IN VITRO ORGANIZATION OF DISSOCIATED RAT CARDIAC CELLS INTO BEATING THREE-DIMENSIONAL STRUCTURES*

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(Received for publication 27 November 1970)

Early in this century, Wilson (1) demonstrated that invertebrate sponges could be dissociated into individual cells, which would reassociate under proper conditions to form complete viable organisms. Since that time, a considerable body of literature has accumulated describing the tendency of dissociated cells from many life forms to re-aggregate and organize into structures reminiscent of the tissues from which they have been derived. In addition to invertebrates such as sponges (2, 3), similar investigations have been performed with reptile, avian, and mammalian tissues. In most of these short-term experiments, reaggregation was encouraged or brought about by means of appropriate agitation of the individual cell suspensions. Thus, Townes and Holtfreter (4) mixed isolated epidermal and mesodermal cells from embryonic amphibian neurula, and after reaggregation the appropriate cells sorted out and eventually formed mesenchyme, coelomic cavities, and blood cells. Moscona (5, 6), as well as Trinkaus and Groves (7), observed that trypsin-dissociated chick embryo mesonephros cells re-aggregated to form structures quite reminiscent of renal tubules. Okada (8), using the same system, and an antiserum apparently specific for renal epithelial cells, was able to demonstrate that only those cells containing the kidney antigen were involved in the formation of tubules. Similar investigations have even recently been carried out with dissociated human fetal pancreas cells, which reaggregated into histotypical masses of α- or β-cells capable of secreting insulin (9).

Yaffe (10) has demonstrated the ability of dissociated newborn rat skeletal muscle cells to become dramatically organized into multinucleated fiber-like units, under "feeder layer" tissue culture growth conditions. Clones of such cells could be transplanted repeatedly, and they retained the tendency to organize and differentiate into muscle fibers for many generations, indicating that this "instinct" could be a stable property of the clones.

Trypsin-isolated embryonic or newborn beating-heart cells of avian or mammalian origins have been studied in tissue culture by a number of investigators. There is general agreement that these usually grow in the form of synchronously pulsating networks or sheets which are generally one cell-layer thick (11–16). A tendency toward fiber like development was briefly described in beating heart cultures of newborn rat cells by Harary and Farley (17).

* This investigation was supported by research grants from the National Heart Institute and the American Heart Association; the latter as part of the Florida Heart Cooperative Research Program.

In previous studies from our laboratory, dissociated beating newborn rat heart cells were cultivated on glass surfaces for investigations of the toxicity of cardiac antibodies or of streptolysin O (18, 19). Under the tissue culture conditions used, the characteristic growth of these cells as beating sheets confirmed the findings of previous investigators. During the course of these experiments, it was found that cells growing under identical conditions on untreated polystyrene surfaces grew similarly for only the first 3 or 4 days, i.e., as networks or sheets of stretched-out cells which beat synchronously. However, after this time subsequent growth and development were significantly different from that observed on the glass surfaces. In scattered areas throughout the cultures, the continuing growth became focally three-dimensional, while beating continued uninterruptedly. These pulsating foci of growth eventually became well circumscribed spherical masses which were completely separated from the underlying cell sheets, and they gradually increased in size. The purpose of this report is to describe the characteristics of these heart cell masses and their development, since they appear to represent an attempt at organization of the dissociated cells into a heart-like structure, analogous to what has been described for other embryonic tissues, such as kidney, pancreas, or skeletal muscle.

Materials and Methods

Cells from cardiac ventricles of 1–4-day old neonatal Sprague-Dawley rats were dissociated with crystallized bovine pancreatic trypsin (Worthington Biochemical Co., Freehold, N.J.) at a concentration of 0.4 mg/ml, in calcium- and magnesium-free Hanks' solution. 20-minute trypsinizations were carried out 7 or 8 times, the first three harvests being discarded because of contamination with erythrocytes and cellular debris. The pooled dissociated cells from approximately 25 animals were centrifuged for 5 min at 800 rpm, and resuspended in complete medium. Their concentration was adjusted to 2–3 $\times$ 10^5 cells/ml. The medium described by Marks and Strasser was used (13),[1] containing specially selected batches of fetal calf serum, penicillin, and streptomycin at final concentrations of 10%, 100 units/ml, and 0.1 mg/ml respectively. The cultures were grown in "Rose" chambers (20), according to the method of Marks and Strasser (13). These sealed units consisted of a microscope cover glass (Arthur H. Thomas Co., Philadelphia, Pa.), and a clear polystyrene sheet, 0.25 mm thick, separated by a medical grade silicone rubber gasket, 2.5 mm thick. The inoculum was 0.2 ml of the final suspension of dissociated cells, which was added to about 1.5 ml of complete medium. In previous experiments with this system (18, 19), the heart cells were grown on the glass surface. In the studies being reported here, the cultures were grown on the opposite polystyrene surface instead. Cultures of the same cell suspensions grown on the glass cover slips always served as rigid controls for the present investigations. In addition, culture chambers fashioned entirely of polystyrene with the same general configuration as the Rose chambers were also used. These experimental units were made through the courtesy of the Cordis Laboratories (Cordis Corp., Miami, Fla.).

A total of three different samples of untreated polystyrene were used for these investiga-

[1] The authors are grateful to Mrs. Carrie Smith for technical assistance in the preparation of these tissue cultures.

tions, and attempts were made to obtain the sheets free of mold-release agent. The chemical identity of only one of these polystyrene specimens was known. This was a general purpose polystyrene lot No. SMD-3500 prepared by Union Carbide Corp., Atlanta, Ga. Most of these units were cleaned with 70% ethanol for 30 min, then rinsed overnight with water. They were then sterilized by immersion in 70% ethanol, followed by three to four rinses with sterile distilled water, rinsed in sterile Ca- and Mg-free Hanks' solution, and aseptically assembled into the units. The units fashioned entirely of polystyrene were cleaned ultrasonically in the presence of Liquinox detergent (Alconox, Inc., New York), thoroughly rinsed with water, then sterilized by ethylene oxide treatment, with outgassing for at least 24 hr under vacuum.

