Cellular Mechanisms of Normal Growth in the Mammalian Heart

I. Qualitative and Quantitative Features of Ventricular Architecture in the Dog from Birth to Five Months of Age

MARIANNE J. LEGATO

SUMMARY This paper describes the qualitative and quantitative composition of dog myocardium over the first 5 months of life. The quantitative composition of dog right and left ventricle over this period does not vary. A stereological analysis of electron micrographs representing 32,000 μm² of tissue surface revealed that 79% of the heart is made up of myofibers, whereas 21% is extracellular space. Twenty-eight percent of the extracellular compartment by volume is vasculature (tissue was preserved by immersion rather than vascular perfusion); 72% is occupied by nonvascular elements and "empty" space. In contrast to the remarkable constancy of quantitative composition of the whole myocardium, myocyte shape and dimensions and the arrangement of intercellular connections vary dramatically over the age period studied. In early postnatal life, the morphology of blood vessels, many of which have completely partitioned lumina, also changes significantly. *Circ Res* 44: 260-262, 1979

THE MECHANISMS of normal mammalian myocardial growth at a cellular level have received surprisingly little attention; only limited descriptive and quantitative data concerning changes in heart size, architecture, and cellular composition in postnatal life are available. Available data concern either a single or at most a few isolated periods in embryonic or postnatal life (Laguens, 1971; Page et al., 1971; Page et al., 1972; Reith and Fuchs, 1973; Page and McCallister, 1973a; Page et al., 1974; Goldstein et al., 1974; Lazarus et al., 1976; Frank and Langer, 1974; Polimeni, 1974). This paper is the first of two which report the qualitative and quantitative (Legato, 1979) changes that occur during normal myocardial growth in the mongrel between the ages of 24 hours and 5 months.

**Methods**

**Preparation of Tissue**

Three separate litters, each from a different set of mongrel parents, were chosen for the study. The dogs were anesthetized by an intraperitoneal injection of pentobarbital (Diabutal), 30 mg/kg, and the chest was opened. We removed the heart and excised a 2-4 mm X 2 cm piece of the central portion of the free ventricular wall, gently stretching the specimen and tying it to an applicator stick at either end with 4-0 silk. The stick was broken off at either end of the specimen, and the tissue, tied to the stick, was dropped into a solution of ice-cold 6.25% glutaraldehyde in phosphate buffer (pH 7.6). After 1 hour, the tissue between the ligatures was cut free and minced with an acetone-cleaned razor blade into 1-mm cubes and replaced in glutaraldehyde for an additional 2 hours. These cubes were subsequently washed in phosphate buffer (pH 7.4) and dehydrated in a series of graded concentrations of acetone. We infiltrated and embedded the tissue with an epoxy resin (Durcopan). Sections were made with a diamond knife on a Sorvall-Porter Blum MT-2 microtome, and were stained with lead citrate and uranyl acetate and examined in a Philips 300 electron microscope.

**Population Studies**

Ten littersmates were studied at 24 hours of age. A second set of two littersmates was taken at 72 hours of age. The siblings of a third litter were killed, one by one, at ages of 2 months, 2.5 months, 3 months, 3.5 months, 4.5 months, and 5 months. Samples from both the right and left ventricles of all dogs were studied. (See Table 1 for a summary of specimen numbers and sources.)

**Stereological Techniques**

**Photography**

We picked up a ribbon of sections on a formvar and carbon-coated 300-mesh copper grid. In the microscope, the first unblemished section was randomly chosen and the entire face of the section systematically photographed (changing fields with
TABLE 1 Summary of Population Studies

<table>
<thead>
<tr>
<th>Litter</th>
<th>Age of litter</th>
<th>No. of dogs</th>
<th>No. of sites sampled</th>
<th>No. of micrographs</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>24 hr</td>
<td>2</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>II</td>
<td>72 hr</td>
<td>2</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>III</td>
<td>2 mo</td>
<td>1</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2.5 mo</td>
<td>1</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>3 mo</td>
<td>1</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>3.5 mo</td>
<td>1</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>4.5 mo</td>
<td>1</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>5 mo</td>
<td>1</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>8 age groups</td>
<td>10</td>
<td>38</td>
<td>160</td>
</tr>
</tbody>
</table>

Table 1 describes the population of dogs studied. Note that eight ages were sampled, with 38 different sites taken from both right and left ventricles. In all, we quantified 32,000 μm² of tissue.

Each exposure so as just to include the appropriate border of the previous photograph. Fourteen pictures covered the whole face of the section. Each set of 14 photographs was calibrated with the 15th picture, taken at the same magnification, of a grating replica (54,864 lines/inch; Ernest F. Fullam, Inc.). Final magnifications of developed micrographs ranged between 12,360 and 12,833x. The actual area of tissue measured was approximately 200 μm² on each micrograph. Ten micrographs were randomly selected from a total number of sections photographed and were counted for each data point. Therefore, 2000 μm² were quantitated for the final values we obtained for each age in each of the two chambers. For the entire study, we quantified 32,000 μm² (8 time periods × 2 chambers × 2000 μm²) of myocardium.

