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Separation of Beating Cardiac Myocytes from Suspensions of Heart Cells

Thomas G. Pretlow II, MD, Melvin R. Glick, MS and
William J. Reddy, PhD

Heart cells were obtained in suspension after incubation with collagenase and hyaluronidase in Saline A. Cardiac myocytes were separated by isopycnic centrifugation in 88.6 to 92.4% purity from other heart cells with different densities, and by velocity or rate-zonal sedimentation, in 92.8 to 97.4% purity from heart cells with different diameters. A previously described computer integration of the differential sedimentation equation was used to determine the centrifugal force, duration of centrifugation and gradient design, which would permit the separation of cardiac myocytes from other heart cells by velocity sedimentation. The myocytes continued to contract rhythmically after being recovered from the density gradients. Velocity sedimentation was superior to isopycnic sedimentation for the separation of cardiac myocytes from heart cell suspensions because it gave the most highly purified myocytes, resulted in recovery of the largest proportion of myocytes in purified fractions from the gradient and required lower centrifugal forces for shorter periods of time. The potential significance of the availability of pure cardiac myocytes is discsused (Am J Pathol 67:215–226, 1972).

BOTH AVIAN AND MAMMALIAN cardiac myocytes can be obtained from minced heart muscle following incubation of the heart muscle with a variety of proteolytic enzymes and chelating agents.[1–4] Numerous investigators have reported that these myocytes contract

From the Departments of Pathology, Engineering Biophysics and Medicine, The School of Medicine and Dentistry, University of Alabama in Birmingham, Birmingham, Alabama.

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Address reprint requests to Dr. Thomas G. Pretlow, Department of Pathology, University of Alabama in Birmingham, The Medical Center, 1919 Seventh Avenue South, Birmingham, Ala 35233.

with an intrinsic rhythmicity when suspended in appropriate physiologic salines, and beat synchronously during extended periods of cultivation *in vitro*.[5-7] DeHaan [8] recently employed beating avian cardiac myocytes as an exquisitely sensitive assay for toxic substances in tissue culture medium. The rhythmicity of myocytes *in vitro* would appear to be a generally useful parameter for the study of the nutritional adequacy of tissue culture medium, response to hormones *in vitro*, etc. While many investigators have not specifically mentioned the composition of these heart cell suspensions, DeHaan [9] noted cells which are similar to fibroblasts in addition to the myocytes; and Harary and Farley[10] described their suspensions as containing blood cells, "stellar cells", myoblasts, and epithelial cells. This model system could be made more specific for the study of myocytes if pure myocytes could be cultivated *in vitro* in the absence of other heart cells. Similarly, the metabolism of the cardiac myocyte could be more specifically studied if pure cells could be obtained. We now report a method for obtaining highly purified beating cardiac myocytes by separating the myocytes from the fibroblasts, endothelial cells, and blood cells which are found together with myocytes in myocardium.

Materials and Methods

Suspension of Heart Cells

Heart cells were obtained in suspension using a modification of the method of Berry, Friend and Scheuer.[4] The heart ventricles were excised from two 150–200 g, male, Sprague-Dawley rats, minced to fragments of approximately 2 mm in greatest dimension, washed and placed in 1.5 ml of Saline A[1] containing 0.1% collagenase and 0.05% hyaluronidase (Sigma Chemical Company, St Louis, Mo 63178). The cardiac muscle fragments were then gently agitated in this disaggregation medium at 100 cycles/min for 15 minutes on a Dubnoff Metabolic Shaker with the water bath at 37 C. The supernatant from this first 15-minute incubation, containing predominantly red blood cells, was discarded. The supernatants from three subsequent incubations under identical conditions were washed to remove the collagenase and hyaluronidase, combined, resuspended and diluted to contain 15–25 $\times$ 10^6 heart cells in 7 ml of Saline A and gently layered over the density gradients.