For histological sections of the structures which were formed, the tissue culture chambers were opened and the cell-bearing surfaces were immersed in neutralized 3% formaldehyde. The specimens were embedded in paraffin, and 3 μ sections were stained with hematoxylin and eosin. For ultrastructural studies, some of the masses were fixed in osmium tetroxide and embedded in Araldite.[2]

RESULTS

Suspensions of the dissociated newborn rat heart cells used for inoculum were virtually completely separated from each other, as shown in Fig. 1 *a*. When these cells were grown on the glass surfaces, by 20–28 hr considerable numbers of them had become attached and stretched out. Within an additional 1–2 days, the cell sheet had begun to form as seen in Fig. 1 *b*. The cell network when fully formed could be maintained as a synchronously beating unit for many weeks with fresh changes of medium every 2–3 days. The typical appearance of such a cell sheet after 3 wk is shown in Fig. 1 *c*. These networks beat synchronously at rates varying from 80 to 140 beats/min, depending upon the health of the culture. It was clear that not all of the cells in the network were functioning myocardial cells. Often localized areas of the sheets did not pulsate, and morphologically the cells in these areas appeared to represent endothelial cells, as described by Marks and Strasser (13), and Harary and Farley (11). The cell network usually was one cell thick, although after longer periods of growth, in some areas it became two or at most three cells deep. However, in almost all instances when the beating cells were grown on glass, the sheet pattern persisted as long as the culture was maintained, even up to 2 months.

When inocula of dissociated cells were grown on the polystyrene surface, the early sequence of events was quite similar to that described above. The attachment of the individual cells to the surface, their appearance after stretching out, the time of onset of pulsation (about 18–24 hr), and the beating rates were similar to those found on glass during the first 3 days. Such an early culture on the polystyrene surface is seen in Fig. 2. However, after about 4 days, the subsequent growth and development on polystyrene was significantly different from that seen on glass. The beating heart cells then began to reveal scattered foci of growth in three dimensions, which often appeared in and on the cell network. These localized accumulations of cells were brought about by

[2] The authors are deeply indebted to Mr. George Musil for his excellent collaboration in preparing these specimens.

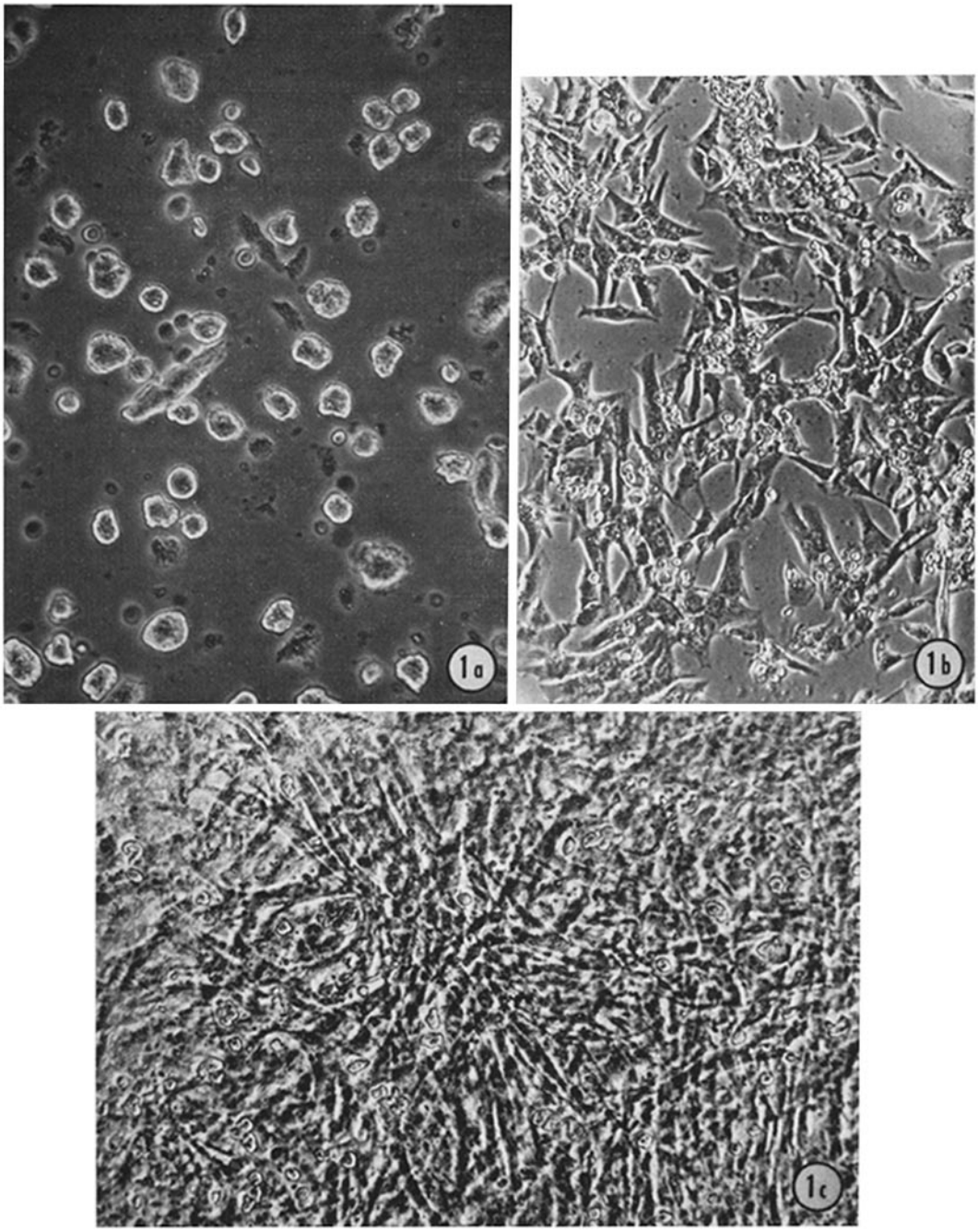


FIG. 1. Dissociated cell suspension of newborn rat heart used as inoculum (*a*), and its subsequent growth on a glass surface after 3 days (*b*) and after 21 days (*c*). Phase contrast. (*a*) × 150; (*b*) and (*c*) × 100.

an apparent "piling-up" process, which occurred without interruption of the beating. It was often clear that the beating of the conglomerate involved cells contracting throughout the depths of the mass. Usually the beating appeared simultaneously in all portions of the unit, although on some occasions the contractions appeared to originate from one side of the structure. Typical examples of such foci of three dimensional growth are shown in Fig. 3. It may be noted that the cells within them tended to be rounded or compact, in contrast to the stretched-out cells which grew on the surface.

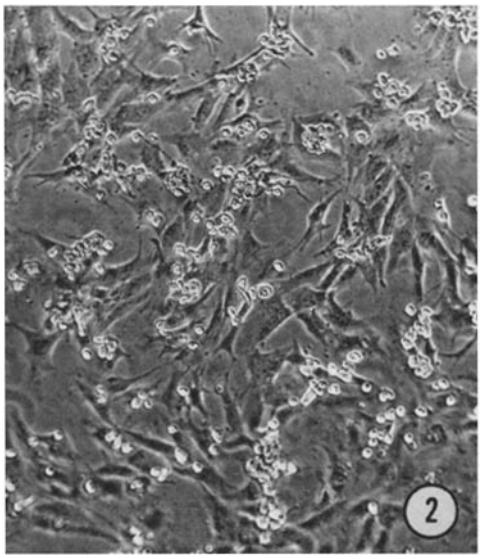


FIG. 2. Early network growth of dissociated beating newborn rat heart cells on polystyrene surface. Beginning cell-sheet formation after 2 days. Phase contrast. × 90.

