Chapter 19

PRELIMINARY OBSERVATIONS OF CELLULAR REACTIONS FOLLOWING PARTIAL RESECTION OF NEWT CARDIAC VENTRICLE*

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IN 1974 BECKER, CHAPIN, AND SHERRY reported that excision of one-third to one-half of the ventricle of the adult newt, *Triturus viridescens,* was followed by complete healing and regeneration in twenty-four hours. Based upon the following observations, they believed the regenerated myocardium derived from dedifferentiated nucleated red cells of the organism: (1) within a few minutes after ventricular myocardial excision, a blood clot had formed on the cut myocardial surface; (2) by thirty minutes, the clot had differentiated into an inner zone adjacent to the cut surface, and a peripheral outer zone (the inner zone was composed of small intensely basophilic cells derived from the nucleated red cells, while the outer zone was composed of normal appearing erythrocytes with eosinophilic cytoplasm); (3) between three and five hours following surgery, the outer zone red cells were degenerating, while the inner zone basophilic cells were diminishing in number with concomitant appearance of slightly basophilic myocardial fibers, (4) by twenty-four hours, histologic sections showed a reconstituted ventricular myocardium, normal in appearance and with no signs of injury other than an occasional small mass of degenerating peripheral erythrocytes external to the myocardium.

The above results should be considered in light of the work of others (Oberpriller and Oberpriller, 1971, 1974; Bader and Oberpriller, 1978, 1979) in which excision of lesser amounts (one-eighth) of newt ventricular myocardium was not followed by such rapid or extensive regeneration. Oberpriller and Oberpriller (1974) reported that, ten and sixteen days after resection of one-eighth of the ventricle, occasional mitotic figures were seen in muscle trabeculae adjacent to the wound area and that, by twenty days after resection, mitoses were more numerous. The above authors believed that new myocytes might now be present in the area adjacent to the wound.

We felt that an attempt should be made to determine whether or not the rapid and extensive regeneration reported by Becker et al. might be

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confirmed. In this preliminary report we present light and electron microscopic observations on changes in the red cells in the inner zone of the blood clot. A second report dealing primarily with myogenesis is in preparation.

Materials and Methods

Aquatic stage *Notophthalmus* (also known as Triturus) *viridescens* were obtained from the Connecticut Valley Biological Supply Company and maintained in tanks containing tap water. They were fed ground beef or ground liver once a week. Animals were anesthetized by exposure to 1 : 500 Tricaine® (ethyl-*m*-benzoate methane sulfonate) for approximately three to five minutes. Midline incisions were then made respectively through the skin, cartilaginous sterum, and pericardial sac, and the heart exposed and delivered to the exterior. One-third to one-half of the ventricle was excised by means of a cut normal to the long axis of the ventricle. The heart was then returned to the pericardial sac, the edges of the sternum were juxtaposed, and the skin wound only was sutured. Recovery from anesthesia usually occurred within fifteen to twenty minutes. Animals were killed at time intervals to be given. For light microscopy the ventricles were fixed in buffered neutral formalin for several minutes, and then cut in half and maintained in the same fixative. Such specimens were embedded in paraffin, sectioned at 6-7 μm, and stained with hematoxylin-eosin. For electron microscopy, after thirty minutes in 2.5% glutaraldehyde in 0.1*M* cacodylate buffer pH 7.3 (with changes of fresh fixative every ten minutes), tissues were washed in 0.1*M* cacodylate buffer pH 7.3 (brought to 220m osmol with sucrose) for thirty minutes, the last ten minutes of which were at 4°. Tissues were then postfixed for one hour in 1% osmium tetroxide in cacodylate buffer at 4°, and processed for embedding in Vestopal® W or in Epon®. Ultra thin sections were stained with uranyl acetate and lead citrate.