Gradient Centrifugation

Previous reports have presented detailed accounts of the theory of gradient centrifugation as applied to mammalian cells,[11-15] detailed descriptions of the technic which we employ[14] for the separation of viable mammalian cells in gradients of Ficoll (polysucrose, average molecular weight 400,000; Pharmacia Fine Chemicals, Piscataway, NJ) in tissue culture medium and the results of specific separations which have been carried out in our laboratory;[16-20] therefore, we shall omit a detailed presentation of technic and theory from this report.

The gradients for isopycnic centrifugation varied linearly from 4.1% (w/w) Ficoll at the sample-gradient interface, 14.9 cm from the center of revolution, to 43.0% (w/w) Ficoll at the gradient-cushion interface, 26 cm from the center of revolution. Isopycnic centrifugation was carried out using a centrifugal force of 950 *g* at 4.0 C for 90 minutes. After isopycnic centrifugation, the gradients were collected in 3-ml fractions. Refractive indices were measured on all gradient fractions in order to confirm the linearity of all density gradients. Cell counts were performed on all gradient fractions using hemocytometer chambers. Slides for microscopic examination were prepared using the Cytocentrifuge (Shandon Scientific Company, Sewickley, Pa) and stained with Wright stain (Chroma-Gesellschaft Schmid and Company, Stuttgart-Unterturkheim, Germany). Differential cell counts were performed, counting 500 cells from each gradient fraction.

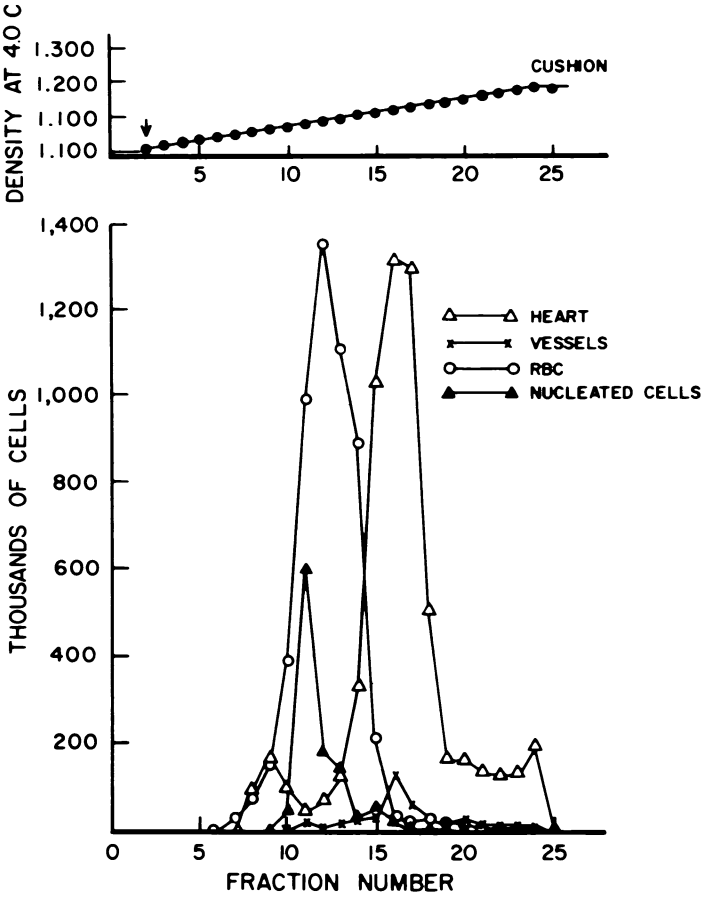
Gradients for the separation of cells by velocity sedimentation were constructed such that the Ficoll concentration varied linearly from 2.4% (w/w) Ficoll at the sample-gradient interface, 13.7 cm from the center of revolution, to 18.5% (w/w) Ficoll at the gradient-cushion interface 26.0 cm from the center of revolution. Preliminary velocity sedimentation was carried out at 18.7 *g* (measured at the sample-gradient interface 13.7 cm from the center of revolution) for short periods of time. Each respective type of cell was located on the gradient. The location of each type of cell, after the described velocity sedimentation, and the density of each type of cell, as determined experimentally using isopycnic sedimentation, were substituted in the computer integration of the differential sedimentation equation in order to calculate effective diameters for each of the modal populations of cells as described previously.[14] The calculated effective diameters and the experimentally determined densities were then used in the computer integration of the differential sedimentation equation in order to ascertain: a) whether or not the described gradient would permit the purification of cardiac myocytes and b) a satisfactory speed and duration of centrifugation for the separation of myocytes from the other heart cells which were present in the cell suspension. Simulated experiments carried out in this fashion indicated that centrifugation at 24 *g* (measured at the sample-gradient interface 13.7 cm from the center of revolution) for 30 minutes would result in separation of the myocytes from the other heart cells; laboratory experiments were carried out using these conditions. After velocity sedimentation, 4-ml fractions were collected from the gradients; refractive indices were measured, cell counts were performed, and slides for microscopic examination were prepared as described above.

