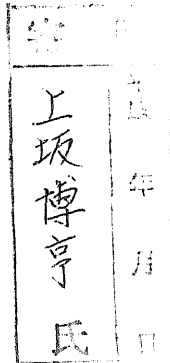Electrophysiological Studies on the Formation of
the Beat Rhythm of the Heart in the Oyster
Crassostrea gigas

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1987

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Science,
in Doctoral Program in Biological Sciences,
University of Tsukuba

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I. INTRODUCTION

1) Automatic activity and pacemaker site

2) Coordination between the auricle and ventricle

1) Automatic activity and pacemaker site

The molluscan heart is stated to be of myogenic nature, i.e. the automatic activity originates in the myocardium (KRIJGSMAN and DIVARIS, 1955 ; HILL and WELSH, 1966).

In some molluscs such as gastropods (OCHIAI, 1959; NOMURA, 1963; KISS and S-RÓZSA, 1973; KUWASAWA, 1979; HILL and YANTORNO, 1979), and bivalves (IRISAWA et al, 1961a,b ; IRISAWA and KOBAYASHI, 1964; EBARA, 1964a,b, 1966, 1967, 1969; IRISAWA et al., 1967; IRISAWA et al., 1968; SHIGETO, 1970; WILKENS, 1972a,b; WILKENS and GREENBERG, 1973; IRISAWA et al., 1973a; IRISAWA et al., 1973b; EBARA and KUWASAWA, 1975; EBARA et al., 1976), the spontaneous electrical activities have been recorded intracellularly by using microelectrodes or the sucrose gap method from the isolated heart, especially from the ventricle. The spontaneous action potentials are preceded by a slow depolarizing potential (pacemaker potential) which is a characteristic of the pacemaker activity seen in the pacemaker region of the vertebrate hearts. Moreover, the membrane currents of the action potentials generated spontaneously in the ventricular myocardia have similar characteristics as that of the vertebrate hearts (KISS, 1980). These indicate that each myocardium of the molluscan ventricle possesses the pacemaker property and has the ability to beat automatically.

The pacemaker activities of the ventricular myocardial cells are integrated to be synchronized through the electrical junctions to function as a whole (EBARA, 1964a,b, 1967: IRISAWA

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et al., 1973a), but the initiation site of the activity may wander from area to area (EBARA and KUWASAWA, 1975). Though the pacemaker cells in vertebrate hearts are smaller than the other myocardial cells, no remarkable differences are found among the myocardia in molluscan ventricles (IRISAWA et al., 1969; NISBET and PLUMMER, 1969; ELEKES et al., 1973; IRISAWA et al., 1973b). Thus, it has been considered that the pacemaker of the molluscan ventricle is diffused (a so-called diffuse pacemaker : IRISAWA, 1978), and that there is no localized area of the heart that consistently dominates the rest of the myocardia (JONES, 1983). In some gastropods, however, some functional differentiation among myocardia has been found in the ventricle (KUWASAWA, 1979; KOESTER et al., 1979). Even though the heart is of diffuse myogenic nature, the auriculo-ventricular (A-V) valve ordinarily acts as the dominant pacemaker of the ventricle (KUWASAWA, 1979).

In contrast to the ventricle, the auricles are not studied so much, nevertheless the automaticity is thought to originate similarly in the individual myocardium (HILL and WELSH, 1966). An isolated oyster auricle can continue to beat by itself with a regular rhythm (JULLIEN and MORIN, 1931; TAKATSUKI, 1932; OKA, 1932), and seems to function as a whole with a similar mechanism as in the ventricle.

Thus, each auricle and the ventricle of the molluscan heart is considered to possess intrinsic automatic activity in itself (JONES, 1983).

2) Coordination between the auricle and ventricle

In spite of the fact that each auricle and ventricle of the molluscan hearts intrinsically have the ability to beat independently with its own rhythm, they beat alternately and regularly with a constant A-V delay in the intact state.

In the hearts of higher vertebrates, the cardiac muscles having pacemaker property are found in a specific site (sino-atrial node) of the heart. The activity of the heart as a whole begins at the pacemaker site and spreads through the specially developed conduction system which propagates the excitation from the atrium to the ventricle. Moreover, due to the characteristics that the conduction velocity of the system appeared very slow at the point from the atrium to the ventricle, the atrium and ventricle beat with a certain time lag, i.e. A-V delay.

A conduction system as found in higher vertebrates has not been reported in molluscs (JONES, 1983), nevertheless there seems to be some sort of interaction between the auricle(s) and ventricle. It was stated in the Helix heart that the electrical conduction pathway as the system was not present between its auricle and ventricle (RIPPLINGER and RIPPLINGER, 1972). On the other hand, in many molluscs, the heart is suspended in the pericardial cavity and is attached to the pericardial wall. Hence, the auricle(s) and ventricle stretch each other alternately by their contractions. Willems (1932) found in the Helix heart that the coordination between the auricle and

ventricle was maintained by connecting them with a silver wire and supposed that the coordination was mediated by stretching. Matsui (1945) also suggested it by similar experiments in the _Dolabella_ heart.

In spite of the suggestion that the coordination of rhythms between the auricle and ventricle might be mediated by mutual stretching, the effect of a briefly lasting stretch caused by the contraction has not yet been examined. Moreover, the mechanisms to integrate the rhythmic activities between the auricle and ventricle and to form the beat rhythm of the whole heart have still remained unsolved. In order to understand the heart not only as a physiological organ but also as a biological oscillator (PAVLIDIS, 1973: WINFREE, 1973a,b: PINSKER, 1977a,b), it is important to know the mechanisms of the heart.

The present study was carried out to elucidate the mechanisms in which the auricle and ventricle in oyster heart achieve the coordinated and alternated beats with a constant and functional A-V delay.

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1) Materials

Oysters, <u>Crassostrea</u> <u>gigas</u> were used throughout this study.
The animals weighing 50 - 150 g were collected at the shore of
Nakaminato (Ibaraki, Japan), and were kept in a tank filled with
aerated and filtrated running artificial sea water (ASW) at a
temperature of 15 - 18°C. For the experiments, the animals
within three weeks in the tank were used.

2) Anatomical observations

The oyster heart consists of two auricles and one ventricle.
The two auricles are narrowly connected near the venous end
(TORIGOE, 1981) and joined to the ventricle. A pair of A-V
valves is at the A-V junctions inside the heart. The blood runs
into the two auricles through the right and left veins, flows
into the ventricle and is sent out through two aortae. The heart
is attached to the pericardial wall at the venous and aortic ends
by the vessels. It is suspended in the pericardial cavity beside
the adductor muscle (Fig. 1) so that the auricle contracts and
thereby stretches the ventricle and <u>vice</u> <u>versa</u>, alternately.

The visceral ganglion lies on the ventral surface of the
adductor muscle. The cardio-regulator nerve fibers contained in
the visceral nerve descend from the ganglion and innervate each
auricle and ventricle (OKA, 1931).

3) Preparations

a) Semi-intact heart in situ

After taking off the right valve (upper valve), small parts of the mantle and the pericardial wall were removed to expose the heart. Nerves innervating from the visceral ganglion to the heart were severed at a portion near the adductor muscle to exclude extrinsic neural regulations. Two aortae and two veins were cut at portions near the pericardial wall to avoid effects of blood pressure.

The preparation was placed in a small experimental chamber and electrical activities of the heart were recorded in situ. Two types of preparations were used. For recording spontaneous activities of the auricles and ventricle, the heart composed of two auricles and one ventricle was left intact. To examine the interaction between the auricle and ventricle, one of the auricles was cut off at the auriculo-ventricular boundary (A-V boundary) and at the venous end to simplify the obtained results, so the heart consists of a ventricle and only one auricle. With the latter preparation, the alteration of beat cycle by electrical stimulation was examined. In either case, the pericardial cavity was continuously perfused with aerated ASW. In such a state, steady and coordinated beating rhythms of the auricle(s) and ventricle were maintained.

b) Isolated and opened heart

The heart was isolated from the pericardial cavity by cutting its aortic and venous ends. After removing the ventricle, the remained auricles were opened by longitudinal incision to expose the inner wall. The auricles were pinned on the bottom of the experimental chamber and electrical connection between them were examined electrophysiologically.

To examine the existence of an electrical pathway between the auricle and ventricle, one of the auricles was removed from the isolated heart. The heart was opened longitudinally and placed in the chamber. The A-V valve was partly removed to expose the A-V boundary.

c) Isolated auricle and ventricle

To examine the effect of a brief stretch on the electrical activity of the myocardium and the beat rhythm of the heart, the isolated auricle or ventricle was prepared by separating the two parts at the A-V boundary. One end of the preparation was pinned on the bottom of the chamber and the other end was tied by a fine thread to the hook of the stretching apparatus. The experimental arrangements are schematically illustrated in Fig. 12.

4) Solutions

ASW was prepared as the physiological solution. The

composition of the ASW was as follows:

$$NaCl \quad ; \quad 461.9 \text{ mM}$$

$$KCl \quad ; \quad 9.4 \text{ mM}$$

$$CaCl_2 \quad ; \quad 9.0 \text{ mM}$$

$$MgCl_2 \quad ; \quad 35.7 \text{ mM}$$

$$MgSO_4 \quad ; \quad 17.4 \text{ mM}$$

$$Tris\text{-}HCl \quad ; \quad 6.0 \text{ mM} \quad (pH \; ; \; 7.2 - 7.4)$$

The ASW was used during the dissection of the preparation and for perfusion throughout the experiments.

To suppress the automatic activity of the preparation, ACh-ASW was prepared by adding acetylcholine chloride (10^{-4} M) to the ASW (Shigeto, 1970).

All the experiments were carried out at a temperature of 20 - 25 $^{\circ}$C.

5) Electrophysiological recordings

The extracellular action potentials representing the beats were recorded from the auricle(s) and the ventricle with glass suction electrodes (inner diameter: 100 - 150 μm). A peristaltic pump was employed to maintain negative pressure inside the electrodes. Under this condition, continuous recording became possible for over 10 hours without remarkable decay in the amplitude of the potential. The action potentials

were amplified by conventional AC-amplifiers (AB-620G, Nihon Kohden Kohgyo) with a time constant of 0.1 - 2.0 sec.

The intracellular recordings were performed by using micro-electrodes filled with 3M KCl having a resistance of 20 - 40 M ohms. The electrical signals were amplified by employing a DC pre-amplifier (MEZ-7101, Nihon Kohden Kohgyo).

The mechanical movements of the heart preparation were recorded employing a mechanoelectro-transducer (TB-612T, Nihon Kohden Kohgyo). A tapered tungsten wire (0.5 mm to ca 0.01 mm in diameter, 50 mm in length) was joined to the tip of the transducer for the attachment to the heart. The current signals from the transducer were converted to the voltage signals by employing a carrier amplifier.

All the signals induced were displayed on a cathode ray oscilloscope (561B, Tektronix) or on a storage oscilloscope (DSS-5020A, Kikusui Electronics Corp.), then photographed. A pen-writing oscillograph (WR3001, Watanabe Instruments Corp.) and a magnetic tape data recorder (RMG-5304, Nihon Kohden Kohgyo) were also employed for recordings.

a) Beat rhythms of the auricle and ventricle in situ

The extracellular potentials of the auricle(s) and ventricle were recorded simultaneously to investigate the beat patterns of the heart in situ. The recording electrodes were placed near the venous end(s) of the auricle(s) and the aortic end of the ventricle to keep the disturbance due to the movement of the heart to a minimum. The current applying electrode was placed by

the recording electrode to evoke an extra action potential.

To record the mechanical movements of the heart, a small part of the ventricular myocardia near the A-V boundary was hooked up by the attachment of the mechanoelectro-transducer. The contractions of the auricle and ventricle were detected by the voltage changes indicated in positive and negative directions, respectively.

b) Electrotonic spread in the heart

Spread of electric current between the two auricles was examined. Anodal current was applied through a suction electrode placed on one of the auricles and electrotonic potentials were recorded with a microelectrode from the other auricle.

To examine the existence of an electrical pathway between the auricle and the ventricle, the current applying electrodes were placed on both the auricle and the ventricle and intracellular recordings were made at various portions around the A-V boundary. In both cases, spontaneous activities of the preparation were suppressed by perfusion of ACh-ASW (SHIGETO, 1970).

c) Beat interval changes in the isolated auricle and ventricle by the stretch

Changes in spontaneous electrical activity caused by a stretch were recorded intracellularly from the isolated ventricles. The heart wall around the portion where the microelectrode was inserted was tightly fixed with pins to avoid

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production of artifacts due to the stretch.

Effects of a stretch on the beat interval were examined also extracellularly in the isolated auricles and ventricles.

The applied stretch was monitored with a mechanoelectro-transducer. The arrangement of the recording devices is schematically illustrated in Fig. 12.

6) Stimulation

a) Electrical stimulation

Electric pulses were applied as stimuli to the cardiac muscle from an electronic stimulator (MSE-3R, SEN-3201, Nihon Kohden Kohgyo) through the suction electrode placed on the auricle or on the ventricle. The stimulus was a rectangular cathodal or anodal pulse (1 - 10 V, 0.1 - 1 sec). Cathodal pulse was applied to the preparation in situ to evoke an extra action potential in the stimulated part and anodal pulse was applied to the isolated preparation to examine the spread of electrotonic potentials in the heart. An Ag-AgCl wire (0.3 mm in diameter) was placed near the stimulating electrode and used as the indifferent electrode.

b) Mechanical stimulation

A galvanometer for a pen-writing oscillograph (WTR751, Watanabe Instruments Corp.) was used for applying stretches to the isolated auricle or ventricle. A stainless wire hook

(diameter of 100 μm) was joined to the galvanometer for the attachment to the heart. The galvanometer was controlled by a feedback amplifier (WA751, Watanabe Instruments Corp.) to give rectangular shaped stretches that are proportional to the rectangular electric pulses given off through the amplifier. The stretches were monitored with a mechanoelectro-transducer (TB-612T, Nihon Kohden Kohgyo) attached to the hook. These stretches were ranged in a duration of 50-500 msec (brief stretches) and a magnitude of 0.4-1.3 mm (5-25 % of the auricular or ventricular length).

7) Oscillator terminology and its definition

The concept of biological oscillator can be applied to the heart which beats with a constant rhythm. An oscillator is a system which continuously alters its values between a maximum and minimum. The value indicates the state of the oscillator at a given moment.

An interval of the oscillator can be defined as the length of time from one state to the next same state. In a constantly oscillating system (isochronous system), the intervals must all be of the same length.

A phase is a point of time which corresponds to one state of the oscillator in the interval. Therefore, in an isochronous system, the phase can be regarded as the set of time of the same states in the sequence of the intervals as defined by the

equation

$$Px=(tx - to) \bmod T$$

Px : Phase of the state "x"

tx : Time showing the state "x"

to : Time defined to be zero

T : Length of free-run interval

The phase can be expressed either in units of time or it can be normalized and expressed as degrees of angle or as a percentage of the interval. In this study, the phase is expressed as the percentage of free-run interval.

If the oscillator is perturbed by a stimulus at a phase in the interval, the next cycle shifted to be advanced or delayed. This change is referred to as the phase shift and is defined by the equation

$$P(x,s)=[t(x,s) - t(x,o)] \bmod T$$

P(x,s) : Phase shift of the state "x" by a stimulus

t(x,s) : Time showing the state "x" after the stimulus

t(x.o) : Time showing the state "x" before the stimulus

T   : Length of free-run interval

The phase shift is not an enduring change of the interval. In this study, the phase shift was also expressed as the percentage

of free-run interval.

A phase response curve (PRC) is a plot of the phase shift versus the phase of the stimulus (stimulation phase). The characteristics of the PRC depend on both the features of the stimulus (e.g. intensity, duration etc.) and its phase relative to the phase of the oscillator.

8) Measurement of beat intervals and PRC

a) The measuring system

A micro-computer system (PC-9801/F, NEC Corp.) was used for automatic measurement of the beat intervals. The action potentials recorded extracellularly were amplified through the AC amplifier and digitalized by an analogue to digital converter (A/D converter; ANALOG-PRO, Canopus Electronics Co.) which has a resolving power of 12 bits (10/4096 V) within the range of -0.5 to 5.0 V. Each digitalized signal was taken into the computer by a delay of 170 μsec then stored in its main memory.

The signals in the memory were used for detecting the peak of the action potential. The mean of the newest 10 signals (new mean) on the memory were compared with the mean of 10 signals of 170 μsec before (old mean). The moment of the peak was defined as the time at which the value of the old mean minus the new mean became positive. The time was read out from the internal clock of the computer which has a resolving power of 1/1024 sec. The beat interval was defined as the duration from a peak of the

action potential to that of the next.

A brief stretch was applied to the preparation under control of the computer. A triggering electric pulse was sent out from the computer to the electronic stimulator to drive the stretching apparatus. The time of the stretch application was read from the clock as the time when the triggering pulse was sent out.

All the programs employed in the experiments were written in Pascal language on an operating system of MS-DOS Ver. 2-11 (see appendix).

b) Procedures for measurements

In order to obtain a PRC, the intervals of 10 free-run beats were measured to check up the stability and to obtain the control interval (100 %) of the heart beat. When the free-run intervals were sufficiently stable (alteration of less than 1 %), a single brief stretch was triggered by the computer and applied to the preparation. The time of the action potential peak was defined as phase 0 (i.e. phase 100). The phase shift of the beat due to the stretch was calculated as the percentage of the control interval and displayed on the CRT as a function of the stimulation phase when the stretch was applied. The stimulation phase was measured as the time of onset of the stretch and indicated by the percentage of the control interval. In order to obtain the curve, the phase shifts were examined at 50-200 different phases of the stretch.

III. RESULTS

1) Beat rhythms of the auricle and ventricle

2) Alteration of beat intervals by electrical stimulation

3) Mediating factor of interaction

    a) Electrical coupling between two auricles

    b) Electrical isolation between the auricle and ventricle

    c) Mechanical interaction between the auricle and ventricle

4) Effects of a single brief stretch

    a) Alteration of electrical activity and beat interval

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5) Entrainment of beat rhythm by repetitive brief stretches

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6) Beat interval alterations in the heart _in situ_

1) Beat rhythms of the auricle and ventricle

Action potentials of two auricles and one ventricle were recorded simultaneously. In the intact heart, the auricles and the ventricle continue to beat rhythmically during perfusion of ASW through the pericardial cavity. The interval between spike potentials (beat interval) ranged from 1.8 sec to 2.8 sec, and the delay time between action potentials of the two auricles was fairly short. The delay between the auricular and the ventricular action potential (A-V delay) widely varied (0.5 sec - 2.3 sec) among preparations at 22°C. An example is shown in Fig. 2.

When one of the experimental conditions was changed (e.g. stop of the perfusion, or rapid change in temperature), the coordination of activities between auricle and ventricle gradually disappeared. As the perfusion was stopped, the beat rhythms, mainly that of the auricle became irregular. At last, the auricles and the ventricle began to beat irregularly. In most cases, though, the beats of the auricles were almost synchronized. When the perfusion was started again, the coordination quickly recovered.

In the course of disappearance of the coordination, two cases of change in spontaneous beat pattern were observed from a number of preparations. One is the case where the length of auricular beat intervals became slightly longer than that of the ventricle, and the other is the reversed case where the auricular beat intervals became shorter as compared with the ventricular interval. An example of the former case is shown in Fig. 3a. In

the coordinated state, the beat intervals were 2.0 sec in this preparation, with a short A-V delay of 0.68 sec. As the coordination disappears, the mean auricular beat interval became 2.3 sec which was longer as compared with that of the ventricle (2.0 sec). In this case, this change in intervals caused the A-V delay to become gradually shorter, beat by beat, until the auricular action potential turned out to succeed the ventricular one. At that moment, the auricular beat interval became remarkably long. In an example of the other case, the beat interval was 2.0 sec with a long A-V delay of 1.46 sec in the coordinated state. As the coordination disappeared, as shown in Fig. 3b, the mean auricular beat interval became 2.2 sec which was shorter than that of the ventricle (2.6 sec). In this case, the A-V delay became gradually longer, beat by beat. Then the auricular action potential turned out to precede the ventricular one. At this instant, the auricular beat intervals became remarkably shortened as compared with the foregoing beat intervals. These remarkable interval changes occurred periodically (Fig. 3).

The results obtained from a number of preparations suggest that there may be two states of heart preparations under the experimental conditions in this study. One is the state where the ventricle possesses the higher intrinsic rhythm and the heart beats with a short A-V delay (shorter than approximately half of the interval), and the other is the state where the auricle has the higher intrinsic automaticity and the heart beats with a long A-V delay (longer than approximately half of the interval).

2) Alteration of beat intervals by electrical stimulation

Extra action potentials were evoked at various moments in
the phase of the auricular or ventricular cycle by application of
cathodal current, to observe the change in the ventricular or
auricular beat intervals, respectively.

When an auricular action potential was forced to occur at an
advanced phase (i.e. prolonged A-V delay) to produce an extra
action potential, the corresponding ventricular beat interval
became shorter (Fig. 4a). Further, the phase of the extra action
potential became more advanced, the ventricular intervals began
to prolong. Thus, the ventricular beat intervals were affected by
the moment of evoked auricular action potential to prolong or to
shorten. On the contrary, when a ventricular action potential
was evoked by a cathodal current pulse, the auricular beat
intervals were also affected to be alternated (Fig. 4b) in a
similar manner. In any case, the length of the auricular beat
interval changed in accordance with the moment of the ventricular
action potential in the auricular cycle as mentioned above.

From the above results, it can be conclusively suggested
that changes (shortening or prolongation) in the length of an
auricular beat interval occurs when the ventricular extra action
potential is evoked during the former period of the corresponding
auricular beat interval or the latter period of the foregoing
auricular beat.

The changes in auricular beat interval were plotted versus
the A-V delay. The curves were obtained to be biphasic i.e.

showing interval prolongation and shortening in correspondence with the A-V delay. In the example shown in Fig. 5, the maximum shortening was 0.49 sec and the maximum prolongation was 0.29 sec. The maximum values of interval change varied among preparations, though, the curves in all cases are more or less biphasic. The effect of the change in A-V delay on the auricular beat intervals was investigated in the same way. Essentially, similar results were obtained, though the effects were smaller than those of the results mentioned above.

3) Mediating factor of interaction

a) Electrical coupling between two auricles

As mentioned above, the two auricles beat synchronously. The mechanisms to achieve this synchronicity were examined electrophysiologically.

The auricles are narrowly joined near the venous ends. The bridge connecting them was ca 300 μm in diameter and ca 500 μm in length in an example shown in Fig. 6a. Some myocardial bundles were found running through the bridge.

Anodal current pulses were applied to one of the auricles through a suction electrode and the resulting electrotonic potentials were recorded intracellularly from the myocardia of both auricles. When the pulse was applied to the left auricle (Fig. 6a, A1), both auricles showed electrotonic potential, though the potential recorded from the right auricle was smaller

(Fig. 6b, left records). Similarly, when the pulse was applied to the right auricle (Fig. 6a, A2), potential was again recorded from both parts (Fig. 6b, right records). Thus, bidirectional electrical coupling was found between the two auricles.