Results

Gross Observations

For reference purposes, Figure 19-1 shows the appearance of the ventricle from an unoperated control animal. The black spots on this heart and those shown in Figures 19-2 and 19-3 are melanocytes. The dotted line indicates the level at which ventricular excisions were made. Within a few minutes after surgery, a large red clot usually formed. If, after fifteen minutes, it was grasped at its periphery and pulled gently, the outer portion of the clot readily detached, leaving an inner portion adherent to the cut myocardial surface. Operated hearts were examined at one, two, three, six, twelve, and twenty-four hours and it was observed that the inner

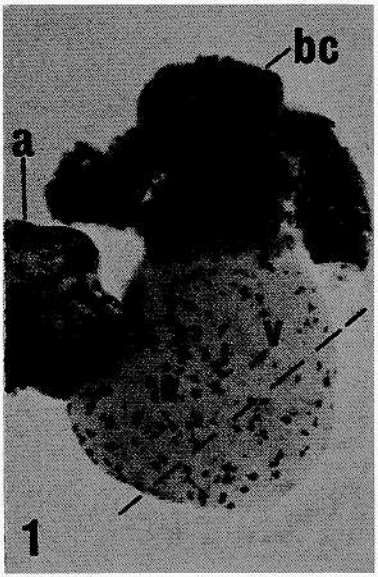

Figure 19-1. A dissected ventricle (v) of the newt *Notophthalmus*. The dotted black line indicates the usual level of experimental excision. The black spots in this figure and in Figures 19-2 and 19-3 are melanocytes. The structures above and to the left of the ventricle are parts of the bulbus cordis *(bc)* and auricles *(a)* (two in number).

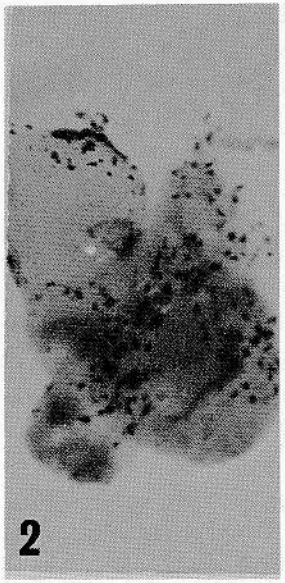

Figure 19-2. Newt ventricle (twenty-four hours postsurgery) showing complete regeneration of excised portion. The amorphous deep grey material within and outside the ventricle is blood. ×6.5.

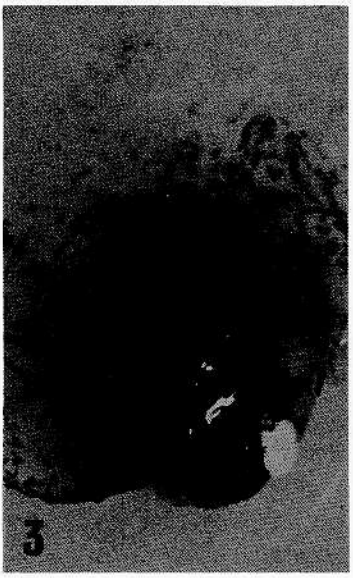

Figure 19-3. Newt ventricle (twenty-four hours postsurgery). An example of incomplete regeneration during the given time interval. This ventricle has been tilted upwards to show the amount of inner clot still remaining. The ventricle is outlined by the dotted line. × 10.

clot decreased or completely disappeared with time elapsed. By twenty-four hours approximately one-third of thirty-two operated hearts had no remaining clot or only extremely small residual clots (Fig. 19-2), and the ventricles appeared completely restored and fully functional. In the remaining animals, varying amounts of inner clot were still evident at twenty-four hours (Fig. 18-3).

Histologic Observations

LIGHT MICROSCOPE OBSERVATIONS: Figure 19-4 shows a cross section of a ventricle three hours after surgery. The mass of very dark cells that forms the wall of the ventricle from the 3 to 7 clock-face positions (arrows) represents the inner clot contoured to form a ventricular continuum, and it is formed of intensely basophilic cells. Figure 19-5 shows a different specimen, three hours postsurgery, in which the basophilic mass of inner clot cells (arrows) is identifiable in the central region of the illustration.