With continued growth, the foci developed into sharply circumscribed units which were often completely independent of the underlying cell sheet. These separate, now usually spherical masses, appeared to increase slowly in size. They usually continued to beat regularly throughout their depth, sometimes vigorously. Typical examples of these isolated structures are shown in Fig. 4. Although they were usually symmetrically spherical in shape when they were small, they often appeared to become somewhat lobulated as they increased in size. They sometimes became intimately associated with lint particles, which had been accidentally introduced into the culture (e.g. see Fig. 4 *c*).

The tendency to form such miniature heart-like structures ("mini-hearts") by cells growing on polystyrene surfaces was generalized throughout such cultures. This is clearly evident in the low-power photograph of Fig. 5. The con-

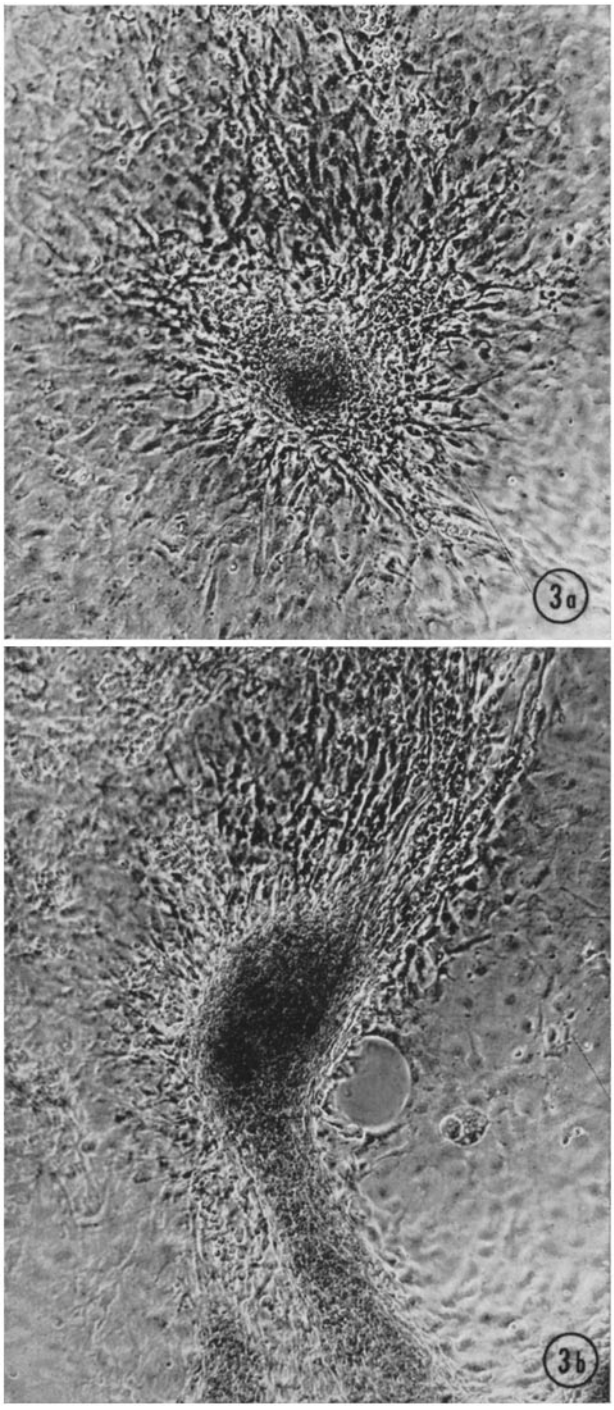
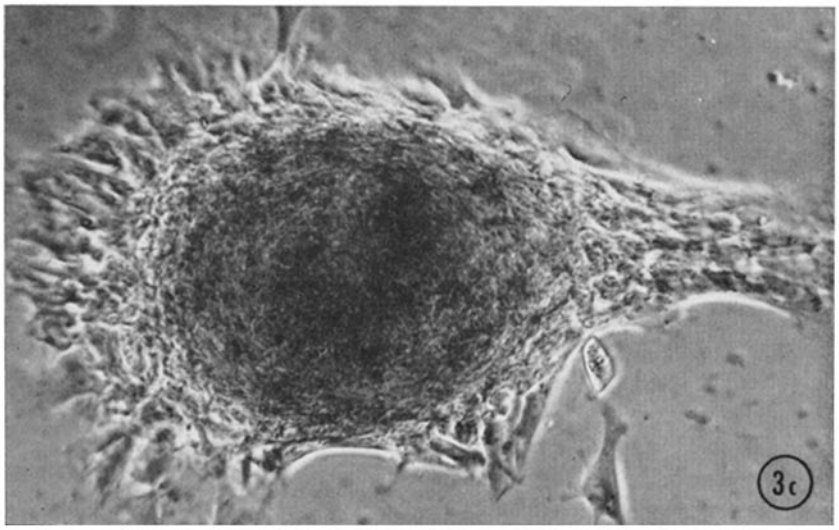


