in contrast to the possibility that the harmonics are present entirely as a traveling wave within the cochlea.

Relative amplitudes of f_1, f_2, and f_3 plotted as a function of signal level for one unit are shown in Fig. 3a. For this unit, and generally, (i) the amplitude of f_1 is proportional to stimulus amplitude at low stimulus levels; (ii) the amplitude of f_2 is greater than that of f_3; and (iii) the amplitudes of f_2 and f_3 rise more rapidly than that of f_1 at low signal levels. Each of these properties corresponds to observations of amplitude characteristics of harmonics of CM (*2*). Figure 3b shows the relative phase plotted as a function of signal level of the fundamental component for three different frequencies for the same unit as that shown in Fig. 3a. Changes of phase of less than 20 degrees over dynamic ranges of 50 to 60 db are not uncommon. The amplitude and phase correspondences reported above suggest that there is a very close relationship between the CM and the discharge activity of cochlear nerve fibers.

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7. The characteristic frequency is the sinusoidal frequency to which the fiber is most sensitive. The precision to which this number can be determined depends on several conditions. Those values in Fig. 1 are approximate to within 100 hz for low CF and to within several hundred hertz for higher CF.
8. E. Wever and M. Lawrence, *Physiological Acoustics* (Princeton Univ. Press, Princeton, N.J., 1954), p. 172. We also have made comparisons between amplitudes of harmonics as a function of signal level and amplitudes of fundamentals mimicking the harmonics in frequency, that is, f_2 compared to responses to $2f_1$, and f_3 compared to responses to $3f_1$. Our results are essentially the same as those reported in figures 4 and 5 of reference (*2*). The data provide further support for the similarity between results for single fibers and CM.
9. It is well known that for click stimulation the microphonic of the round window potential occurs ahead of the N_1N_2 component. Therefore, in an attempt to obtain quantitative agreement between CM and single fiber results, we have incorporated in the plots of Fig. 1 an estimated 0.5 msec delay between initiation of the CM and the initiation of the spike discharges. (To change back to the original single fiber results, one merely adds 1.0π of the lag per kilohertz). The differences remaining are perhaps due to the difference in species.
10. Supported by PHS research grants NS 07498, FR-396, FR-504, and FR-6115 from the National Institutes of Health. We thank K. Williams, M. A. Kelly, R. Cox, and T. DeWoskin for their assistance and support.

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Preparation of Beating Heart Cells from Adult Rats

Abstract. *Suspensions of cardiac cells have been prepared from fragments of hearts from adult rats by prolonged incubation with trypsin and collagenase. In these preparations, about 50 percent of the viable cells routinely exhibit spontaneous contractions ranging from 20 to 120 beats per minute at room temperature. The contractile activity is essentially lost at 2°C but returns to normal as the temperature is increased. The rate of cellular contractions and the percentage of total cells exhibiting contractility are influenced by experimental conditions of cell preparation and the composition of the suspending medium.*

The routine preparation of component cells from various tissues can be accomplished by tissue perfusion or incubation with a variety of proteolytic enzymes. In 1955, Cavanaugh (*1*) reported on the preparation of suspensions of heart cells obtained by incubation of neonatal chick heart (5 days old) with trypsin. However, spontaneous contractions were observed in only a portion of the cells and only after several hours, when the cells attached to the glass culture tube. Similarly, individual cells isolated from heart tissue of neonatal rats exhibit spontaneous beating after attachment of the cells to the glass surface of the culture vessel (*2*). These cells continue to beat in culture as they increase in size, and, as the cells come into contact with each other, they begin to beat in synchrony (*1*, *3*). Harary (*4*) recently reviewed the various factors influencing contractility and certain metabolic activities of neonatal rat heart cells in culture.

Until now, there has not been a report of the preparation of spontaneously beating cells from adult mammalian tissue. Kono (*5*) recently reported on the preparation of a cell suspension from cardiac muscle tissue from adult rats but did not describe the viability of the cells. Harary and Farley (*3*) indicated that cells prepared from hearts of 6-week-old rats are relatively inactive, and those prepared from hearts of 16-week-old rats are essentially all spherical cells with no contractile properties.

The present report describes a procedure for preparation of cardiac cells from adult heart tissue and certain factors influencing contractility of these cells in dispersed suspensions.