Serial sections showed this blastema-like aggregate to be an extension of inner clot from the cut outer surface of the ventricle into its interior. Such basophilic cell aggregates were also seen on ventricular trabeculae not apparently connected with inner clot, although in the latter instances the aggregates were not as large and extensive as those in Figures 19-4 and 19-5.

In Figure 19-6, resemblance of the upper aggregate (shown in Fig. 19-5)

to a blastema is evident because of its marked cellularity and basophilia. An interesting phenomenon that contributed to formation of the basophilic blastema-like cell mass is indicated in this figure by arrows. It is ejection of nuclei from red cells. This process occurred throughout the inner clot mass and was also observed to occur in free red cells near muscle trabeculae in the wound vicinity (see Fig. 19-8). The intensely basophilic nuclei liberated in this fashion had a thin rim of cytoplasm containing cell organelles, as will be seen later (Fig. 19-14 through 19-18). A second phenomenon, also contributing to formation of basophilic cells of the blastema-like masses, was dedifferentiation of inner clot red cells to form erythroid precursor and erythroblast stages. Such changes may be seen in Figure 19-7 showing (from the lower inner clot mass of Fig. 19-5) red cells in various stages of dedifferentiation. Erythroid precursor cell (epc) types and blast (bl) forms predominate. Criteria for assignment of such changes as being indicative of formation of precursor and blast cell types are taken from the earlier work of Jordan and Speidel (1939), Holtfreter (1947), and Grasso (1973): enlarged and intense basophilia of nuclei; a change from eosinophilic to basophilic cytoplasm; a change from the characteristic disc

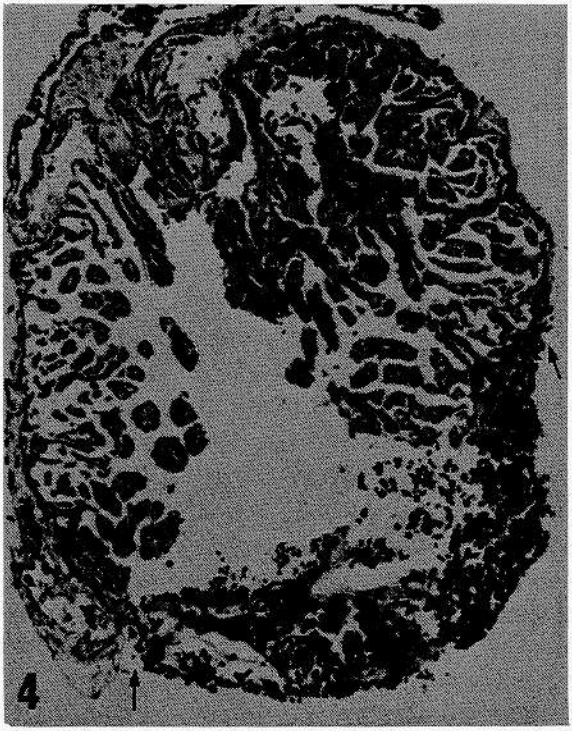

Figure 19-4. Newt ventricle (three hours postsurgery). Cross section showing inner clot (between *arrows*) forming a basophilic, blastema-like cell mass that reestablishes ventricular wall continuity. H&E stain; ×20.