The distance from the current applying electrode to the recording electrode was progressively increased to examine the degree of potential decay through the connecting bundle (Fig. 7). The electrotonic potential decreased exponentially within the auricle in which the current was applied (Fig. 7, A1) and within the other auricle (Fig. 7, A2), with a space constant of 0.7-0.9 mm. Furthermore, the potential decay within the two auricles was found to fit into each other to form a single linear function of distance (p<0.02), thus the space constant within the connecting bridge was considered to be the same as that of within the auricles.

b) Electrical isolation between the auricle and ventricle

The possibility of an electrical pathway between the auricle(s) and the ventricle was investigated. There is a pair of A-V valves at each junction of the ventricle with the two auricles. From morphological observations, myocardial bundles in the ventricle seemed to be continued with those in the A-V valve, whereas, bundles in the auricles were seen to be terminated at the portion of the A-V boundary. No bundle running through the boundary was detected in histological sections.

Electrical connections among the myocardia in these parts were examined electrophysiologically. Anodal current pulses were

applied to the auricle and the ventricle through suction electrodes and the resulting electrotonic potentials were recorded intracellularly from myocardia in various portions around the A-V boundary (Fig. 8a). When the pulse was applied to the auricle, the eletrotonic potential was recorded only from the auricle and not from the ventricle nor the A-V valves (Fig. 8b left records). On the contrary, when the pulse was applied to the ventricle, an electrotonic potential was recorded from the ventricle and the A-V valves but not from the auricle (Fig. 8b, right records), indicating that myocardia in the ventricle and in the A-V valves were electrically coupled.

Electrical isolation between the auricles and the ventricle was further investigated. When the distance from the current applying electrode to the recording electrode was progressively increased, the amplitude of the electrotonic potentials decreased exponentially within the auricle (Fig. 9a) and within the ventricle (Fig. 9b). No electrotonic potential, however, was recorded over the A-V boundary from the auricle to the ventricle and vice versa in spite of the many recordings along the boundary (Fig. 9). Moreover, an action potential induced in the auricle or ventricle did not cause any potential change in the other.

c) Mechanical interaction between the auricle and ventricle

When the A-V boundary of the regularly beating heart was tied by a fine thread and fixed with fine forceps to interfere with the mutual stretching, the auricle and the ventricle began to beat with individual rhythms. In the case

shown in Fig. 10a, the mean beat interval was 2.11 sec in the auricle and 2.81 sec in the ventricle, but the rhythm can be faster in the auricle or in the ventricle depending on the preparation. As the forceps were released, the auricle and the ventricle stretched each other again and the coordinated beats were recovered (Fig. 10a). These results suggested that the coordination of individual rhythms between the auricle and ventricle is mediated by mutual stretching.

This was further confirmed in the following experiments. The boundary between the auricle and ventricle which beat in a coordinated rhythm was tied at two portions and cut in the middle. After recovery from the effect of cutting (i.e. 30-60 min after the separation), the auricle and the ventricle began to beat in individual rhythms. When the auricle and ventricle were connected again by tying the threads left at the two portions, they mutually stretched and beat in a coordinated rhythm (Fig. 10b).

From the above results, the coordination of the beat rhythms between the auricle and ventricle was considered to be mediated by mutual stretching and not by electrical interaction.

4) Effects of a single brief stretch

The mechanical movements of both the auricle(s) and ventricle in the coordinated state were recorded simultaneously. Contraction in the auricle implies stretching in the ventricle and vice versa. The action potentials of the auricle and ventricle appeared alternately, thus alternate contractions were developed. As shown in Fig. 11, each contraction was seen with a certain time lag from the corresponding action potential. The time from the peak of the action potential to that of each contraction was measured to be 400-600 msec in the auricle and 500-700 msec in the ventricle. As mentioned above, these brief lasting stretches due to the contractions were considered to be the mediating factor to achieve the coordinated beats of the heart. In order to elucidate the function of the stretch, the effects of an artificial brief stretch on the beat interval and on the membrane potential were examined.

a) Alteration of electrical activity and beat interval

An isolated auricle or ventricle usually continues to beat with a regular rhythm. The effect of a brief stretch on the spontaneous electrical activity of the myocardium was examined intracellularly in the isolated ventricles. The myocardium exhibited periodic firing of the action potential preceded by a slow depolarizing potential (pacemaker potential). As shown in Fig. 13, a brief stretch applied to the myocardium altered the periodic changes of the membrane potential and exerted a negative

or a positive chronotropic effect on the beat interval depending on the phase of the application in the beat cycle.

A stretch of 500 msec in duration applied at a moment during the falling phase of the action potential caused prolongation of the beat interval compared to the preceding control interval (Fig. 13-1 and 13-2). As the applied stretch was progressively in a later phase, the degree of prolongation gradually increased until a certain point and then decreased. The maximum prolongation was induced by the stretch applied at the plateau of the action potential (Fig. 13-1).

Alterations of the membrane potential by the stretch is shown in Fig. 14a. The action potential increased in duration by elongation of the plateau and was followed by decreases in the maximum hyperpolarization and in the rate of rise of the pacemaker potential to the next action potential. (Fig. 14a). More or less of these features of the potential changes were observed with a stretch applied at any moment during the falling phase as described above and caused prolongation of the beat interval.

In contrast, stretch applied around the phase between the maximum hyperpolarization and the peak of the next action potential increased the rate of rise of the pacemaker potential (Fig. 14b) and caused shortening of the beat interval (Fig. 13-4 and 13-5). The turning point from interval prolongation to shortening was found at a point just before the maximum hyperpolarization (Fig. 13-3). As the applied stretch was progressively in a later phase of the periodic activity, the

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degree of interval shortening was gradually increased and after a certain point, it was decreased. The maximum shortening was induced by the stretch applied at the beginning of the pacemaker potential (Figs. 13-4 and 14b).

Thus, a brief stretch altered the beat interval in which it was applied. The following interval was usually not affected when the magnitude of the applied stretch was within ca 25 % of the preparation length. However, when the stretch was applied sufficiently close to the next action potential, a detectable prolongation was always seen in the following beat interval by slight elongation of the action potential duration (Fig. 13-5).

b) Membrane potential responses of the myocardium

The brief stretch applied to the ventricle in which the automatic activity was suppressed by application of 10^{-4}M ACh caused a small depolarization in the membrane potential of the ventricular myocardium (Fig. 15). When the stretches were applied repetitively, the depolarizing potentials were summated, then an action potential was evoked (Fig. 15a). The amount of the depolarizing potential was detected to be within a range of 0.4-5.0 mV with a slow decay of 1 - 10 sec by the stretch of 0.4-1.0 mm in magnitudes (Fig. 16). Moreover, in some preparations, there were cases where the depolarization last and/or developed even after the stretch was removed (Fig. 16b).

From the results, the stretch was suggested to possess depolarizing effects on the membrane potential to cause alteration of the spontaneous electrical activity.

c) PRC obtained by single brief stretches

A PRC is a plot of the phase shift versus the stimulation phase. To analyze the rhythmic characteristics of the auricle and ventricle, the brief stretches as stimuli were applied in a random order in the beat cycle to obtain a PRC. Examples of the PRC are shown in Figs 17 and 18. The control beat intervals were ranged within 2.45-4.82 sec in the auricle (8 preparations) and 2.01-4.60 sec in the ventricle (12 preparations). The curves are obtained to be biphasic, showing phase delay and phase advance. In all preparations, the stretch applied in the former phase of the cycle (ca 0-40 %) induced phase delay and the stretch in the latter phase (ca 40-90 %) caused phase advance. The stretch applied at 90-100 % induced a little phase delay in many preparations. Thus, the stretch can produce phase advance or phase delay as a function of the phase of the beat cycle in which it was applied. The PRCs of the auricle and the ventricle showed no remarkable difference which suggests that they both have similar rhythmic characteristics (Fig. 17). When the magnitude of the stretch was increased, the amount of phase shift became larger (Fig. 18). The maximum values of the phase delay and of the phase advance were ca +20 % and ca -30 %, respectively under the experimental conditions. With increasing duration of the stretch, the PRCs tended to shift to the left (advanced side) in many cases.

5) Entrainment of beat rhythm by repetitive brief stretches

When repetitive brief stretches were applied to isolated auricle or ventricle, the spontaneous rhythm changed and its frequency became equal to that of the stretches (stable entrainment) as long as the frequency of the stretches were not too different from that of the free-run beat (Fig. 19-2,3). By increasing the magnitude of the stretches, the stable entrainment could be induced even when the frequency of the stretches was different by more than 20 % from that of the free-run beat. On the other hand, when the frequency of the repetitive stretch was sufficiently different from that of the free-run, the beat frequency could not be stably entrained and altered periodically (incomplete entrainment, Fig. 19-1,4).

In both cases, the entrained beat frequency returned quickly to that of the free-run when the application of the stretches was terminated (Fig. 20).

a) Stable entrainment and phase-locking

In the state where the stable entrainment occurred, the action potential and the brief stretch were in a constant phase relationship (phase-locking). Four examples of phase-locking with different phase relationships are shown in Fig. 20. In the case where the frequency of the stretch was lower than that of the free-run beat, each stretch positioned just after the action potential (Fig. 20-2). As the frequency of the stretch was decreased, the locked phase of the stretch was at a more delayed

position within the period of ca 0-30 % of the free-run interval
(Fig. 20-1). In the opposite case where the frequency of the
stretch was higher, the stretch positioned before the action
potential within the period of ca 50-90 % (Fig. 20-3,4).
Furthermore, when the frequency of the stretch was close but
slightly longer than the free-run frequency, the constant phase
of the stretch was near the action potential (ca 90-100 %). The
stretches, however, were not locked around the middle of the
interval (ca 30-50 %).

In the course of getting to the stable entrainment,
different patterns of the beat rhythm alterations were observed
depending on the phase at which the first stretch was applied.
Fig. 21 shows the examples of the different patterns in two
entraining frequencies. Of these entraining frequencies, one of
them adjusts the beat frequency to that of the applied repetitive
stretches by slowing down (Fig. 21a-1,2) and the other, by
speeding up (Fig. 21b-1,2). In the course of slowing down, the
beat interval became prolonged when the first stretch was applied
at an early period of the beat interval (Fig. 21a-1), then
quickly entrained with only little changes in the phase
relationship. A different pattern was seen when the first
stretch was at a late period. The beat intervals were at first
shortened and then gradually prolonged beat by beat with gradual
shift of the phase relationship until stable entrainment was
achieved. Conversely, in the course of speeding up, similar
results but reversed patterns were obtained (Fig. 21b-1,2).

b) Incomplete entrainment and change in the phase relationship

When the frequency of applied repetitive stretch was much lower or higher than that of the free-run, stable entrainment was not established (incomplete entrainment, Fig. 22).

In the case where the frequency of the stretch was sufficiently low, the phase of the stretch gradually shifted toward the delayed position in the beat interval (Fig. 22-1). When the stretch was at a position in the early period of the interval (Fig. 17), the beat interval became longer (hence, became close to that of the stretch), and then, the phase relationship appeared fairly stable temporarily. However, this fairly stable state did not continue for a long time. At an instant, the phase relationship quickly changed. At this moment, the beat interval became remarkably short compared to that of the free-run. Then the phase of the stretch gradually returned into a position in the early period of the interval. According to above changes in the phase relationship, the beat intervals were prolongated and shortened to induce periodic alterations of the rhythm (Fig. 23a-2). When the frequency of the repetitive stretches was further lowered, the periodical interval alteration became more frequent (Fig. 23a-1).

On the other hand, in the case where the frequency of the stretches was sufficiently high, the phase of the stretch became gradually shifted toward the advanced position (Fig. 22-2). Similarly, the interval alteration was induced (Fig. 23b-1,2).

6) Beat interval alterations in the heart in situ

The auricle and the ventricle in the heart in situ beat
alternately with a regular rhythm (coordinated beat) in the state
where the ASW was continuously perfused into the pericardial
cavity. When the perfusion was stopped, the coordination
gradually disappeared. In the course of the coordination
disappearing, two types of periodical rhythm alterations were
noticed (Fig. 24). Fig. 24a shows a pattern where the
ventricular beat interval shortened, and the auricular interval
prolonged. A fairly stable state of the beat intervals was
temporarily achieved when the auricular and ventricular intervals
became close to each other. In this state, the phase relationship
between the auricular and ventricular beats altered gradually
with gradual shortening of the ventricular beat interval and
prolongation of the auricular interval. Following the stable
state, the auricular beat intervals largely prolonged
associated with fairly small interval shortenings of the
ventricle. During this time, the phase relationship between the
action potentials of the auricle and ventricle altered rapidly.
These two states appeared periodically and resembled the
phenomena of rhythm alteration shown in Fig. 23. Fig. 24b shows
the case where the auricular beat interval shortened and the
ventricular interval prolonged. The reverse pattern of rhythm
alterations in the auricle and ventricle occurred in the similar
manner.

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IV.   DISCUSSION

1) Beat rhythms in the intact heart

2) Interaction of activities in the heart

  a) Electrical interaction between two auricles

  b) Electrical isolation between the auricle and ventricle

  c) Mechanical interaction between the auricle and ventricle

3) Effects of a single brief stretch

  a) Change in beat interval

  b) Change in electrical activity

      and responses in membrane potential

  c) PRC obtained by single brief stretches

4) Entrainment by repetitive brief stretches

  a) Stable entrainment and PRC

  b) Incomplete entrainment and PRC

5) Mechanisms of coordination between

      the auricle and ventricle

  a) Achievement of the same beat frequencies

  b) Development of constant A-V delay

1) Beat rhythms in the intact heart

In the intact heart, the auricles and ventricle beat regularly and alternately (Fig. 2). When a current pulse which could evoke an action potential was applied to the auricle, the alteration of the beat intervals appeared not only in the auricle but also in the ventricle (Fig. 4a). Similarly, when the current was applied to the ventricle, alteration of the beat intervals appeared also in the auricle (Fig. 4b). In both cases, the alteration of beat rhythms gradually returned to be coordinated within several beats. These results suggest that there may be some sort of mutual interaction to coordinate the beat rhythms of the auricle and ventricle.

The auricle and the ventricle, each intrinsically possesses an automatic activity and has the ability to beat independently (TAKATSUKI, 1949). The rate of the intrinsic rhythm of the ventricle is always higher than that of the auricle in Dolabella (MATSUI, 1945). The present results of the oyster heart, however, suggest that such a finding is not always applicable. Under experimental conditions, the intrinsic rhythm is faster in the ventricle or auricle depending on the preparations. In the coordinated state, the case in which the intrinsic rhythm is faster in the ventricle has a tendency to take a short A-V delay, and the case in which the rhythm of the auricle has a faster rate is apt to take a long A-V delay (Fig. 3; UESAKA et al., 1987a).

With this result together with the curves shown in Fig. 5, it can be supposed that, in the case where the preparation has a

short A-V delay, the ventricular action potential produced during the former period of the auricular beat interval is in the position to shorten the auricular interval thus accelerating the auricular beat, while the auricular action potential is in the position which prolongs the ventricular interval thus inhibiting the ventricular beat oppositely. On the other hand, in the case with a long A-V delay, the similar phenomena can be obtained but the position of the auricle and the ventricle reversed (UESAKA et al., 1987a).

2) Interaction of activities in the heart

a) Electrical interaction between two auricles

The myocardia of the two auricles, joined together near the venous end were electrically coupled (Figs 6 and 7). The coordination of beat rhythms mediated by electrical interaction has been reported in the connected half-ventricles of the oyster (EBARA, 1964a,b; 1967; 1969). In the reports, when the connecting bridge of myocardia was thin (containing several myocardia), the halves of the ventricle showed mutual perturbation in the beat rhythms by a spread of electrotonic potential. When the bridge was fairly thick, however, the beat rhythms of both halves became synchronized.

The synchronized beat of the two auricles was considered to be achieved by the similar mechanisms as in the connected half-ventricles. The diameter of the connecting bridge between the

auricles was measured to be ca 300 μm, which is sufficiently thick compared with that of the ventricle prepared by Ebara. Furthermore, the space constant within the bridge was 0.7-0.9 mm which was the same within the auricle. These are considered to be sufficient to spread the activity for synchronization between the two auricles considering the length of the connecting bridge of ca 500 μm.

b) Electrical isolation between the auricle and ventricle

The synchronization of beating rhythms mediated by electrical interaction was found between the two auricles. However, it is unlikely that the coordination of rhythms between the auricle and ventricle is mediated through an electrical connection (Figs 8 and 9). The space constant of the ventricular myocardia of the oyster is reported to be 1.1 mm (IRISAWA et al., 1973a). In this study, it was 0.7-0.9 mm and 1.1-1.2 mm in the auricle and in the ventricle respectively, with intense electrical coupling within each part. However, no evidence of electrical connections was obtained between the auricle and the ventricle morphologically nor electrophysiologically. On the other hand, Kuwasawa (1979) has shown in Dolabella that the A-V valve has characteristics of dominant pacemaker of the ventricle. Myocardia within the A-V valves of the oyster heart were electrically coupled to those of the ventricle, but not to the auricle (Fig. 8). Thus, the auricle and the ventricle of the oyster heart are electrically isolated and neither possibility of a conduction pathway nor of

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an electrical connection between them has been found.

c) Mechanical interaction between the auricle and ventricle

In the heart of Helix (WILLEMS, 1932) and Dolabella (MATSUI, 1945), the auricle and ventricle were suggested to interact mechanically. In oyster, the coordination of beat rhythms between the auricle and ventricle was with-held when the mutual stretches are interfered (Fig. 10a), whereas it could be restored by connecting the auricle and the ventricle by tying a fine thread (Fig. 10b). These indicate that the coordination is maintained, at least when the mechanical interaction is present. Conclusively, it can be stated in the oyster heart that the interaction to coordinate the individual intrinsic beating rhythms of the auricle and ventricle must be mediated by the mutual stretches without any direct interaction in their electrical activities.

3) Effects of a single brief stretch

a) Change in beat interval

The molluscan heart responds to continuous stretch by increasing the beat frequency (KRIJGSMAN and DIVARIS, 1955; HILL and WELSH, 1966; JONES, 1983). In Dolabella, the beat frequency of the heart increases while the muscle fibers of the ventricle are continuously stretched (MATSUI, 1961; MATSUI et al., 1961; NOMURA, 1963). In oyster heart as well, continuous stretch

increases the beat frequency (TAKATSUKI, 1949; UESAKA et al., 1987b). Thus, the effect of continuous stretch is stated to cause a positive chronotropic effect which could control the cardiac output.

In contrast to the continuous stretches, a brief stretch applied to the isolated auricle or ventricle of the oyster resulted in more complex changes in the beat interval (Fig. 13). When the stretch was applied during the former period (0-40 %) in an interval, a prolongation of the interval occurred. But when the stretch was in the latter period (40-90 %), the interval was shortened. Thus, the effect was to cause not only a positive but also a negative chronotropic effect depending on the phase where the stretch was applied. Furthermore, the change was usually obtained in the interval in which the brief stretch was applied, although a little change appeared in the following interval when the stretch was applied close to the next action potential.

From these considerations, the effect of brief stretch seems to imply the mechanisms which control the auricular and ventricular beats to be coordinated.

b) Change in electrical activity and responses in membrane potential

Change of electrical activity by continuous stretch has been recorded intracellularly in single muscle bundles of the Dolabella ventricle (NOMURA, 1963) and extracellularly in strips of Helix ventricle (ALMQVIST, 1973). They found that the stretch increased the rate of rise of the pacemaker potential and

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resulted in increase in beat frequency. And Nomura (1963) also found that the plateau of action potential increased in duration by stretching.

In this study, a brief stretch applied to the oyster ventricle caused different changes on the membrane potential of the myocardium depending on the phase of its application and caused prolongation or shortening of the beat interval (Figs. 13 and 14). When the stretch was applied during the falling phase of the action potential, the plateau of action potential was elongated. Though the maximum membrane potential was decreased, it was followed by a decrease in the rate of rise of the pacemaker potential to the next action potential and resulted in interval prolongation (Fig. 14a). Contrarily, a stretch applied during the pacemaker potential increased the rate of rise of the pacemaker potential and resulted in shortening of the beat interval (Fig. 14b).

These potential alterations and the resulting interval changes by the brief stretch were very similar to the effects of a brief electrical stimulation (EBARA, 1967: EBARA and SATO, 1971), as well as to the effects of small potential propagated electrotonically (EBARA, 1964b, 1966, 1969). These suggest that the brief stretch may induce depolarizing effects on the membrane potential of the myocardium. In fact, a small depolarizing potential with slow decay was obtained by the brief stretch in the isolated ventricle in which its spontaneous activity was suppressed by application of 10^{-4} M ACh (Fig. 15 and 16). The slow decay of the potential is thought to

be the cause of prolongation of the next interval when the stretch was applied sufficiently close (90-100 %) to the next action potential (Fig. 13-5).

c) PRC obtained by single brief stretches

The beat rhythm of the auricle and the ventricle showed phase shift by a single brief stretch with phase delay or advance, and their PRCs were remarkably alike (Fig. 17). This implies that the two are similar electrically-oscillating organs having automatic activity.

The PRCs obtained by application of the stretch resembled those obtained by application of depolarizing currents (EBARA, 1967; EBARA and SATO, 1971). Hence, brief stretch and depolarizing currents have similar effects on the cell membrane in relation to the coordination found in the oyster heart. On the other hand, some differences between the above mentioned PRCs were as follows. A current applied at the action potential (phase 0 or 100 %) showed no phase shift whereas a brief stretch tended to cause a slight phase delay (Figs 17 and 18), i.e. the PRC was shifted a little (within 10 %) toward the advanced side (left side). Secondly, the turning point from phase delay to phase advance was reported to be at the phase where the current was applied just after the maximum hyperpotential (EBARA, 1967; EBARA and SATO, 1971), whereas the point was seen just before instead of after when the stimulation was a brief stretch, thus a little time difference was found. Hence, the effect of stretch was suggested to take more time to appear upon observation. This

shift of the PRCs became more evident with longer durations of the stretch (500 msec).

As shown in Fig. 5, the auricle responded to the extra action potential evoked in the ventricle by prolongation or shortening of the beat interval depending on the A-V delay. The same response was obtained also in the reverse direction, i.e. from the auricle to the ventricle. The relation between the change of beat interval and the A-V delay was biphasic showing interval prolongation and shortening, and resembled the PRCs obtained by the brief stretches (Figs 17 and 18). However, the response to the extra action potential was delayed about 500-700 ms compared with that of the brief stretches. The delay was considered as the time taken from the peak of the action potential to that of its corresponding contraction (400-600 msec in the auricle, 500-700 msec in the ventricle: Fig. 11).

