as the end of a heart to which a train of stimuli is applied contracts in the rhythm of the stimulus train. As soon as the frequency for beat initiation of the driver drops below that of the driven end, the latter slips into the driving role. The recorded levels of frequencies in the isolated halves of the heart would thus directly reflect the levels of excitability during a series of beats. The periods of transition between levels of high and low frequency, however, will not directly reflect the excitability of the active pacemaker regions. Applying trains of stimuli to the ends of the heart showed that several stimuli were required before the heart responded to each stimulus of the train; after stimulation was stopped, the heart beat for two or three cycles at the imposed frequency before resuming its natural frequency. It is thus possible to detect only relative changes in the excitability of a given region by recording changes in its frequency.

Collisions, pauses, and single beats escaping from the non-dominant end during a beat series depend upon the slope and direction of frequency changes at the two ends. If the slopes of the changes in the two frequencies are very different, immediate reversals take place as the driven end slips into the driving state. If, however, the slopes are nearly superposed, the frequency levels of both ends will be similar for a period and collisions will occur. Collisions are, in fact, often observed as a series of events in which the driven end gradually takes over as driver.

An unresolved question concerns the meaning of these highly regular variations, or oscillations, in frequency level of viscerally initiated beats. It seems reasonable to consider the nearly square-wave oscillations as integrals of multiple oscillations in levels of autogenic activity in the pacemaker units of a given region. The area bounded by the nearly square waves would thus represent the sum of the areas bounded by the curves of the oscillations in autogenic activity of all local, active pacemaker sites.

The ligation experiments have shown that, in addition to the pacemaker rhythm which generates contractile activity, there is another, longer-period rhythm produced by the isolated visceral end of the heart. This rhythm alternates between periods of high and low frequency with a periodicity of a few minutes. Such a period, in terms of its duration, is an unusual one among described biological rhythms. In addition to exhibiting these regular variations, the visceral end is also characterized by a non-linear increase in threshold for one-to-one following when trains of stimuli are applied at increasing frequencies (see Fig. 6). The hypobranchial end, however, does not exhibit regular variations in frequency levels, and it is characterized by a linear increase in threshold for one-to-one following of stimulus trains at increasing frequencies. There are thus stable physiological differences between the two ends of the heart.

Though in failing preparations contractions occasionally began spontaneously at some point along the length of the heart, beats were usually initiated at the ends. Isolated pieces from along the length of the heart tend to beat at nearly equal frequencies, indicating that equivalent pacemaker capability is widely distributed. A likely explanation is that current spreads along the low-resistance pathways provided by the close membrane contacts in the myocardium. Excitation developed at a point along the length of the heart will spread radially and decrementally from its point of origin. If regions of close membrane apposition are absent in the raphe and in the walls of the sinuses with which the ends of the heart communicate, then excitation developed at an end will not spread radially in all directions; rather, it will encounter high resis-

tance at the raphe and at the junction between the heart and the sinuses. An active focus at an end will therefore draw its current preferentially from one direction, whereas one in the middle of the heart would draw the same amount of current from sources arranged in 360° around the focus. Therefore, when a beat is initiated at an end, the available current is concentrated more effectively on cells on one side of the focus of excitability. This concentration establishes a direction of spread toward the centre of the heart. A study of the fine structure of the heart and of the sinuses is required to test this hypothesis.

Several proposals have been put forward to account for reversal in tunicate hearts. The four postulated mechanisms for reversal found in the recent literature—back pressure, extrasystole, pacemaker fatigue and partial pressure of CO_2—will be considered in the light of the present findings.

1. *Back pressure.* Many early workers (see Krijgsman, 1956) suggested that resistance in the capillary bed is gradually built up during a series of beats, compelling the beat to reverse when the active end can no longer work against the capillary load. Haywood and Moon (1950, 1953) proposed that back pressure causes the heart to stop beating in a particular direction: '...pressure is built up by the action of the heart until the heart muscles are unable to act against it. The heart labours for a short time and then stops.' They did not concern themselves with the problem of initiation of contractions in the opposite direction. Krijgsman (1956), Krijgsman & Krijgsman (1957) and Millar (1952, 1953*b*) have argued that the back pressure hypothesis is untenable; their main points are that reversals take place in isolated hearts whose walls have been cut open to ensure equal pressures inside and outside the tube and that the leading centres do not necessarily exhibit a gradual slowing as a result of a gradual increase in pressure.

Kriebel (1964) showed that pressure changes of 2 mm. of water in cannulated isolated hearts caused reversals; he suggested that a pressure difference across the heart triggers them. He also showed (cf. von Skramlik, 1926*b*) that localized heating and cooling of the ends of the heart affected the frequency of beat initiation, and was able to determine the direction of beat propagation through dominance of the warmer, higher-frequency end.

In the present experiments, however, it was shown that reversals can take place in pieces of heart tissue transversely and longitudinally cut, in which no pressure changes could have occurred. Ligation of the centre of the heart in the intact circulatory system produced a marked swelling of the heart tube at the ligature, toward which the ends continued to beat for periods of 2–3 hr. In these preparations, in which pressure differences obviously existed, reversals in the two halves seldom took place; when they did, only one or two beats were initiated at the ligature and conducted to the ends, and a second reversal occurred before the pressure at the ligature was completely relieved. Mechanical stimuli can, however, affect excitability. Stretching the heart causes an increase in the frequency of beat initiation, and touching the tissue with a glass probe usually evokes a contractile response in quiescent hearts.