We printed each negative with a grid placed over the photographic paper to be exposed. Thus the final micrograph had a probe composed of squares, each side 1 cm long, printed over its surface. We prefer this technique of printing the grid directly onto the picture, as it avoids artifact due to shrinkage of the developed and dried photographic paper. If the probe is printed on the paper, any shrinkage or distortion of the paper obviously is not confined to the micrograph of the tissue, but extends to the probe as well. In counting, we chose always to quantify and match samples from the right and left ventricles of the same dog.

**Counting Techniques**

We considered all methods used by workers reporting quantitative data about striated muscle ultrastructure before settling on our own stereological techniques. There is universal agreement about how to measure compartment volumes. To do this, we counted the number of points (a “point” was defined as the place at which the lines making up the grid intersected) lying within or on the border of the object to be measured. These are recorded in our tables of data as, for example, P, with the subscript representing the tissue component being quantified [P myofiber (P_m), P extracellular space (P_esp), etc.].

**Statistical Analysis**

Statistical analyses were performed on all the data accumulated. We did a one-way analysis of variance, testing the significance of changes with time for each ventricle. We also did a two-way analysis of variance, to test the significance of changes in the data obtained with respect to time and with respect to the chamber sampled, and to see whether the chambers behaved in a similar or different fashion as growth proceeded. Student’s t-test was applied to evaluate the significance of differences observed in parameters tested as development proceeded.

**Results**

**General Tissue Architecture: Changes in Cell Size and the Development of Intercellular Connections**

Newborn (24- to 72-hour-old) myocardium has a heterogeneous pattern. Rarely (more commonly in the right ventricle than in the left), there are areas where cells look like those of the adult heart: they are longer than they are wide, and are in some cases quite narrow, with a centrally placed oval nucleus and regularly arranged myofibrils filling the volume of the cell. For the most part, however, cells are crowded together to form islands of myocytes abutting on one another around their entire circumference in a mosaic arrangement quite characteristic of this age (Fig. 1). The close juxtaposition of cells often ensures commingling of the perimembranous component (the glycocalyx) of adjacent sarcolemmas along the entire circumference of the cell. Mitotic nuclei are seen occasionally at this age (Fig. 2). Such cells show considerable disorganization of their interior architecture. Myofibrils are disrupted and only fragments of sarcomeres exist; in particular, Z bands are either attenuated or not present at all.
Nexal junctions are present often as isolated connections between cells from the first day of postnatal life. They appear with approximately the same frequency in neonatal as in adult heart (Page and McCallister, 1973b); nexuses make up about 12% of the surface membrane of the cell. They occasionally have unusual configurations; one nexal junction in a 72-hour-old left ventricular sample...
formed a complete circle (Fig. 3a). Desmosomes, on the other hand, appear frequently only as the intercalated discs begin to grow and increase in complexity: as isolated connections they remain rare and are often incompletely developed. No classic intercalated discs are present. Occasionally, a short sequence of connections consisting largely of zonae adhaerentes interspersed with nonspecialized portions of the sarcolemmas is apparent. These are found most frequently joining cells end to end, and they form the earliest intercellular junctions.

As the cells grow longer and narrower, the lateral distances between them increase; the right ventricular pattern is different than that of the left in this respect from birth onward. Even at 24 hours of age, some portions of the right ventricular tissue are remarkable for the amount of space between cells, in marked contrast to the much closer packing of myofibers observed in the newborn left-sided chamber.

Spaces between cardiac cells are prominent by 2 months of age in both chambers. Contacts between cells are in general of a specialized type. (Side-to-side connections between cells still are infrequent in either ventricle, but generally are desmosomes.) End-to-end intercellular linkages are well established now; they are easily recognizable as intercalated discs but still do not have the usual stepwise pattern often (although not exclusively) found in

<table>
<thead>
<tr>
<th>Age</th>
<th>LV cell diameter (μm)</th>
<th>RV cell diameter (μm)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hr</td>
<td>3.7 (11) ±0.6</td>
<td>7.7 (22) ±0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>72 hr</td>
<td>3.8 (47) ±0.3</td>
<td>4.6 (29) ±0.4</td>
<td>NS*</td>
</tr>
<tr>
<td>2 mo</td>
<td>7.4 (25) ±0.7</td>
<td>4.2 (69) ±0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2.5 mo</td>
<td>3.9 (23) ±0.2</td>
<td>5.7 (34) ±0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3 mo</td>
<td>8.9 (12) ±1.2</td>
<td>8.7 (17) ±1.1</td>
<td>NS</td>
</tr>
<tr>
<td>3.5 mo</td>
<td>6.4 (34) ±0.6</td>
<td>8.8 (37) ±0.4</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>4.5 mo</td>
<td>4.4 (14) ±0.6</td>
<td>7.4 (20) ±0.6</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>5 mo</td>
<td>6.0 (17) ±0.5</td>
<td>5.3 (25) ±0.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 2 illustrates the changes in cell diameter with development. A total of 440 cells was measured; on the average, we surveyed 23 cells for each age period in the LV and 31 in the RV. The actual number of cells measured for each sample is in parentheses. The figures in this and subsequent tables are mean ± standard error. P values for significant differences between the diameters are listed.

* NS = not significant.
electron micrographs of adult ventricular muscle (Fig. 3b). Most are straight, with the area of specialized junctions perpendicular to the long axis of the cell, especially in the left ventricle. In the right ventricle, discs are longer and pursue a more undulating course.