4) Entrainment by repetitive brief stretches

a) Stable entrainment and PRC

Stable entrainment of the beat rhythm by the repetitive stretches was established when the frequency of the stretch was not so much different from that of the free-run (Figs 19, 20 and 21). In the entrained state, the phase of the stretch was locked in the period of 90-100 % or 0-30 % of the free-run beat interval when the frequency of the stretch was lower (Figs 20-1,2 and 21-1,2), and in the period of 50-90 % when the frequency of the

stretch was higher (Figs 20-3,4 and 21-3,4). These locked phases coincide well with the phase where phase delay or advance was induced as shown in the PRCs (Fig. 17). With increasing or decreasing the frequency of the stretch, the locked phase of the stretch shifted toward the phase inducing more phase shift in the PRCs (cf. Figs 17 and 20). From these findings, it is strongly suggested that the state of stable entrainment might be regarded as a train of single phase responses.

This was further confirmed from the results of beat interval changes in the course of getting to the stable entrainment (Fig. 21). In the case where the frequency of the stretch was lower than that of the free-run and the first stretch was applied at the position of phase delay, the beat interval was smoothly entrained without remarkable change in the phase relationship (Fig. 21a-1). On the contrary, when the first stretch was at the position of phase advance, the beat interval became even shorter than that of the free-run in spite of the attempt to lower the frequency of the beat (Fig. 21a-2). Opposite but similar changes of beat interval were obtained according to the phase of the first stretch when the frequency of the stretch was higher than that of the free-run (Fig. 21b-1,2).

b) Incomplete entrainment and PRC

When the frequency of repetitive stretch was much lower or higher than that of the free-run, periodic interval alterations were obtained instead of stable entrainment (Figs 22 and 23). In these cases, a gradual shift of the phase relationship was

observed according to the difference between the two frequencies (Fig. 22). Because the phase relationship might determine the interval length according to the PRC, the frequency of the periodic interval change is suggested to be related to the difference between the two frequencies.

The amount of interval alteration in each state could be predicted from the PRCs. During the shift of the phase relationship, when the stretch was applied in a position of phase delay in the PRC, the corresponding interval prolonged by an amount indicated by the PRC. Oppositely, when the stretch was situated in the phase of advancing position, the beat interval shortened by the amount shown in the PRC. Thus, the range between the maximum prolongation and shortening could be expected from the range of the maximum phase shift indicated in the PRC at a given magnitude and duration of stretch.

From the above considerations, it can be conclusively stated in general that: 1) The frequency of the periodic interval alterations might be a function of frequency differences between the free-run and repetitive stimuli, i.e. the stretches. 2) The range of the interval alterations is related to the intensity of the stimuli rather than the frequency differences. Whether the frequency is higher or lower than the free-run does not make any difference.

5) Mechanisms of coordination between
the auricle and ventricle

a) Achievement of the same beat frequencies

In the intact heart, the auricle and ventricle beat
alternately with a regular rhythm. The mechanism in achieving
the coordinated beat may be expected from the results of
entrainment as follows.

The ventricle is found to possess characteristics of being
entrained by repetitive stretches. In the state of stable
entrainment, the ventricle turned out to have the same beat
frequency as the stretches. The stretch, in the intact heart, is
produced by the repetitive contraction of the auricle.
Therefore, the ventricle is regarded to be entrained by the
auricular contraction in the intact state. On the other hand,
the auricle might possess similar characteristics as the
ventricle (Fig. 17). This indicates that the auricle is also
reversely entrained by the ventricular contractions. Hence,
mutual entrainment can be expected in the intact heart. Thus,
the frequencies of the auricle and the ventricle became equal
though they were intrinsically different. In fact, in the course
of the coordination disappearing (Fig. 3), the beat interval
prolongation of the auricle was associated by the ventricular
interval being shortened (Fig. 24a), and conversely the
shortening of the auricular beat interval was associated by the
prolongation of the ventricular interval (Fig. 24b). These
corresponded interval alterations were observed in many

preparations.

The amount of interval alteration of the auricle was much larger than that of the ventricle (Fig. 24). This result seems to correspond to the fact that the amount of the phase shift became large when the applied stretch was increased in magnitude or in duration (Fig. 18). Because the ventricle contracted more powerfully than the auricle and moreover because one of the auricles was removed, the auricle must have been largely stretched.

Conclusively, in the mechanism to establish the coordinated beat of the oyster heart: the intrinsic frequencies of the auricle and ventricle should not be too different, and each of them must have the ability to produce sufficient power of contraction. The automatic activities of the auricle and ventricle might be altered by the mutual stretches to change the beat interval which could be predicted from the PRCs. In this instance, the faster part (auricle or ventricle) may be affected to be slowed-down by the slower part, whereas the slower part is speeded-up by the faster one, depending on the achieved phase relationship between the two parts (mutual entrainment). Thus, in the coordinated state, the heart as a whole is considered to beat with a rhythm that was formed as a result of mutual entrainment rather than to beat with the intrinsic rhythm of the auricle or the ventricle itself.

b) Development of constant A-V delay

The A-V delay is the time lag from the peak of the auricular

action potential to that of the ventricular one. According to this lag, the blood is effectively pumped-out through the auricle to the ventricle. In the oyster, the A-V delay was reported to be widely ranged (0.5-2.3 sec) among preparations when the beat interval was 1.8-2.8 sec (UESAKA et al., 1987a). But the delay was constant in one preparation when the experimental condition was fixed. The mechanisms in which this constant A-V delay is established might be considered from the results of the entrainment.

When the ventricular rhythm was entrained by applied repetitive stretches, a constant phase relationship between the action potential and the stretch was established (Fig. 20 and 21) according to the difference in frequencies between the two. In this state, the locked phase of the stretch could be predicted from the PRC (Fig. 17). The shape of the PRC, furthermore, depends on the magnitude of the stretch (Fig. 18). Therefore, the locked phase of the stretch could be predicted when both the frequency differences and the magnitude of the stretch are known. In the intact heart, the auricle and the ventricle are considered to be entrained by each other in the coordinated state as mentioned above. So the relationship between the action potential and the applied stretch could be regarded as the correspondence between the auricular (or ventricular) action potential and the ventricular (or auricular) contraction. This suggests that the phase relationship between them might be dependent on the degree of the difference between the intrinsic frequencies and the power of the contraction of the auricle and

the ventricle. On the other hand, as well known, the timing of the contraction and the action potential is directly coupled. In the oyster, it takes 400-600 msec in the auricle and 500-700 msec in the ventricle from the peak of the action potential to the corresponding contraction. The delay from action potential to contraction must be one of the factors which determines the A-V delay. Therefore, by shifting the locked phase of the stretch to the advanced side by this delay time, the phase relationship between the action potentials of the auricle and the ventricle could be predicted.

In conclusion, the A-V delay of the oyster heart can be considered to be the phase relationship between the action potentials of the auricle and the ventricle in the state of mutual stable entrainment (i.e. the coordinated state). It was dependent on both the degree of the difference in the intrinsic beat frequencies between the auricle and the ventricle and the power (both magnitude and duration) of contraction. Further, the time delay from the action potential to the contraction is thought to be one of the factors.

V. SUMMARY

1) Coordination of activities between the auricle and ventricle

2) Mediating factors of the interaction

3) Effects of a brief stretch

4) Mechanisms of coordination between the auricle and ventricle

1) Coordination of activities between the auricle and ventricle

a) Coordination of activities between the auricle and ventricle was studied in the heart of oyster.

b) Some lines of evidence showed that the auricle and ventricle possess individual intrinsic rhythms, whereas they beat coordinately with a constant auriculo-ventricular (A-V) delay. The A-V delay widely varied among preparations.

c) In the coordinated state, the extra action potential evoked electrically in the auricle prolonged or shortened the beat interval of the ventricle depending on its moment in the ventricular cycle. Oppositely, the extra action potential evoked in the ventricle also changed the auricular beat interval similarly.

d) The mutual interaction was suggested to produce the coordination of beat rhythms between the auricle and ventricle.

2) Mediating factors of the interaction

a) The two auricles beat synchronously because there is an electrical pathway in the connecting bridge which contains some myocardial bundles.

b) No electrical connection was found between the auricle and ventricle.

c) The coordination between the auricle and ventricle was maintained even in the state where only mutual stretching was kept.

d) It was assumed that the interaction to coordinate the beat rhythms of the auricle and ventricle is mediated by mutual stretching without any direct interaction in their electrical activities.

3) Effects of a brief stretch

a) A brief stretch altered the periodic membrane potential changes of the myocardium and caused prolongation or shortening of the beat interval depending on the phase of its application.

b) The beat interval was prolonged when the stretch was applied during the plateau phase of the action potential. On the contrary, the interval was shortened when the preparation was stretched during the phase of pacemaker potential.

c) The turning point from interval prolongation to shortening was just before the maximum hyperpolarization of the action potential.

d) These effects of the brief stretch were considered to be fundamentally produced by the depolarizing membrane potential response of the myocardium.

4) <u>Mechanisms</u> <u>of</u> <u>coordination</u> <u>between</u> <u>the</u> <u>auricle</u> <u>and</u> <u>ventricle</u>

   a) The phase response curves (PRCs) of the auricle and
      ventricle by single brief stretches were very similar to
      each other showing biphasic property of phase delay and
      advance.

   b) By increasing the magnitude of the stretch, the amount of
      phase shift increased.

   c) The beat rhythm could be entrained by repetitive stretches
      when its frequency was not so much different from the free-
      run beat frequency (stable entrainment).

   d) In the state of stable entrainment, the moment of applied
      stretches was locked in a specific phase in the beat
      interval (phase-locking).

   e) The locked phase in the entrained state depended on the
      frequency differences between the applied stretches and
      free-run, and coincided with the phase which could be
      predicted from the PRC.

   f) When the frequency of the stretch was very different from
      that of the free-run beat, the beat rhythm could not always
      be entrained and altered periodically (incomplete
      entrainment).

   g) In the state of incomplete entrainment, alteration pattern
      of the beat intervals depends on the shift of the phase
      relationship. The phenomena resembled the uncoordinated
      auriculo-ventricular beats in which the auricle and
      ventricle interact with each other in the uncoordinated
      state.

h) It can be concluded that the coordinated beat rhythm of the oyster heart is achieved as the result of mutual stable entrainment between the auricular and ventricular beat rhythms, the intrinsic rates of which is considered to be different from each other.

i) The A-V delay is regarded as the phase relationship between the auricular and ventricular action potentials during mutual stable entrainment.

ACKNOWLEDGMENTS

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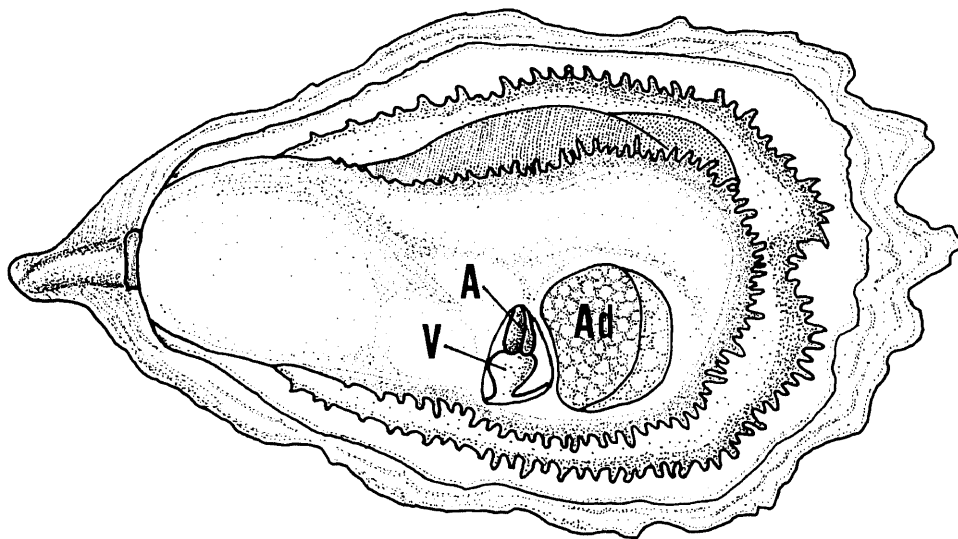
VIII. FIGURES AND LEGENDS

Fig. 1. Schematic illustration of the oyster. The right valve, parts of the mantle and the pericardial wall were removed to expose the heart; A, Auricles; V, Ventricle; Ad, Adductor muscle.
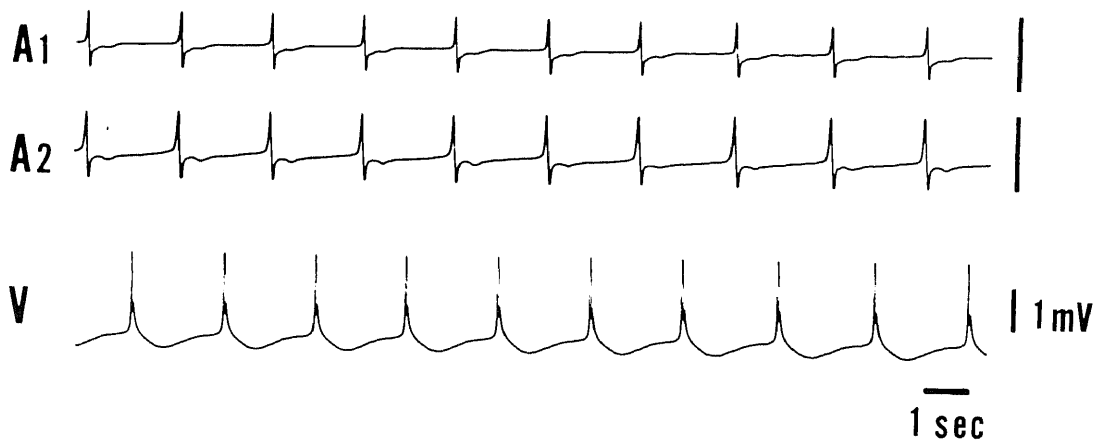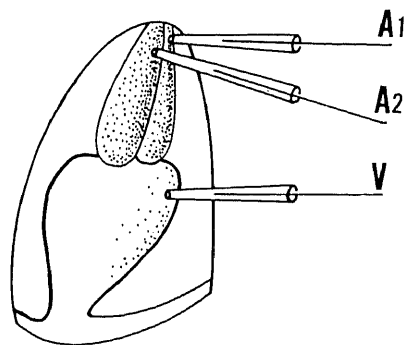
Fig. 2. Spontaneous rhythmic activities recorded simultaneously from two auricles (A_1, A_2) and one ventricle (V) with continuous perfusion of ASW into the pericardial cavity. Records were obtained extracellularly from the auricles and the ventricle of the intact heart. Electrical activities between the auricles and the ventricle show a constant A-V delay, and that of the auricles is almost synchronized.

a

A

V

b

A

V

1 min   1 sec
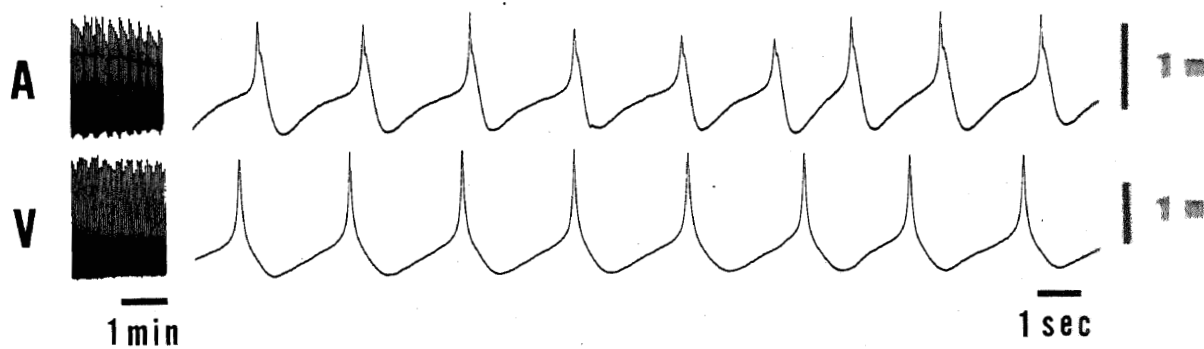
1 =

1 =
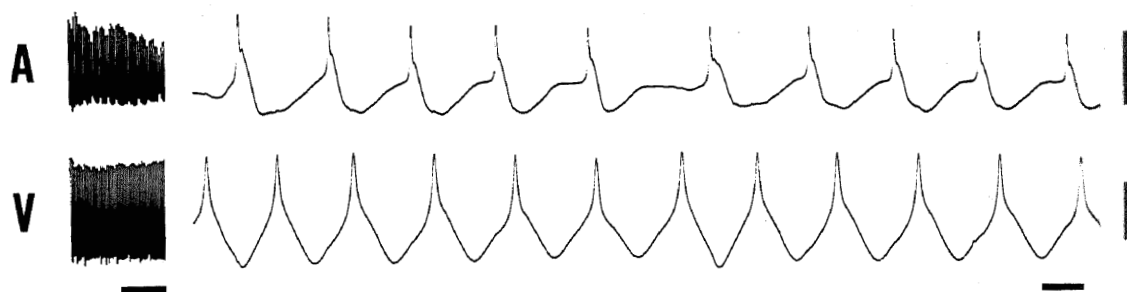
Fig. 3. Spontaneous activities recorded simultaneously from one auricle (A) and one ventricle (V) several minutes after the stoppage of perfusion. Records show two cases of change in beat interval. (a): As auricular beat interval becomes slightly longer than that of the ventricular one, the A-V delay turns out to be shorter, beat by beat. Finally, the auricular beat manifests and succeeds the ventricular one. At this instant, the auricular beat interval becomes remarkably long and the ventricular one slightly short. (b): As auricular beat interval becomes slightly shorter than that of the ventricular one, the A-V delay turns out to be longer, beat by beat. Finally, the auricular beat precedes the ventricular one. At this instant, the auricular interval becomes remarkably short and the ventricular one slightly long. These remarkable interval alternations appear periodically as shown in the slow records (left traces in each column).
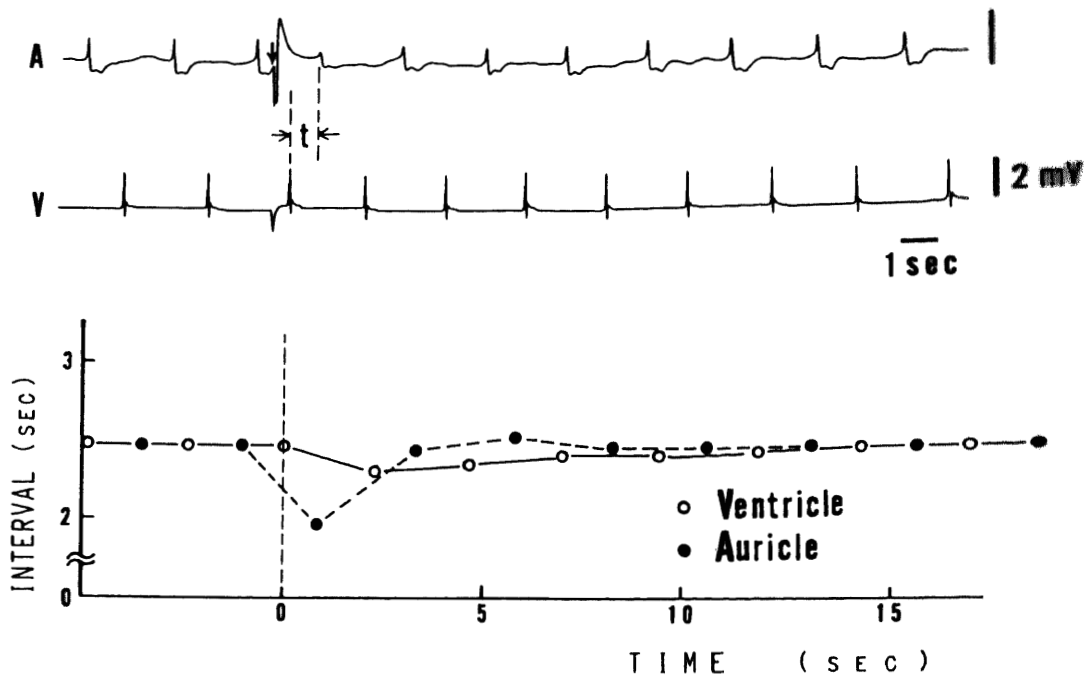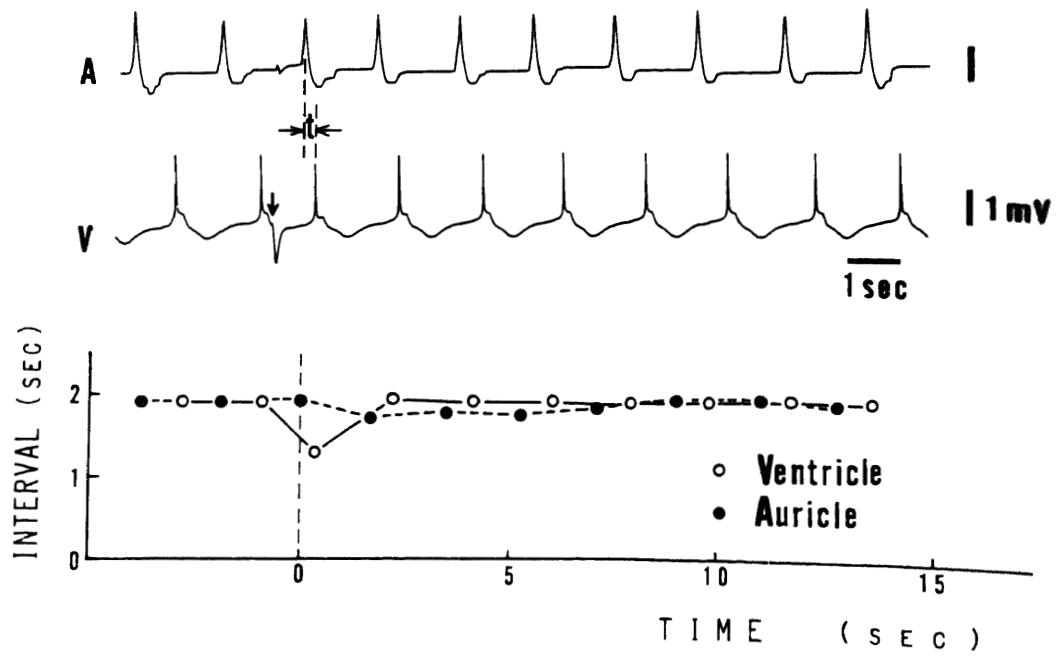
a

A

V

$|2\,mV$

$\overline{1\,sec}$

○ Ventricle
● Auricle

INTERVAL (SEC)

TIME (SEC)

b

A

V

$|1\,mV$

$\overline{1\,sec}$

○ Ventricle
● Auricle

INTERVAL (SEC)

TIME (SEC)