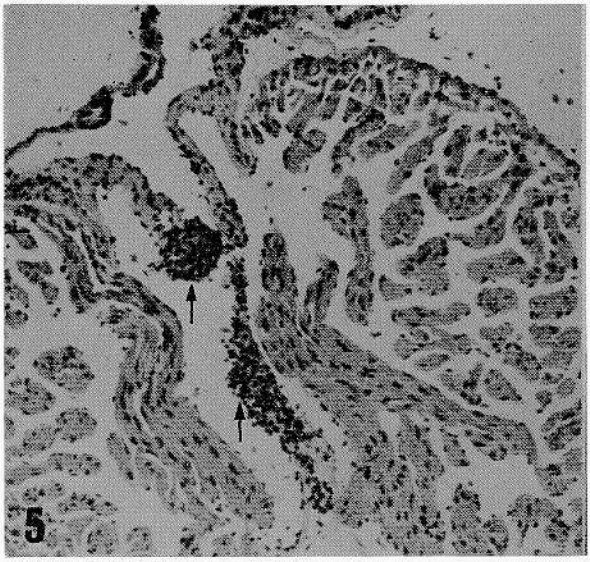

Figure 19-5. Newt ventricle (three hours postsurgery) from a different animal than that shown in Figure 19-4. *Arrows* point to blastema-like inner clot that has extended down from the outer cut surface into the ventricular interior. H&E stain; ×29.

shape of mature red cells to more spherical forms with variation in cell size; intense nucleic acid synthesis and loss of hemoglobin, the latter two phenomena being reflected in the intense basophilia and decreased cytoplasmic eosinophilia of the changing cells. Figures 19-8 and 19-9 show sections of ventricular muscle (m) trabeculae near, but not in direct contact with, the inner clot. In Figure 19-8, arrows point to red cells that have ejected or are ejecting their nuclei. Some liberated nuclei lie free within ventricular space, others are clustered on and others have penetrated into the muscle. A mycocyte nucleus (mn) is readily distinguished from the red cell-derived nuclei by its characteristic elongated shape and lesser basophilia. Under higher power and oil immersion (not shown), the cluster of ejected nuclei on the surface of the trabeculum appeared to be surrounded by a thin cytoplasmic continuum (cc), a portion of which can be seen. At (r) two altering red cells, slightly out of focus in this exposure, are attached to the muscle surface. In Figure 19-9, in addition to ejected red cell nuclei (arrows), there is a dedifferentiating red cell resembling a polychromatophilic erythroblast (pce) attached to the muscle surface. The red cell nucleus, although slightly out of focus, is distinctly enlarged and altered in shape, as is the cell itself, in assuming a more rounded form. The occurrence of melanocytes at and near wound sites was frequently observed, and in this field dendritic melanocyte (md) processes are evident. Muscle (m) trabeculae in Figure 19-9 are lightly stained and not in focus. The attach-

ment interface between red cells and muscle surfaces, viewed by light microscopy, had no unusual features. However, when viewed by electron microscopy, a variety of interesting and unusual features emerged, to be described later (see Fig. 19-11 through 19-18).

Electron Microscopic Observations

The characteristic discoid appearance of the newt mature red cell is illustrated in Figure 19-10, in which portions of four red (r) cells are shown at a ventricular endothelial surface. In such cells, cytoplasm was characteristically homogeneous, with no evidence of cytoplasmic organelles except for an occasional small mitochondrial profile (not shown). Parts of a