Hearts from adult male rats (250 g) were dissected free of major blood vessels and atria and were minced into coarse fragments (3 mm) with a razor blade. These were incubated for 30 minutes at 34° to 36°C in saline A (*6*) containing 0.1 percent trypsin and 0.1 percent collagenase (2 to 3 ml per heart). The supernatant was discarded and the remaining tissue fragments were reincubated for 30 minutes in 2 to 3 ml of saline A solution containing 0.1 percent trypsin and 0.05 percent collagenase. At 30-minute intervals thereafter, the supernatants were collected and the residual tissue fragments were reincubated in fresh enzyme solution. Heart cells were obtained from the supernatants by gentle centrifugation, and these were resuspended in various media. Total number of cells and the percentage of total cells with contractile properties were determined by repeated counting of samples of the cell suspensions on hemocytometer slides. A qualitative measurement of viability of the nonbeating cells was obtained by vital staining with neutral phenol red.

The incubation of adult heart tissue fragments with either trypsin or collagenase alone was ineffective in yielding a large number of cardiac cells with spontaneous contractile behavior (Fig. 1). Cells obtained during the initial 30-minute incubation in the first enzyme solution described below consisted largely of blood cells and damaged cardiac cells. The total number of cells and the proportion of contracting cardiac cells progressively increased as the incubation was continued. Cell suspensions obtained from the 90- to 120-minute incubation contained primarily cardiac cells, of which approximately 80 percent were rod-shaped (Fig. 2) and of these 40 to 60 percent exhibited spontaneous contractions ranging from 20 to 120 beats per minute. This same incubation procedure has been applied successfully to the preparation of contracting cardiac cells from adult human heart (*7*).

The cells generally range in size from 10 to 50 μm in diameter and from 50

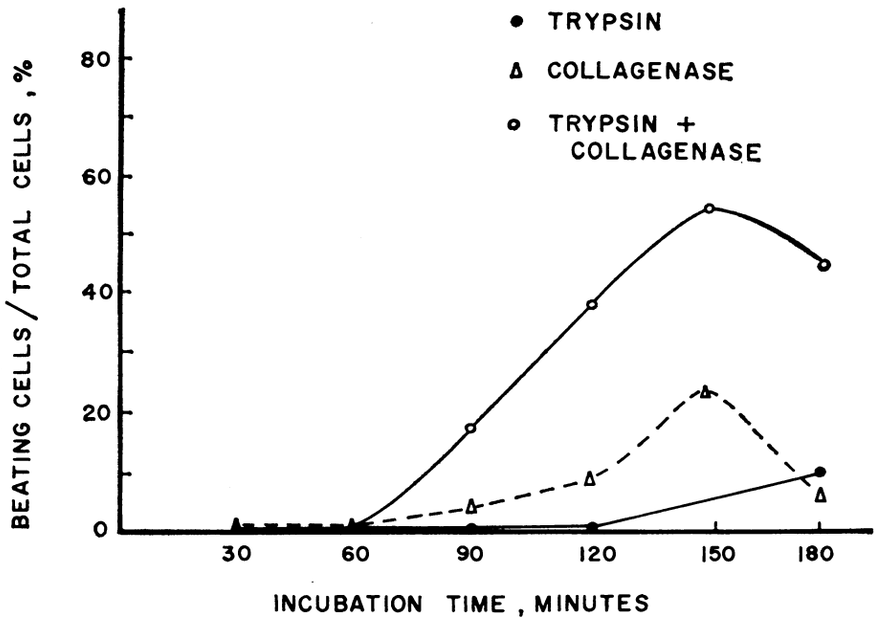

Fig. 1. Composition of enzymes and time of incubation. For the first 30 minutes, the heart fragments were incubated at 37°C with an enzyme solution consisting of either 0.1 percent trypsin, 0.1 percent collagenase, or a mixture of the two in saline A (*6*). Subsequent incubations were performed with the same enzyme solution used initially except that the collagenase level was reduced to 0.05 percent.