These data suggest that pressure increases in the tunicate heart may have an effect similar to that of other mechanical stimuli. Because tissue pieces reverse the direction of contraction in the absence of a pressure differential, and because temperature and electrical stimuli affect the activity of the heart as significantly as pressure, it is un-

likely that pressure is the primary cause for reversal. Instead, it may superimpose an effect on the inherent pacemaker activities of the heart.

2. *Extrasystole.* Mislin (1964, 1965) and Mislin & Krause (1964) suggested that spontaneous extrasystoles, which were observed prior to reversal, disturbed the automaticity of the active end and reversed the direction of contractile activity. They mention, however, that reversals were not always preceded by an extrasystole but instead took place immediately or were preceded by a pause (Mislin & Krause, 1964). It appears that the 'extrasystoles', which Mislin recorded with suction electrodes similar to those used in the present experiments, actually correspond to collisions. These extrasystoles are thus epiphenomena of the reversal process, observed when active pacemaker centres at opposite ends of the heart produce similar levels of autogenic activity.

3. *Pacemaker fatigue.* Krijgsman (1956) and Krijgsman & Krijgsman (1957) assumed that there are two intrinsic pacemakers in the tunicate heart, one at each end. [They did not agree with von Skramlik (1926*a*, 1929) who proposed the existence of a third pacemaker in the centre of the heart.] They suggested that the reversals of the direction of contraction are determined by fatigue of the leading centres which causes periodic arrests of pacemaker activity, and proposed that this fatigue represents a rise in the threshold of response to a metabolite or ion during periods of activity.

The cutting experiments performed in this series of experiments, as well as in the work of Mislin & Krause (1964) and of Mislin (1965), have shown that pacemaker properties are not isolated at the ends of the heart but are present in cells over the entire length of the heart. Therefore, it may be concluded that pacemaker centres are not confined to a given region. Indeed, Krijgsman (1957) decided that: '...all parts of the tunicate heart have the power of autonomism.'

The word 'fatigue' can be interpreted as a decrement in response; the decrease in the output of an autogenic unit may result from changing conditions within the unit. The ligation experiments demonstrated that variations in the frequency of beat initiation take place. A substance which affects the level of automaticity of a pacemaker unit could be produced in periodically varying concentrations by intrinsic cellular metabolic processes. Strumwasser (1963, 1965, 1966) has postulated that such events account for the rhythmic activity of the parabolic burster cell in the parietovisceral ganglion of *Aplysia*.

4. *Partial pressure of* CO_2. Brocas *et al.* (1966*a*, *b*) tested the effects of increasing the partial pressure of the CO_2 in the bathing medium of *Ciona intestinalis*. Their results indicated that the activity of the hypobranchial end of the heart is slowed by increasing the P_{CO_2} while the visceral end is not greatly affected. These workers concluded that the level of activity of the hypobranchial centre determines the direction of circulation, and that this level of activity is a function of the P_{CO_2}.

No direct comparison of the data presented by Brocas *et al.* and the results obtained in this study can be made. It is, however, not clear from their account that the phenomena observed were not merely a result of the broad variability in frequency exhibited by tunicate hearts.

The present results indicate that none of these explanations is necessary in view of the demonstrated periodic variations in frequency of the pacemaker systems. The mechanical phenomena observed in the heart of *Ciona intestinalis* can be predicted

entirely on the basis of intrinsic pacemaker properties of its constituent cells; extrinsic sources of stimulation are not required to account for variations in the frequency of activity, reversals or collisions.

SUMMARY

1. Electrophysiological techniques have been used to define pacemaker characteristics and organization in the heart of the tunicate *Ciona intestinalis*. The frequency of spontaneous beat initiation was regular or irregular for a given period of time; in any single heart, these frequency modes intermittently changed from one to the other in no regular order. Reversals could take place immediately, after a pause, or after collisions.

2. Trains of electrical stimuli applied to the ends of the heart could drive propagated contractions at a frequency of up to 2·6/sec., and dominance could be controlled by altering the frequency and/or intensity of the stimuli. Each end gave a characteristic response to increasing frequency of driving, and the threshold for one-to-one driving was frequency-dependent.

3. When the middle of a heart was ligatured, the two ends were independently active. Visceral ends exhibited regularly varying levels of high and low frequency with a period of several minutes, whereas the frequency levels of the hypobranchial ends varied irregularly.

4. Intracellular resting potentials were approximately −50 mV., and propagated action potentials associated with contractile events did not show 'overshoot.' Partial electrical responses were affiliated with weak or failing mechanical events, and complex, double-peaked intracellular potentials were often associated with collisions. In some cases a cell could show electrical activity that was completely dissociated from the mechanical response of the whole heart. Excitation thus probably spreads passively between cells, and multiple foci of activity may co-exist at any given time.

5. From electrophysiological and anatomical data, an hypothesis is proposed to explain the observed mechanical activity on the basis of the interactions of individual cells and higher-order interactions among groups of cells.

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