FIG. 3. Foci of beating three-dimensional growth of newborn rat heart cells, originating from the underlying cell sheet. Phase contrast. (*a*) 7 day old culture, × 160; (*b*) 14 day old culture, × 160; (*c*) 14 day old culture, × 260; (*d*) 21 day old culture, × 320.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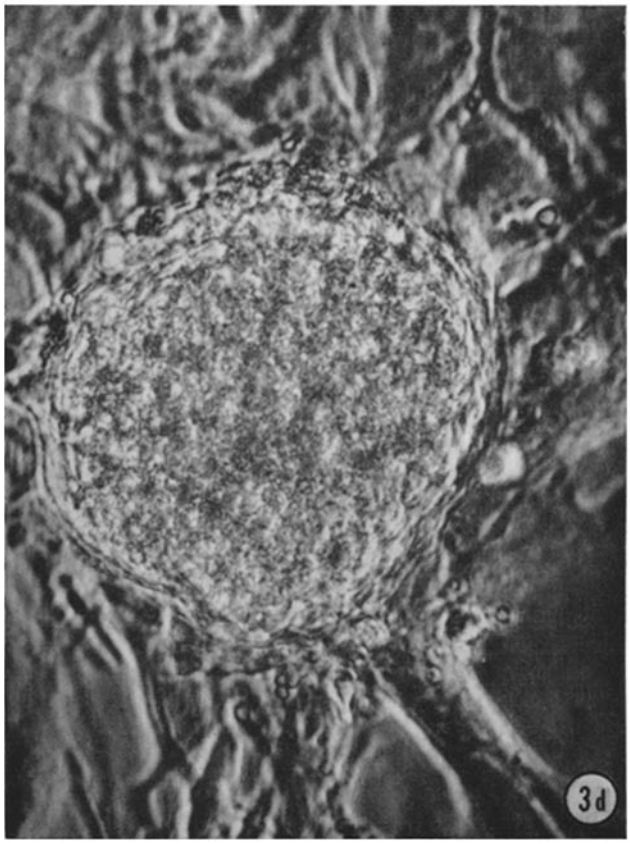
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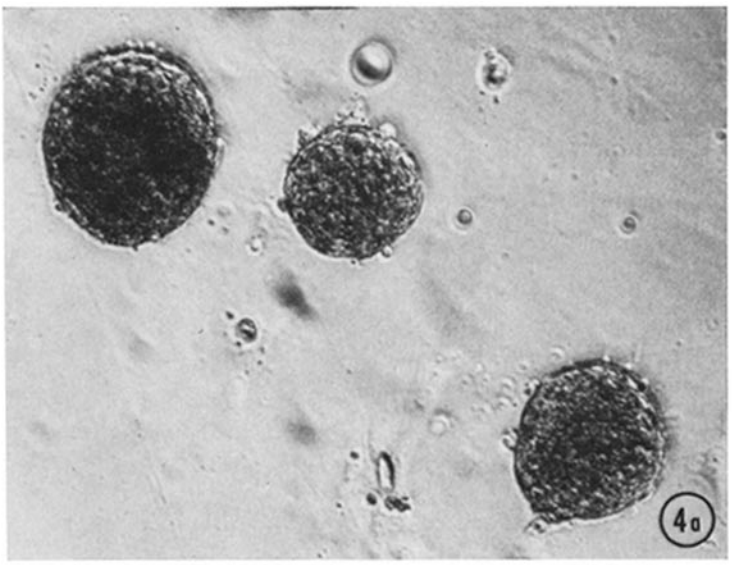
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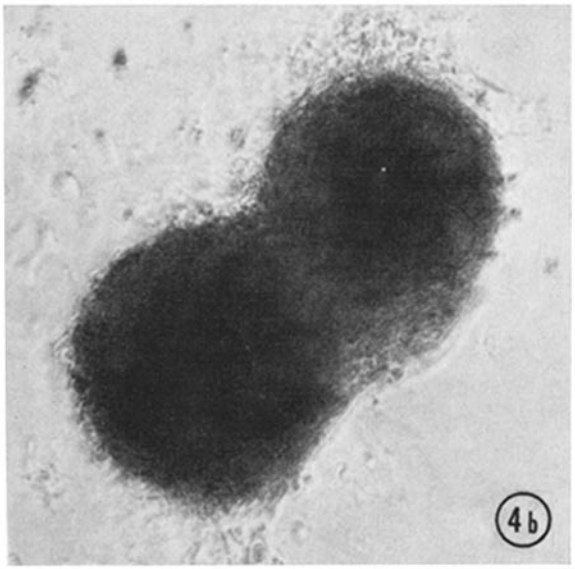
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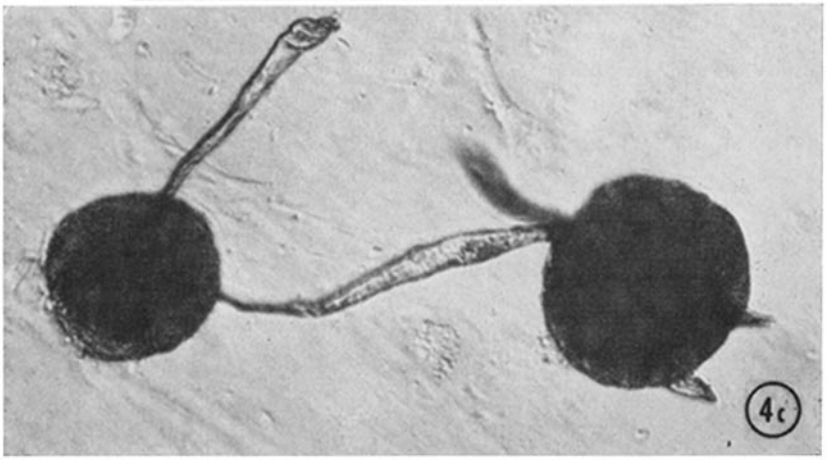
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trol culture grown in parallel on the glass surface was revealed as a diffuse haze under these conditions (Fig. 5 *b*), while the cells growing on polystyrene (Fig. 5 *a*) showed numerous intense spots of lights, each of which represents a mini-heart tissue mass.

Some of the mini-hearts grew to rather large size, occasionally reaching diameters of over 2 mm. One such unit is shown in Fig. 6, and the lobulations of this structure are clearly evident. One edge of this mass pulsated intermittently, although the unit was approximately 2 months old. Under the low-power steromicroscope its color was a muddy light tan, and small dark specks were evident at numerous sites on the surface or within the depth of the structure. These could have represented contaminant particles entrapped in the unit. As the mini-hearts grew in size during the culture period, it was not uncommon for them to completely lose their attachment to the surface of the plastic.

Histological sections of some of the mini-hearts are shown in Fig. 7. It may be seen that intact cells are apparent throughout the depths, and in certain areas there appeared to be some cell-to-cell orientation resulting in fiber-like formation. These are indicated by the arrows on Figs. 7 *a* and *c*. Patches were seen within the depths of the mini-hearts which appeared to represent deteriorated cells. It is not yet clear whether these were due to inadequate fixation, since this proved to be troublesome, or whether it was caused by incomplete nutrition of myocardial cells in the interiors of units. Mitotic figures were rarely seen within these structures.

Several mini-hearts grown under these conditions were prepared for electron microscopy (Fig. 8), and it was found that intercalated disc-like structures often appeared between myocardial cells which were in intimate contact at multiple points. An enlargement of these structures is shown in Fig. 9. These sections also showed appreciable cell detritus in the interior of the "mini-hearts", but the relative roles of inadequate nutrition or fixation are not possible to define as yet.