Fig. 4.   Beat interval alternations in the auricle (A) and ventricle (V) obtained by artificial changes in the beat interval by application of cathodal current (50 msec, 2V). The arrow indicates the moment of current application and t indicates the A-V delay. a; beat interval of the auricle was changed. b; beat interval of the ventricle was changed. The beat intervals of both the auricle and ventricle are plotted in each lower graph; the beat intervals in the ordinate and the time in the abscissa. Note that the interval changes were also seen in the unstimulated part.
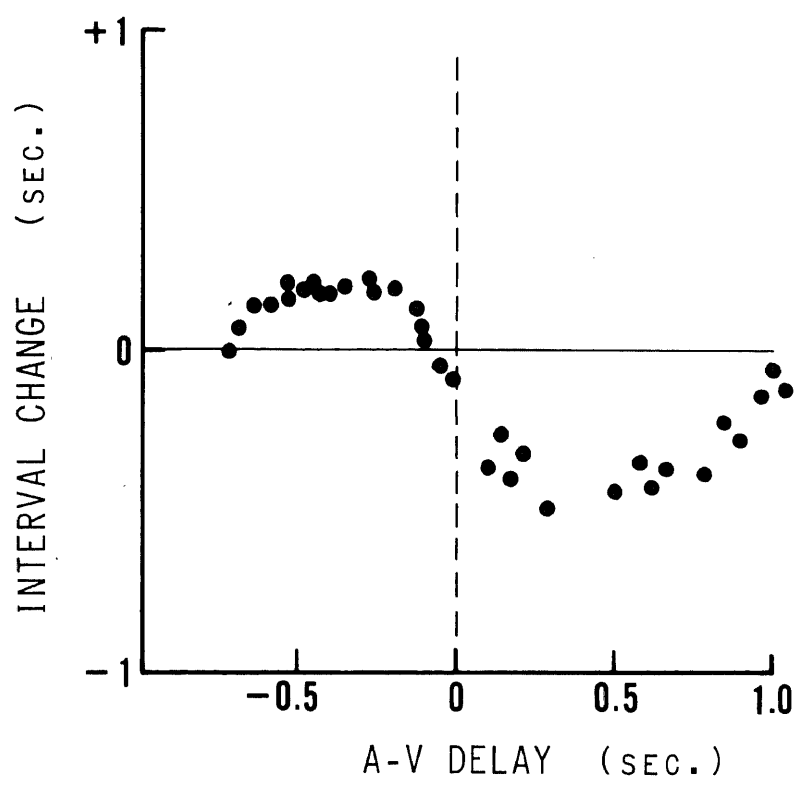
Fig. 5. Changes in the auricular beat interval versus A-V delay. The control beat interval was measured as the length of time during which no extra-action potential of the ventricle was evoked. The interval change (ordinate) shows the difference between the control interval and the altered interval. The A-V delay (abscissa) shows the time difference between the auricular action potential and the ventricular extra-action potential. Time zero (broken line) indicates the moment when the auricular action potential appeared.
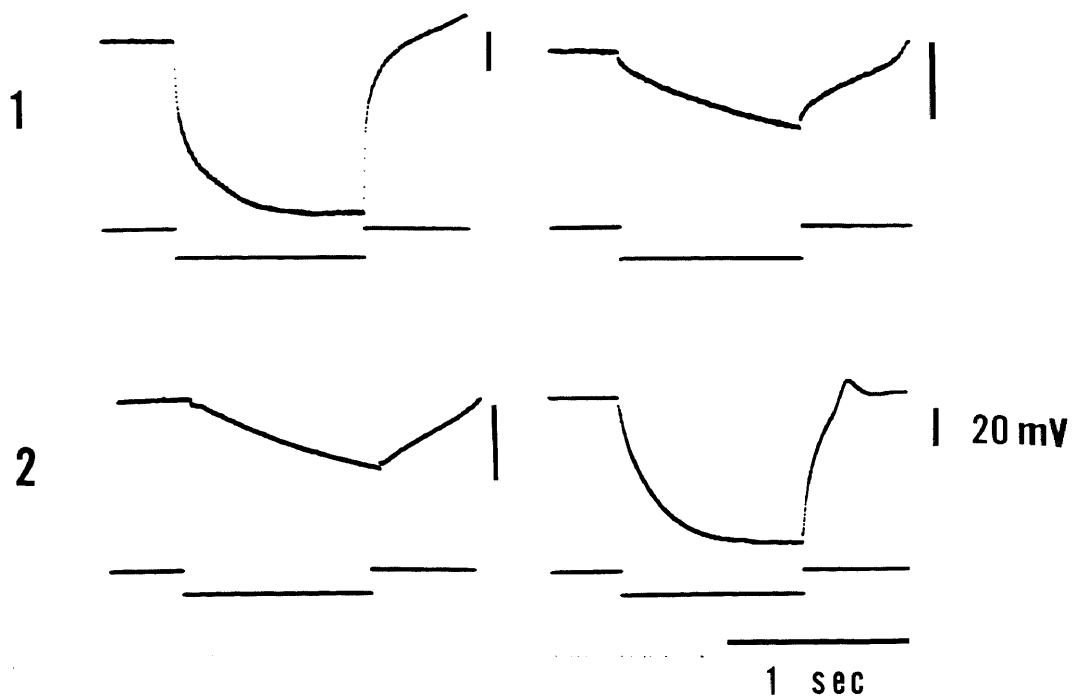
a

A₁ 1 2 A₂

b

1

2

20 mV

1 sec
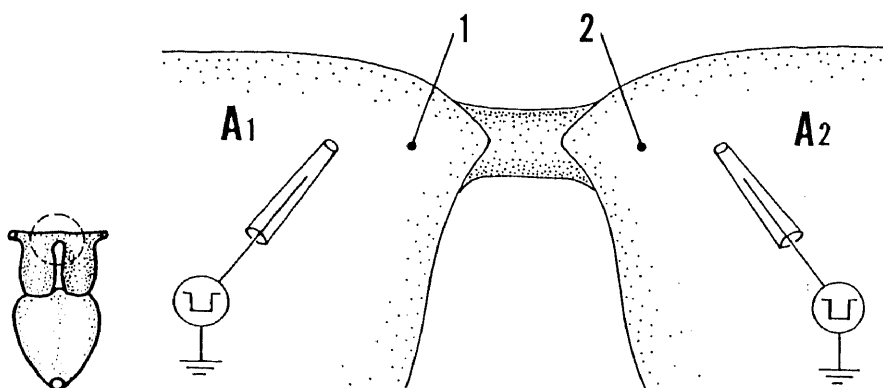
Fig. 6. Electrical connection between the two auricles. a: Schematic illustration of the opened auricles connected by the bridge. A_1, A_2; the auricles. Anodal current pulses were applied through the suction electrodes placed on the auricles and the recording electrode was inserted at points indicated by 1 and 2. b: Electrotonic potentials recorded intracellularly. Pulse was applied to one of the auricles (A_1) in the left recordings and to the other auricle (A_2) in the right recordings. The resting membrane potential was 58-60 mV.
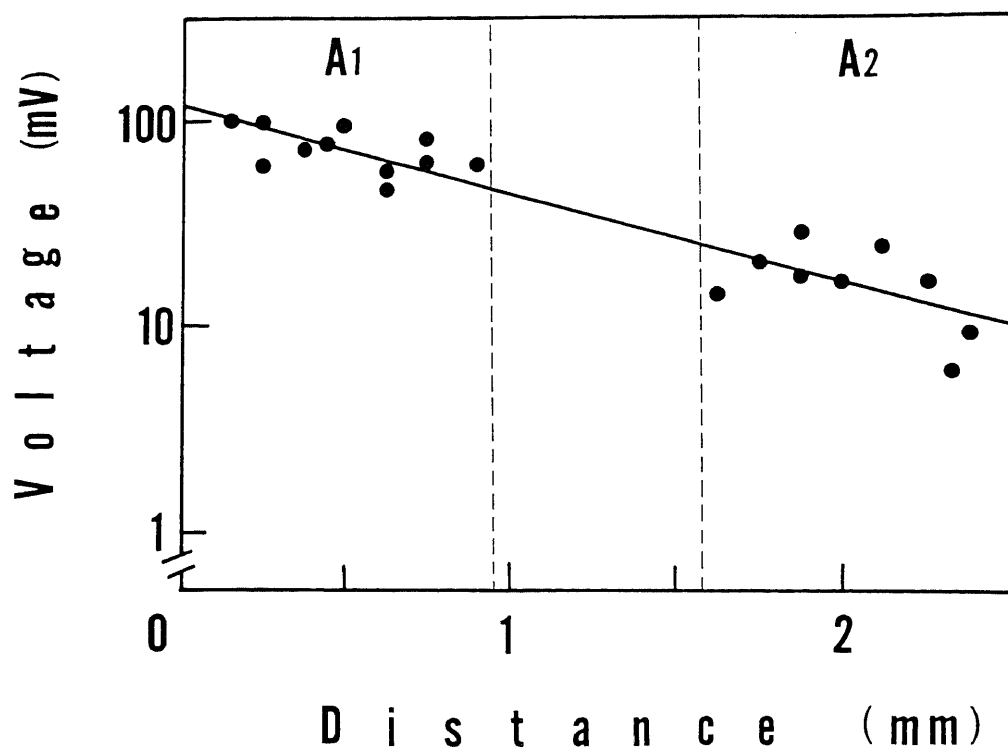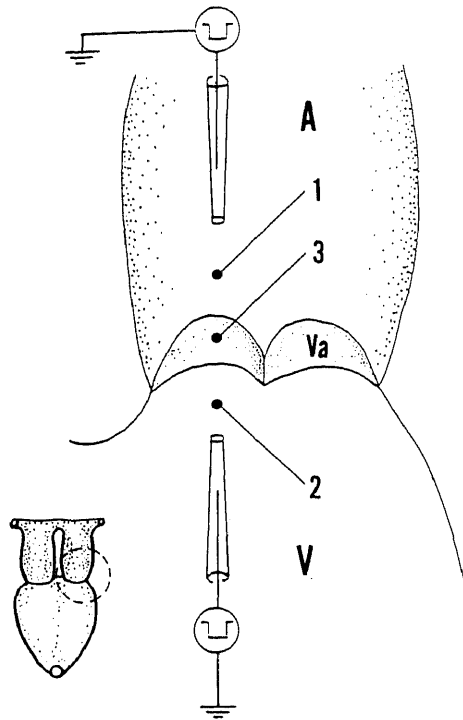
Fig. 7. Spread of electrotonic potentials. Distance from the stimulating electrode is shown in the abscissa and the log of the potential, in the ordinate. A_1 and A_2 are within the auricles, and the area between the vertical broken lines indicates the connecting bridge between the auricles. Notice that exponentially decaying electrotonic potentials were obtained beyond the connecting bridge. The line was drawn by the least square method.
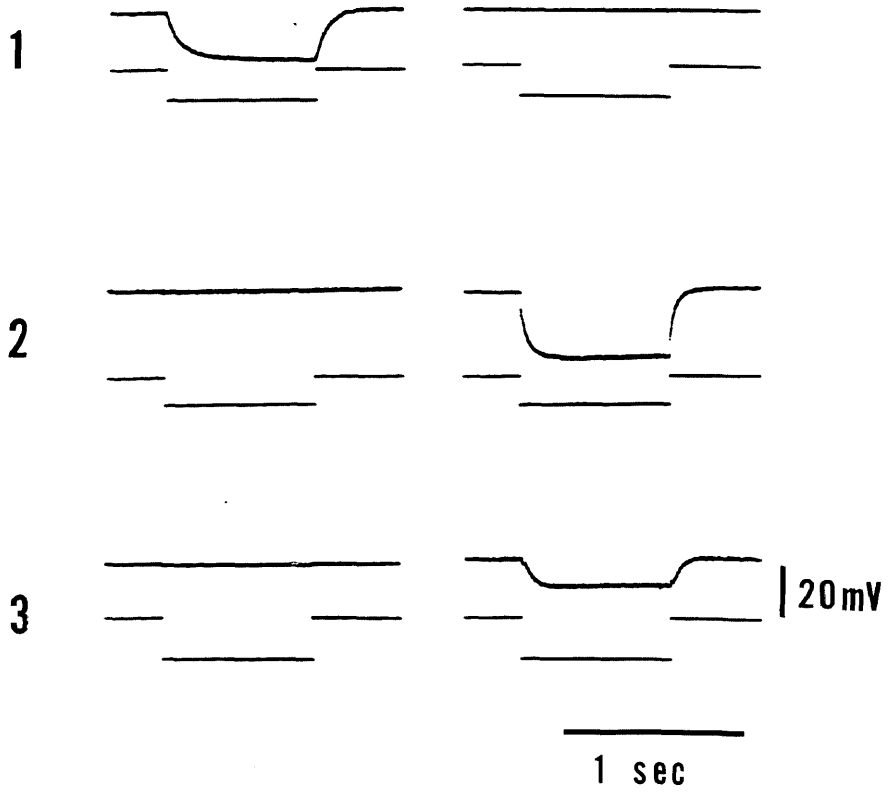
a

A

Va

V

b

1

2

3

20mV

1 sec

Fig. 8. Electrical connection around the A-V boundary. a: Schematic illustration of an opened heart. A;auricle, V;ventricle, Va;A-V valve. Anodal current pulses were applied through the suction electrodes placed on the auricle and the ventricle, and the recording electrode was inserted at points indicated by 1-3. b: Electrotonic potentials recorded intracellularly. Pulse was applied to the auricle in the left recordings and to the ventricle in the right. Records 1-3 correspond to the positions shown in a. The resting membrane potential was $42.7 \pm 3.4 mV$.

a
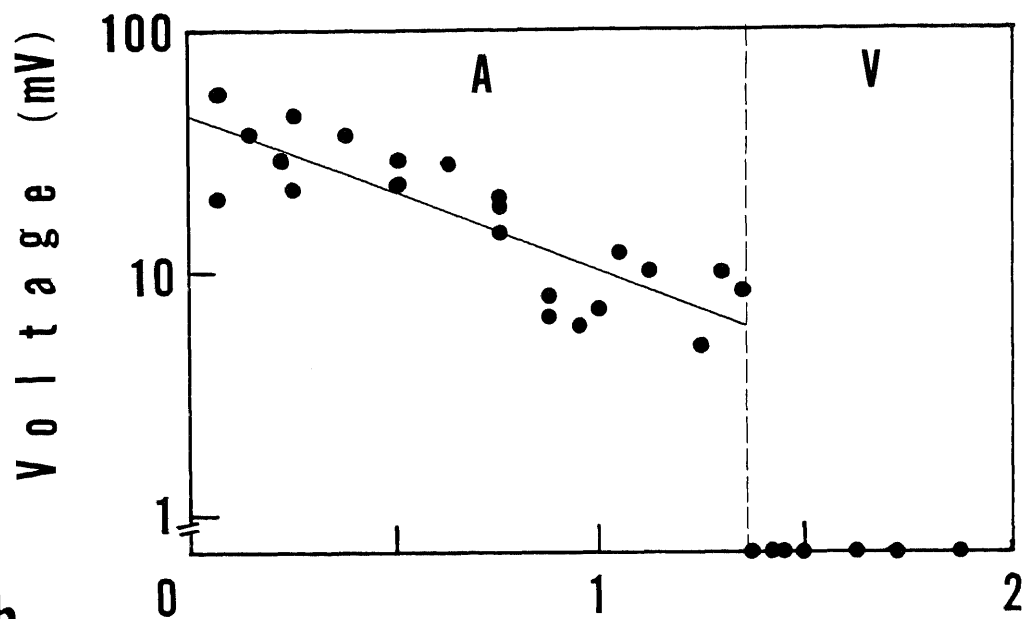
b
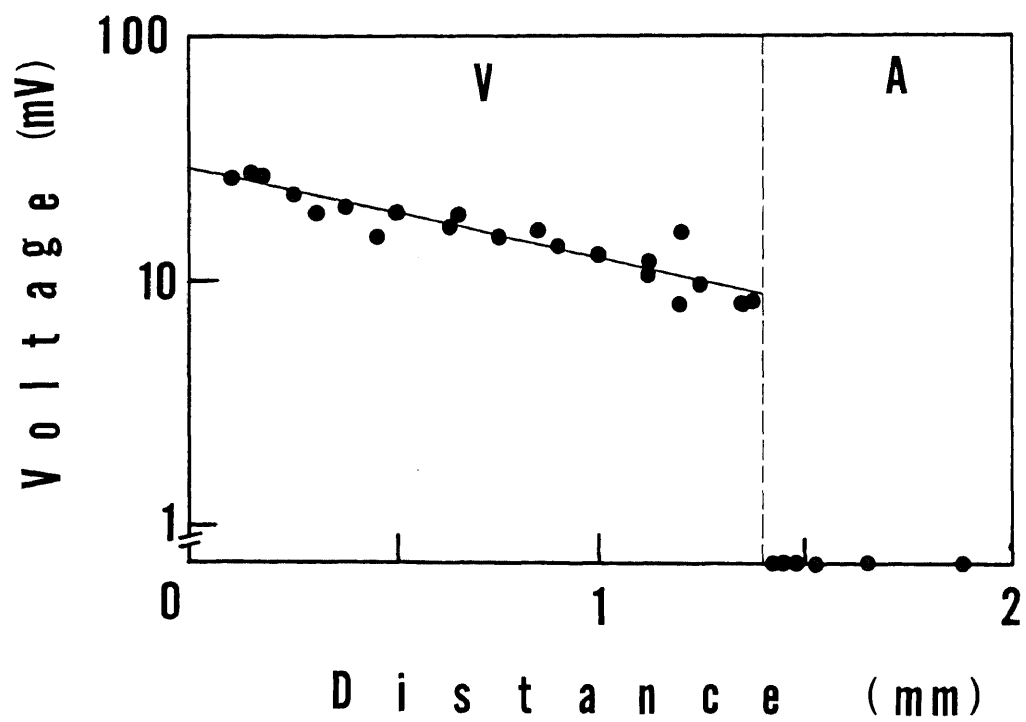
Fig. 9. Spread of electrotonic potentials. Distance from the stimulating electrode is shown in the abscissa and the log of the potential, in the ordinate. A is within the auricle, V is within the ventricle and the vertical broken line indicates the A-V boundary. a: Spread of electrotonic potential from the auricle. b: Spread from the ventricle. Notice that no electrotonic potential was obtained over the A-V boundary in both cases. The lines were drawn by the least square method.

a

A

V

A

V

A

V

1mV

3 sec

b

A

V

A

V

A

V

1mV

3 sec
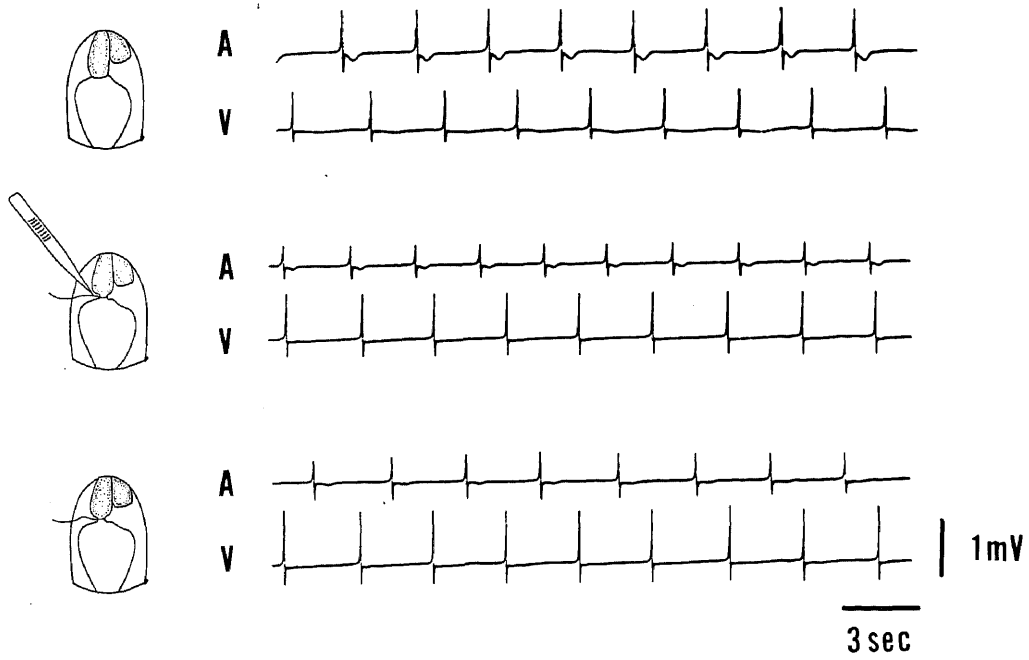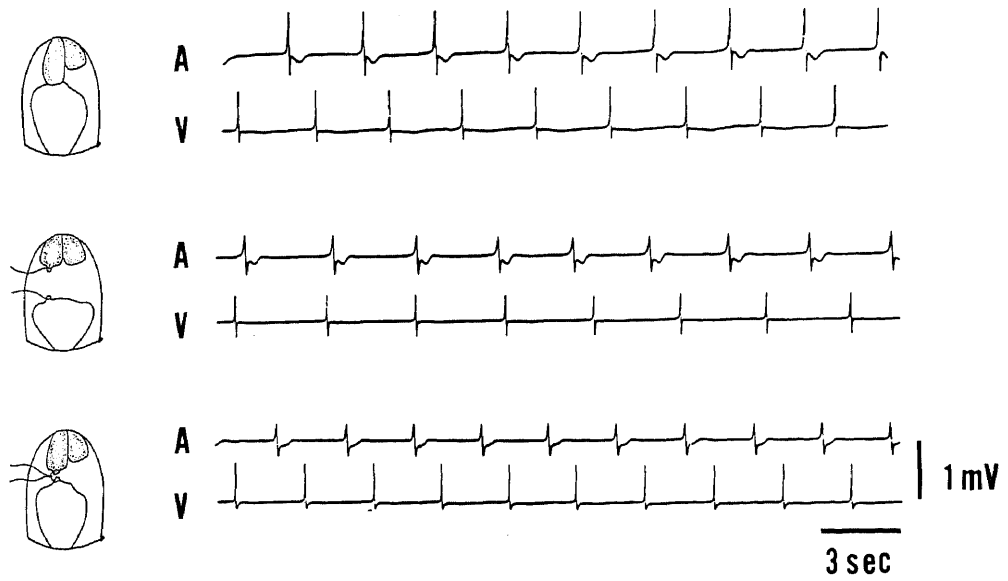
Fig. 10. The role of mutual stretching in the coordination of rhythms between the auricle and ventricle. Illustrations on the left show the heart in the pericardial cavity. One of the auricles was cut at the A-V boundary. Right traces show electrical activities recorded extracellularly from the auricle (A) and the ventricle (V). a: A fine thread was tied at the A-V boundary and fixed with forceps, then the forcepts was released. b: The auricle and ventricle were each tied and cut in the middle. Notice when the threads were connected, the coordinated beats recovered.