Results

Sample Composition

Suspensions of cells, which were obtained using the described procedure modified after Berry *et al*[4] for the digestion of myocardium, contained consistently less than 23% cardiac myocytes. Preliminary purification of these heart cells could be accomplished by centrifuging them to form a pellet at 65 *g* for 3 minutes and selectively discarding the top portion of the pellet which was found to be rich in red blood cells. This preliminary step resulted in a maximum myocyte purity of 46%. Even after this preliminary purification step was repeated several times, the cardiac myocytes usually constituted considerably less than 46% of the

heart cells in this suspension. In some experiments, the hearts from heparinized rats (100 units intraperitoneally 20 minutes before sacrifice) were perfused with saline A before being minced and digested with the described enzymes. While this perfusion step removed many of the red blood cells, some red blood cells always remained and the myocytes were never obtained in greater than 46% purity. Berry *et al*[4] described two-thirds of the myocytes examined with the electron microscope as being "morphologically intact." Using only light microscopy,



TEXT-FIG 1—Separation of rat heart cells by isopycnic sedimentation. In this experiment, 22.8 × 10^6 cells were separated on a linear, 11.1 cm, 4.1–43.0% w/w Ficoll gradient. Centrifugation was carried out at 950 *g* (measured at the sample-gradient interface) at 4.0 C for 90 minutes. In isopycnic sedimentation, cells are separated according to differences in density. It can be observed that there is an appreciable overlap between the densities of the various kinds of cells. An *arrow* marks the sample-gradient interface on the density plot.

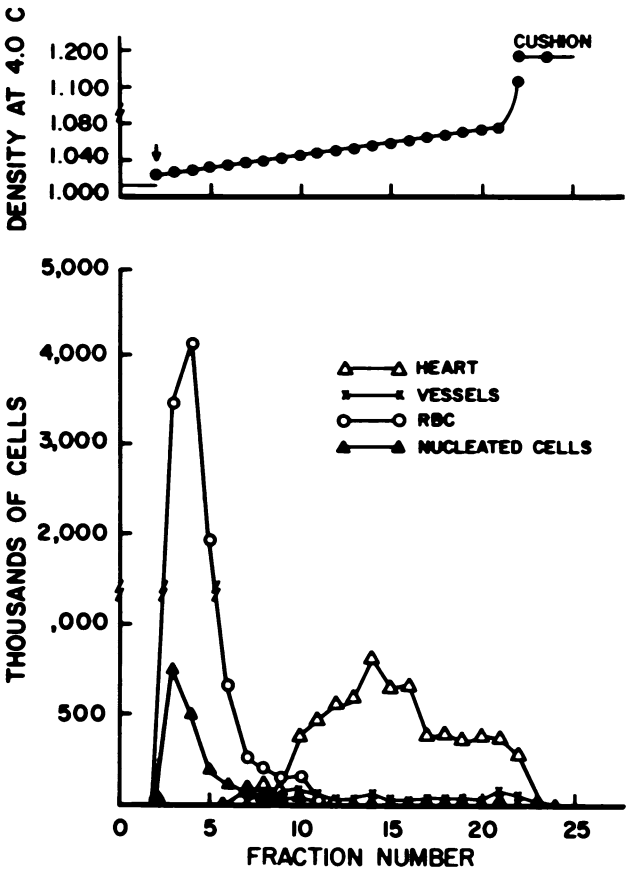
a very small proportion of the cells in our experiments appeared to be damaged.