DISCUSSION

Most studies involving tissue culture of dissociated mammalian cells utilize their growth on glass or specially treated plastic surfaces. These usually result in cells which are stretched out into thin and flattened configurations, with rather firm attachment to the surface. It is clear that for many of the cells studied, such a pattern is not typical of their growth in vivo. The flattened

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FIG. 4. Sharply circumscribed beating masses of newborn rat heart cells growing on polystyrene surfaces. These structures were no longer associated with the flat cell sheet, which persisted in some areas of the culture. Phase contrast. (*a*) 9 day old culture, × 160; (*b*) 13 day old culture, × 160; (*c*) 21 day old culture, × 160.

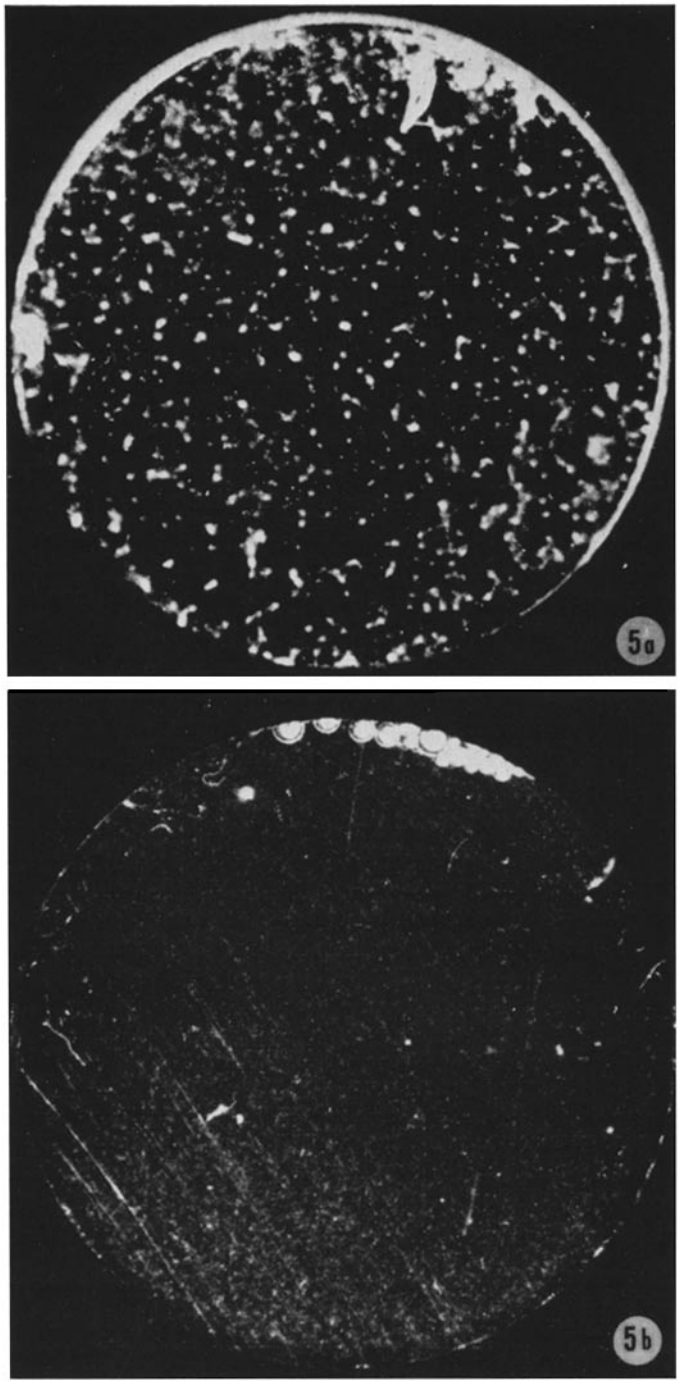


FIG. 5. Low-power photograph of cultures of newborn rat heart cells growing for 15 days on polystyrene (*a*) and on glass (*b*). Dark field. × 3. Each spot of intense light in (*a*) represents a three dimensional mass of cells. In contrast, the control parallel culture grown on glass (*b*) is revealed as a diffuse haze.

configuration is readily visualized microscopically, however, and is therefore valuable for many types of investigations, even if it represents an unnatural state. Most cells achieve their final three dimensional in vivo configurations and cell-to-cell relationships by forces and chemical determinants at the cell surface which are poorly understood at the present time.

It is now clear that under certain conditions, some dissociated cells cultivated in vitro retain their instinct to associate with similar cells in an apparent effort to organize into the ultimate tissue structure for which these cells were

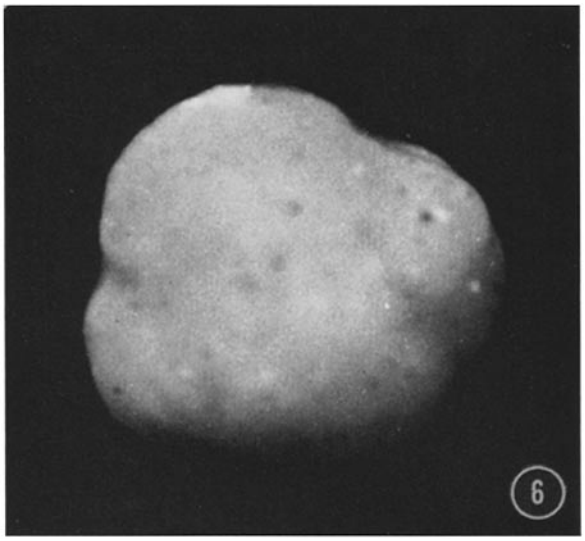
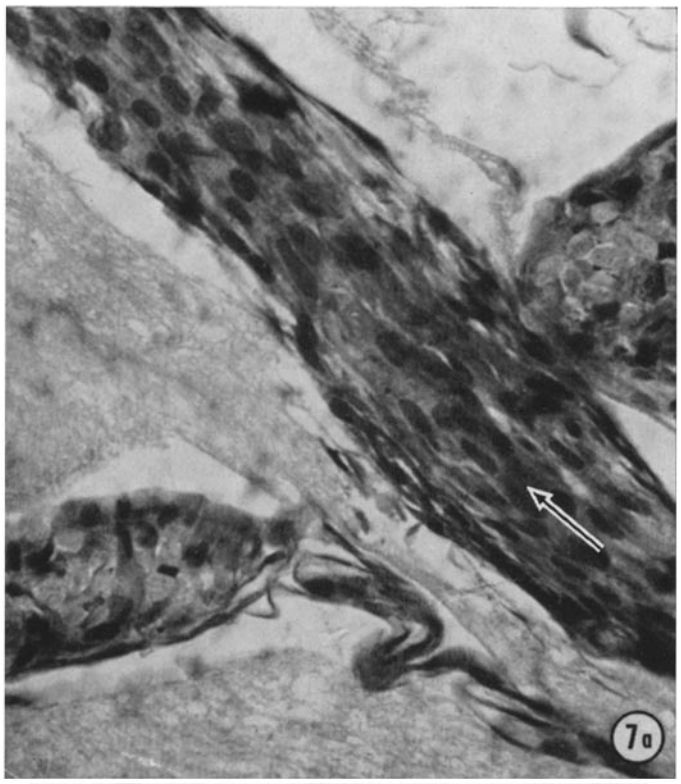


FIG. 6. A single large mini-heart, approximately 2 mm in diameter, grown from dissociated newborn rat heart cells on a polystyrene surface for about 2 months. Note the lobulated appearance, reflected light, × 25.

destined. In the case of plants, Steward has found that dissociated cells from mature carrot tissue could even grow into a complete embryo and plantlet under certain conditions (24). The work of Yaffe (10) convincingly demonstrated that in the case of skeletal muscle cells, this tendency to organize in vitro may be a rather stable characteristic of cultivated cell lines.

