J. Membrane Biol. 34, 313–329 (1977)

Growth Orientation of Heart Cells on Nylon Monofilament

Determination of the Volume-to-Surface Area Ratio and Intracellular Potassium Concentration

C. Russell Horres, Melvyn Lieberman, and Joyce E. Purdy

Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710

Received 1 September 1976

Summary. A new method is described for orienting the growth of embryonic chick heart cells as thin annuli about nylon monofilament. Analytical measurements of cell water, intracellular potassium, cell volume, and cell surface area incorporate several new techniques and provide the quantitative basis for characterizing the respective cell types in the preparation. The measurements support the hypothesis that tissue culture methodology does not alter the morphological and physiological properties of cardiac muscle cells. The preparations are ideally suited for radiotracer studies since tissue mass can be increased while retaining a relatively short diffusional distance.

The membrane properties of cardiac cells derived from electrical measurements have not been successfully correlated with those derived from ionic flux studies [2, 7]. Moreover, the complex structure and cellular heterogeneity of naturally occurring cardiac muscle may complicate the interpretation of ionic fluxes obtained from radio-tracer experiments [24]. Therefore, by applying tissue culture techniques we attempted to develop preparations of simplified geometry and defined cell type that subsequently would provide the basis for investigating ionic movements across cardiac cell membranes [12]. Previous studies described a technique for growing small preparations ("synthetic strands") of embryonic cardiac muscle cells within narrow grooves cut in an agar film [17] and subsequently demonstrated the suitability of such relatively simple preparations in electrophysiological studies [16, 18, 19]. However, there were serious limitations for ionic flux studies (*cf.* Discussion). In the present study, we describe the growth orientation of heart cells about a thin nylon monofilament and demonstrate the usefulness of the preparation for obtaining several measurements, namely, cell water, intracellular potas-

sium, cell volume and cell surface area, all of which are necessary to evaluate properly transmembrane potassium flux [15].

Materials and Methods

Tissue Culture

Suspensions of cardiac cells used for preparing growth-oriented strands were obtained by enzymatic disaggregation of either 11-day-old embryonic chick hearts (White Rock, Pittsboro Hatchery) or primary mass cultures of embryonic heart cells. Cells derived from the disaggregation of intact embryonic hearts were used to produce contractile preparations, while those obtained from primary mass cultures were used to develop noncontractile preparations.

Finely minced heart tissue was exposed to 0.1% trypsin in modified GIBCO-A (*see* Table 1) at 37 °C. Agitation and gentle pipetting were employed to assist in the separation

Table 1. Tissue culture solutions

Solution[a]	Modified GIBCO-A	Medium, R	Medium, F
NaCl (mM)	116.0	118.0	118.0
KCl (mM)	5.3	5.0	5.4
$NaH_2PO_4 \cdot H_2O$ (mM)	–	0.94	0.94
$MgSO_4 \cdot 7\ H_2O$ (mM)	–	0.81	0.81
$CaCl_2$ (mM)	–	2.7	2.7
$NaHCO_3$ (mM)	26.2	26.2	26.2
Glucose (mM)	5.6	5.6	5.6
Phenol red (mg/liter)	0.125	0.125	0.125
CO_2/air (%)	4/96	4/96	4/96
pH	7.5	7.4	7.4
Trypsin[b] (g/liter)	1.0	–	–
Medium 199[c] (%)	–	65	65
Penicillin[d] (units/ml)	–	100	100
Streptomycin[e] (μg/ml)	–	50	50
Fetal Calf Serum[f] (%)	–	10	10
Embryo Extract[g] (%)	–	–	2

[a] All solutions were prepared from double-distilled water and sterilized by filtration (0.45 μm, Millipore).

[b] 1:300 Trypsin – Nutritional Biochemical Corp.

[c] Vitamin and Amino Acid Supplement in Earle's Solution (1X) – Grand Island Biological Co.

[d] Penicillin G – E.R. Squibb and Sons.

[e] Streptomycin Sulfate – Eli Lilly and Company.

[f] Fetal Calf Serum – Grand Island Biological Co.

[g] Embryo Extract – 12 day chick embryo (50% in K-free salt solution). (*See* Kaighn *et al.* [13] for details.)

of cells. At periodic intervals of 6–10 min, the entire enzyme solution containing free cells was drawn off and immediately added to an equal volume of cold (0 °C) medium (R, Table 1). In this manner, isolated cells were exposed to activated trypsin for no longer than 10 min. After completion of the disaggregation, the cells were filtered through four layers of gauze to remove residual clumps of tissue, and the suspension was centrifuged at $225 \times g$ for 6 min. The supernatant was discarded and the cell pellet was resuspended in cold culture medium (R). A cell count was made with a hemocytometer and the suspension appropriately diluted to obtain the desired cell concentration (1×10^7 cells/ml). Viability of cells in the suspension was usually found to be greater than 95% when evaluated by the trypan-blue exclusion method [6].

The proportion of muscle cells in culture was increased by utilizing the differential adhesive properties of the cells, i.e., muscle *vs.* nonmuscle [26, 35]. One ml of the cell suspension from the initial disaggregation was placed into a 100-mm culture dish (No. 3003, Falcon Plastics) containing 4 ml of culture medium (F, Table 1) and incubated for 1 hr at 37 °C. During this period, the quiescent fibroblastlike cells preferentially attached to the dish with proportionally more of the muscle cells remaining in the supernate. A new cell pellet was then prepared from the muscle-enriched supernate and appropriate dilutions were made for seeding the growth chambers (*see below*).

The cells which remained attached to the culture dish were incubated for one week, the medium being replaced after four days. To retrieve these cells, the culture medium (F) was removed, the dish rinsed with 10 ml of balanced salt solution, and 7 ml of 0.05% trypsin added for 10 min at 37 °C. After 5 min, the trypsin solution was pipetted gently to aid disaggregation. Trypsin activity was then quenched by the addition of culture medium. The resultant cell suspension was processed in the same manner as in the initial disaggregation procedure and then used as the seeding population for noncontractile preparations.