Isopycnic Centrifugation

Isopycnic centrifugation resulted in an incomplete separation of the modal population of myocytes from the modal population of red blood cells (Text-figure 1). In repeated experiments, 76.4 to 79.8% of myocytes were recovered in five 3-ml fractions (Fractions 14 through 18) from the 72-ml gradient. These myocytes varied in density from 1.12 to 1.14 g/ml. Cardiac myocytes were obtained in a maximum purity of 88.6 to 92.4% myocytes in Fraction 18 after isopycnic centrifugation (Text-figure 1). Five adjacent 3-ml fractions from the gradient (Fractions 10 through 14) contained 90.4 to 92.5% of the erythrocytes recovered from the gradient. These erythrocytes varied in density from 1.10 to 1.12 g/ml. Erythrocytes were obtained in 76.2 to 83.0% purity in Fraction 12 (Text-figure 1). There are two well-defined modes of myocytes which are quite different from each other in density. As noted above, the overwhelming majority of myocytes is found in the mode having a density of 1.12 to 1.14 g/ml; these myocytes appear to be intact when examined microscopically. The less dense modal population of myocytes, which is present at a density of 1.07 through 1.1 g/ml, are fragmented and considered to have been severely damaged by mincing and enzyme digestion steps used for disaggregation.

Velocity Sedimentation

After velocity sedimentation at 24 *g* (measured at the sample-gradient interface 13.7 cm from the center of revolution) for 30 minutes, the cardiac myocytes were almost completely separated from other heart cells (Text-figure 2). As noted previously, the starting sample suspension of heart cells never contained more than 46% myocytes. In repeated experiments, 82.8 to 86.2% of the separated cardiac myocytes were recovered in Fractions 12 through 22, varying in purity between 92.8 and 97.4% myocytes. Both the maximum purity (Figure 1) and the largest number of myocytes were found in Fraction 15 ($\pm$ 1 gradient fraction). Since our purpose was to prepare pure myocytes, no effort was made to separate red blood cells from nucleated cells. This separation has been demonstrated previously [19] and requires a gradient having a lesser slope (g/ml/cm), such as the isokinetic [15] gradient. Myocytes were not present in the fraction that contained erythrocytes and nucleated cell peak (Figure 2). When gradients were made of Ficoll and Joklik's modification of minimum essential medium, cardiac myocytes failed to



TEXT-FIG 2—Separation of rat heart cells by rate-zonal or velocity sedimentation. In this experiment, 24.7 × 10^6 cells were separated on a linear, 12.3 cm, 2.4–18.5% w/w Ficoll gradient. Centrifugation was carried out at 24 g (measured at the sample-gradient interface) at 4.0 C for 30 minutes. Rate-zonal sedimentation results in the separation of cells primarily because of differences in diameter. Cardiac myocytes are both more highly purified and more widely separated from the other heart cells than was observed after isopycnic centrifugation.

beat after their entry into the gradients; this cessation of myocyte contraction in media containing magnesium or calcium has caused most investigators to use media such as Saline A [1] which are free of calcium and magnesium. When gradients were made of Ficoll in Saline A, cardiac myocytes continued to contract rhythmically after they were separated by density gradient centrifugation.

Velocity sedimentation offers several advantages over isopycnic sedimentation for the separation of cardiac myocytes since: a) the modal population of myocytes is more widely separated from the other kinds of cells, b) the myocytes are obtained in higher purity, c) the cells are

subjected to much lower centrifugal forces and d) the cells are available more rapidly for subsequent experiments.