The data presented here indicate that suspensions of individual dissociated rat heart cells may also possess a tendency to organize in vitro into functional units suggestive of their ultimate destiny in vivo. In this case, retention of function was unequivocal, as evidenced by the continued rhythmic beating. Healthy mini-heart units tended to pulsate at rates comparable to that seen in the whole heart of the animal from which the cells were derived, as was the case with the stretched-out flattened myocardial cells growing on glass sur-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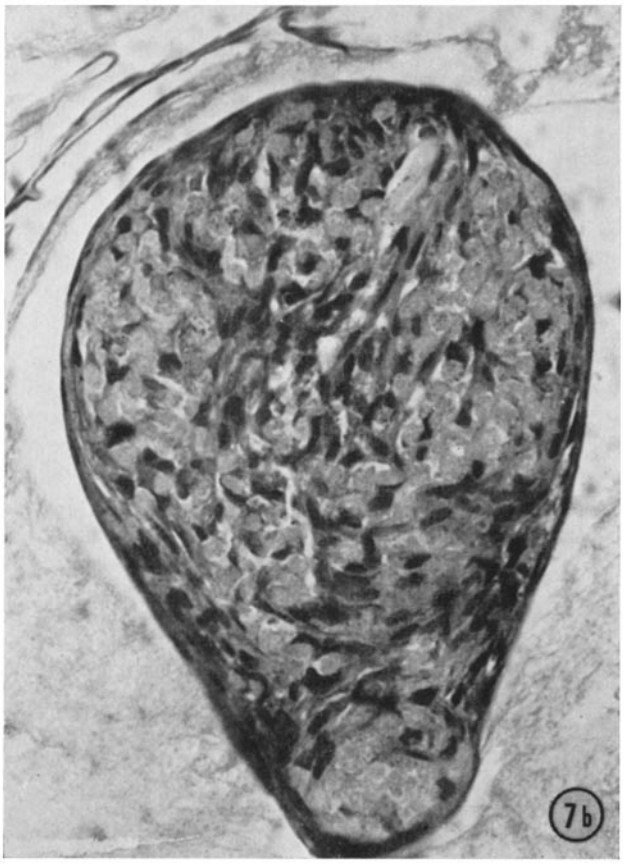
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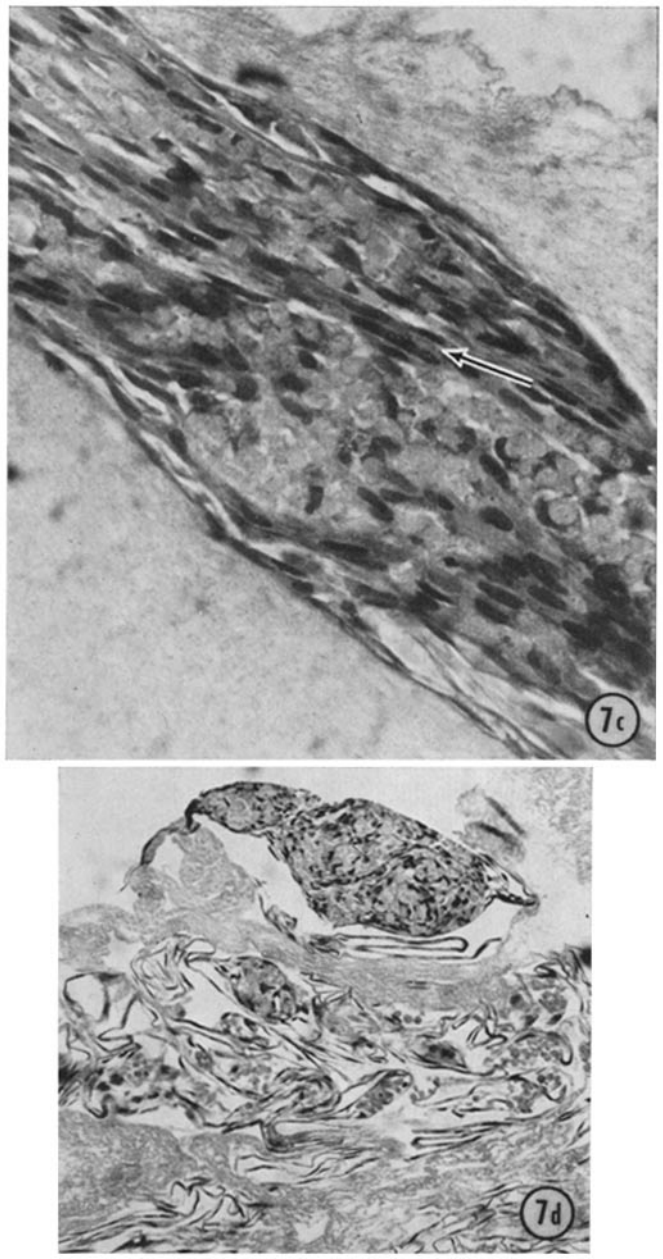
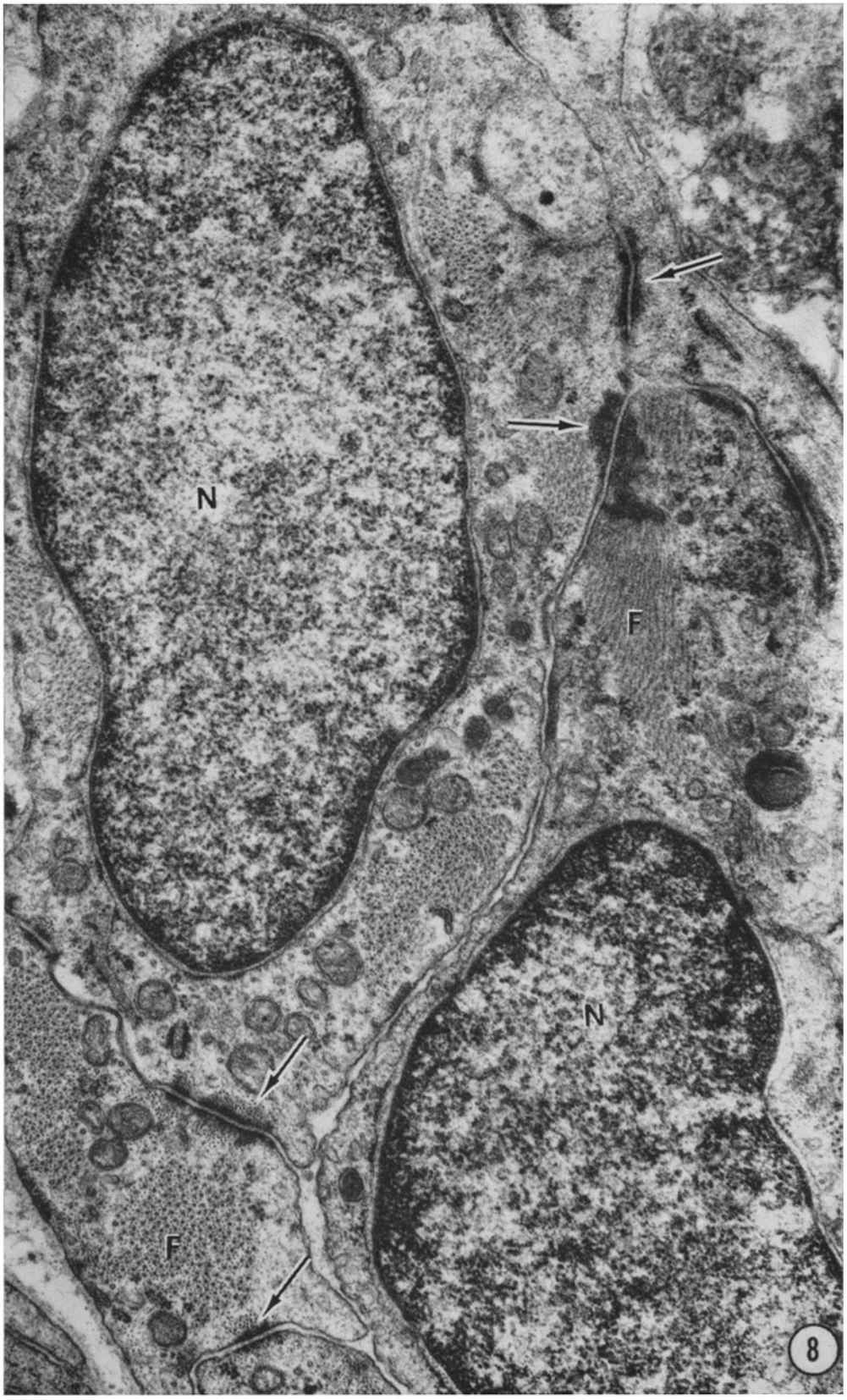