Growth Orientation Chamber

A nylon monofilament (20 μm OD)[1] was wound around a U-shaped support (11 × 33 mm, *see* Fig. 1 *C*) formed by bending silver wire (1.27 mm OD) on a mandrel (8 mm OD). To automate the winding operation, an engine lathe was designed to include a thread guide and spool holder mounted on a power-driven tool post.[2] The wire support was secured in the lathe chuck such that its axis of rotation was parallel to the travel of the tool post. As the support turned and the monofilament was drawn from its spool, a travelling guide spaced the filament evenly along the support at a pitch of twelve threads per centimeter. During winding, tension on the monofilament was maintained by the frictional resistance created by the unwinding of the spool. After 35 windings of the monofilament, a small rubber clamp was placed on the side opposite the tying point to prevent the monofilament from shifting while it was cut and tied with a fine glass hook.

Growth of the preparations was restricted to the nylon monofilament by a chamber (Fig. 1) which was designed to prevent contact between the silver support and the cells. The chamber was fabricated from silicone rubber (Sylgard 184, Dow Corning) and consisted of two molded parts: a bottom section (Fig. 1 *E*) containing recessed areas into which the silver support could be fitted and an insert section (Fig. 1 *B*) which pressed the silver support into the recesses and clamped the threads ca. 100 μm above the central compartment that was separated from the support. The recessed area of this compartment contained a sterile glass cover-slip (30.10 mm × 5.21 mm, No. 1 thickness; Corning Glass Co.) that

1 Multistranded cord of nylon-66 monofilament was generously provided by the Monsanto Research Corporation, Research Triangle Park, N.C.

2 A detailed photograph of this apparatus will be provided upon request.

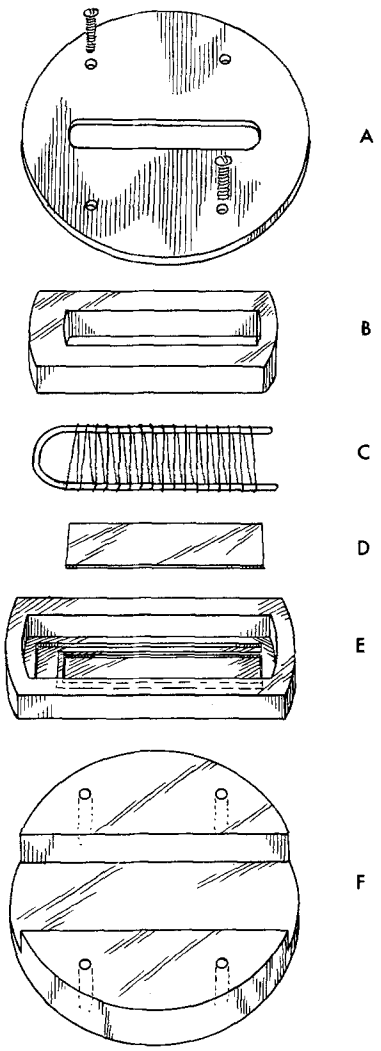

Fig. 1. Exploded view of chamber assembly. (*A*) clamp top; (*B*) chamber insert; (*C*) support assembly; (*D*) cover glass insert; (*E*) chamber bottom; (*F*) clamp base

was covered with a thin, dry layer of 1% agar (Purified Agar, Difco Laboratories; *see* Fig. 1 *D*). Chamber accessories included a methyl methacrylate base (Fig. 1 *F*) to house the silicone rubber chamber bottom. This base, in combination with a stainless steel lid (Fig. 1 *A*), served as a clamp to secure the rubber insert.[3] The lid, which contained a channeled opening to allow optical access into the chamber, was covered by the bottom portion of a snugly fitting inverted culture dish to prevent contamination of the culture.

3 Future chambers should be made with a metal clamping base since the methacrylate base tends to become distorted with frequent use at 37 °C.

Fig. 2 shows a cross-section of the assembled growth chamber (upper panel) and a detailed drawing of the methyl methacrylate mold used in the fabrication of the chamber bottom (lower panel).[4]

The component parts of the growth chamber were boiled in a nontoxic detergent (7X, Linbro Chemical Co.) and rinsed thoroughly with distilled water. After drying, the components were sterilized by autoclaving and the chamber was then assembled under sterile conditions.

Twenty-four hours prior to cell inoculation, 1 ml of culture medium was added to the assembled chamber. At the beginning of the growth period, the medium was replaced with a 1-ml suspension of cells (1×10^5 to 10^6 cells depending on the size of the desired preparation). In most cases, preparations were obtained from a population of 4×10^5 cells per ml. The chambers were stored undisturbed in a water-jacketed incubator (Model 3341, National Appliance Co.) set at 37 °C and gassed with 4% CO_2 and 96% air. Preparations could only remain in the growth chamber for a limited period of time because cellular bridges would tend to form between the nylon strands. Therefore, after 72 hr of incubation, the chambers were disassembled under sterile conditions and the preparations were quickly transferred to culture dishes (60 mm) which contained a silicone rubber ring with indentations for suspending the clip above the bottom of the dish. Preparations were then incubated for an additional one to two days.

Analytical Determinations

Cell water and intracellular potassium. Cell water content of the preparation was determined by taking the difference between the wet weight (corrected for extracellular water) and the dry weight as determined below. The contribution of the extracellular and extraneous water, i.e., that which adhered to the surface of the preparation, to tissue wet weight was accounted for in the following manner. The entire wet weight procedure was performed in a high humidity environment by modifying a tissue culture incubator (Model 329, Forma Scientific) to contain glove ports and interior lighting. The preparations were first equilibrated for 15 min in culture medium containing 29 μM/liter of the extracellular marker, ^{125}I-iothalamic acid (Abbott Laboratories), a compound shown in previous studies to be an effective substitute for inulin [28]. To remove the excess solution adhering to the surface, the preparations were totally immersed in fluorocarbon (Medifluor FC-80, 3M Company) contained in 13×100 mm test tubes. By tapping and shaking the tubes, excess culture medium was made to rise to the surface of the fluorocarbon where it was removed by blotting with cotton-tipped applicators. The monofilament was then removed from the silver support as an intact loop, transferred within the incubator to the weighing chamber