By 2.5 months of age, the myofibers throughout both ventricles have become longer and narrower than in the newborn heart, but the left ventricular cell is many times longer and only one-third to one-half as wide as those of the right-sided chamber (Table 2). Indeed, at this age, many right ventricular myocytes have a rounded, almost "squared-off" appearance, whereas in contrast, in the same dog at
FIGURE 3  A: This electron micrograph of a portion of a 72-hour-old left ventricle illustrates an apparently intracellular nexus (arrow), a frequent finding in the early neonatal period. (See text for possible interpretations of this phenomenon.) 22,000×.  B: This micrograph from the right ventricle of a 2.5-month-old dog illustrates the type of obliquely oriented intercalated disc characteristic of this age (arrows). The disc has not yet assumed the step-like appearance it has in older myocardium (compare to Figure 3C). 12,000×.  C: The sample of right ventricular tissue shows the step-like, “riser-tread” pattern of the intercalated disc characteristic of adult myocardial tissue. Note that the specialized portions of the disc (arrows) are perpendicular to the long axis of the cell, whereas the nonspecialized portion lies parallel to it. 7,350×.

the same period, myocytes of the left side are delicate, long, ribbon-like cells which not infrequently are only two sarcomeres in diameter.

By 3.5 months of age, left ventricular cells, although they continue to be much longer than those of the right side, are two to three times thicker than they were just 6 weeks earlier. Not infrequently, they approach the right ventricular myocyte in diameter (about 6 μm). By 5 months of age, there is no significant difference in cell width between the two chambers (Table 2). However, even by 5 months of age, the oldest period studied, left ventricular cells always were substantially longer (three to four times on the average) than the cells of the right ventricle. Intercalated discs, especially in the left ventricle, assumed their characteristic stepwise pattern by 5 months in many instances (Fig. 3c). Isolated side-to-side connections between cells still are rare and, as before, are almost exclusively desmosomes.

The Extracellular Compartment

The extracellular compartment in the newborn heart has all the elements of the adult ventricle except that recognizable nerve fibers are very infrequent. Fibroblasts, capillaries, and undifferentiated mesenchymal cells are all present. Collagen fibrils, although present, are not abundant in the neonatal period in comparison to the myocardium of older animals.

Capillaries are much thicker-walled during the newborn period than in adult myocardium. The walls themselves are remarkable for the abundance of micropinocytotic vesicles contained within them (Fig. 4a). The latter are of all sizes; some are quite small (60–80 Å in diameter) whereas others are four to five times larger. They are much less frequent in the walls of the capillaries of older myocardium. Partitioning of the capillary lumen by strands of endothelial cell cytoplasm is a common finding in newborn vessels. Rough endoplasmic reticulum and free ribosomes are abundant in the newborn capillary wall; this is no longer seen in either chamber by 2 months of age (Fig. 4b).

Another finding unique to 24- to 72-hour-old myocardium is that the capillary lumen in many instances is full of an amorphous, electron-dense material which is not seen in the much larger vessel lumina of older tissue. Mitochondria are sparse and arranged in randomly placed pools of from two to
Figure 4  A: This capillary has the thick wall containing many vesicles that is characteristic of the early (72-hour-old) postnatal period. Note the partitioning of the lumen (L) by a finger of cytoplasm; this is another common phenomenon in these early vessels. F = fibroblast. 11,500x. B: Note the much thinner wall (arrow) of this capillary from the left ventricle of a 2.5-month-old dog. Partitioned lumina are not seen in the vasculature at this age. 9,100x.

The cytoplasm of fibroblasts, like that of the capillaries during the first days of postnatal life, is filled with rough endoplasmic reticulum (Fig. 4a), indicating the intense secretory activity of the cell during this period. Mitochondria are quite infrequent; they are much fewer than in myocytes, for
example. Collagen fibrils are present but are short, infrequent, and not, for the most part, arranged in bundles, but scattered randomly throughout the extracellular compartment.

By 2 months of age, the extracellular compartment is full of collagen fibrils arranged in the whorls and bundles characteristic of adult myocardium. Nerve fibers are more frequent at this state of development; these were rare in the newborn period. The vesiculation of capillary walls is not nearly as striking as it was in hearts 24-72 hours of age, but it is still very apparent, although on the whole, the vesicles are much larger and less frequent than in the 24- to 72-hour-old vessel. By 2-2.5 months of age, the amount of rough endoplasmic reticulum in both capillary walls and fibroblasts is strikingly less than that of the newborn period.

Quantitative Data

The most remarkable feature of normal growth is the constancy of myocardial composition as the heart enlarges with increasing age (Table 3). Whole myocardium is 79% myofibers and 21% extracellular space by volume. The vasculature takes up 28% of the extracellular space (7.2% of whole tissue). Fifty percent of the vasculature is lumen; the vessel walls occupy the other half of its volume. Student's t-test and a two-way analysis of variance show that the values obtained were not significantly changed by time or with respect to the chamber sampled.

Discussion

There is need for data obtained at frequent and sequential intervals which describe the events that occur at a cellular level with the normal increase in cardiac size that occurs after birth. The descriptive data that are available do not cover extensive time periods, and are, for the most part, from embryonic tissue (Hibbs, 1956; Muir, 1957; Wainrach, 1961; Challice and Edwards, 1961; Olivo et al., 1964; Cedergren and Harary, 1964; Przybylski and Blumbert, 1966; Manasek, 1968, 1969, 1970; Manasek and Monroe, 1972; Lemanski, 1973; Chacko, 1976). In contrast, there is abundant information in the literature about the composition and properties of both the right and left hypertrophied ventricles (Alpert, 1971).