FIG. 7. Histological sections of typical mini-hearts. Hematoxylin and eosin, 3 μ. Note the tendency toward cell-to-cell orientation in *a* and *c* (arrows), and the flattened cells at the surface of these units. The latter is most clearly shown in *b*. In *d*, the remnants of the cell sheet may be seen, folded back and forth on itself. *a*, *b*, and *c*, × 420; *d*, × 150.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faces. No information is yet available regarding the precise factors which induced this tendency to organization in the cultures investigated here. The characteristics of the surface on which the cells first adhered must partly be responsible for this effect.

It is quite probable that the mini-heart units consisted of more than one cell type. No data has yet been obtained regarding the localization of beating myocardial cells within these structures. The tendency of cells at their surfaces to be stretched out does not necessarily indicate that the superficial layer consists of endothelial or other nonmyocardial cells. It would be possible to unequivocally identify the beating myocardial cells in these masses by use of immunofluorescence, with specific antisera to myosin.

It is almost certain that these heart-like structures are not related to viral transformations, which are known to be associated with the development of malignant change in tissue-cultured cells, and are also characterized by a piling-up process of multiplication. That this was not the case in the above experiments is indicated by the following facts:

(*a*) The mini-heart changes were quite reproducible with each batch of animals; using identical inocula and otherwise identical growth conditions, they were only observed on the plastic surfaces, never on glass; (*b*) cytopathogenic effects seen with viral infections, were never apparent in these cultures; (*c*) the mini-hearts showed parallel and orderly arrangements of some myocardial cells, which is not characteristic of the transformations in vitro associated with malignant change; (*d*) and viral inclusion bodies were not found microscopically in the cells within the mini-heart masses.

It is apparent that increased detailed knowledge of the precise stimuli which induce the organization of isolated cells in tissue culture might ultimately make it possible to harvest a small number of cells from certain organs, grow them into relatively organized tissue masses in vitro, and then use them for transplantation, as required. In addition to the heart studies reported here, the formation of gland-like masses with human pancreatic cells suggests the general potential for such long-term future development.

It is also probable that detailed information regarding the conditions and stimuli which bring about organization of dissociated cells in vitro could shed light on the factors involved in the normal process of morphogenesis. In this regard, one might recall the dramatic effects of mouse submaxillary gland nerve-growth factor (21). This protein or protein complex, in extremely low concentrations, is able to induce the differentiation of certain types of nerve cells with fiber formation. Analogous tissue substances may have been encoun-