Recovery of Cells

After isopycnic centrifugation, 61.2 to 74.6% of the heart cells layered over the gradient were recovered in fractions from the gradient. After velocity or rate-zonal sedimentation, 64.4 to 75.2% of the cells in the starting sample suspension were recovered from the gradient. As noted above, the relative proportions of red blood cells and myocytes in the starting sample suspension varied as a function of the technic used for disaggregating the myocardium and according to the number of preliminary centrifugations carried out before gradient centrifugation. The peak heights and the areas under the peaks for each respective cell type varied as a function of the composition of the starting cell suspension. There did not appear to be a disproportionate loss of either red blood cells or cardiac myocytes. The numbers of neutrophils, vessel fragments and other nucleated cells in the starting sample suspension were too small to permit a rigorous evaluation of the recovery of each of these kinds of cells. The 24.8 to 38.8% of heart cells, which were present in the starting sample suspension and not recovered in the fractions from the gradient, represent a larger loss of cells than can be accounted for by experimental counting error. Much of this cell loss could have resulted from the wall effect artifact observed in all centrifugations carried out in cylindrical tubes. The wall effect artifact has been discussed in previous reports.[12,14]

Discussion

The described technic for the purification of cardiac myocytes would appear to have considerable significance in several areas of investigation. Pure cultures of cardiac myocytes have not been obtained by the currently available technics for dissociating and culturing heart cells. Mark *et al* [21] emphasize the changing proportions of endothelioid and muscle cells observed during the growth of heart cells in culture, and warn about interpreting enzyme or respiratory metabolic changes when such heterogeneous and changing cell populations are cultured together. The method reported here not only permits the inoculation of purified myocytes in culture, but allows immediate separation of millions of adult, differentiated heart muscle cells for metabolic and physiologic studies. One of our laboratories (Wm R and MG) has recently found that certain enzyme activities formerly attributed to heart muscle are, in fact, present in previously studied myocardium primarily because

the cardiac myocytes were adulterated with incompletely removed red blood cells.

Clark [22] measured glucose utilization in dissociated heart cells under various experimental conditions. Schreiber *et al* [23] reported specific alterations in protein synthesis in the "overloaded" heart. Both the metabolism of cardiac myocytes under normal circumstances and the response of the cardiac myocyte to pathogenic stimulation could be more specifically elucidated using preparations free of other kinds of cells. Purified cardiac myocytes should proved particularly useful in the study of nutritional requirements of the heart cell. Gerchenson, Harary and Mead [24] reported that cultured heart cells require an exogenous supply of linoleic and palmitic acids for optimal growth and mitochondrial integrity. While, by definition, whole animals must be able to synthesize all nonessential amino acids, certain adult cells are not able to synthesize all of the required nonessential amino acids; for example, normal human bone marrow is not capable of synthesizing adequate amounts of serine and asparagine.[25] The availability of pure cardiac myocytes permits the investigator to study the nutritional requirements of this single cell type *in vitro* while excluding the possibility that other kinds of cells in the culture are altering the experimental conditions by exporting nutrients for use by the cardiac myocytes. Halle and Wollenberger [26] recently described a defined medium without macromolecules for the culture of avian myocytes. Cultures of pure, mammalian cardiac myocytes would be particularly suited to the development of a defined medium for mammalian cells. The intrinsic rhythmicity of the cardiac myocyte provides a very sensitive assay of the cell's milieu which is not available in work with other kinds of cells.

DeHaan [8] recently detected a toxic substance readily eluted from disposable intravenous administration sets, that had been used to perfuse cultured chick heart cells. The toxicity of the medium delivered through these sets was detected before cell death occurred by a decrease in the fraction of cells actually beating. Other investigators [27,28] studied the response of heart cells to drugs and other toxic substances. The information derived from this kind of study could be much more specific if the studies were carried out using only cardiac myocytes.