It is important to realize that, although a quantitative analysis of the normal growth process can be valuable and can provide important insights into the mechanisms of the cellular response to workloads of different types (Page et al., 1972; Reith and Fuchs, 1973; Page and McCallister, 1973), it alone is not enough to give a complete picture of normal development. In spite of a remarkable constancy in the relative volumes occupied by various components of the myocardium, profound changes are occurring in the geometric arrangement of the tissue, in the shape of the myocytes, in the distribution of their intercellular connections, and in the amount of surface area in intimate contact between adjacent muscle cells. The tailoring of the entire intracellular compartment, in particular, is in constant progress as the animal develops (Legato, 1979). The vasculature undergoes important changes; the degree of myocyte surface area in close juxtaposition to capillary walls increases dramatically as maturation progresses. Collagen fibrils begin to appear and fill in the extracellular compartment.

The Intracellular Compartment

General Organization and Myocyte Shape

We observed important differences in the postpartum period between right and left ventricular cell shape and in the arrangement and apposition of one myofiber to another in the two chambers. Although our study samples only a tiny fraction of the entire ventricle, that is a limitation common to all ultrastructural investigations. It is also true that in the case of cell shape and tissue geometry we do not base our observations on extensive quantitative data (see Table 3). That would be worthy of a separate study. However, we examined several hundred micrographs of right and left ventricular myocytes to reach our conclusions, and there is no question that the general architecture of the myocardium is quite different in the two chambers at birth and that these differences are largely eliminated by 5 months of age.

The changing pattern of myocardial architecture may well reflect the differences in the work done by the ventricles in postnatal life, when one chamber operates against a much lower pressure than the other, a fact reflected in the striking regression of right wall thickness and the equally striking increase in left ventricular mass during the first weeks of postpartum existence. The left ventricle at birth has many areas composed of cells that are long and narrow, although the intercellular connections are not those of the mature chamber, and the cells are much smaller than the adult left ventricular myofiber (2-3 μm in diameter). The right ventricular cell population, on the other hand, is a mosaic of closely packed myocytes with few myofibrils and a large nucleus that dominates the cell. By 3 days after birth, the situation has changed, and the left myocardium in many areas is indistinguishable from that of the right ventricle. The left ventricle may well adjust to the gradually increasing pressure load occasioned by the closure of the ductus arteriosus by producing more cells. Although we have no direct evidence to support this idea, the 72-hour-old ventricle has many more primitive-looking myocytes than the 24-hour-old chamber. By 2 months of age, these are no longer in evidence.

By 5 months, both ventricles have the same general pattern. There is no significant difference between cell diameters at this time (see Table 2),
Table 3  Quantitative Composition of the Right (A) and Left (B) Ventricular Myocardium