4 The chamber bottom was created by casting silicone rubber over the methyl methacrylate mold placed in the center of a 60-mm culture dish (No. 3002, Falcon Plastics). The insert section of the chamber was made by injection molding. For this operation, a blank mold was fabricated of similar dimensions to the one previously described, but 3.20 mm thicker and lacking the surface features of the first mold. The blank was then used to create another chamber bottom with deeper sides than the first. This new bottom served as the injection mold for the insert section when a top, containing a plug having the dimensions of the glass insert, was clamped over the opening. Silicone rubber was injected into one side of the assembled mold which contained a needle at the opposite side to provide a vent. All surfaces were lightly coated with liquid detergent (7X-Linbro Chemical Co.) to enhance release after curing. The rubber was allowed to cure for 24 hr at room temperature and was post-cured by boiling in water for about 30 min. The chamber bottom was trimmed to a width of 25 mm.

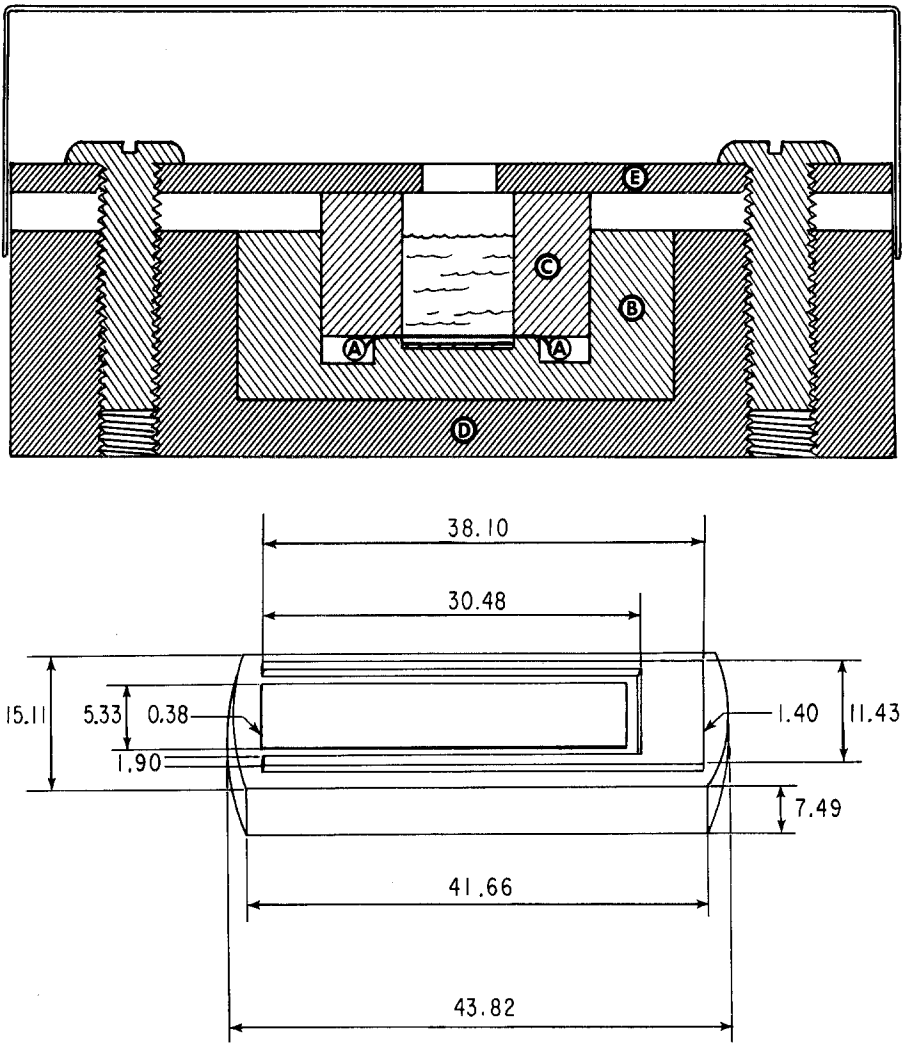

Fig. 2. *Upper panel.* Cross-section of an assembled growth chamber. (*A*) silver wire (thick line between support arms represents nylon monofilaments); (*B*) silicone rubber chamber bottom with glass insert shown beneath nylon monofilaments; (*C*) silicone rubber insert; (*D*) methylmethacrylate clamp base; (*E*) stainless steel clamp top with overlying protective cover in place. *Lower panel.* Details of methylmethacrylate chamber mold (dimensions are in millimeters)

of an electrobalance (Model G2, Cahn) and snared on the hangdown of the balance beam. The weight was then monitored at fixed intervals during a 10-min period.

The dry weight of the preparation was determined by weighing after storage overnight at 110 °C in a vacuum drying oven (Model 5821, NAPCO) with subsequent cooling to room temperature under vacuum. Measurements were made quickly after removing the preparations from the vacuum. To tare the weight of the monofilament, it was necessary to remove the dried tissue. Since this procedure was impossible to accomplish without

altering the mass of the monofilament, an average weight of 0.299 ± 0.005 mg (SD) was obtained from 15 cell-free monofilament supports and used in all mass determinations.

The preparations were then placed in low alkali test tubes (Vycor, Corning Glass Co.), dissolved in 1 ml nitric acid (Reagent A.C.S., Fisher Scientific) and counted in a well type NaI scintillation crystal (Model 1200, Nucleus Co.) by photopeak analysis to reduce the background count (250 cpm). The volume of extracellular space was derived by comparing the gamma activity of the preparations with that obtained from a known volume of ^{125}I-labelled incubation medium dissolved in 1 ml nitric acid.