A

mec

V

1 mV

1 sec
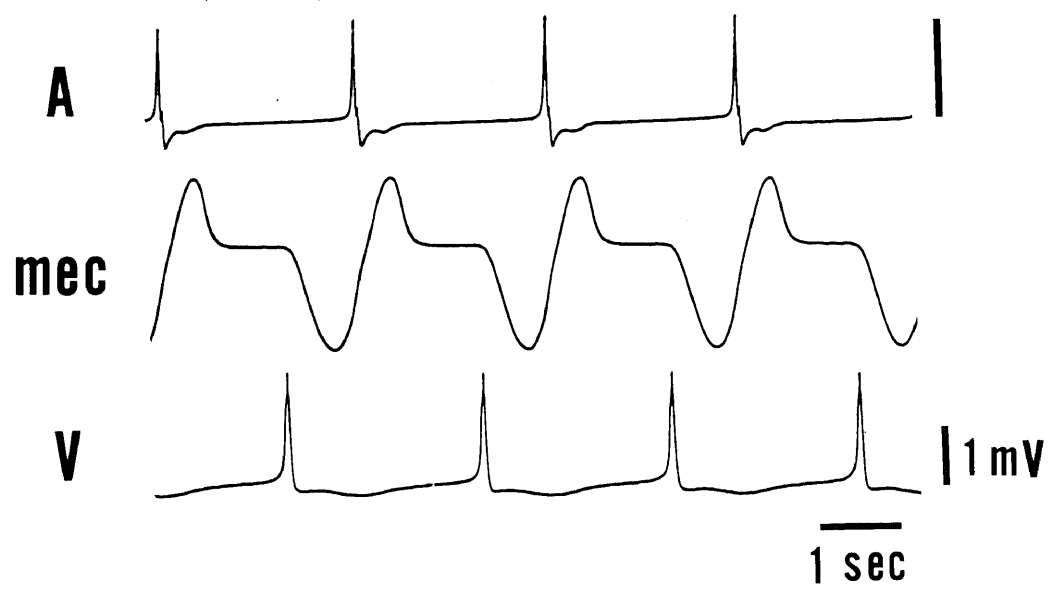
Fig. 11. Mutual stretching of the auricle and ventricle in the
coordinated heart.   A, Auricular action potential; V, Ventricular
action potential; Mec, Mechanical contractions of the auricle
(upward direction) and ventricle (downward direction).
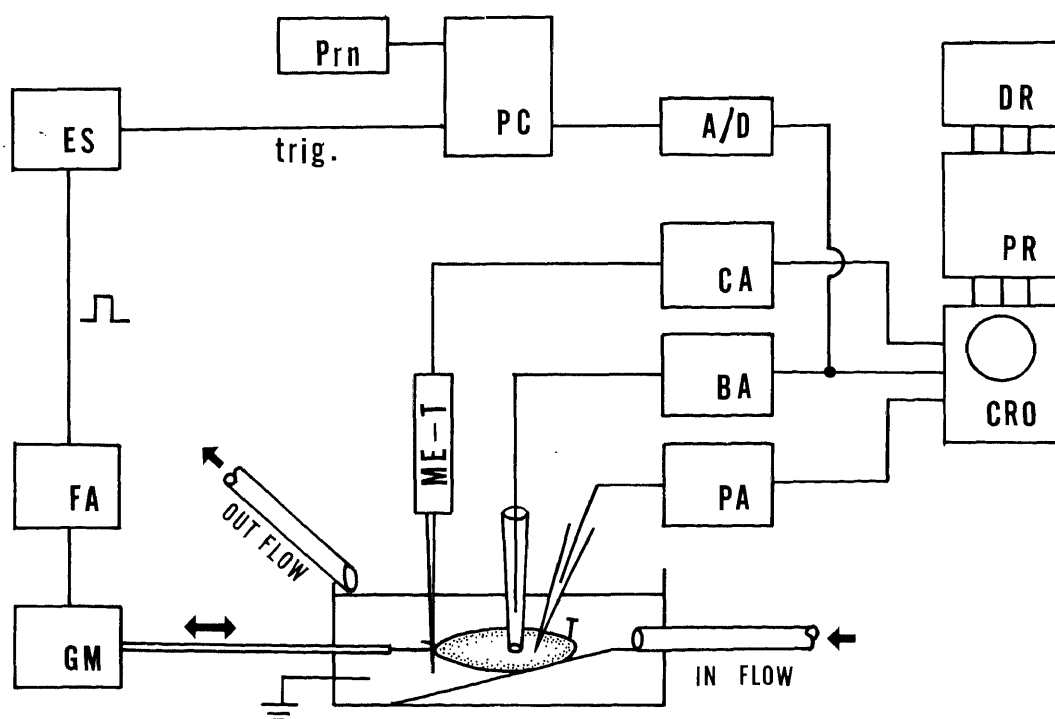
Fig. 12. Schematic representation of the experimental arrangement; ME-T, mechanoelectro-transducer; CA, carrier amplifier; BA, biological AC amplifier; PA, pre-amplifier; CRO, cathode ray oscilloscope; PR, pen-writing oscillograph; DR, magnetic data recorder; A/D, analogue to digital converter; PC, micro-computer; Prn, dot-impact printer; ES, electronic stimulator; FA, feed-back amplifier; GM, galvanometer.

1 110

2 104

3 94

4 87

5 97 103

|20 mV

|1 mm

5 sec
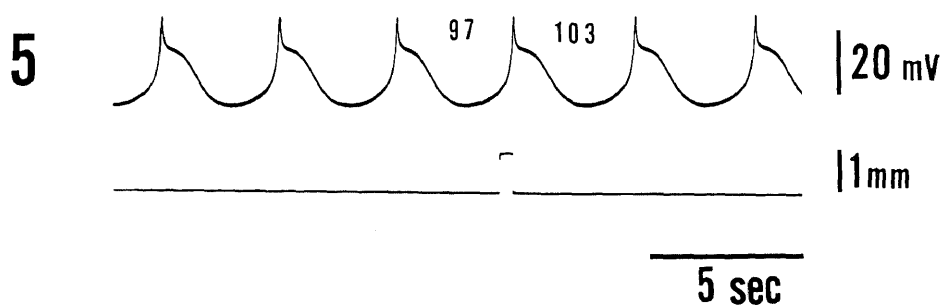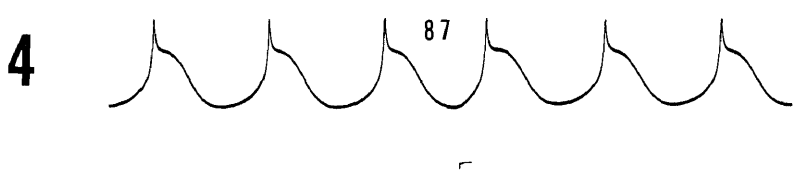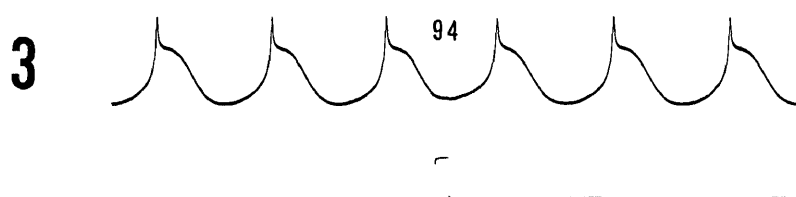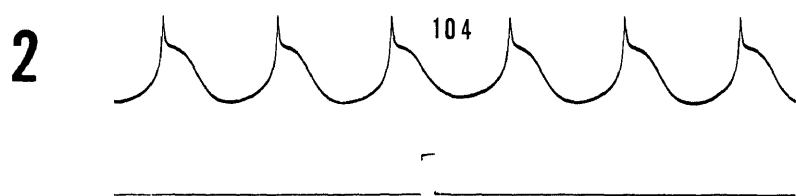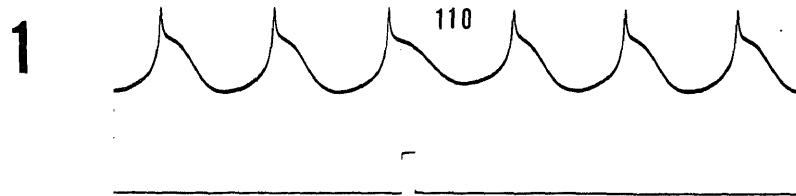
Fig. 13. Effect of a brief stretch on the electrical activity and on the beat interval. Records were taken intracellularly from the isolated ventricle. Upper trace; membrane potential, lower trace; monitor of stretch. The phases (%) where the stretch was applied are 12, 28, 45, 66 and 90 % in records 1-5, respectively. Interval change is shown by % relative to the preceding control interval (100 %).
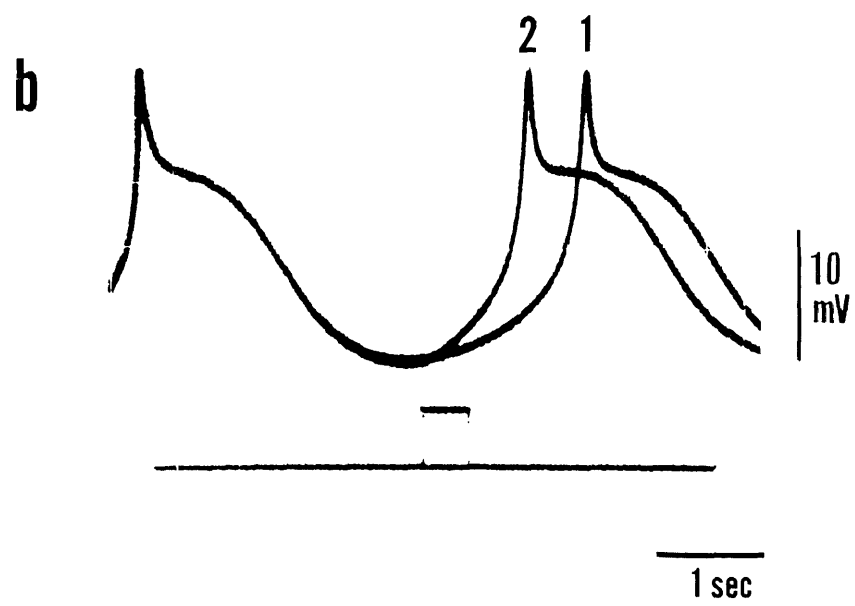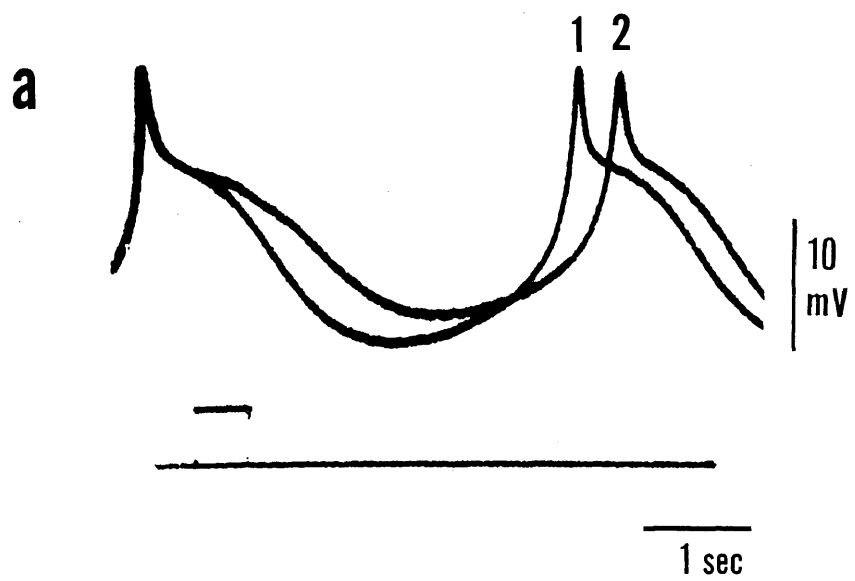
Fig. 14. Effect of a brief stretch on membrane potential. Maximum interval prolongation (a) and shortening (b) in Fig. 14 are shown. Potential change of the preceding control interval (1) is superimposed onto that of the stretch applied interval (2). Upper trace; membrane potential, lower trace; monitor of stretch.

10 mV

1 s

5 mV

1 s
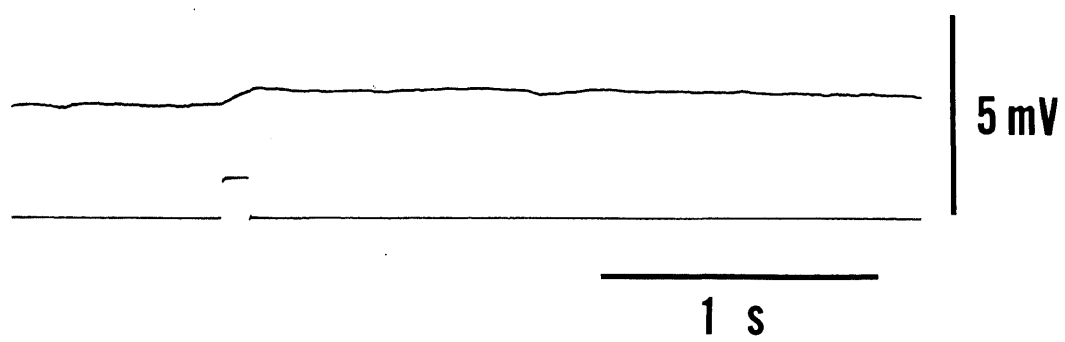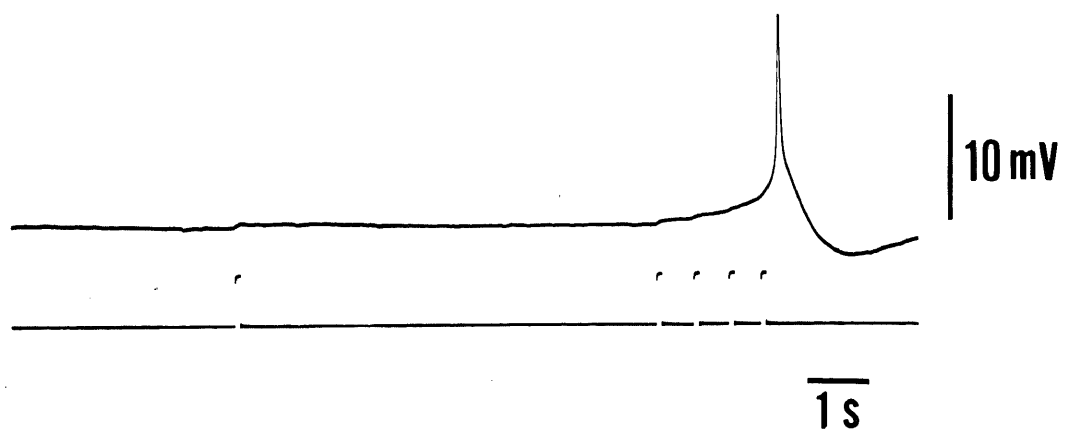
Fig. 15. Effect of a brief stretch on the membrane potential of the ventricular myocardium. Spontaneous activity of the ventricle was suppressed by 10^{-4}M ACh. Upper trace, intracellular records; Lower trace, monitor of stretch. The stretches were 100 msec in duration and 1.0 mm in magnitude.

10 mV

5 S

10 mV

5 S
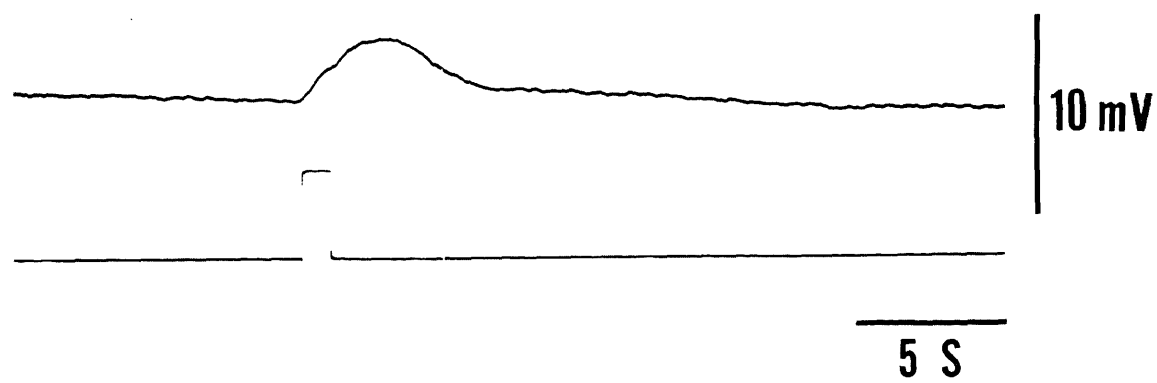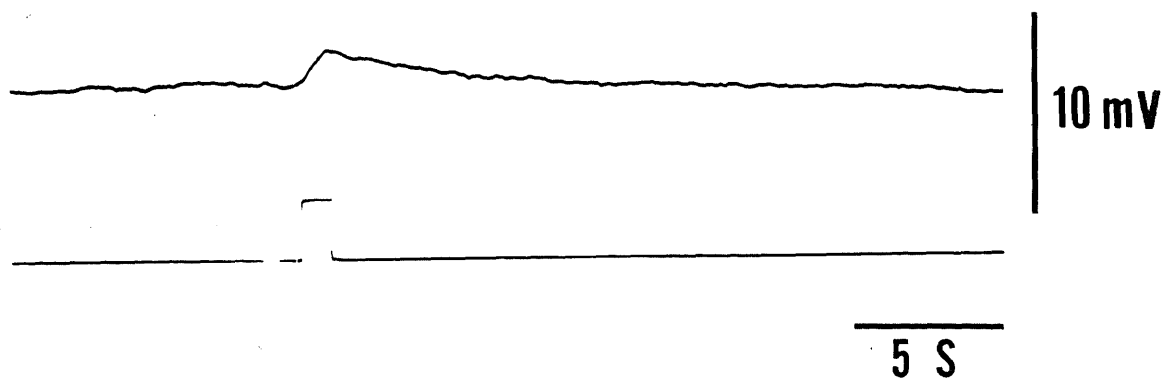
Fig. 16 Effect of a brief stretch on the membrane potential of the ventricular myocardium. Upper trace, intracellular records; Lower trace, monitor of stretch. Duration of he stretches was 1.0 sec. The magnitudes were 0.8 mm in the upper record (a) and 1.2 mm in the lower record (b).

A

PHASE SHIFT (%)

+30

0

-30

0   50   100

STIMULATION   PHASE (%)

V

PHASE SHIFT (%)

+30

0

-30

0   50   100
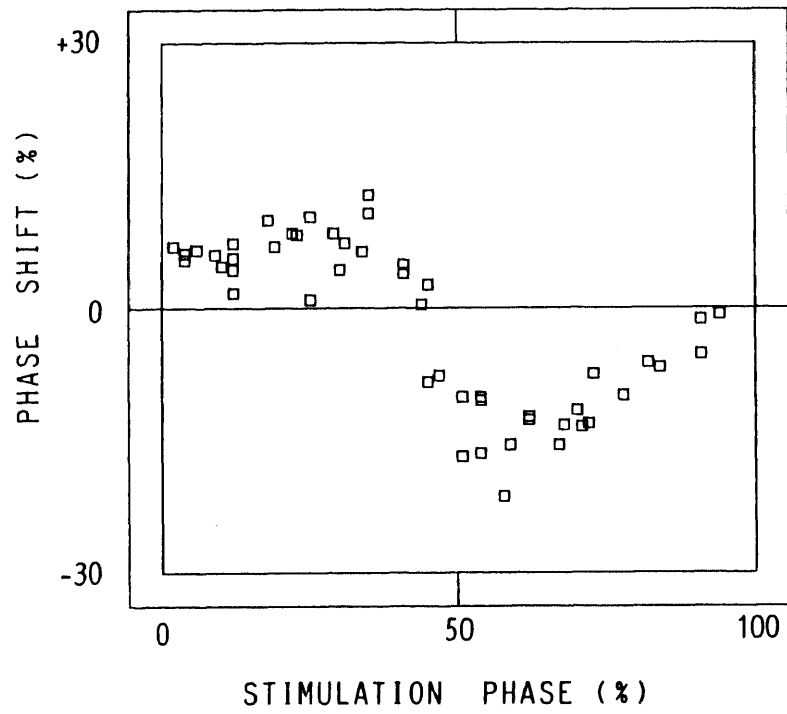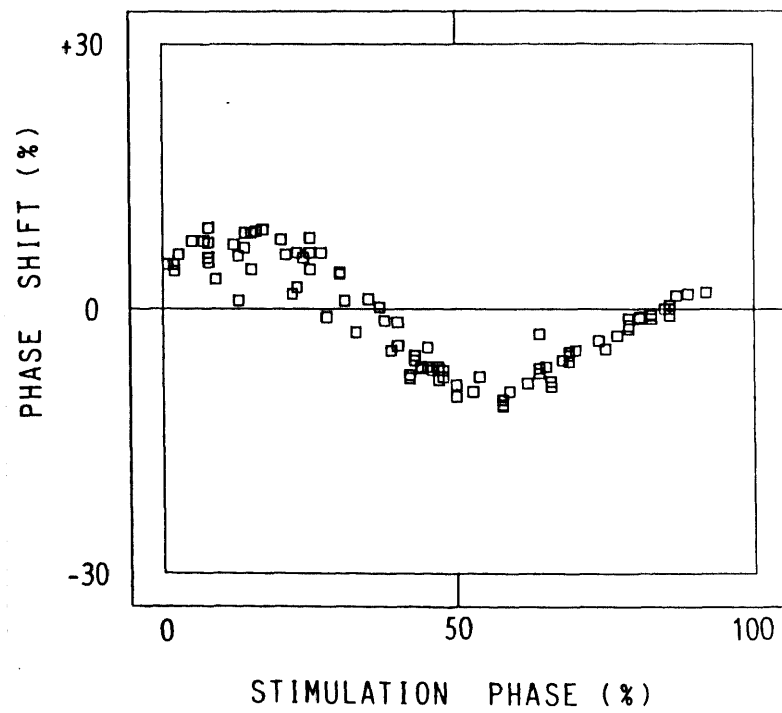
STIMULATION   PHASE (%)

Fig. 17. Phase-response curves (PRCs) of the auricle (A) and ventricle (V) obtained by the application of single brief stretches as stimuli. Ordinate, phase shift (%); Abscissa, stimulation phase (%), relative to the control interval (100 %). The stretches were 50 msec in duration and 0.6 mm in magnitude in A, 100 msec and 0.8 mm in V. Auricular control interval was 3.3-3.6 sec and ventricular control was 2.1-2.3 sec.