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Acknowledgments

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[*Illustrations follow*]

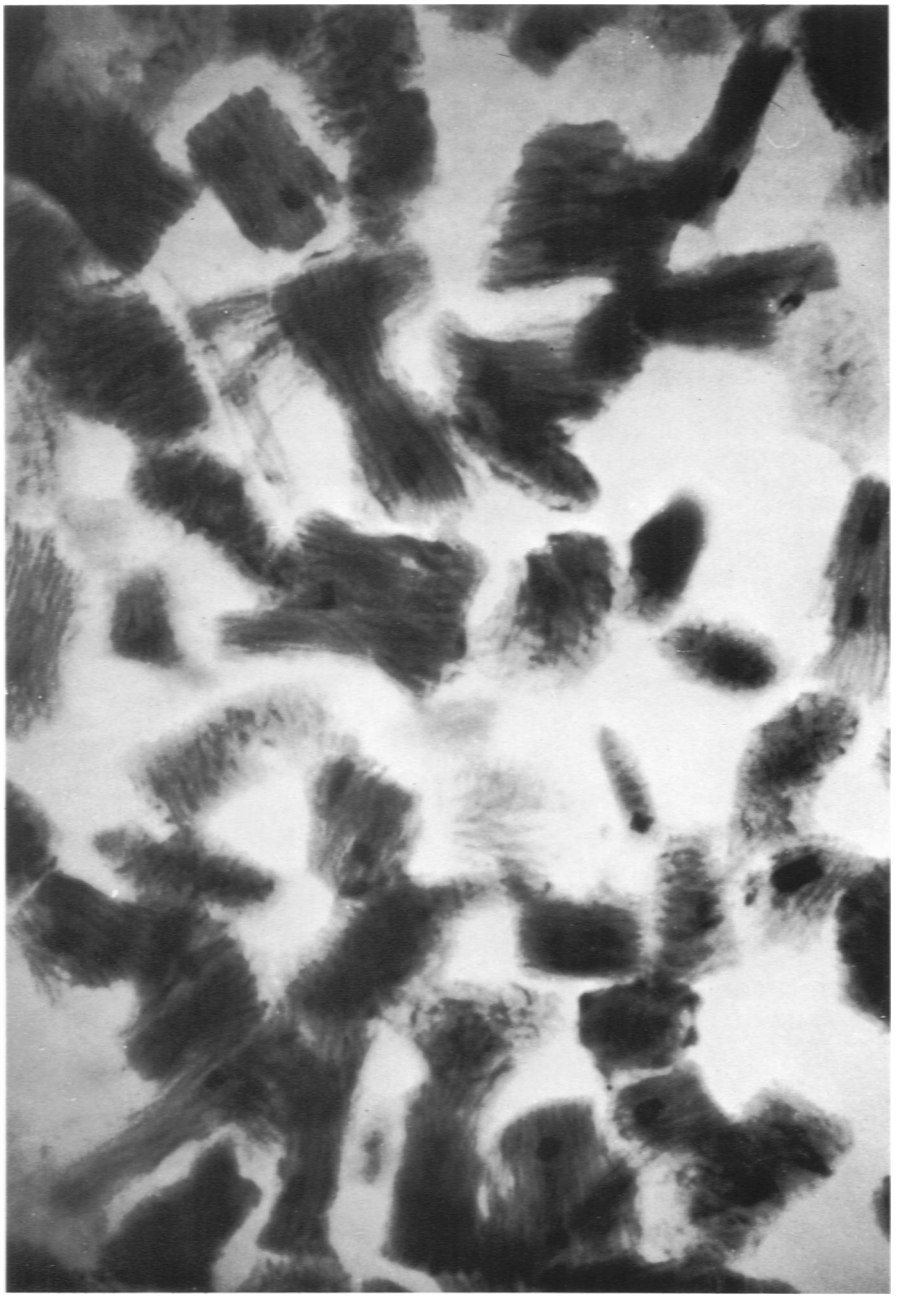

Fig 1—Fraction 15 from the rate-zonal separation of rat heart cells. This fraction contains purified cardiac myocytes (Wright stain, × 100).