Intracellular potassium was determined by evaporating the acid-digested preparations to dryness in an aluminum test tube heater under a negative draft hood. After cooling, 2 ml of a solution containing 0.125 ml/liter Acationox (Scientific Products) and cesium chloride (0.685 gm/liter) was added to the preparations which then remained in the test tubes at room temperature for 1 hr. Potassium concentration of the resulting solution was measured with an atomic absorption flame spectrophotometer (Model 303, Perkin-Elmer) set at a wavelength of 383 μm. Standardization curves were obtained before and after every 15 samples, and each sample was measured twice, nonconsecutively. Standards contained equimolar amounts of sodium. The potassium readings in the sample tubes ranged from 0.050 to 0.143 mM/liter.

Volume/surface area (V/A) measurements. Growth-oriented strands were prepared for ultrastructural and stereologic analysis by a modification of the methods reported by Purdy *et al.* [27]. The preparations were rinsed in cold balanced salt solution and fixed for 15 min with cold 2.5% glutaraldehyde buffered to pH 7.6 with 0.025 M cacodylate or fixed at room temperature for 1 hr in Karnovsky's [14] fixative at half-strength (2.5% glutaraldehyde, 2.0% formaldehyde buffered to pH 7.2 with 0.1 M cacodylate buffer). Fixative was removed by a rapid wash in cold buffer solution and the preparations were placed in 1% osmium tetroxide for 1 hr after which they were dehydrated in graded ethanols and embedded in Epon 812.

Thin sections (silver-gold interference colors) were cut with a diamond knife on an ultramicrotome (Cambridge-Huxley, Cambridge Instrument Co., Inc.) and mounted on 300 or 400 mesh copper grids. The sections were then double stained with saturated uranyl acetate and lead citrate and examined with an electron microscope (Model EM6B, AEI).

Electron microscopic measurements of V/A used a stereologic technique which had been described in detail for analyses of embryonic chick hearts [3]. A test grid containing lines every 15 mm (8 horizontal lines, 7 vertical lines) was superimposed over micrographs (final magnification, 13,800 ×) and the volume-to-surface area ratio of the cells was obtained by point counting and line integration based on the method of Sitte [29] as described by Page *et al.* [25]. Although the structural orientation (anisotropy) of adult muscle fibers complicates stereological analyses [33], heart cells in tissue culture are relatively simple in structure and seemingly could be analyzed by isotropic methods. Nevertheless, we tested for tissue anisotropy by using an analytical procedure adapted from the study of Eisenberg *et al.* [5]. In brief, measurements were taken at grid orientation angles of 0°, 30°, 45°, 60°, and 90° and when normalized with respect to those obtained at 0°, were nearly equal to 1.0. As a consequence, we were justified in analyzing the data by isotropic methods [34].

Results

Growth Orientation and Ultrastructure

Each nylon-supported preparation of heart cells in tissue culture consisted of 70 separate strands, 5 mm in length and having an approx-

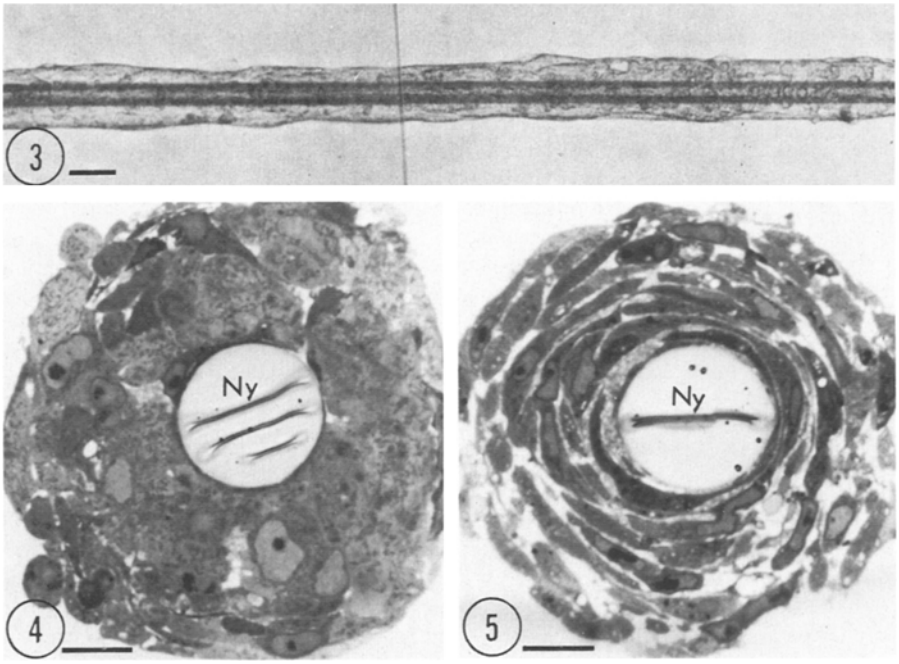

Fig. 3. Phase-contrast photomicrograph of a segment of a contractile preparation oriented about a nylon filament. Scale, 50 μm, 150×

Fig. 4. Photomicrograph of a cross-section of a contractile preparation. Note the outer sheath of flattened cells. *Ny*, nylon monofilament. Scale, 10 μm, 1100×

Fig. 5. Photomicrograph of a cross-section of a noncontractile preparation. *Ny*, nylon monofilament. Scale, 10 μm, 1100×

imate mean diameter of 60 μm (including the nylon core of 20 μm diameter) that was reasonably uniform along its entire length. The diameter of the strands in the preparation could be varied (between 40 and 200 μm) by altering the initial seeding density of the cells within given limits, i.e., cell densities less than 1×10^5 cells/ml rarely produced uniformly confluent strands whereas densities greater than 1×10^6 cells/ml promoted bulbous aggregates as well as the development of cellular bridges between linear portions of the strands. The preparations contracted spontaneously in a direction parallel to the axis of the nylon support. The contractions were generally synchronous along the entire length of the strand indicating extensive electrical coupling between the muscle cells. A representative segment of one such preparation is shown in Fig. 3.