a
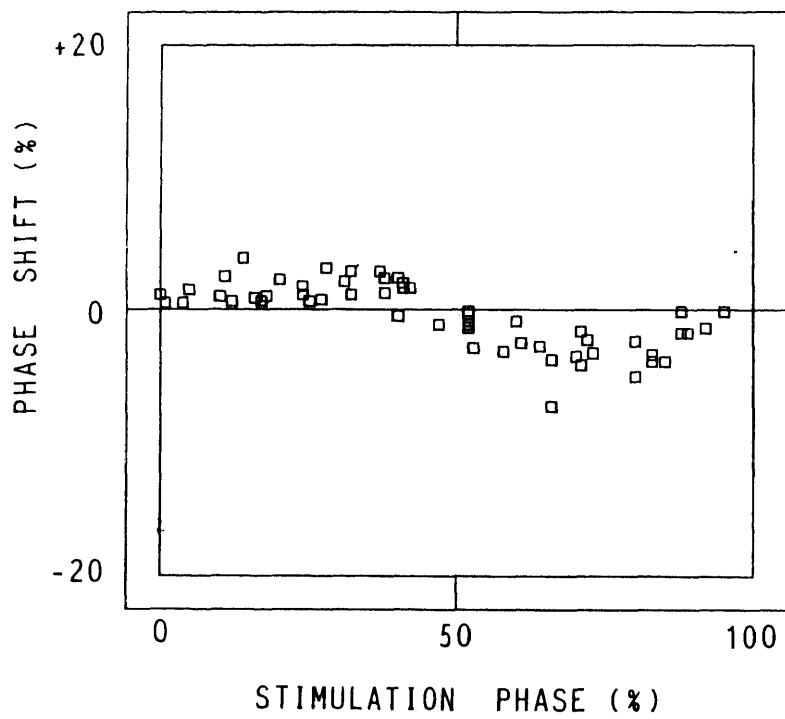
b
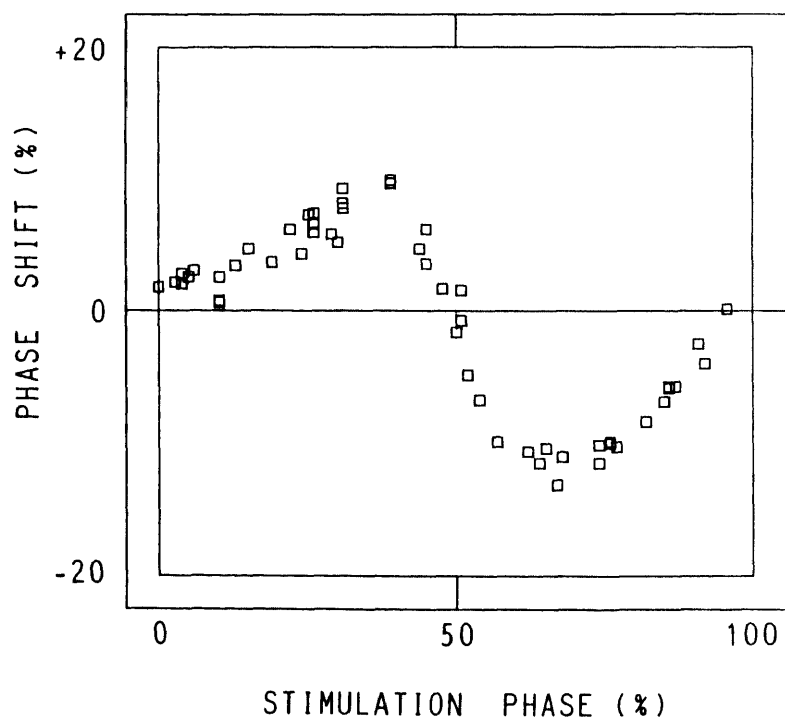
Fig. 18.   PRCs of the auricle obtained by the application of single brief stretches of different magnitudes. Duration of the stretches was 50 msec. Magnitudes were 1.5 mm in a and 0.7 mm in b.   Control interval was 2.5-2.6 sec.

1

2

3

4

| 1 mV
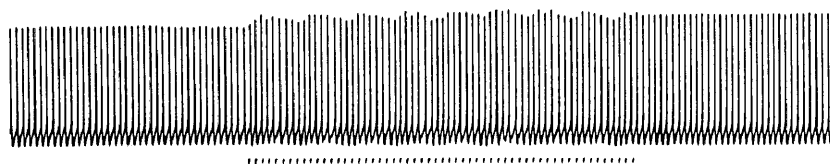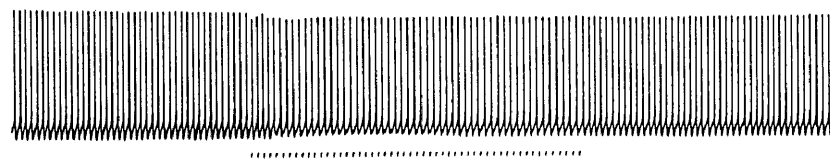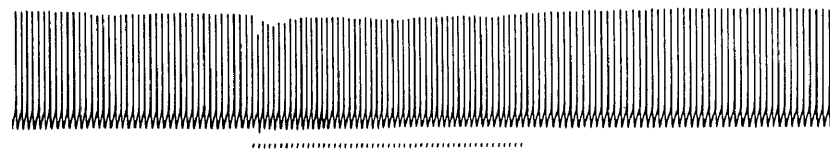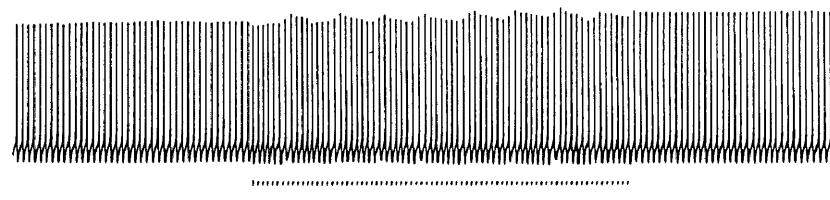
ı 1 mm

1 min

Fig. 19.   Effects of repetitive brief stretches on the beat rhythm of the ventricle. Upper traces, extracellular potentials; Lower traces, monitor of stretch. Intervals of the stretches were 3.9 sec in 1, 3.6 sec in 2, 3.1 sec in 3 and 2.9 sec in 4. Stable entrainment was achieved in both cases when the frequency of repetitive stretches was either lower (2) or higher (3) than the free-run frequency.   However, stable entrainment could not be achieved when the frequency of the stretches was too low (1) or too high (4). Free-run control interval was 3.4 sec, and the stretches were 500 msec in duration, 1.2 mm in magnitude.

1

2

3

4

| 1 mV

| 1 mm

3 sec
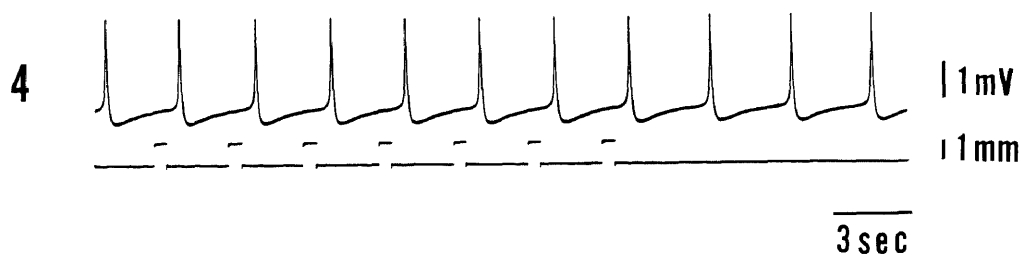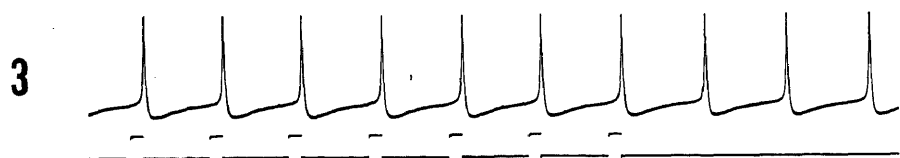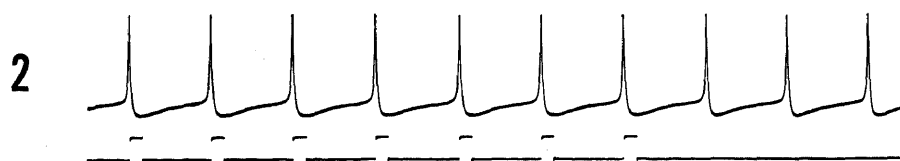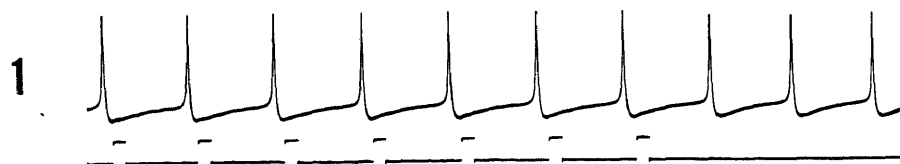
Fig. 20. Stable entrainment of the ventricular beat rhythm. Upper traces, extracellular record; Lower traces, monitor of stretch. The stretches were 500 msec in duration and 1 mm in magnitude. Intervals of the repetitive stretches were 3.40 sec in 1, 3.20 sec in 2, 3.10 sec in 3 and 2.90 sec in 4. Free-run control interval was 3.14-3.18 sec. Note that phase-locking of the stretches with the action potentials occurred in different phase relationships depending on the frequencies of the stretches.
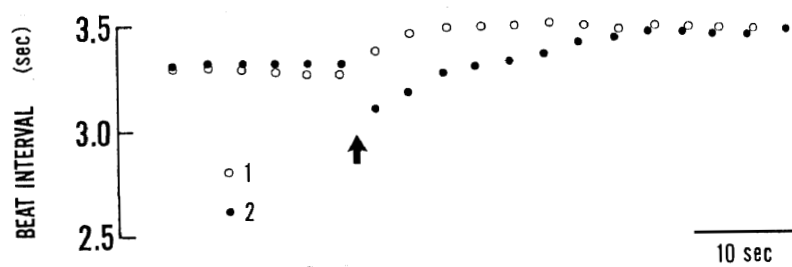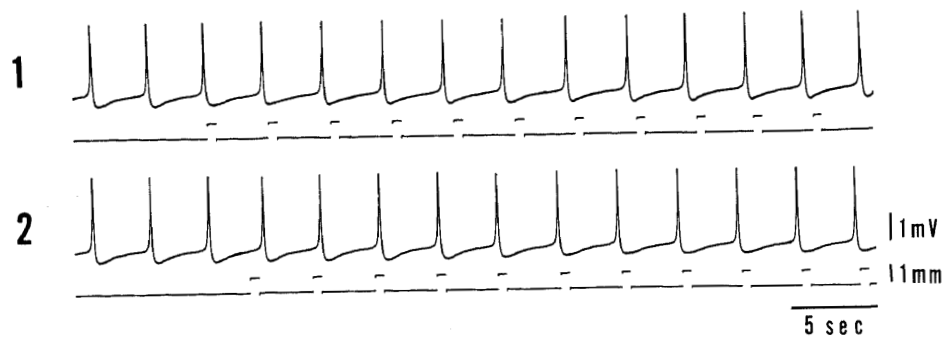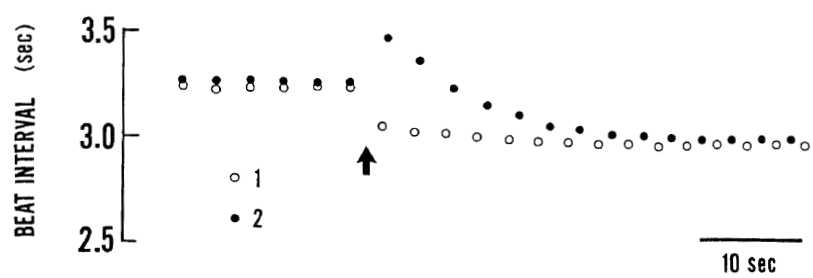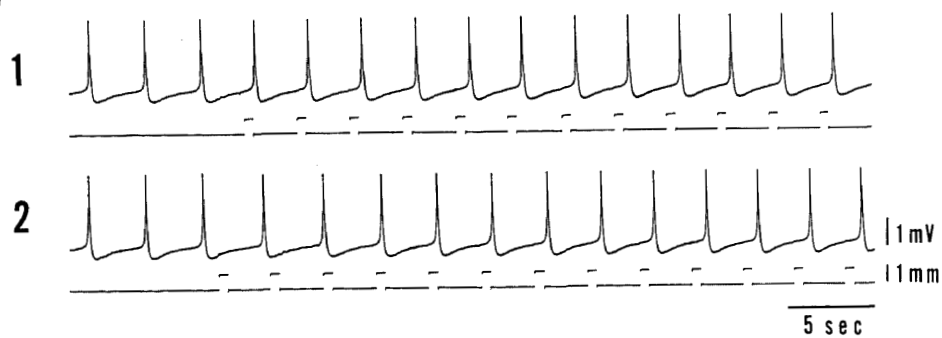
Fig. 21. Patterns of beat interval alterations in the course of becoming stably entrained, according to the phase in which the first stretch was applied. a: Different patterns obtained by a lower entraining frequency of stretch. Interval of the stretches was 3.45 sec, whereas the free-run beat interval was 3.11 sec. Phase of the first stretch was 5 % in 1 and 69 % in 2. b: Different patterns obtained by a higher frequency of stretches. Interval of the stretches was 2.95 sec whereas the free-run interval was 3.26 sec. Phase of the first stretch was 76 % in 1, and 25 % in 2. The stretches were 500 msec in duration and 1 mm in magnitude. Graphs show the alteration of beat intervals in the time course as the free-run beats become entrained. Plots of open circles (1) and closed circles (2) correspond to the upper records, 1 and 2, respectively. Arrows in the graph indicate the interval in which the first stretch was applied.

1

2

1 mV

1 1 mm

10 sec
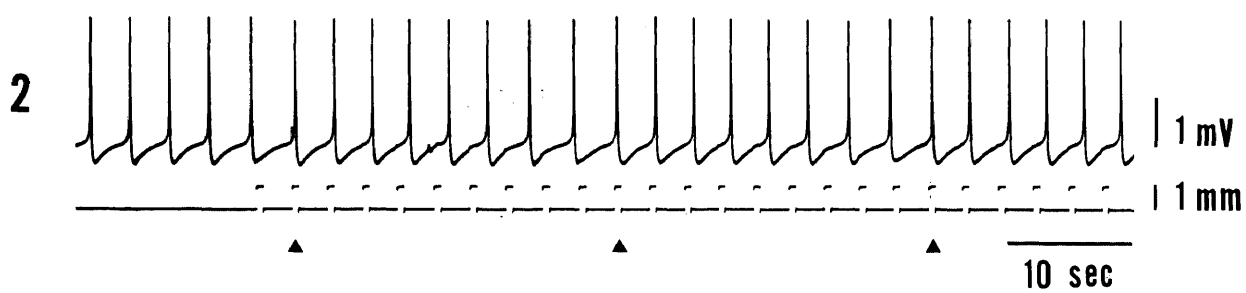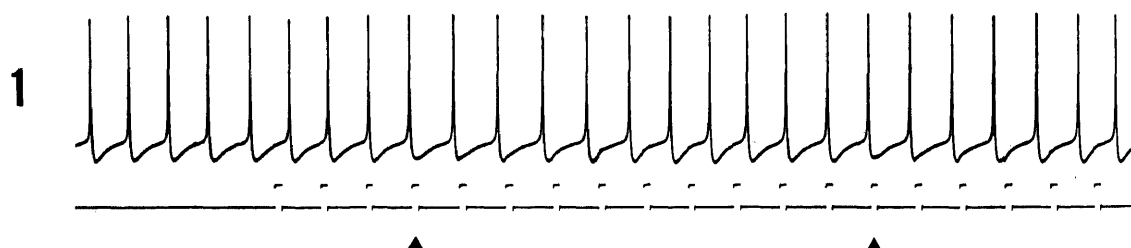
Fig. 22.   Beat rhythm alterations and shifts of phase relationship between the stretch and action potential. Frequencies of the stretches were too low (1) and too high (2) compared to that of the free-run, thus stable entrainment was not achieved. Upper traces, extracellular record; Lower traces, monitor of stretch.   Intervals between the stretches were 3.40 sec in 1 and 2.80 sec in 2.   Free-run interval was 3.18 sec in both cases. The stretches were 500 msec in duration, 1 mm in magnitude.   Arrow heads indicate the same phase relationship within the records.
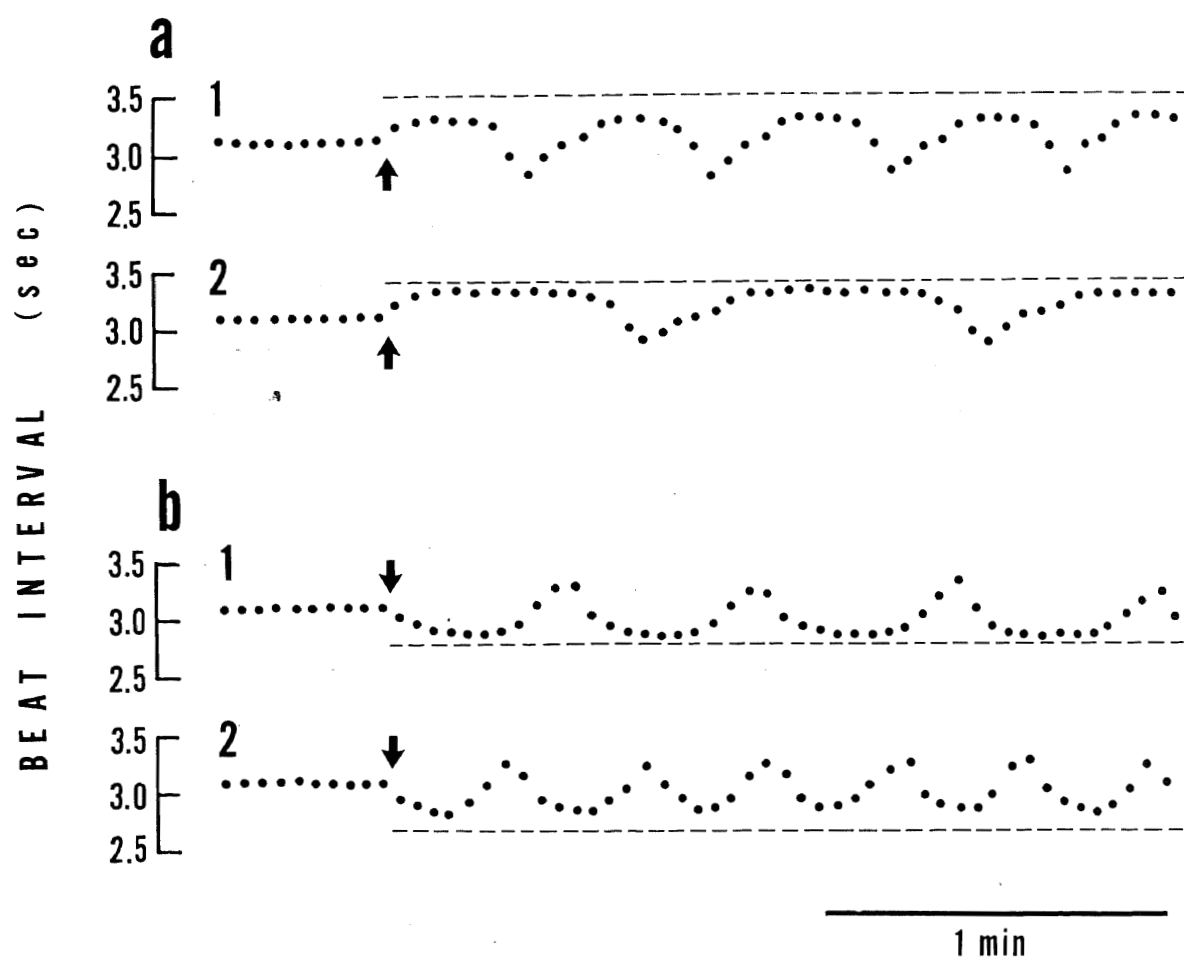
Fig. 23.   Alteration of beat intervals when stable entrainment could not be achieved. a, Frequency of the stretches was too low compared to that of the free-run. The intervals of the stretches were 3.50 sec in 1 and 3.40 sec in 2. b, Frequency of the stretches was too high. The intervals were 2.80 sec in 1 and 2.70 sec in 2. Free-run interval was 3.18 sec. The stretches were 500 msec in duration, 1 mm in magnitude. Broken line shows the interval of the stretches, and arrow indicates the beat interval in which the repetitive stretch was started. (Graphs a-2 & b-1 are plots of the recordings of Fig. 8 - 1 & 2, respectively.)
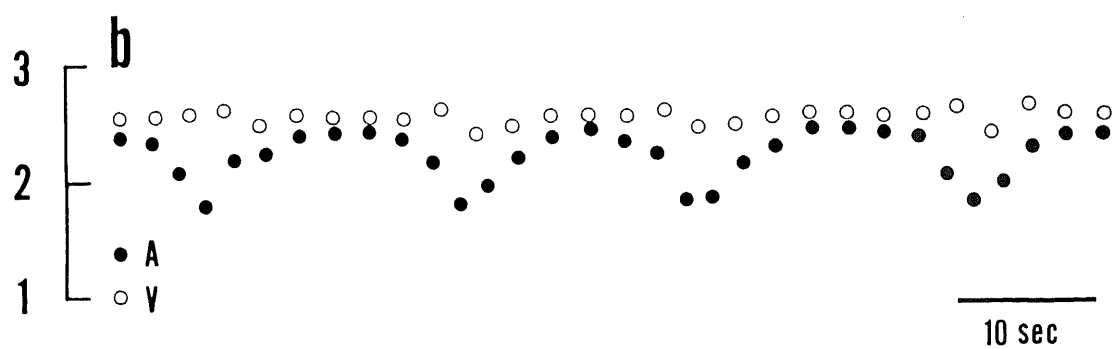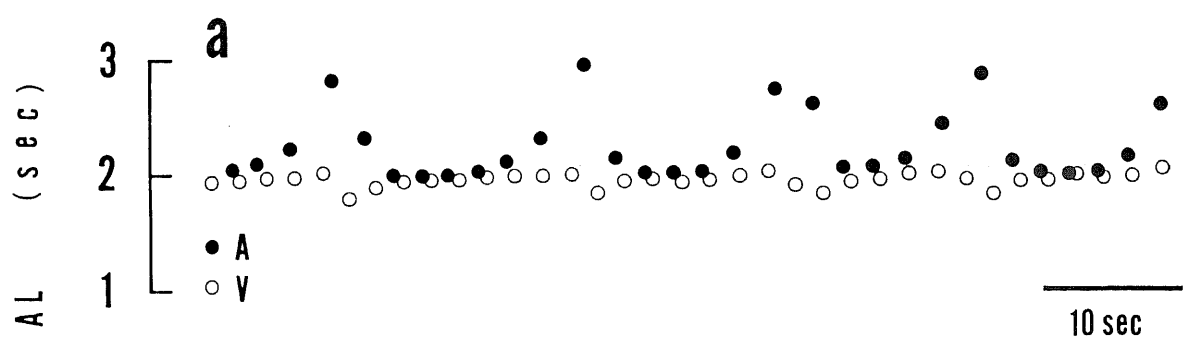
Fig. 24. Alteration in auricular (A) and ventricular (V) beat intervals in the heart in situ. Graphs show the beat interval versus the time. Two different patterns are shown in a and b. a: Pattern in which the auricular beat interval prolonged periodically whereas the ventricular one shortened. b: Reverse pattern of a. Interval alterations were obtained by stoppage of perfusion into the pericardial cavity.