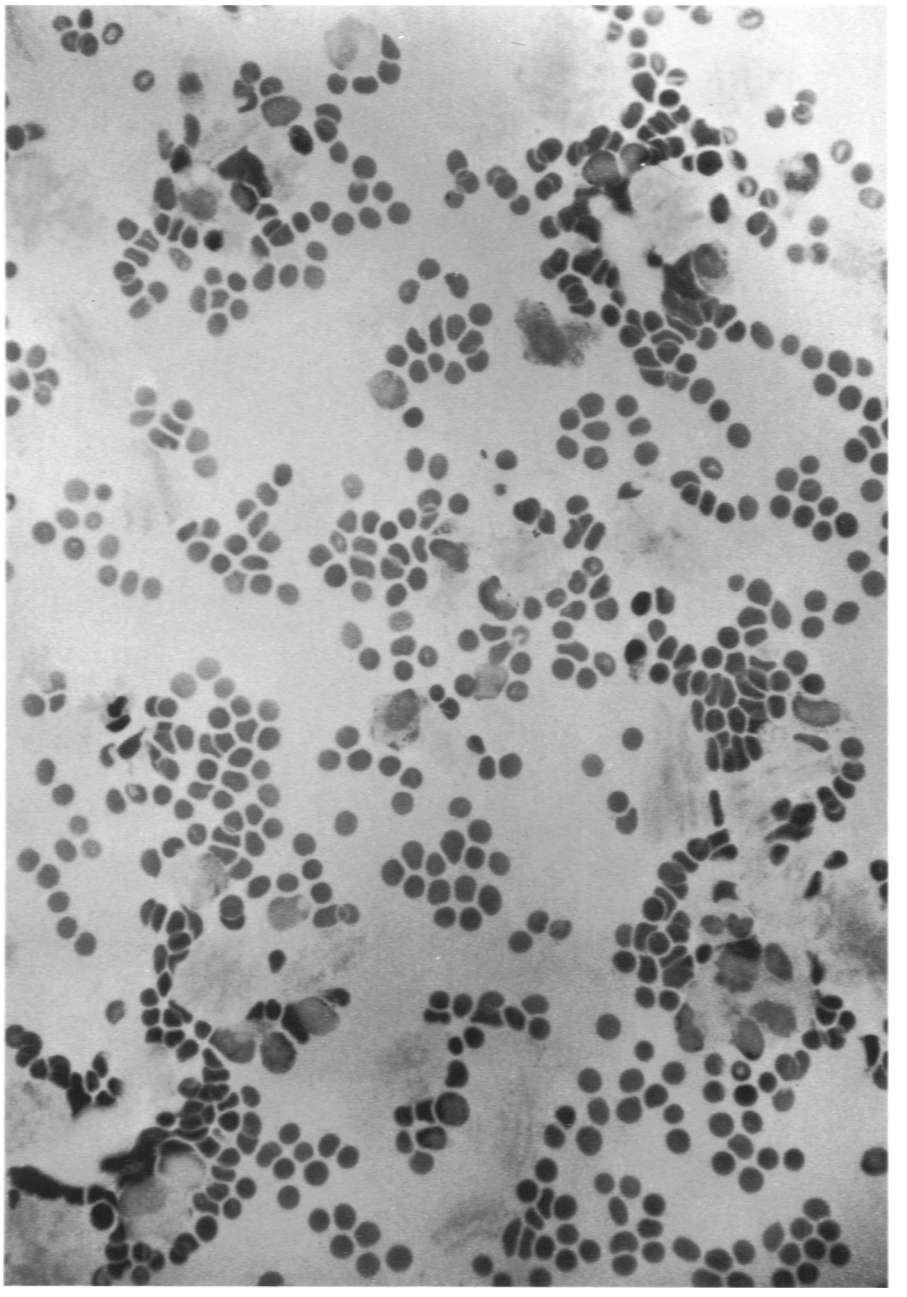

Fig 2—Fraction 4 from the rate-zonal separation of heart cells. This fraction contains the modal population of erythrocytes as well as nucleated cells and damaged cells. Red blood cells were not resolved from nucleated cells because of the very steep (g/ml/cm) gradient which was found to be optimal for the separation of cardiac myocytes from other cells (Wright stain, × 100).