A cross-section of a typical strand (Fig. 4) shows the cells formed a continuous annulus about the nylon substrate and the longitudinal

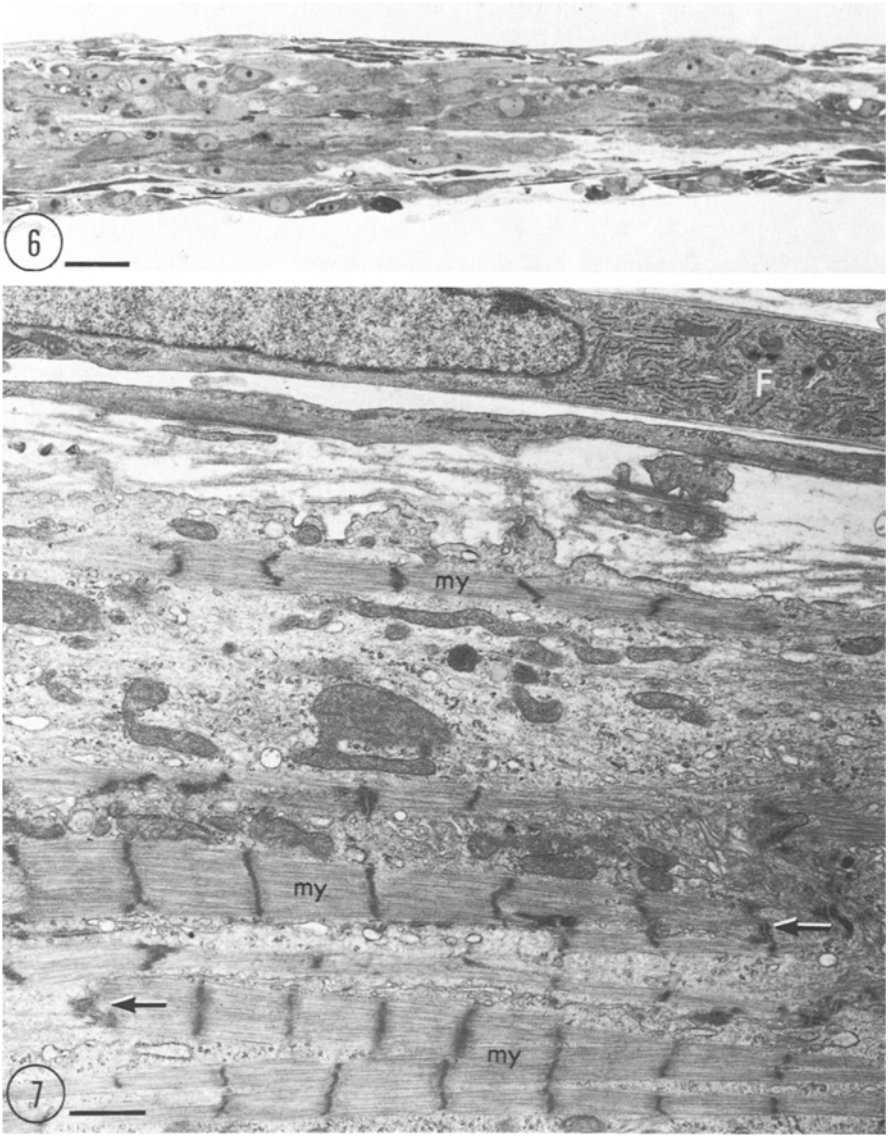

Fig. 6. Photomicrograph of a portion of a contractile preparation sectioned longitudinally near the nylon monofilament. Scale, 25 μm, 400 ×

Fig. 7. Electron micrograph of a longitudinal section from a portion of a contractile preparation. A fibroblastlike cell (*F*) characteristic of those preferentially located at the periphery of these preparations is seen at the upper edge of the micrograph. The inner segment of the preparation, seen in the middle and lower portions of the micrograph, contains muscle cells with junctional specializations (arrows) and well-formed myofibrils (*my*). Scale, 1 μm, 12,000 ×

section (Fig. 6) shows the orientation of the cells along the axis of the nylon support. The preparations are comprised of two morphologically distinct cell types, a core of muscle cells enveloped by a sheath of fibroblastlike cells that were distinguished from muscle cells by the absence of myofibrils and the presence of large amounts of rough endoplasmic reticulum (Fig. 7). The same two cell types were arranged in a similar fashion in synthetic strands of heart cells formed along channels cut in an agar substrate [27]. Longitudinal sections of the preparations (Fig. 7) show the cross-striated myofibrils and junctional specializations (e.g., intercalated discs and gap junctions) which are typical of cardiac muscle. As in embryonic [20] and adult chicken [31] cardiac muscle, the cells lack transverse tubules. The deep, narrow intercellular clefts characteristic of Purkinje strands from dog and ungulate hearts were minimal as was the organized extracellular collagen typically present in adult cardiac muscle [30].

Since the contractile preparations typically contained both muscle and fibroblastlike cells, it was necessary to grow preparations entirely of fibroblastlike cells in order to establish a quantitative basis for sorting-out and evaluating the contribution of this cell type to the radioisotopic flux characteristics of the contractile preparations [12]. Fig. 5 shows a noncontractile preparation composed almost entirely of flattened, elongated, fibroblastlike cells similar in appearance to those found in the cortical region of the contractile preparations (cf. Fig. 7).

Cell Water and Intracellular Potassium

The dry weight of the preparations was dependent on the seeding density of the initial cell suspension. The dry weight for the contractile preparations was 0.27 ± 0.04 mg (mean $\pm$ SD) and 0.26 ± 0.03 mg (mean $\pm$ SD) for noncontractile preparations (*see* Table 2). To reduce variations produced by differences in tissue culture conditions, measurements of cell water and intracellular potassium concentration were made on preparations from the same cell suspension (Table 2).