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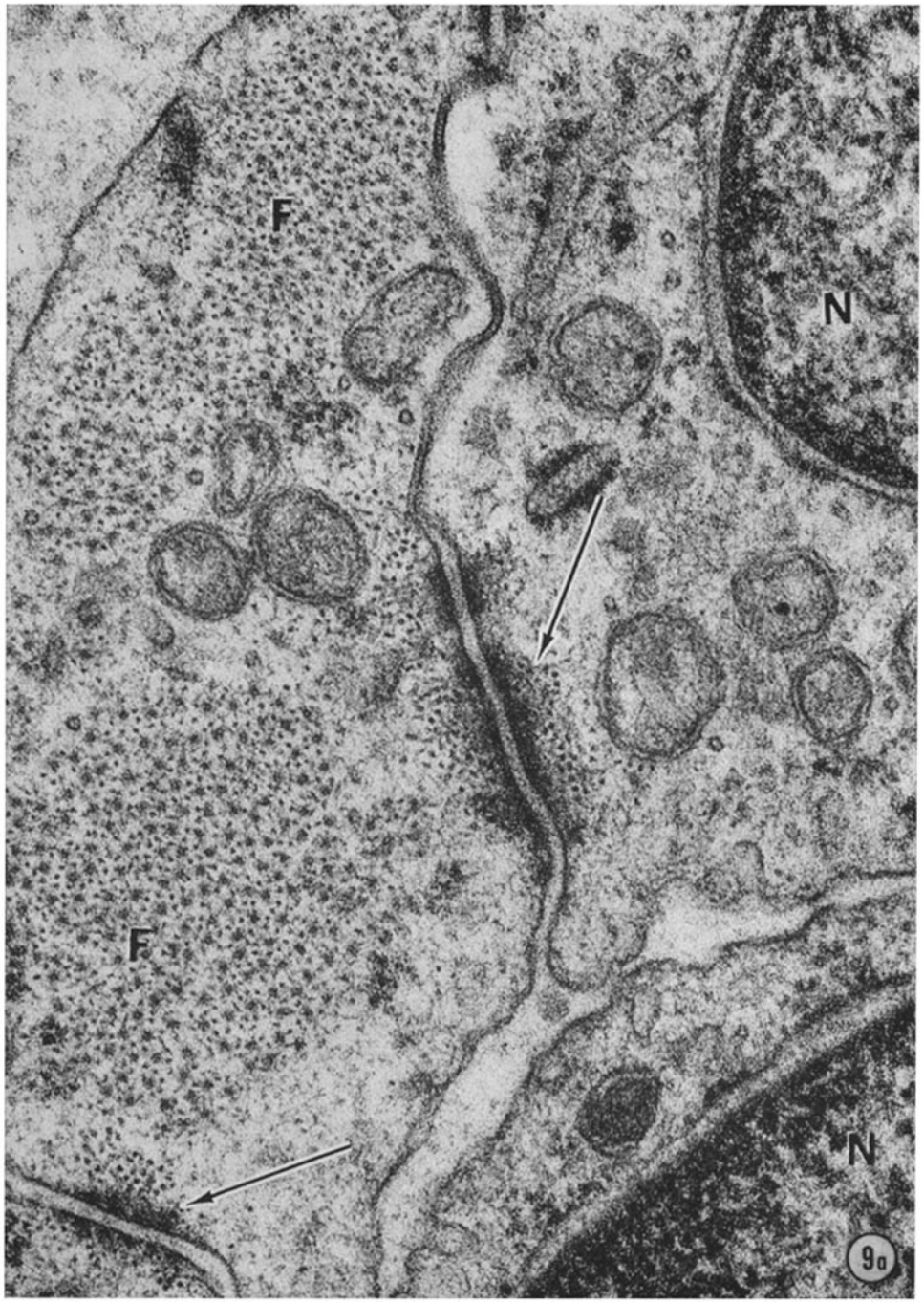
FIG. 8. Electron micrograph of myocardial cells within a mini-heart. Intercalated disc-like structures shown by the arrows. × 57,000. *N*, nucleus; *F*, myofibrils.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tered, but not yet characterized, which stimulate the organization of mammalian cells (22, 23). It is conceivable that many such biological substances exist and that they have potent enhancing activities for the type of process reported here.

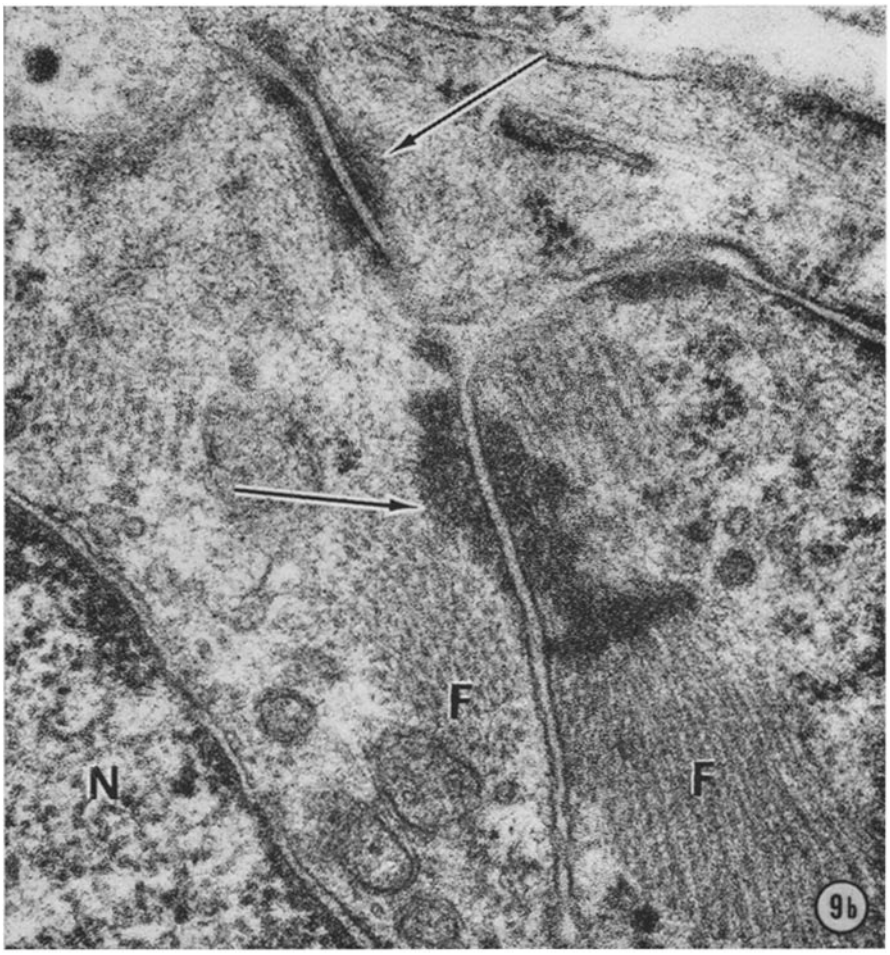

FIG. 9. Higher magnification electron micrograph of a myocardial cell within a mini-heart showing details of intercalated disc-like structures (arrows) *a*, × 155,000; *b*, × 115,000. *N*, nucleus; *F*, myofibril.

SUMMARY

When isolated beating ventricular heart cells from newborn rats were grown in tissue culture on untreated polystyrene surfaces, they showed a striking

tendency to grow focally in three dimensions from the single layer cell sheets which were formed early in growth. During this process, they frequently formed miniature spherical heart-like masses, which continued to beat and grow in size. These often were somewhat lobulated in appearance, and grew up to 2 mm in diameter. Histological sections of such structures sometimes revealed evidence of appreciable orientation of the cells to each other, in fiber-like units. Electron microscope sections of such mini-hearts showed structures resembling intercalated discs between myocardial cells. The precise factors which induced the cardiac cells to apparently organize into these heart-like structures are not presently known.

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