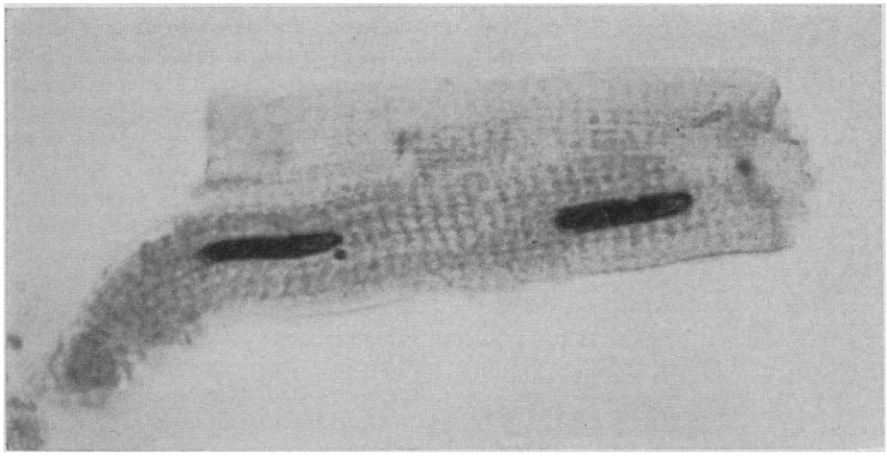

Fig. 2. Photomicrographs of individual cardiac cells stained with hematoxylin and eosin (× 760).

to 100 μm in length. They are highly refractile and exhibit characteristic cross-striations; it is also possible to visualize the intercalated discs by phase microscopy. Approximately 80 percent of the cells in suspension are binucleate (Fig. 2), as shown by hematoxylin and eosin staining, and include both rod-shaped and rounded cells. The remainder of the cells are mononucleated, although it is possible in most preparations to find fragments containing three nuclei.

Although the cells will become attached to the glass surface of the slide during microscopy, attachment is not a requisite for the spontaneous contractions of adult cardiac cells. Thus, the cells can be maintained as a highly dispersed suspension for several hours and exhibit contractile behavior during this period.

The presence of spherical cardiac cells has been described by several investigators (see *6*, *8*); prior to settling and attachment to the glass surface, cells prepared from hearts of 3- to 10-day-old rats are spherical (*3*). We have observed a "rounding up" process with beating cardiac cells, and this change in morphology is commonly associated with the termination of cellular contractions. As the process occurs there is a shortening in cell length, increase in the rate of contraction, loss of cross-striations, and, eventually, the cells become spherical and appear granular. These rounded cells no longer contract and finally die, as evidenced by vital staining. Infrequently cells will cease contracting without apparent structural alterations.

Cardiac cells can be maintained for several hours at 2°C, but at this temperature they do not exhibit spontaneous contractions. At 25°C about 50

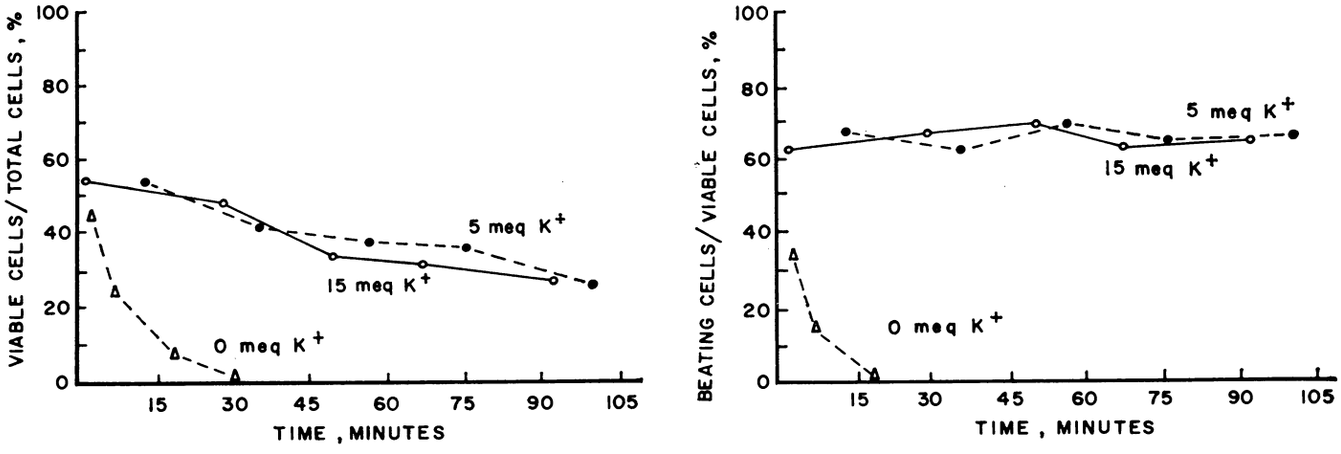

Fig. 3. Effect of K^+ concentration on cardiac cell viability and contractility.

percent of the cardiac cells are contracting at rates ranging from 20 to 120 beats per minute with a mean rate of 30 to 40 beats per minute. Approximately one-half of the cells that are resuspended in 0.9 percent sodium chloride solution or Krebs-Ringer buffer (minus Ca^{2+}) containing glucose lose contractile activity over a 2-hour period at room temperature. This does not appear to be related to a limitation of an energy source, since the further addition of glucose or albumin-bound palmitic acid to the medium does not have marked influence on contractile activity. Furthermore, with the conditions employed at present, addition of adenosine triphosphate (5 to 100 μM) does not affect either the initiation or maintenance of cardiac cell contractions.