Gravimetric and isotopic methods were combined to determine cell water. The weight of each preparation was monitored for 10 min, at 1-min intervals, and the wet weight determined by extrapolation to the time at which the preparation was removed from the fluorocarbon (Fig. 8). Because of the relatively low water absorption of nylon, the water content of the nylon monofilament could be ignored. It is evident

Table 2. Chemical and stereological analyses of growth-oriented heart cells

Preparation	Dry weight (mg)	E-H_2O[a] (%)	Cell H_2O[b] (%)	K Content (moles/kg dry wt)	K Concentration (mM)	Volume/surface area[c] ($cm \times 10^{-4}$)
Contractile						
(Mean ± SD)	0.27 ± 0.04 ($n=12$)	51.2 ± 3.0 ($n=12$)	81.1 ± 1.4 ($n=12$)	0.57 ± 0.12 ($n=12$)	133 ± 21 ($n=12$)	1.06 ± 0.25 ($n=10$)
Noncontractile						
(Mean ± SD)	0.26 ± 0.03 ($n=8$)	58.5 ± 5.0 ($n=8$)	78.2 ± 2.0 ($n=8$)	0.56 ± 0.06 ($n=8$)	156 ± 25 ($n=8$)	0.48 ± 0.08 ($n=10$)

[a] Extracellular and extraneous water as a percentage of tissue wet weight.
[b] Cell water as a percentage of cell wet weight.
[c] Each determination represents the mean of five measurements.

that moisture loss from the preparation was rapid even though the procedure was carried out under conditions of high humidity. "Blotting" the preparation with liquid fluorocarbon reduces the amount of fluid adhering to the cells without causing cell damage, loss of tissue and possible weighing errors due to adherence of blotting material as would be the case, for example, if filter paper were used. Since the procedure does not completely remove all of the extraneous fluid, we used ^{125}I-iothalamic acid, a marker for water in renal experiments [28]. This compound, rather than inulin, was chosen because of diffusional limitations associated with the equilibration of inulin in the extracellular space of adult cardiac tissue [23]. Fig. 9 shows that incubation of the preparation for as long as 15 hr in iothalamic acid is not accompanied by intracellular uptake of the marker.

Cell water was measured in 12 contractile and 8 noncontractile preparations (Table 2). The cell water determined for the contractile preparations was 81.1 ± 1.4% (mean ± SD) of the cell wet weight with the extracellular and extraneous water amounting to 51.2 ± 3.0% (mean ± SD) of the wet cell weight. Contractile preparations contain between 0.09 and 0.182 μM of potassium (corrected for extracellular potassium, ca. 5%). From the cell water content, a potassium concentration of 133 ± 21 mM (mean ± SD) was calculated. Noncontractile preparations contained approximately the same amount of potassium per dry weight of tissue, but the slightly lower water content resulted in a higher calculated intracellular potassium concentration 156 ± 25 mM (mean ± SD).

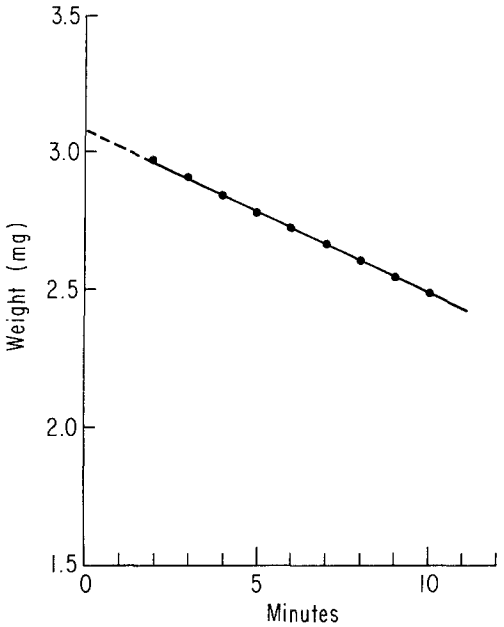

Fig. 8. Extrapolation of preparation wet weight. Measurements were obtained in a humidified incubator at 37.5 °C. Actual cell wet weight (preparation wet weight-extracellular water) was 1.29 mg

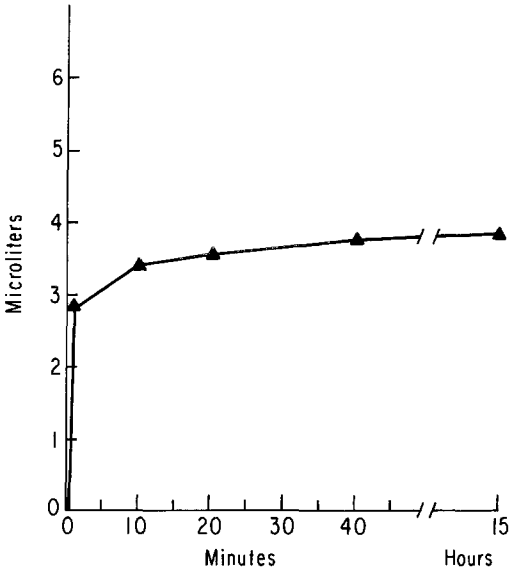

Fig. 9. Equilibration of ^{125}I-iothalamic acid in a nylon-supported preparation. The preparation was subsequently exposed to culture medium containing 29 μM/liter of the tracer for the period of time indicated, blotted briefly in fluorocarbon, and counted for 1 min. Extracellular volume was calculated by ratio with the counts obtained with a known volume of incubation media

Cell Volume-to-Surface Area

The V/A ratios obtained by stereologic analysis from electron-micrographs of the nylon-supported strands are summarized in Table 2. It is apparent that the method is capable of distinguishing between the cell types in the different preparations since a value of $1.06 \pm 0.25 \times 10^{-4}$ cm (mean $\pm$ SD) was obtained for the muscle cells whereas the flatter, noncontractile cells had a value of $0.48 \pm 0.08 \times 10^{-4}$ cm (mean $\pm$ SD).

Discussion

Growth Orientation of Heart Cells

We chose to grow heart cells around a fine monofilament of nylon (20 μm diameter) which was wound around a silver wire support so as to form a preparation of cardiac muscle comprising 70 short thin strands, the total mass of which (roughly equivalent to a small papillary muscle from the cat) is sufficient to provide a high level of total radioactivity [10–12] equivalent to that obtainable only in thicker natural preparations—yet avoiding the problems of extracellular diffusion gradients that are inescapable with thick preparations [12]. Furthermore, the cells in these growth-oriented preparations are highly differentiated with a well-organized structure comparable to that of the naturally-occurring parent cell. The individual strands are composed of a core of muscle cells surrounded by a sheath of fibroblastlike cells; the contributions of the two cell types (which are inseparable in natural preparations) to the overall measured flux are assessable [12] since preparations can be grown with only fibroblastlike cells and which are otherwise identical in overall geometry to the muscle-containing preparations.