<table>
<thead>
<tr>
<th>Age</th>
<th>P_nun</th>
<th>P_nu</th>
<th>P_nw</th>
<th>P_end</th>
<th>P_cap</th>
<th>P_cap/P_nw</th>
<th>P_cap/P_cap</th>
<th>P_cap/P_cap</th>
<th>P_cap/P_cap</th>
<th>P_cap/P_cap</th>
<th>P_cap/P_cap</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>24 hr</td>
<td>312.0</td>
<td>362.4</td>
<td>119.3</td>
<td>70.9</td>
<td>42.8</td>
<td>21.1</td>
<td>22.5</td>
<td>7.4</td>
<td>20.3</td>
<td>13.1</td>
<td>0.724</td>
</tr>
<tr>
<td></td>
<td>±19.3</td>
<td>±11.5</td>
<td>±11.7</td>
<td>±13.0</td>
<td>±6.3</td>
<td>±8.3</td>
<td>±2.9</td>
<td>±5.9</td>
<td>±4.6</td>
<td>±0.050</td>
<td>±0.027</td>
</tr>
<tr>
<td>72 hr</td>
<td>342.6</td>
<td>319.7</td>
<td>107.8</td>
<td>88.4</td>
<td>28.3</td>
<td>48.5</td>
<td>15.6</td>
<td>22.0</td>
<td>12.6</td>
<td>26.5</td>
<td>0.793</td>
</tr>
<tr>
<td></td>
<td>±7.7</td>
<td>±14.7</td>
<td>±11.5</td>
<td>±10.9</td>
<td>±8.1</td>
<td>±7.9</td>
<td>±2.3</td>
<td>±3.2</td>
<td>±6.5</td>
<td>±0.035</td>
<td>±0.029</td>
</tr>
<tr>
<td>2 mo</td>
<td>341.6</td>
<td>374.7</td>
<td>107.8</td>
<td>88.4</td>
<td>91.6</td>
<td>9.3</td>
<td>9.0</td>
<td>18.5</td>
<td>0.791</td>
<td>0.825</td>
<td>0.209</td>
</tr>
<tr>
<td></td>
<td>±15.4</td>
<td>±14.7</td>
<td>±11.5</td>
<td>±10.9</td>
<td>±8.1</td>
<td>±7.9</td>
<td>±2.3</td>
<td>±3.2</td>
<td>±6.5</td>
<td>±0.035</td>
<td>±0.029</td>
</tr>
<tr>
<td>2.5 mo</td>
<td>333.6</td>
<td>347.4</td>
<td>93.9</td>
<td>73.9</td>
<td>31.0</td>
<td>28.3</td>
<td>22.0</td>
<td>9.3</td>
<td>9.0</td>
<td>18.5</td>
<td>0.791</td>
</tr>
<tr>
<td></td>
<td>±18.3</td>
<td>±14.7</td>
<td>±11.5</td>
<td>±10.9</td>
<td>±8.1</td>
<td>±7.9</td>
<td>±2.3</td>
<td>±3.2</td>
<td>±6.5</td>
<td>±0.035</td>
<td>±0.029</td>
</tr>
<tr>
<td>3 mo</td>
<td>350.0</td>
<td>335.0</td>
<td>82.1</td>
<td>88.7</td>
<td>26.4</td>
<td>23.3</td>
<td>9.1</td>
<td>14.1</td>
<td>17.1</td>
<td>9.2</td>
<td>0.810</td>
</tr>
<tr>
<td></td>
<td>±13.2</td>
<td>±8.7</td>
<td>±13.3</td>
<td>±9.8</td>
<td>±9.3</td>
<td>±7.1</td>
<td>±3.3</td>
<td>±4.3</td>
<td>±6.3</td>
<td>±3.8</td>
<td>±0.031</td>
</tr>
<tr>
<td>3.5 mo</td>
<td>340.1</td>
<td>341.8</td>
<td>92.4</td>
<td>90.4</td>
<td>18.7</td>
<td>36.6</td>
<td>12.6</td>
<td>16.6</td>
<td>6.1</td>
<td>20.0</td>
<td>0.786</td>
</tr>
<tr>
<td></td>
<td>±11.2</td>
<td>±14.6</td>
<td>±11.2</td>
<td>±14.6</td>
<td>±4.9</td>
<td>±9.0</td>
<td>±3.9</td>
<td>±3.5</td>
<td>±2.0</td>
<td>±6.5</td>
<td>±0.027</td>
</tr>
<tr>
<td>4.5 mo</td>
<td>319.3</td>
<td>324.8</td>
<td>112.7</td>
<td>105.4</td>
<td>24.1</td>
<td>20.7</td>
<td>8.2</td>
<td>6.0</td>
<td>30.3</td>
<td>14.7</td>
<td>0.739</td>
</tr>
<tr>
<td></td>
<td>±8.5</td>
<td>±14.0</td>
<td>±13.9</td>
<td>±6.0</td>
<td>±4.3</td>
<td>±2.0</td>
<td>±1.2</td>
<td>±3.7</td>
<td>±0.020</td>
<td>±0.030</td>
<td>±0.261</td>
</tr>
<tr>
<td>5 mo</td>
<td>315.5</td>
<td>365.8</td>
<td>82.9</td>
<td>66.1</td>
<td>26.0</td>
<td>18.0</td>
<td>16.7</td>
<td>9.9</td>
<td>9.3</td>
<td>8.9</td>
<td>0.806</td>
</tr>
<tr>
<td></td>
<td>±8.9</td>
<td>±7.6</td>
<td>±9.3</td>
<td>±7.6</td>
<td>±5.9</td>
<td>±4.8</td>
<td>±2.7</td>
<td>±3.1</td>
<td>±0.021</td>
<td>±0.018</td>
<td>±0.020</td>
</tr>
</tbody>
</table>

This is a summary of all the quantitative data on right (A) and left (B) ventricular composition. For each age period, the mean value for all 10 micrographs counted for each parameter studied was calculated. These are tabulated with standard errors of the mean. The age periods are listed in the first column. The abbreviations are as follows: P = the number of points lying over the contours of the organelle; myo = myocyte; vas = vasculature; end = endothelial cell; lum = capillary lumen; ecs = extracellular space; wt = whole tissue.
although left ventricular cells continue to be substantially longer than those of the right chamber.

**Intercellular Connections: Mechanism of Formation of the Intercalated Disc**

The development of the intercalated disc might be considered a prime example of tissue remodeling in postnatal life. In fortuitous sections of mature working ventricular myocardium, the disc has a stepwise or "riser-tread" pattern like that of a staircase (see Fig. 3c). The so-called specialized portions of the disc are perpendicular to the long axis of the myocyte and include nexal and desmosomal connections between cells as well as the fascia adhaerens, into which the terminal sarcomeres of the myofibrils insert. The nonspecialized portions of the disc, which consist of the adjacent but separate sarcolemmas of the connected myocytes, run parallel to the long axis of the cell.

As we have pointed out, this pattern of intercellular connections is not seen in the newest areas of the postpartum ventricle. The earliest discs between the rounded cells of the newborn heart are short, linear clusters of specialized connections between cells. In somewhat older myocardium, they have a zigzag pattern (see Fig. 3b). The thin filaments of the terminal sarcomeres of the myofibril insert into the fascia adhaerens. The myofibrils increase in length and, by the addition of new sarcomeres, in register with the rest, the once obliquely oriented, specialized portions of the disc become more and more perpendicular to the long axis of the cell. Consequently, mature working ventricular myocytes, many times longer than they are wide, are connected by intercalated discs with the classic stepwise pattern characteristic of the adult heart. This sequence of changes, which is complete by about 2 months after birth, is so universal that the age of the animal can be estimated quite accurately by the pattern of the intercalated discs in the working ventricular myocardium.