Alterations in the osmolality of the suspending medium (0.9 percent NaCl) had marked effects on the proportions of contracting cardiac cells to total cells. Increasing the osmolality from 305 to 320 meq/liter with sucrose resulted in a sharp decrease (from 55 to 18 percent) in the percentage of beating cells. Any further increase in osmolality resulted in complete cessation of contractile activity; it could be restored to the original level by reducing osmolality to 305 meq/liter.

The absence of potassium in the suspension medium resulted in a rapid loss in both total viable cells and contracting cells (Fig. 3). At concentrations of 5 and 15 meq/liter of K^+, the percentage of contracting cardiac cells was initially high and remained constant throughout the 100-minute experimental period. During this same period, the percentage of viable cells decreased from 55 percent initially to approximately 30 percent. The presence of calcium (0.5 to 5.0 meq/liter) caused immediate cessation of cardiac cell contractions.

The procedure for tissue dissociation reported here results in a cell suspension containing a high percentage of viable cells, of which approximately half exhibit contractile properties. To date, the most important determinant for maintenance of both cell viability and contractility appears to be the composition of the medium in which the cells are resuspended after dissociation. Preliminary studies have indicated that this type of preparation can be useful in investigating metabolic pathways and control mechanisms in cardiac tissue.

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Asexual Reproduction in a Sipunculan Worm

Abstract. *The sipunculan worm* Aspidosiphon brocki *reproduces asexually by transverse fision into two unequal parts, the smaller part comprising the posterior fifth of the animal. Prior to fission each part regenerates the structures essential to the formation of a new individual. The smaller posterior part (daughter) regenerates an anterior body, including introvert, anterior gut, retractor muscles, and nephridia, whereas the larger anterior part (parent) regenerates only the posterior body wall.*

Asexual reproduction was unknown in sipunculan worms until Rajulu and Krishnan (*1*) reported "budding" in four specimens of *Sipunculus robustus* after they had maintained the animals in stale seawater in the laboratory. I now report that a small species of *Aspidosiphon* from the Caribbean Sea and the Straits of Florida undergoes asexual reproduction both in the field and in the laboratory by constriction and subsequent detachment of the posterior end to form a new individual. The constriction occurs at a distance approximately one-fifth of the total length of the trunk from the posterior extremity. The internal organs which are severed by the constriction and thereby segregated into the posterior body are incorporated along with a regenerated anterior end in the formation of the "daughter" individual. When the narrow neck of the constriction closes, the posterior portion of the "parent" animal drops off and is at once replaced by the eversion of a new posterior end that is formed during constriction. At the time of its detachment or within 1 to 3 days the separated posterior entity or daughter individual everts the regenerated anterior end, formed prior to detachment, thus completing the formation of the "juvenile" (*2*).

The specimens observed in this study, tentatively identified as *Aspidosiphon brocki* Augener 1903 (*3*), were collected in Jamaica, Puerto Rico, and the Florida Keys from intertidal coralline limestone where they inhabit burrows, presumably of their own formation (*4*). Adult specimens range from 3 to 10 mm long with introvert retracted. Characteristic of the genus, horny cuticular shields are present on the anterior and posterior ends of the trunk, although in this species the posterior shield is weakly developed. The introvert, when extended, is nearly as long as the trunk. A single pair of retractor muscles that function in the withdrawal of the introvert originates from the body wall in the posterior quarter of the trunk.

Twenty-three specimens were collected in which posterior constrictions were present. Sixteen were preserved or fixed for histological study. The remaining 7 were maintained alive in the laboratory until detachment of the posterior end occurred (5 to 14 days after collection) and the juvenile was fully formed. At one station in the Florida Keys, where all specimens were counted and carefully examined for asexually reproducing individuals, 15 percent (8 of 53) had posterior constrictions. Moreover, in two different observations, a juvenile, apparently recently detached, and a detached posterior end were found, each occupying the posterior portion of a burrow of an adult worm.

In the constricted animal, the area of the constriction is encircled externally by a blackened band, presumably of cuticular origin (Fig. 1a). Internally, the stricture is traversed by a noncellular sheet of unknown composition (Fig. 2). The constriction occurs immediately caudad to the origin of the re-