Although the concept of using fibers as a substrate to manipulate the growth and attachment of cells has been previously documented [4, 8, 22], the present study has demonstrated the inherent advantages of the preparation for experimental manipulation of the cells. For example, the integrity of the cell annulus and physiological state of the cells were maintained throughout the various preparative manipulations as well as during perfusion for 1 hr at a fluid velocity of 3.2 cm/sec. This observation contrasts sharply with the results obtained from perfusion of monolayer preparations of cultured heart cells (e.g., 1) and the synthetic strand of cardiac muscle (Lieberman, *unpublished observation*), which in both cases, promoted cell detachment from the growth substrate.

The growth-oriented preparations also retain the major advantage of tissue culture preparations for tracer studies, namely the high surface area-to-tissue volume ratio [1]. Finally, radioisotopic flux measurements [10–12] show the preparations can provide data which correlate with that obtained from electrical studies of oriented heart cells [18].

Cell Water

The difficulties of measuring cell water in preparations of cardiac muscle have been outlined by Page [24]. The problems that especially apply to the growth-oriented preparation and the solutions that were used for this measurement were discussed earlier in this report. There is good agreement between the average values for cell water found in the contractile preparations in this study (81.1%) and those previously reported for tissue-cultured and intact embryonic heart cells. Calculations from the data of McDonald and DeHaan [21], for monolayer cultures of 7- to 8-day chick hearts, gave a value of 79% (cell water as a percentage of cell wet weight) while data from aggregates of heart cells gave a value of 80.3%. Harsch and Green [9] and McDonald and DeHaan [21] reported data for intact 11-day embryonic chick heart, from which values for cell water of 80.8% and 78%, respectively, were calculated. More recently, Carmeliet *et al.* [3] obtained a value of 80.5% for 6- to 8-day embryonic chick hearts. The value of 78.2% for noncontractile cells obtained in this study is not significantly different from that for contractile preparations (Student's t-test, $0.60 < p < 0.70$).

Cell Potassium

Cell potassium content is compared in terms of the moles of potassium per unit dry weight of tissue rather than in terms of the potassium concentration in cell water to accommodate for slight differences in the determination of cell-water (*see above*). Recalculating the cell potassium content of tissue-cultured aggregates of chick heart cells from 7–8 day embryos reported by McDonald and DeHaan [21] resulted in a value of 602 mM/kg dry weight, which is in relatively good agreement with the values of 570 and 560 mM/kg found in the present study for contractile and noncontractile preparations, respectively. Although a direct age correlation of the data cannot be made between cells grown in culture and those of the intact embryonic heart, the values obtained

from the cultured heart cells are within the range reported for developing embryonic hearts. For example, data of Carmeliet *et al.* [3] can be calculated to give a potassium content for 6- to 8-day embryonic chick heart of 636 mM/kg dry weight; whereas, Harsch and Green [9] and McDonald and DeHaan [21] give data for the 11-day heart from which values of 578 and 471 mM/kg dry weight, respectively, can be calculated. Indeed, the results cited above substantiate the ability of embryonic heart cells in tissue culture to recover fully from possible damage to the cell membranes caused by enzymatic disaggregation of the intact tissue [32].

Cell Volume-to-Surface Area

In preparations with comparable overall dimensions and growth orientation, the values obtained from stereologic V/A analysis show a large difference between those of nonmuscle cells (0.48×10^{-4} cm) and cardiac muscle cells (1.06×10^{-4} cm). This difference reflects the flatness of the fibroblastlike cells. The cells in the muscle core of oriented strands of embryonic chick cardiac cells previously were shown by morphometric analysis from electron-micrographs to have a V/A of 1.14×10^{-4} cm [17], which compares favorably with the stereologic value of 1.06×10^{-4} cm for muscle cells in the present study. Recently, Carmeliet *et al.* [3] reported a value of 1.24×10^{-4} cm for V/A ratio for 6–8 and 18–20 day embryonic chick hearts.

Conclusion

A multistranded preparation of cultured heart cells grown as thin annuli about nylon monofilament provides a mechanically stable, electrically stimulatable preparation with short diffusional distances and a morphologically simple extracellular space. The ability to increase tissue mass without compromising surface area is of unquestionable value to quantitative determinations of ion transport across cell membranes. The analytical measurements support the hypothesis that cell properties are not permanently altered by tissue culture methodology. Although developed for studying the cellular properties of cardiac muscle, the nylon-supported preparation of cultured cells has broad applicability to physiological, anatomical, and biochemical investigations of excitable and nonexcitable cells.

The authors wish to thank Drs. E.A. Johnson and J.B. Chapman for their critical review of the manuscript and Dr. V. Dennis for supplying the iothalamic acid used in this study. We thank Mr. Thomas Hughes, Ms. Linda Halperin, and Mr. Owen Oakeley for their technical assistance.

This work was part of a dissertation submitted by C.R. Horres in partial fulfillment of the requirements for the Ph.D. degree in Physiology and Pharmacology at Duke University.

A preliminary report was presented at the FASEB Symposium on Excitable Cells in Tissue Culture: Application to Pharmacological Studies, sponsored by the American Society for Pharmacology and Experimental Therapeutics, April, 1975, and the 48th Annual Meeting of the American Heart Association, Fall, 1975 [11].

This research was supported in part by grants from the National Institutes of Health (5T01 GM 00929 and HL 12157), grants-in-aid from the North Carolina Heart Association, and the Walker P. Inman Fund. This research was conducted during the tenure of an Established Investigatorship of the American Heart Association to M. Lieberman.