Muir (1957) and McNutt (1970), among others, believe that the perpendicular orientation of the specialized portion of the intercalated disc to the long axis of the cell is the consequence solely of mechanical traction exerted on it by the long linear array of sarcomeres in the myofibril, which ends by insertion of the terminal sarcomere into the Z substance of the fascia adhaerens. This mechanical action of the contractile apparatus on the disc may well play a role in its final arrangement in myocardium. It is of interest that the stepwise pattern of the disc is very pronounced in the left chamber, as opposed to the right, in which cells have fewer sarcomeres and are shorter than in the left ventricle. In the right ventricle, the disc often has a continuous sequence of nexus, desmosomes, and fascia adhaerens in a linear or oblique orientation between adjacent cells, with few, if any, unspecialized portions of the disc separating them. In contrast, the left ventricular cell, with its much longer array of sarcomeric units anchored at either end in the substance of the fasciae adhaerentes, invariably has the specialized portion of the disc positioned at right angles to its long axis. This is an optimal mechanical arrangement for force development by the myocyte and, together with the much longer myofibrils of the left ventricular myocyte, ensures that this cell will develop more force per beat than the shorter and not much wider right ventricular myocyte—an important factor in relation to the different afterloads against which the two chambers are working.

The interesting finding of the circular nexus illustrated in Figure 3a may be the result of a fortuitous section of a link between the projecting finger of one cell and a complementary, enclosing indentation in an adjacent one. Alternatively, however, it may represent a totally intracellular nexus. This suggests that the final system of intercellular junctions may be formed not only by the synthesis of specialized connections but also by their selected ingestion and destruction. If this interpretation is correct, the phenomenon may be part of the larger process of cell remodeling as development proceeds, as first suggested by Larsen (1970). Ferrans and his co-investigators described a totally intracellular desmosome in diseased human and canine cardiac muscle cells, and also viewed it as part of an overall process of cell remodeling (Buja et al., 1974).

**The Extracellular Compartment**

**The Vasculature**

*Formation of the Vascular Lumen.* Our data are consonant with the idea that the capillary lumen forms by the liquefaction of the cell cytoplasm and parallel some of the observations Manasek has made on embryonic hearts. The walls of early (24- to 72-hour-old) capillaries are much thicker than those seen later in the postnatal period (see Fig. 3a), and the cytoplasm of endothelial cells is filled with vesicles that become larger as age increases. It is not difficult to imagine that these larger vesicles are formed by the coalescence of smaller ones, and that, eventually, large empty spaces are formed in what was the body of the cell. Also in support of this idea are the very frequent examples of threads of cytoplasm bridging the lumen to partition the vessel, sometimes into three apparently separate compartments (Fig. 4a). These too eventually disappear; no such partitioned vessels are seen in older (2 months or more) myocardium. It is a real possibility that blood flow through the vasculature is a molding force in the final construction of the vascular lumen; it may be this which finally breaks down the intravascular septa of cytoplasm characteristic of these very early vessels.

*Does the Vasculature have a Secretory Role in Neonatal Myocardium?* A secretory function for early vessels is suggested by the abundant rough
endoplasmic reticulum that fills the cytoplasm at 24 and 72 hours of age; Manasek has made a similar interpretation of the role of blood vessels in the chick embryo heart because of their prominent Golgi apparatus and the well-developed rough endoplasmic reticulum that characterizes the endothelial cells (Manasek, 1971). Like the vessels of embryonic myocardium, the lumina of these 24- to 72-hour-old capillaries are filled with a finely granular, moderately electron-dense precipitate which may (as Manasek suggests) represent secretory products of the endothelial cell. Of interest in this regard is that older vessels (2-5 months of age) have empty lumina. Hence, the presence or absence of precipitated material in the vasculature may be purely the consequence of luminal size; the smaller vessels of younger tissue may trap precipitated proteins that are not washed out as the tissue is preserved and processed for examination.

Quantitative Data. There is a discrepancy between the values we obtained for the fractional volumes occupied by the vasculature in dog heart and those Frank and Langer (1974) describe for rabbit myocardium. Their tissue, however, was fixed by vascular perfusion at a pressure of 55-66 mm Hg and a flow rate of 0.75-0.9 ml/min, whereas ours was preserved by immersion in glutaraldehyde. Consequently, the lumina of our vessels were smaller than theirs; this is attested to by the fact that their lumen-endothelial cell ratio was approximately 3, while ours was 1. If we correct for this difference by multiplying our luminal values by 3, our figures correspond very closely to those of Frank and Langer; i.e., 56% of the extracellular space is occupied by vasculature (compared to 59% reported by Frank and Langer), whereas the vasculature makes up 14.4% of whole tissue (14% is the figure Frank and Langer report).

Two important considerations emerge from this comparison of data between Langer's and our laboratories. First, it is very important to consider the method of tissue preservation in analyzing the data obtained by stereological analysis. Moreover, the near-identical values reported for the rabbit and the dog attest to the constancy of mammalian myocardial composition, even between different species, with respect to the vasculature.