IX. APPENDIX

```
Program    PRC;

   {$I  c:turtle2.lib}
   {$I  c:hcopy.lib}
   {$I  c:filio.lib}


{***************************************************************}
{***********  PRC Experiment Program  ********  by H. Uesaka  **}
{*******************************************  1984. 8. 8    **}
{***************************************************************}


{ Definition of variables }


   label    START;

   const    MAXDATANUM = 500;
            MAXPERCENT = 100;

            timerport    =$20;
            stb_off_test=$7;
            stb_on_test =$F;
            stb_off_std =$8;
            stb_on_std  =$0;

   type     dataname    = string[14];
            percent      = 0..MAXPERCENT;
            elements     = array [0..10] of integer;
            datarec      = record
                            dataNO        :integer;
                            contcycle     :integer;
                            stimphase     :integer;
                            phaseshift    :real;
                          end;

   var      datanum                 : 0..MAXDATANUM;
            expdata                 : array [0..MAXDATANUM] of datarec;
            timebuf                 : array [0..10] of real;
            registparam             : register;
            maxPS                   : percent;
            maxinput,slicelevel  : integer;
            msec                    : real;
            select                  : char;
            datafilename            : dataname;
```

```
{ PRC Procedures }

{********************   Data I/O Procedures   ********************}

   Procedure flushexpdata;

      var      i   : integer;

      begin
        for i:=0 to MAXDATANUM do
          begin
            with expdata[i] do
              begin
                dataNO     :=0;
                contcycle  :=0;
                stimphase  :=0;
                phaseshift :=0;
              end;
          end;
      end;




   Procedure dataIO (func : char);

      var      datafilevar  : file of datarec;
               dumpdest     : char;



      Procedure savedata;

         var      i   :integer;

         begin
           writeln('Save data file  : ',datafilename);
           if exist(datafilename)=false
             then
               begin
                 writeln('record number = ':34,datanum);
                 assign(datafilevar,datafilename);
                 rewrite(datafilevar);
                 for i:=0 to datanum-1 do
                   write(datafilevar,expdata[i]);
                 close (datafilevar);
               end
             else
               begin
                 writeln;
                 writeln (datafilename,'  already exists !');
               end;
           writeln;
         end;
```

```
Procedure loaddata;

    var      i    :integer;

    begin
      write  ('Work data file ? [*.DAT ]  : ');
      readln (datafilename);
      if pos('.',datafilename)=0
        then datafilename:= datafilename+'.DTA';
      if pos(':',datafilename)=0
        then datafilename:=currentdrive+':'+datafilename;
      if pos(':.',datafilename)<>0
        then datafilename:='';
      uppercase(datafilename); writeln;
      writeln('Work data file  : ',datafilename);
      datanum:=0;
      flushexpdata;
      if exist(datafilename)=true
        then
          begin
            assign(datafilevar,datafilename);
            reset(datafilevar);
            datanum :=filesize(datafilevar);
            writeln('record number = ':34,datanum);
            for i:=0 to datanum-1 do
              read(datafilevar,expdata[i]);
            close(datafilevar);
          end
        else
          begin
            writeln;
            writeln('New file');
          end;
      writeln;
    end;



Procedure Consoleout;

    var      j          :integer;

    begin
      writeln('dataNO':15,'control':15,'Stim Phase':15,
              'Phase Shift':15);
      writeln('            (msec)' :30,'( % )'      :15,
              '( % )'      :15);
      writeln;
      for j:=0 to datanum do
        begin
          with expdata[j] do
            writeln(dataNO:15,contcycle:15,stimphase:15,
                    phaseshift:15:1);
        end;
    end;
```

```
Procedure Printerout;

   var      j    :integer;

   begin
     writeln(lst,'< '+datafilename+' >':20);
     writeln(lst);
     writeln(lst,'dataNO':15,'control':15,'Stim Phase':15,
                'Phase Shift':15);
     writeln(lst,'          (msec)'  :30,'( % )'     :15,
                '( % )'       :15);
     writeln(lst);
     for j:=0 to datanum-1 do
       begin
         with expdata[j]  do
           writeln(lst,dataNO:15,contcycle:15,stimphase:15,
                  phaseshift:15:1);
       end;
   end;




   Procedure outdata;

   begin
     writeln('Dump out the data to Console   (''c'')  ');
     write  ('                    or Printer ? (''p'')  ');
     readkeyboard (dumpdest);
     writeln; writeln;
     case dumpdest of
        'C','c' : Consoleout;
        'P','p' : Printerout;
     end;
   end;



begin
  case func of
    's' : savedata;
    'w' : loaddata;
    'o' : outdata;
  end;
end;
```

```
{*****************  PRC Graphic Procedures  *******************}


   function    vwx(x:integer) : integer;
      begin
        vwx := round(x/640*32767+13000);
      end;



   function    vwy(y:integer) : integer;
      begin
        vwy := round(y/400*32767+16000);
      end;




Procedure drawframe;

   var        maxst      :strings;

   begin
     opengraph;
     linecolor(blue);
     box (vwx(0)   ,vwy(-160),vwx(360),vwy(160));
     box (vwx(-20),vwy(-180),vwx(380),vwy(180));
     line(vwx(-20),vwy(0)    ,vwx(380),vwy(0)   );
     line(vwx(180),vwy(-180),vwx(180),vwy(180));
     textcolor(cyan);
     writetext(vwx(0)   ,vwy(-195),'0');
     writetext(vwx(350),vwy(-195),'100');
     writetext(vwx(175),vwy(-195),'50');
     str(maxPS,maxst);
     writetext(vwx(-60),vwy( 157),'+'+maxst);
     writetext(vwx(-60),vwy(-  3),'  0');
     writetext(vwx(-60),vwy(-162),'-'+maxst);
   end;



Procedure dispinfo;

   begin
     clrscr;
     gotoXY(1,2);
     lowvideo ; write  ('Data file : ');
     highvideo; writeln(datafilename);
     gotoXY(1,4);
     lowvideo ; write  ('Data number : ');
     highvideo; writeln(datanum);
     gotoXY(1,7);
     lowvideo ; write  ('A');lowvideo;write('bsc MAX  :');
     highvideo; write  (maxPS:3);
     lowvideo ; writeln(' % ');
```

```pascal
      gotoXY(1,10);
      lowvideo ; writeln('< Data records > ');
      lowvideo ; writeln('  data NO.     : ');
      lowvideo ; writeln('  cont. cycle : ');
      lowvideo ; writeln('  stim. phase : ');
      lowvideo ; writeln('  phase shift : ');
      highvideo;
   end;




Procedure currentinfo(maxin,level : integer);

   begin
     gotoXY(1,17);
     lowvideo ;writeln('< Monitor >');
     lowvideo ;write  ('  max in (<2047) : ');
     highvideo;writeln(maxin:4);
     lowvideo ;write  ('  silce level    : ');
     highvideo;writeln(level:4);
     lowvideo ;
     writeln('                                ');
     writeln('                                ');
     writeln('< Message >                     ');
     highvideo;
   end;




Procedure dispdata(dn : integer);

   begin
     with expdata[dn] do
       begin
         gotoXY(17,11);write(dataNO:5);
         gotoXY(17,12);write(contcycle:5);
         gotoXY(17,13);write(stimphase:5);
         gotoXY(17,14);write(phaseshift:5:1);
       end;
   end;
```

```
Procedure getmax(var maxval : percent);

   var      max    : percent;

   begin
     repeat
       gotoXY(21,20);write('        ');
       gotoXY(1,20);
       highvideo;write('Max phase shift ? : ');
       read(max);
       gotoXY(1,20);
       lowvideo ;write('Max phase shift ? : ');
     until max in [O..MAXPERCENT];

     if max<>0
        then maxval:=max
        else maxval:=maxPS;
     gotoXY(12,7);
     highvideo;
     write(maxPS:3);
   end;




Procedure plot(stph:integer; phsh:real; mark:markertype);

   var      coeffx,coeffy,mk        :integer;

   begin
     coeffx:=3600 div 100;
     coeffy:=16000 div maxPS;
     gsxx1:=vwx(coeffx*stph div 10);
     if abs(phsh)<=maxPS
        then gsxy1:=vwy(round(coeffy*phsh/100))
        else
          if phsh<-maxPS
             then gsxy1:=vwy(-170)
             else gsxy1:=vwy( 170);
     if abs(phsh)<=maxPS
        then  polymarkercolor(yellow)
        else  polymarkercolor(red);
     polymarkertype (mark);
     marker(gsxx1,gsxy1);
   end;
```

```
Procedure volumeplot(mk : integer);

   var      i    : integer;
            mark : markertype;

   begin
     mark:=markertype(mk);
     for i:=0 to datanum-1 do
       begin
         dispdata(i);
         with expdata[i] do plot(stimphase,phaseshift,mark);
       end;
   end;



Procedure monoplot(dn : integer);

   begin
     dispdata(dn);
     with expdata[dn] do
       plot(stimphase,phaseshift,asterisk);
   end;



Procedure hardcopy;

   begin
     writeln (lst,'<'+datafilename+'>':20);
     copygraph;
   end;



Procedure graphmain;

   var      answer    : char;
            mk        : integer;

   begin
     drawframe;
     dispinfo;
       repeat
         getmax(maxPS);
         drawframe;
         repeat
           gotoXY(24,21);write('   ');
           highvideo;
           gotoXY( 1,21);write('Marker type ? [1..5] : ');
           readln(mk);
           lowvideo;
           gotoXY( 1,21);write('Marker type ? [1..5] : ');
         until mk in [1..5];
```

```pascal
        gotoXY(21,23);write('  ');
        highvideo;
        gotoXY(1,23);write('Begin plot ? (y/n): ');
        readkeyboard(answer);
        lowvideo;
        gotoXY(1,23);write('Begin plot ? (y/n): ');
        highvideo;
      until answer in ['Y','y'];
    volumeplot(mk);
    gotoXY(1,24); writeln('                              ');
    gotoXY(1,24);
  end;




{***************   PRC Experiment Procedures   ******************}


  Procedure initalldata;

    begin
      flushexpdata;
      datafilename:='';
      datanum:=0;
      maxPS:=0;
    end;




  Procedure testsin;

    var     MPS,k     :integer;

    begin
      write('Max data number?  ');readln(datanum);
      write('Max phase shift?  ');readln(MPS);
      flushexpdata;
      for k:=0 to datanum-1 do
        begin
          with expdata[k] do
            begin
              dataNO     :=k;
              contcycle :=2*k;
              stimphase :=random(100);
              phaseshift:=MPS*sin(stimphase*3.6*pi/180);
            end;
        end;
      writeln;
    end;
```

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```
Procedure settime(hset,mset,sset:integer);

  begin
    with registparam do
      begin
        ax  := $2D00;
        cx  := hset*256+mset;
        dx  := sset*256;
        MsDos(registparam);
      end;
  end;




Procedure puttime( i : integer);

  begin
    with registparam do
      begin
        ax:= $0;
        bx:= ofs(timebuf[i]);
        es:= seg(timebuf[i]);
        intr($1C,registparam);
      end;
  end;




Function readtimebuf(i:integer):real;

  var       yy,11,dd,hh,mm,ss          : byte;
            day,minute,second          : integer;
            hour                       : real;

  begin
    yy:=mem[seg(timebuf[i]):ofs(timebuf[i])+0];
    11:=mem[seg(timebuf[i]):ofs(timebuf[i])+1];
    dd:=mem[seg(timebuf[i]):ofs(timebuf[i])+2];
    hh:=mem[seg(timebuf[i]):ofs(timebuf[i])+3];
    mm:=mem[seg(timebuf[i]):ofs(timebuf[i])+4];
    ss:=mem[seg(timebuf[i]):ofs(timebuf[i])+5];
{ year    :=(yy div 16)*10+(yy mod 16);}
{ month   :=(11 div 16);                 }
    day     :=(dd div 16)*10+(dd mod 16);
    hour    :=(hh div 16)*10+(hh mod 16);
    minute  :=(mm div 16)*10+(mm mod 16);
    second  :=(ss div 16)*10+(ss mod 16);
    readtimebuf := hour*3600 + minute*60 + second;
  end;
```

```
Procedure timerstart;

  begin
    settime(0,0,0);
    Port[timerport]:= stb_off_std;
    Port[timerport]:= stb_on_std;
    Port[timerport]:= stb_off_std;
    Port[timerport]:= stb_off_test;
    Port[timerport]:= stb_on_test;
    Port[timerport]:= stb_off_test;
  end;




Procedure timerstop;

  begin
    Port[timerport]:= stb_off_std;
    Port[timerport]:= stb_on_std;
    Port[timerport]:= stb_off_std;
  end;




Function ADread(ch : byte):integer;

  const   chsel  = $D0;
          cnvst  = $D2;
          oscsel = $D4;
          rword  = $D2;

  var     data      : integer;

  begin
    Port[chsel]  :=ch;
    Port[cnvst]  :=ch;
    repeat
      data:=PortW[rword];
    until (data and $8000) <> 0;
    data   :=data and $0FFF;
    ADread:=data-$800;
  end;




Procedure DigitalOut(ch : integer);

  const   oscsel = $D4;
          ONout0 = $48;    { DO0  on  , 1KHz }
          ONout1 = $88;    { DO1  on  , 1KHz }
          OFFout = $08;    { DO   off , 1KHz }
```

```
      var       d         : integer;

   begin
     case ch of
        0 : Port[oscsel]:=ONout0;
        1 : Port[oscsel]:=ONout1;
     end;
     for d:=0 to 10 do begin end;
     Port[oscsel]:=OFFout;
   end;




Procedure getslicelevel(SL:percent; var maxin,level:integer);

   var       live,i    : integer;
             maxpot    : real;

   begin
     maxpot:=0;
     for i:=-10000 to 10000 do
       begin
         live:=ADread(0);                        { channel 0 selected }
         if maxpot<live then maxpot:=live;
       end;
     level:=round(maxpot*SL/100);
     maxin:=round(maxpot);
   end;




Procedure getpeak(var flag:boolean);

   var       databuffer            : array [0..10] of integer;
             oldsum,newsum,i       : integer;

   begin
     if ADread(0) < slicelevel
        then
          begin
            repeat until ADread(0)>=slicelevel;
            for i:=0 to 10 do   databuffer[i]:=ADread(0);
            repeat
              oldsum:=0;
              newsum:=0;
              for i:=0 to 9 do   databuffer[i]:=databuffer[i+1];
              for i:=0 to 9 do   oldsum :=oldsum+databuffer[i];
              databuffer[10]:=ADread(0);
              for i:=1 to 10 do   newsum :=newsum+databuffer[i];
            until oldsum - newsum >=0;
            flag:=true;
          end
        else flag:=false;
   end;
```

```
Function  intervalSE(num : integer ; data : elements):real;

  var      i                                  :integer;
           sum,sigma,varience,mean            :real;

  begin
    sum:=0;
    varience:=0;
    sigma:=0;
    for i:=0 to num-1 do sum:=sum+data[i];
    mean:=sum / num;
    for i:=0 to num-1 do sigma:=sigma+sqr(mean-data[i]);
    varience:=sigma / (num-1);
    intervalSE:= sqrt(varience);
    writeln (lst,'SE=',sqrt(varience));
  end;




Function  mean10int(num : integer ; data : elements):real;

  var      i                                  :integer;
           sum,sigma,varience,mean            :real;

  begin
    sum:=0;
    varience:=0;
    sigma:=0;
    for i:=0 to num-1 do sum:=sum+data[i];
    mean:=sum / num;
    mean10int:= mean;
  end;




Function  seqdelay(sequence,cyclelength:integer):integer;

  var      i,unit,unitdelay : integer;

  begin
    unit:=cyclelength div 10;
    unitdelay:=unit*(sequence mod 10);
    seqdelay:=unitdelay + random(unit);
  end;
```

```
Procedure Interval;

  const  CYCLEMAX          ` = 100;

  var    i,j                  : integer;
         found                : boolean;
         realmsec             : array [0..1] of real;
         cycle                : array [0..CYCLEMAX] of integer;
         POans                : char;

  begin
    ClrScr;
    write ('Printer Out ? [Y/N] : ');
    readkeyboard(POans);
    if POans in ['Y','y'] then writeln(lst);
    writeln;
    write('HIT ANY KEY ');
    repeat until keypressed;
    writeln(^G);
    getslicelevel(70,maxinput,slicelevel);lowvideo;
    write(^G);
    writeln('Max input = ',maxinput:5,' *
            slice level = ',slicelevel:5);highvideo;
    i:=0;
    repeat getpeak(found) until found;
    timerstart;
        repeat
          repeat getpeak(found) until found;
          puttime(0);
          timerstart;
          write(^G);
          realmsec[0]:=readtimebuf(0);
          cycle[i]:=round(realmsec[0]);
          write (i:2,':',cycle[i]:4,' ');
          if (i mod 10)=9 then writeln;
          if i=99 then writeln;
          if POans in ['Y','y'] then
            begin
              write (lst,i:2,':',cycle[i]:4,' ');
              if (i mod 10)=9 then writeln(lst);
              if i=99 then writeln(lst);
            end;
          if i=99 then i:=-1;
          i:=i+1;
        until (i=CYCLEMAX) or (keypressed);
    timerstop;
  end;
```

```
Procedure Experiment;

    var     i,j,DM,DN                     : integer;
            msecstr                       : string[12];
            found                         : boolean;
            realmsec                      : array [0..10] of real;
            interval                      : elements;
            answer                        : char;
            maxst                         : strings;
            Rcontcycle                    : real;
            SL                            : percent;

    begin
      dispinfo;
      drawframe;
      getmax(maxPS);
      drawframe;
      highvideo;
      gotoXY( 1,21);write('Data Numbers ? [0,.500]: ');
      read(DN);
      lowvideo ;
      gotoXY( 1,21);write('Data Numbers ? [0..500]: ');
      highvideo;
      gotoXY( 1,22);write('Slice Rate(%)? [0..100]: ');
      read(SL);
      lowvideo ;
      gotoXY( 1,22);write('Slice Rate(%)? [0..100]: ');
      highvideo;
      gotoXY( 1,23);write('HIT ANY KEY ');
      repeat until keypressed;
      currentinfo(0,0);
      FOR j:=0 TO DN DO
        BEGIN
          gotoXY(1,23);
          write('Get SLICE LEVEL      '); write(^G);
          getslicelevel(SL,maxinput,slicelevel);
          currentinfo(maxinput,slicelevel);
          repeat
            gotoXY(1,23);
            write('Test the STABILITY. '); write(^G);
            timerstart;
            for i:=0 to 10 do
              begin
                repeat getpeak(found) until found;
                puttime(i);
                realmsec[i]:=readtimebuf(i);
              end;
            write(^G);
            writeln(lst);
            write  (lst,'interval :');
            for i:=0 to 9 do
              begin
                interval[i]:=round(realmsec[i+1]-realmsec[i]);
                write  (lst,interval[i]:5,' *');
              end;
```

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```
      writeln(lst);
      timerstop;
   until intervalSE(10,interval) < 30;
   for i:=0 to 1 do
      begin
         write(^G);
         delay(10);
      end;
   gotoXY(1,23);
   write('STIM. &  EXP.        ');
   DM:=round(mean10int(10,interval)*0.95);
   timerstart;
   repeat
      getpeak(found);
      puttime(0);                  { Zero time action  potential}
   until found;
   repeat
      getpeak(found);
      puttime(1);                  { 1st action potential time }
   until found;
   delay(seqdelay(j,DM));
   DigitalOut(0);                   { Stimulation trigger out   }
   puttime(10);                     { Stimulation time          }
   repeat
      getpeak(found);
      puttime(2);                  { 2nd action potential time }
   until found;
   repeat
      getpeak(found);
      puttime(3);                  { 3rd action potential time }
   until found;
   repeat
      getpeak(found);
      puttime(4);                  { 4th action potential time }
   until found;
   timerstop;
   for i:=0 to 4 do realmsec[i] := readtimebuf(i);
                    realmsec[10]:= readtimebuf(10);
   with expdata[datanum] do
      begin
         dataNO     :=datanum;
         Rcontcycle:=mean10int(10,interval);
         contcycle :=round(Rcontcycle);
         stimphase :=round((realmsec[10]-realmsec[1])
                           *100/Rcontcycle);
         phaseshift:=(realmsec[4]-realmsec[0]-Rcontcycle*4)
                     *100/ Rcontcycle;
      end;
   monoplot(datanum);
   with expdata[datanum] do
      writeln(lst,'NO ',dataNO:3,contcycle:9,stimphase:5,
              phaseshift:5:1);
      writeln(lst);
   datanum:=datanum+1;
END;
```

```
      gotoXY(1,23);lowvideo ;writeln('Experiment end.      ');
        repeat
          highvideo;write('Save data ? (y/n):');
          readkeyboard(answer);
        until answer in ['Y','y','N','n'];
      clrscr;
      if answer in ['Y','y'] then dataIO('s');
    end;
```