References

1. Burrows, R., Lamb, J.F. 1962. Sodium and potassium fluxes in cells cultured from chick embryo heart muscle. *J. Physiol. (London)* **162**:510
2. Carmeliet, E.E. 1961. Chloride and Potassium Permeability in Cardiac Purkinje Fibres. Editions Arscia S.A., Bruxelles
3. Carmeliet, E.E., Horres, C.R., Lieberman, M., Vereecke, J.S. 1975. Potassium permeability in the embryonic chick heart: Changes with age, external K and valinomycin. *In*: Developmental and Physiological Correlates of Cardiac Muscle. M. Lieberman and T. Sano, editors. p. 103. Raven Press, New York
4. Edelman, G.M., Rutishauser, U., Millette, C.F. 1971. Cell fractionation and arrangement on fibers, beads, and surfaces. *Proc. Nat. Acad. Sci. USA* **68**:2153
5. Eisenberg, B.R., Kuda, A.M., Peter, J.B. 1974. Stereological analysis of mammalian skeletal muscle. I. Soleus muscle of the adult guinea pig. *J. Cell Biol.* **60**:732
6. Girardi, A.J., MacMichael, H., Jr., Henle, W. 1956. The use of HeLa cells in suspension for the quantitative study of virus propagation. *Virology* **2**:532
7. Haas, H.G., Kern, R. 1966. Potassium fluxes in voltage clamped Purkinje fibres. *Pfluegers Arch.* **291**:69
8. Harrison, R.G. 1914. The reaction of embryonic cells to solid structures. *J. Exp. Zool.* **17**:521
9. Harsch, M., Green, J.W. 1963. Electrolyte analyses of chick embryonic fluids and heart tissues. *J. Cell. Comp. Physiol.* **62**:319
10. Horres, C.R. 1975. Potassium tracer kinetics of growth oriented heart cells in tissue culture. Ph.D. Dissertation, Duke University, Durham, N.C.
11. Horres, C.R., Lieberman, M. 1975. Compartmental analysis of potassium-42 exchange kinetics in embryonic heart. *Circulation* **52** (Suppl. II):20 (*abstr.*)
12. Horres, C.R., Lieberman, M. 1977. Compartmental analysis of potassium efflux from growth-oriented heart cells. *J. Membrane Biol.* **34**:331
13. Kaighn, M.E., Ebert, J.D., Stott, P.M. 1966. The susceptibility of differentiating muscle clones to Rous sarcoma virus. *Proc. Nat. Acad. Sci. USA* **56**:133
14. Karnovsky, M.J. 1965. A formaldehyde-paraformaldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27**:137a
15. Keynes, R.D., Lewis, P.R. 1951. The sodium and potassium content of cephalopod nerve fibres. *J. Physiol. (London)* **114**:151

16. Lieberman, M. 1973. Electrophysiological studies of a synthetic strand of cardiac muscle. *Physiologist* **16**:551
17. Lieberman, M., Roggeveen, A.E., Purdy, J.E., Johnson, E.A. 1972. Synthetic strands of cardiac muscle: Growth and physiological implication. *Science* **175**:909
18. Lieberman, M., Sawanobori, T., Kootsey, J.M., Johnson, E.A. 1975. A synthetic strand of cardiac muscle. Its passive electrical properties. *J. Gen. Physiol.* **65**:527
19. Lieberman, M., Sawanobori, T., Shigeto, N., Johnson, E.A. 1975. Physiologic implications of heart muscle in tissue culture. *In*: Developmental and Physiological Correlates of Cardiac Muscle. M. Lieberman and T. Sano, editors. p. 139. Raven Press, New York
20. Manasek, F.J. 1970. Histogenesis of the embryonic myocardium. *Am. J. Cardiol.* **25**:149
21. McDonald, T.F., DeHaan, R.L. 1973. Ion levels and membrane potential in chick heart tissue and cultured cells. *J. Gen. Physiol.* **61**:89
22. Okada, T., Nakamori, J., Hirayama, M. 1971. Primary tissue culture method using glass fibre. *Hiroshima J. Med. Sci.* **20**:269
23. Page, E. 1962. Cat heart muscle in vitro. III. The extracellular space. *J. Gen. Physiol.* **46**:201
24. Page, E. 1965. Ion movement in heart muscle: Tissue compartments and the experimental definition of driving forces. *Ann. N.Y. Acad. Sci.* **127**:34
25. Page, E., McCallister, L.P., Power, B. 1971. Stereological measurements of cardiac ultrastructures implicated in excitation contraction coupling. *Proc. Nat. Acad. Sci. USA* **68**:1465
26. Polinger, I.S. 1970. Separation of cell types in embryonic heart cell cultures. *Exp. Cell Res.* **63**:78
27. Purdy, J.E., Lieberman, M., Roggeveen, A.E., Kirk, R.G. 1972. Synthetic strands of cardiac muscle. Formation and structure. *J. Cell Biol.* **55**:563
28. Schmid, H.E., Meschan, I., Watts, F.C., Hosick, T., Muelbaecher, C.A. 1970. Effect of free ^{131}I on renal excretion of diatrizoate-^{131}I, an inulin substitute. *Am. J. Physiol.* **218**:903
29. Sitte, H. 1967. Morphometrische Untersuchungen an Zellen. *In*: Quantitative Methods in Morphology. E.R. Weibel and H. Elias, editors. p. 167. Springer-Verlag, New York
30. Sommer, J.R., Johnson, E.A. 1968. Cardiac muscle. A comparative study of Purkinje fibers and ventricular fibers. *J. Cell Biol.* **36**:497
31. Sommer, J.R., Johnson, E.A. 1969. Cardiac muscle: A comparative ultrastructural study with special reference to frog and chicken hearts. *Z. Zellforsch.* **98**:437
32. Waymouth, C. 1974. To disaggregate or not to disaggreegate. Injury and cell disaggregation, transient or permanent? *In Vitro* **10**:97
33. Weibel, E.R. 1972. A stereological method for estimating volume and surface of sarcoplasmic reticulum. *J. Microscop.* **95**:229
34. Weibel, E.R. 1973. Stereological techniques for electron microscopic morphometry. *In*: Principles and Techniques of Electron Microscopy Biological Applications. M.A. Hayat, editor. Vol. 3, p. 239. Van Nostrand Rheinhold Company, New York
35. Yaffe, D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Nat. Acad. Sci. USA* **61**:477