The Role of the Myocyte in the Tailoring of the Extracellular Space

As in the embryonic heart, the abundant rough endoplasmic reticulum and prominent Golgi apparatus so obvious in the neonatal myocyte in our study is indirect evidence for ongoing protein synthesis even in the well-differentiated cell of the growing heart—protein that is presumably manufactured for export from the cell as development proceeds. Indeed, Manasek proposes an important and even central role for the myocyte as a source of components for the extracellular compartment as well as for the proteins coating the myocyte which determine the migration of cell populations and the pattern of cell-to-cell adhesion during embryonic myocardial development.

Comparison of Quantitative Data with Those of Other Investigators

If we compare the results of this study to other published quantitative data on myocardial composition (see Table 4), several points bear mentioning. First, the osmolarity and nature of the fixative solutions used vary widely. Much has been made of the necessity for using isosmotic solutions during the processing of tissues for stereological work. In actual practice, the majority of workers do not do this: Frank and Langer's initial fixative, a 2.0% glutaraldehyde solution, has an osmolarity of 424 milliosmoles; subsequent osmolarities of solutions used are not mentioned (Frank and Langer, 1974). Goldstein used a standard 4% paraformaldehyde-5% glutaraldehyde fixative (Goldstein et al., 1974). The osmolarity of such a solution might be expected to be greater than 843 milliosmoles. Even when investigators take the trouble to keep fixative osmolarity at 300-500 milliosmoles, subsequent solutions used are not isosmotic. The assumption implicit in all these instances is that the initial fixative renders the tissue impervious to further osmotic shock; there is good evidence that this is not the case. The data of Krames (Krames and Page, 1968) suggest that, contrary to what might be expected, fixation does not completely destroy the semipermeability of the plasma membrane and that, if fixed tissue is subsequently perfused with solutions containing electrolytes, osmotic gradients can be established by the selective migration of certain ions through the plasma membrane, which will cause H2O shifts across the cell wall, thus altering the volume of intracellular compartments. Eisenberg and Mobley (1975), whose paper refers to most of the available data on changes in cell shape and volume artifactually produced by tissue fixation, have actually outlined the changes in diameter of a single frog muscle fiber that occur during fixation, dehydration, and embedding. Remarkably enough, glutaraldehyde fixation shrinks the fiber volume by 20%, osmium restores it to a near-normal level, and staining en bloc with uranyl acetate causes fiber reshrinkage of 20%. Even more significantly, these workers point out that the osmotic gradients across the membranes of organelles within the cell (such as the sarcoplasmic reticulum or mitochondria), because of differences in the structure of their membranes, may be very different than the gradient across the sarcolemmal membrane. There may be quite different changes in the volumes and shapes of subcellular organelles than those in the volume and shape of the cell as a whole. It is probable that not only the osmolarity but the electrolyte composition of the solutions used in tissue preparation is

[Note: The text continues with further details and scientific analysis.]
relevant in determining the final volume of the organelle, cell, or tissue compartment, as well as in determining the surface-volume ratio.

From the data in Table 4, we can consider the influence on stereological data of the type of initial fixation used. The studies of Laguens (1971) and Reith and Fuchs (1973), both of which report that 87% of mammalian myocardium is made up by the myocytes and 13% is extracellular space, use osmium as the initial and only fixative. This study, and those of Frank (Frank and Langer, 1974) and Lazarus (Lazarus et al., 1976), all report a 21-24% value for the extracellular compartment. All used glutaraldehyde as the primary fixative; yet osmolarities varied from 300 (Lazarus et al., 1976) to 925 (the present study). We, therefore, considered the possibility that it is not osmolarity that is essential here, but the nature of the primary fixative. To confirm this, I have tested glutaraldehyde in phosphate buffer at osmolarities from 1.18% (about 447 milliosmoles) to a 6.25% solution (970 milliosmoles), and did not observe substantial differences in the data obtained from the tissues so prepared. Moreover, the work of Pentilla and colleagues (1974) with Ehrlich ascites tumor cells shows that total fixative osmolarity had "little or no effect with glutaraldehyde or glutaraldehyde-OsO₄." This was not the case when OsO₄ was used alone; then the fixative osmolarity had a marked effect on cell volume. These results support our own findings using glutaraldehyde-OsO₄ (double) fixation, and we have concluded that this technique does not permit significant changes in cell volume to occur when followed by dehydration and Epon embedding in the usual sequence. On the other hand, an earlier study on rats (Legato, 1976) used hearts preserved with a 6.25% glutaraldehyde solution, exactly as in the present study. The values we obtained for within the intracellular compartment were almost exactly what Laguens and Reith reported; the value for sarcomere-myofiber volume was exactly what Page reported (Page et al., 1971). It is more likely that the differences in quantitative data reported in Table 4, therefore, are not due to artifacts of the method but to real species differences: rabbit, dog, and hamster seem different from rats with regard to the relative proportions of intracellular and extracellular compartments in the myocardium and also to myocyte composition.