{*********************   PRC Main Routine   *********************}

```
  Begin
    initalldata;
    START:
    ClrScr;
    lowvideo;
    writeln;    highvideo;
    write('L');lowvideo ; write  ('ogged drive    : ');
              highvideo; writeln(currentdrive+':');
    write('W');lowvideo ; write  ('ork data file   : ');
              highvideo; writeln(datafilename);
    writeln;
    writeln('* OPERATIONS *');
    writeln;    lowvideo ;
    write  ('< main >         ');
    write  ('< graph >        ');
    write  ('< data I/O >     ');
    writeln('< file I/O >     '); highvideo;
    writeln;
    write('E');lowvideo; write  ('xperiment        '); highvideo;
    write('G');lowvideo; write  ('raph             '); highvideo;
    write('S');lowvideo; write  ('ave data         '); highvideo;
    write('D');lowvideo; writeln('ir               '); highvideo;
    write('I');lowvideo; write  ('nterval          '); highvideo;
    write('H');lowvideo; write  ('ard copy         '); highvideo;
    write(' ');lowvideo; write  ('                 '); highvideo;
    write('Y');lowvideo; writeln('erase files      '); highvideo;
    write(' ');lowvideo; write  ('                 '); highvideo;
    write('F');lowvideo; write  ('lush CRT         '); highvideo;
    write('O');lowvideo; write  ('ut  data         '); highvideo;
    write('C');lowvideo; writeln('opy a file       '); highvideo;
    write('Q');lowvideo; write  ('uit              '); highvideo;
    write(' ');lowvideo; write  ('                 '); highvideo;
    write('T');lowvideo; write  ('est data         '); highvideo;
    write('R');lowvideo; writeln('ename a file     '); highvideo;
    writeln;
```

```pascal
   repeat
     write('>>') ;
     readkeyboard(Select); writeln;
     writeln;
     Case Select of
          'L','l' : changedrive;
          'W','w' : dataIO('w');
          'F','f' : opengraph;
          'H','h' : hardcopy;
          'D','d' : directory;
          'Y','y' : fileerase;
          'C','c' : filecopy;
          'R','r' : filerename;
          'I','i' : Interval;
          'E','e' : begin
                        if datafilename='' then dataIO('w') ;
                        experiment;
                    end;
          'S','s' : begin
                        if datafilename='' then dataIO('w') ;
                        dataIO('s');
                    end;
          'O','o' : begin
                        if datafilename='' then dataIO('w') ;
                        dataIO('o');
                    end;
          'G','g' : begin
                        if datafilename='' then dataIO('w') ;
                        graphmain;
                    end;
          'T','t' : begin
                        if datafilename='' then dataIO('w') ;
                        testsin;
                    end;
          'Q','q' : begin end;
       else
         goto START;
       end;
   until select in ['Q','q'];
End.
```

```
{**********************************************************}
{***   Graphic Extension Procedures                    ***}
{***                       The 2nd version of TURTLE.PAS  ***}
{**********************************************************}
{***   GSX-86 Sample Procedures                        ***}
{***          ( Copyright (c) 1984, Microsoftware Associates ) ***}
{***                              were used in part.   ***}
{**********************************************************}

const
   MAXPOINT = 100;
   MAXCONTRL= 5;
   MAXINTIN = 80;
   MAXPTSIN = 200; { MAXPOINT * 2 }
   MAXINTOUT= 45;
   MAXPTSOUT= 12;

type
   color   = (black,red,green,blue,cyan,yellow,magenta,white,none);

   linestyle = (noneline,solidline,dash,dotline,dashdot,longdash);

   markertype = (nonemarker,dot,plus,asterisk,ring,cross);

   fillint  = (hollow,solid,harftone,hatch);

   filltype = (nonefilltype,vertical,horizontal,slash,backslash,
               cross2,xxx);

   pblock = record
               contrlptr : ^integer;
               intinptr  : ^integer;
               ptsinptr  : ^integer;
               intoutptr : ^integer;
               ptsoutptr : ^integer;
           end;
   register = record
               ax,bx,cx,dx,bp,di,si,ds,es,flag : integer
              end;
   positionelement = record
                       x,y     : integer;
                     end;
   position = array [1..MAXPOINT] of positionelement;
   strings = string[80];

var
   parameter : pblock;
   contrl    : array [1.. MAXCONTRL]  of integer;
   intin     : array [1.. MAXINTIN ]  of integer;
   ptsin     : array [1.. MAXPTSIN ]  of integer;
   intout    : array [1.. MAXINTOUT]  of integer;
   ptsout    : array [1.. MAXPTSOUT]  of integer;
   currentcolor,gsxx1,gsxy1,gsxx2,gsxy2,      : color;
   gsxx3,gsxy3,gsxx4,gsxy4,iangle             : integer;
   bdosparam                                  : register;
```

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```
procedure gdos;
begin
  with bdosparam do
    begin
      CX  := $473;
      DX  := ofs(parameter);
      DS  := seg(parameter);
      intr(224,bdosparam)
    end
end;

procedure setwritemode(mode:integer);
begin
  contrl[1]  := 32;
  contrl[2]  := 0;
  intin [1]  := mode;
  gdos;
end;

procedure opengraph;
begin
  with parameter do
  begin
    contrlptr  := addr(contrl[1]);
    intinptr   := addr(intin[1]);
    ptsinptr   := addr(ptsin[1]);
    intoutptr  := addr(intout[1]);
    ptsoutptr  := addr(ptsout[1]);
  end;
  contrl[1]  := 1;
  contrl[2]  := 0;
  contrl[4]  := 10;
  intin [1]  := 1;
  intin [2]  := 1;
  intin [3]  := ord(white);
  intin [4]  := ord(dot);
  intin [5]  := ord(white);
  intin [6]  := 1;
  intin [7]  := ord(white);
  intin [8]  := ord(solid);
  intin [9]  := ord(hollow);
  intin[10]  := ord(white);
  gdos;
  iangle := 0;
  gsxxl  := 0;
  gsxyl  := 0;
  setwritemode(1);
end;

procedure cleargraph;
begin
  contrl[1]  := 3;
  contrl[2]  := 0;
  gdos;
end;
```

```
procedure line(x1,y1,x2,y2:integer);
begin
  contrl[1] := 6;
  contrl[2] := 2;
  ptsin [1] := x1;
  ptsin [2] := y1;
  ptsin [3] := x2;
  ptsin [4] := y2;
  gdos
end;

procedure fillcolor(cl:color);
begin
  contrl[1] := 25;
  contrl[2] := 0;
  intin[1]  := ord(cl);
  gdos
end;

procedure circlefill(x,y,r:integer);
begin
  contrl[1] := 11;
  contrl[2] := 3;
  contrl[6] := 4;
  ptsin[1]   := x;
  ptsin[2]   := y;
  ptsin[5]   := r;
  ptsin[6]   := 0;
  gdos
end;


procedure arc(stdeg,eddeg,x,y,r:integer);
begin
  contrl[1] := 11;
  contrl[2] := 2;
  contrl[4] := 3;
  contrl[6] := 2;
  intin[1]   := stdeg;
  intin[2]   := eddeg;
  ptsin[1]   := x;
  ptsin[2]   := y;
  ptsin[7]   := r;
  ptsin[8]   := 0;
  gdos
end;
```

```
procedure move(distance:integer);
var
  angle : real;
begin
  if iangle mod 90 = 0
    then
      case iangle of
        0   : gsxx2 := gsxx1 + distance;
        90  : gsxy2 := gsxy1 + distance;
        180 : gsxx2 := gsxx1 - distance;
        270 : gsxy2 := gsxy1 - distance;
      end
    else
      begin
        angle := iangle * 0.017453292;
        gsxx2 := gsxx1 + trunc(distance*cos(angle));
        gsxy2 := gsxy1 + trunc(distance*sin(angle));
      end;
  if currentcolor <> none
    then line(gsxx1,gsxy1,gsxx2,gsxy2);
  gsxx1 := gsxx2;
  gsxy1 := gsxy2
end;

procedure moveto(x,y:integer);
begin
  gsxx2 := x;
  gsxy2 := y;
  if currentcolor <> none
    then line(gsxx1,gsxy1,gsxx2,gsxy2);
  gsxx1 := x;
  gsxy1 := y
end;

procedure where(var x,y : integer);
begin
  x := gsxx1;
  y := gsxy1;
end;

procedure linecolor(cl : color);
begin
  contrl[1] := 17;
  contrl[2] := 0;
  intin[1]  := ord(cl);
  gdos;
  currentcolor := cl
end;
```

```
procedure turn(a : integer);
begin
  iangle := iangle + a;
  if iangle >= 360
    then iangle := iangle mod 360
    else
      if iangle < 0
        then
          while iangle <= 0 do
            iangle := iangle + 360;
end;

procedure turnto(a : integer);
begin
  iangle := a;
  if iangle >= 360
    then iangle := iangle mod 360
    else
      if iangle < 0
        then
          while iangle <= 0 do
            iangle := iangle + 360;
end;

procedure polyline(var cordinate:position; number:integer);
var
  i : integer;
begin
  contrl[1] := 6;
  contrl[2] := number;
  for i := 1 to number do
    begin
      with cordinate[i] do
      begin
        ptsin[i*2-1] := x;
        ptsin[i*2]   := y;
      end;
    end;
  gdos;
  gsxxl := cordinate[number].x;
  gsxyl := cordinate[number].y;
end;
```

```
procedure polymarker(var cordinate:position; number:integer);
var
  i : integer;
begin
  contrl[1] := 7;
  contrl[2] := number;
  for i := 1 to number do
    begin
      with cordinate[i] do
        begin
          ptsin[i*2-1] := x;
          ptsin[i*2]   := y;
        end;
    end;
  gdos;
  gsxxl := cordinate[number].x;
  gsxyl := cordinate[number].y;
end;


procedure marker(x,y :integer);
begin
  contrl[1] := 7;
  contrl[2] := 1;
  ptsin [1] := x;
  ptsin [2] := y;
  gdos;
end;


procedure polygon(var cordinate:position; number:integer);
var
  i : integer;
begin
  contrl[1] := 9;
  contrl[2] := number;
  for i := 1 to number do
    begin
      with cordinate[i] do
        begin
          ptsin[i*2-1] := x;
          ptsin[i*2]   := y;
        end;
    end;
  gdos;
  gsxxl := cordinate[number].x;
  gsxyl := cordinate[number].y;
end;
```

```
procedure writetext(x,y:integer; txt:strings);
var
  i : integer;
begin
  contrl[1] := 8;
  contrl[2] := 1;
  contrl[4] := length(txt);
  for i := 1 to contrl[4] do
    intin[i] := ord(txt[i]);
  ptsin[1] := x;
  ptsin[2] := y;
  gdos
end;

procedure characterheight(height:integer);
begin
  contrl[1] := 12;
  contrl[2] := 1;
  ptsin[1]  := 0;
  ptsin[2]  := height;
  gdos
end;

procedure fillstyle(style:filltype);
begin
  contrl[1] := 24;
  contrl[2] := 0;
  intin[1]  := ord(style);
  gdos
end;

procedure fillinterior(style:fillint);
begin
  contrl[1] := 23;
  contrl[2] := 0;
  intin[1]  := ord(style);
  gdos
end;

procedure polymarkercolor(cl:color);
begin
  contrl[1] := 20;
  contrl[2] := 0;
  intin[1]  := ord(cl);
  gdos;
end;

procedure polymarkertype(marker:markertype);
begin
  contrl[1] := 18;
  contrl[2] := 0;
  intin[1]  := ord(marker);
  gdos;
end;
```

```
procedure characterupvector(angle:integer);
begin
   contrl[1] := 13;
   contrl[2] := 0;
   intin[1]  := angle*10;
   gdos;
end;

procedure textcolor(cl:color);
begin
 contrl[1] := 22;
 contrl[2] := 0;
 intin[1]  := ord(cl);
 gdos;
end;

Function randomcolor : color;

   var
     t                : integer;
     coldetermin : 1..7;
     cl               : color;

   begin
     randomize;
     coldetermin:=trunc(random*7+1);
     cl:=black;
     for t:=1 to coldetermin do cl:=succ(cl);
     randomcolor:=cl;
   end;

Procedure box(x1,y1,x2,y2 :integer);
   begin
     line(x1,y1,x1,y2);
     line(x1,y2,x2,y2);
     line(x2,y2,x2,y1);
     line(x2,y1,x1,y1);
   end;

procedure circle(x,y,r:integer);
var        i      : integer;
           theta : real;
begin
   polymarkercolor(red);
   polymarkertype(dot);
   for i:=0 to 360 do
     begin
       theta:=Pi*i/180;
       gsxx1:=round(r*sin(theta));
       gsxy1:=round(r*cos(theta)*64/40);
       marker(x+gsxx1,y+gsxy1);
     end;
end;
```

```
{***************************************************}
{************        G-VRAM Hard Copy Procedures        ************}
{***************************************************}



Procedure copygraph;

   const Copy8bit: array[0..5] of char=(#$1B,'S','0','6','4','0');
         CopyMode: array[0..1] of char=(#$1B,'D');
         NatvMode: array[0..1] of char=(#$1B,'M');
         PrntMode: array[0..1] of char=(#$1A,'C');
         LFcopy  : array[0..3] of char=(#$1B,'T','1','6');
         LFnative: array[0..1] of char=(#$1B,'A');
         PrnBusy = $42;
         PrnWrite= $40;
         PrnCtrl = $46;

   type  bitblock = array[0..7] of byte;
         VRAM     = array[0..399,0..79] of byte;

   var   BluV                      : VRAM absolute $A800:0;
         RedV                      : VRAM absolute $B000:0;
         GrnV                      : VRAM absolute $B800:0;
         h,i,j,k,ix,kx             : integer;
         gbuf,pbuf                 : bitblock;




   procedure BitPatTxfer(gbuf:bitblock; var bitimage:bitblock);

      const    mask : byte = $01; {0000-0001}

      var      i, j                : integer;
               Pout,maskedGB       : byte;

      begin
        for i:=7 downto 0 do
           begin
             for j:=7 downto 0 do
                begin
                  Pout:= Pout shl 1;
                  maskedGB:= gbuf[j] and mask;
                  Pout:=Pout or maskedGB;
                  gbuf[j]:= gbuf[j] shr 1;
                end;
             bitimage[i]:=Pout;
           end;
      end;
```

```
Begin
  write(lst,LFcopy);
  write(lst,PrntMode);
  write(lst,CopyMode);
  for i:=0 to 49 do
    begin
      write(lst,Copy8bit);
      ix:=i*8;
      for j:=0 to 79 do
        begin
          for k:=0 to 7 do
            begin
              kx:=ix+k;
              gbuf[k]:= BluV[kx,j];
              gbuf[k]:= gbuf[k] or RedV[kx,j];
              gbuf[k]:= gbuf[k] or GrnV[kx,j];
            end;
          BitPatTxfer(gbuf,pbuf);
          for h:=0 to 7 do
            begin
              repeat until 164=port[PrnBusy];
              port[PrnWrite]:=pbuf[h];
              port[PrnCtrl] :=14;
              port[PrnCtrl] :=15;
            end;
        end;
      writeln(lst);
    end;
  write(lst,NatvMode);
  write(lst,LFnative);
End;
```

```
{ ************************************************************ }
{ *************        File I/O Procedures        ************* }
{ ************************************************************ }

    label   OPE_START;

    type    name    = string[14];
            regist  = record
                          ax,bx,cx,dx,bp,si,di,ds,es,flag : integer;
                      end;

    var     select_ope ,drive        : Char;
            direxist                 : boolean;
            registparameter          : regist;
            FCB                      : string[38];
            DTA                      : string[128];



Function Exist(filename : name) : boolean;

    var    filevar : file;

    begin
      assign(filevar,filename);
        {$I-}
      reset (filevar);
      close (filevar);
        {$I+}
      exist:=(IOresult=0);
    end;



Procedure UpperCase (var strg : name );

    begin
      inline
        ($C4/$BE/Strg/        {            LES    DI,Strg[BP]           }
         $26/$8A/$0D/          {            MOV    CL,ES:[DI]            }
         $FE/$C1/              {            IND    CL                    }
         $FE/$C9/              { L1:        DEC    CL                -   }
         $74/$13/              {            JZ     L2                    }
         $47/                  {            INC    DI                    }
         $26/$80/$3D/$61/      {            CMP    ES:BYTE PTR [DI],'a'  }
         $72/$F5/              {            JB     L1                    }
         $26/$80/$3D/$7A/      {            CMP    ES:BYTE PTR [DI],'z'  }
         $77/$EF/              {            JA     L1                    }
         $26/$80/$2D/$20/      {            SUB    ES:BYTE PTR [DI],20H  }
         $EB/$E9);            {            JMP    SHORT L1              }
                              { L2:                                     }
    end;
```

```pascal
Procedure readkeyboard(var character : char);

  var      charcode  : integer;

  begin
    with registparameter do
      begin
        ax:=$0100;
        MsDos(registparameter);
        charcode := ax and $00ff;
        character:= char(charcode);
      end;
  end;




Function currentdrive : char;

  var     driveNO   : integer;

  begin
    with registparameter do
      begin
        ax:= $1900;
        MsDos(registparameter);
        driveNO:=ax and $00ff;
        currentdrive:=char(driveNO+65)
      end;
  end;




Procedure selectdrive(newdrive: char);

  begin

    with registparameter do
      begin
        ax:= $0E00;
        dx:= integer(upcase(newdrive))-65;
        MsDos(registparameter);
      end;
    end;
```

```
Procedure setFCB (filename : name);

  begin
    with registparameter do
      begin
        ax:=$2900;
        ds:=seg(filename);
        si:=ofs(filename)+1;
        es:=seg(FCB);
        di:=ofs(FCB);
        MsDos(registparameter);

        if ax=$29FF
           then
              begin
                writeln ('Invalid file name or drive. ');
              end;
      end;
  end;




Procedure setDTA;

  begin
    with registparameter do
      begin
        ax:=$1A00;
        ds:=seg(DTA);
        dx:=ofs(DTA);
        MsDos(registparameter);
      end;
  end;




Procedure searchfirstentry;

  begin
    with registparameter do
      begin
        ax:=$1100;
        ds:=seg(FCB);
        dx:=ofs(FCB);
        MsDos(registparameter);

        if ax=$1100
           then direxist:= true
           else direxist:= false
      end;
  end;
```

```pascal
Procedure searchnextentry;

   begin
     with registparameter do
       begin
         ax:=$1200;
         ds:=seg(FCB);
         dx:=ofs(FCB);
         MsDos(registparameter);

         if ax=$1200
            then direxist:= true
            else direxist:= false
       end;
   end;




Procedure deletedirentry;

   begin
     with registparameter do
       begin
         ax:=$1300;
         ds:=seg(FCB);
         dx:=ofs(FCB);
         MsDos(registparameter);
       end;
   end;




Procedure memoryout (segment,offset,bytenum : integer);

   var      asciichar  : char;
            bytedump,i : integer;

   begin
     for i:=0 to bytenum-1 do
       begin
         bytedump:=mem [segment:offset+i];
         asciichar:=chr(bytedump);
         write(asciichar);
         if i mod 16 = 15  then writeln;
       end;
   end;
```

```
Procedure dumpfilename (dumpfilename : name ; wide : boolean);

  label    DUMPEND;

  var      j        : integer;

  begin
    setFCB(dumpfilename);
    setDTA;
    j:=0;

    searchfirstentry;
    if direxist=false
      then
        begin
          writeln ('No files.');
          goto DUMPEND;
        end;
    memoryout (seg(DTA),ofs(DTA)+1,11);
    if wide=true
      then write ('  /  ')
      else writeln;

    repeat
      begin
        searchnextentry;
        if direxist=true
          then
            begin
              memoryout (seg(DTA),ofs(DTA)+1,11);
              if wide=true
                then
                  begin
                    write ('  /  ');
                    j:=j+1;
                    if j mod 5 = 4  then writeln;
                  end
                else  writeln;
            end;
      end;
    until direxist=false;
    DUMPEND:
    writeln;
  end;
```

```pascal
Procedure directory ;
  var      dirfilename : name ;
  begin
    write ('File name : ');
    readln (dirfilename);
    if dirfilename=''
      then dirfilename:='*.*';
    if dirfilename='a:'
      then dirfilename:='a:*.*';
    if dirfilename='b:'
      then dirfilename:='b:*.*';
    if dirfilename='c:'
      then dirfilename:='c:*.*';
    if dirfilename='d:'
      then dirfilename:='d:*.*';
    if pos(':',dirfilename)=0
      then dirfilename:=currentdrive+':'+dirfilename;
    dumpfilename (dirfilename,true);
    writeln;
  end;




Procedure filerename;
   var    filevar        : file;
          oldfilename,
          newfilename  : name;
   begin
     write('File name to rename : ') ; readln(oldfilename);
     if pos(':',oldfilename)=0
       then oldfilename:=currentdrive+':'+oldfilename;
     Uppercase(oldfilename);
     write('            new  name : ') ; readln(newfilename);
     if pos(':',newfilename)=0
       then newfilename:=currentdrive+':'+newfilename;
     Uppercase(newfilename);
     writeln;
     if exist(oldfilename)=false
        then
          writeln('File  ',oldfilename,'  not found. ')
        else
          if exist(newfilename)=true
            then
              writeln('File  ',newfilename,'  already exists !')
            else
              begin
                writeln('Rename  ',oldfilename,'  as  '
                         ,newfilename);
                assign(filevar,oldfilename);
                rename(filevar,newfilename);
              end;
     writeln;
     writeln('rename end.');
     writeln;
   end;
```

```
Procedure fileerase;

   var       erasefilename : name ;
             erasefilevar  : file ;
             answer        : char ;

   begin
     repeat
       begin
         write  ('File name to erase : ');
         readln (erasefilename);
         if pos(':',erasefilename)=0
           then erasefilename:=currentdrive+':'+erasefilename;
         uppercase (erasefilename);
         assign (erasefilevar,erasefilename);
         setFCB (erasefilename);
         writeln;
         dumpfilename (erasefilename,false);

         repeat
           begin
             write ('correct ? (y/n)  ');
             readkeyboard(answer); writeln;
             writeln;
           end;
         until answer in ['Y','y','N','n'];
       end;
     until answer in ['y','Y'];

     deletedirentry;

     writeln ('file erase end.');
     writeln;
   end;




Procedure filecopy;

   const     bufrecsize  = 200;      { 200 records in a buffer }
             bufbytesize = 25600;    { 200 records * 128 byte   }

   var       sourcefilename,
             destinationfilename  : name ;
             sourcevar,
             destinationvar       : file ;
             buffer               : array [1..bufbytesize] of byte;
             MFR,{Max File Records}
             EOR {End Of Records} : integer ;
```

```
begin
  write ('source file name       : ');
  readln( sourcefilename );
  if pos(':',sourcefilename)=0
        then sourcefilename:=currentdrive+':'+sourcefilename;
  uppercase( sourcefilename );
  write ('Destination file name : ');
  readln( destinationfilename );
  if pos(':',destinationfilename)=0
     then destinationfilename:=currentdrive+':'
                                +destinationfilename;
  uppercase( destinationfilename );
  writeln;
  if exist(sourcefilename)=false
     then
       writeln('File  ',sourcefilename,'  not found.')
     else
       if exist(destinationfilename)=true
          then
            writeln('File  ',destinationfilename,
                            '  already exist !')
          else

    begin
      writeln('Copying  ',sourcefilename,'  to  ',
                          destinationfilename);
      assign(sourcevar,sourcefilename);
      reset(sourcevar);
      assign(destinationvar,destinationfilename);
      rewrite(destinationvar);
      MFR:=filesize(sourcevar);
      while MFR>0 do
        begin
          if MFR<=bufrecsize
             then  EOR:=MFR
             else  EOR:=bufrecsize;
          blockread(sourcevar,buffer,EOR);
          blockwrite(destinationvar,buffer,EOR);
          MFR:=MFR-bufrecsize;
        end;
      close(sourcevar);
      close(destinationvar);
    end;
  writeln;
  writeln('filecopy end.');
  writeln;
end;
```

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```
Procedure changedrive;

  begin
    write  ('New Drive : ');
    readln (drive);
    selectdrive(drive);
    writeln;
  end;
```