The sole data mitigating against the conclusion that any variations in the figures given for extracellular-intracellular partitioning of the myocardium are true species differences and not artifact are Polimeni's morphological data (which he confirmed with radioactive isotopic tracer studies). These data show that, in the rat, the extracellular space occupies 19.5 ± 0.3% of myocardium by volume (Polimeni, 1974). Frank and Langer (1974) also measured the extracellular space with radioactive isotopes with various electrical charges, but found they could

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Species</th>
<th>Initial fixative</th>
<th>P_myo/PTT</th>
<th>P_extr/PTT</th>
<th>P_myo/PTT</th>
<th>P_extr/PTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laguens (1971)</td>
<td>Rat, male Adult RV, LV</td>
<td>1% osmium in phosphate buffer: 256 mOsmol</td>
<td>87%</td>
<td>13%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reith (1973)</td>
<td>Rat, male Adult LV</td>
<td>Osmium in collidine buffer</td>
<td>86.8%</td>
<td>13.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frank (1974)</td>
<td>Rabbits, sex? Adult Septum</td>
<td>2% glutaraldehyde in 0.1M Na cacodylate buffer: 424 mOsmol</td>
<td>(75.4%)</td>
<td>24.6%</td>
<td>14%</td>
<td>59.1%</td>
</tr>
<tr>
<td>Polimeni (1974)</td>
<td>Rat, female Adult RV and LV combined</td>
<td>2% glutaraldehyde in cacodylate buffer: &quot;isosmolar&quot;</td>
<td>(80.5%)</td>
<td>19.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Legato (1976)</td>
<td>Rat, sex? 25 days old RV and LV combined</td>
<td>6.25% glutaraldehyde in phosphate buffer: 925 mOsmol</td>
<td>88%</td>
<td>12%</td>
<td>5%</td>
<td>41.2%</td>
</tr>
<tr>
<td>Lazarus (1976)</td>
<td>Syrian hamster LV</td>
<td>1.5% glutaraldehyde in cacodylate buffer: 300 mOsmol</td>
<td>Control Myopathic</td>
<td>(76.1%)</td>
<td>23.9%</td>
<td>(69.6%)</td>
</tr>
<tr>
<td>Legato (present study)</td>
<td>Dog, sex? 5 months old LV, RV</td>
<td>6.25% glutaraldehyde in phosphate buffer: 925 mOsmol</td>
<td>79%</td>
<td>21%</td>
<td>7.2%</td>
<td>28%</td>
</tr>
</tbody>
</table>

This table summarizes quantitative analyses of mammalian myocardial composition from seven studies by six investigators. P = the number of points lying over the contours of the organelle described; myo = myocyte; TT = total tissue; ecs = extracellular space, vas = vasculature.

This study, and those of Frank (Frank and Langer, 1974) and Lazarus (Lazarus et al., 1976), all report a 21-24% value for the extracellular compartment. All used glutaraldehyde as the primary fixative; yet osmolarities varied from 300 (Lazarus et al., 1976) to 925 (the present study). We, therefore, considered the possibility that it is not osmolarity that is essential here, but the nature of the primary fixative. To confirm this, I have tested glutaraldehyde in phosphate buffer as a fixative at osmolarities from 1.18% (about 447 milliosmoles) to a 6.25% solution (970 milliosmoles), and did not observe substantial differences in the data obtained from the tissues so prepared. Moreover, the work of Pentilla and colleagues (1974) with Ehrlich ascites tumor cells shows that total fixative osmolarity had "little or no effect with glutaraldehyde or glutaraldehyde-OsO₄." This was not the case when OsO₄ was used alone; then the fixative osmolarity had a marked effect on cell volume. These results support our own findings using glutaraldehyde-OsO₄ (double) fixation, and we have concluded that this technique does not permit significant changes in cell volume to occur when followed by dehydration and Epon embedding in the usual sequence. On the other hand, an earlier study on rats (Legato, 1976) used hearts preserved with a 6.25% glutaraldehyde solution, exactly as in the present study. The values we obtained for within the intracellular compartment were almost exactly what Laguens and Reith reported; the value for sarcomere-myofiber volume was exactly what Page reported (Page et al., 1971). It is more likely that the differences in quantitative data reported in Table 4, therefore, are not due to artifacts of the method but to real species differences: rabbit, dog, and hamster seem different from rats with regard to the relative proportions of intracellular and extracellular compartments in the myocardium and also to myocyte composition.

The sole data mitigating against the conclusion that any variations in the figures given for extracellular-intracellular partitioning of the myocardium are true species differences and not artifact are Polimeni's morphological data (which he confirmed with radioactive isotopic tracer studies). These data show that, in the rat, the extracellular space occupies 19.5 ± 0.3% of myocardium by volume (Polimeni, 1974). Frank and Langer (1974) also measured the extracellular space with radioactive isotopes with various electrical charges, but found they could
get a quite different value than for their morphological data (35.7% compared to 24.6%).

In any case, although some variations exist in the reported proportions of myocardium occupied by the intra- and extracellular compartment, the breakdown is about 80% intracellular and 20% extracellular space. No significant changes occur in these proportions as growth proceeds, and there is no significant difference between the values obtained for right and left ventricles at any age.

References


Eisenberg BR, Mobley BA: Size changes in single muscle fibers during fixation and embedding. Tissue Cell 7: 383-387, 1975


Goldstein MA, Sordahl L, Schwartz A: Ultrastructural analysis of left ventricular hypertrophy in rabbits. J Mol Cell Cardiol 6: 265-273, 1974


Cellular mechanisms of normal growth in the mammalian heart. I. Qualitative and quantitative features of ventricular architecture in the dog from birth to five months of age.

M J Legato

*Circ Res.* 1979;44:250-262
doi: 10.1161/01.RES.44.2.250

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1979 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/44/2/250

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/