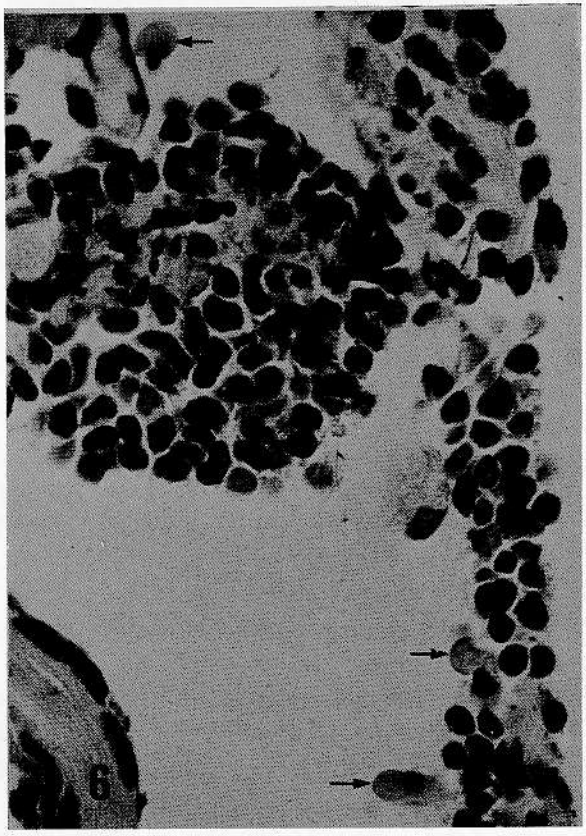

Figure 19-6. Newt ventricle (three hours postsurgery). Detail of blastema-like mass indicated by the upper *arrow* in Figure 19-5. The intensely basophilic cells within the mass are derived from mature red cells entrapped inner clot via two processes: (1) ejection of nuclei *(arrows)* following which the nuclei, surrounded by a thin rim of cytoplasm, assume erythroid precursor and blast forms, and (2) dedifferentiation of mature red cells, with retention of nuclei, to precursor and blast forms. The second process is shown at slightly higher magnification in Figure 19-7.

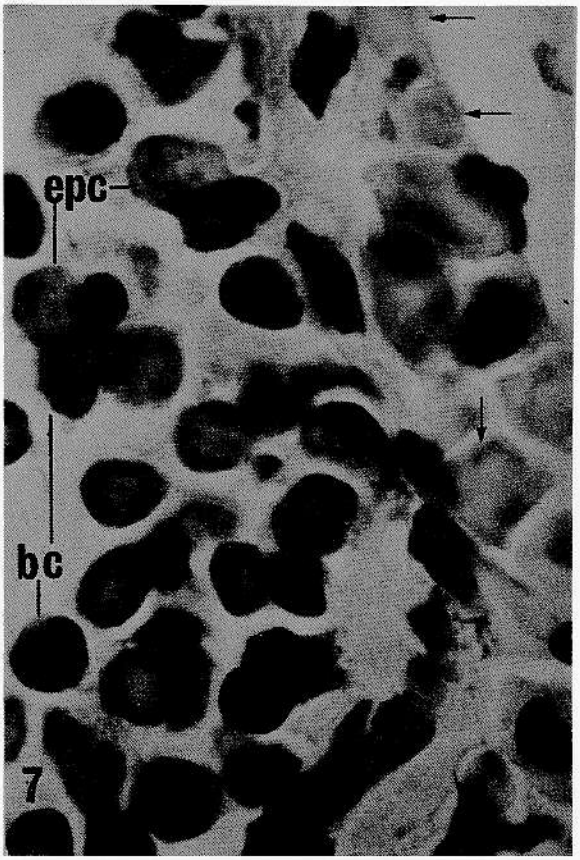

Figure 19-7. Newt ventricle (three hours postsurgery). Detail of blastema-like mass indicated by the lower *arrow* in Figure 19-5. The deeply basophilic cells represent erythroid precursor *(epc)* and blast cell *(bc)* forms. Also present are cytoplasmic remains of red cells that have ejected nuclei *(arrows)*. H&E stain; ×540.

thromboblast (t), a melanocyte (mc), and an endothelial (e) cell are also present in this field.

In Figure 19-11, wound edge myocardium and an adherent inner clot red (r) cell are shown two hours after surgery. The red cell, undergoing dedifferentiation, has assumed an amoeboid form characteristic of erythroblast stages of amphibian red cells (Holtfreter, 1947) and has extended two pseudopodia. One is applied to, the other directed towards, the wound surface. The outermost component of the wound surface is a narrow cell process (cp) whose origin is not detectable. At the interface between red cell and wound surface, tight junctions (tj) are evident. The altering red cell cytoplasm now contains cytoplasmic channels (cc), vesicles (v), and mitochondrial profiles (mi), and its nuclear outline is irregular and indented. The ventricular wound edge shows the profiles of three myocar-

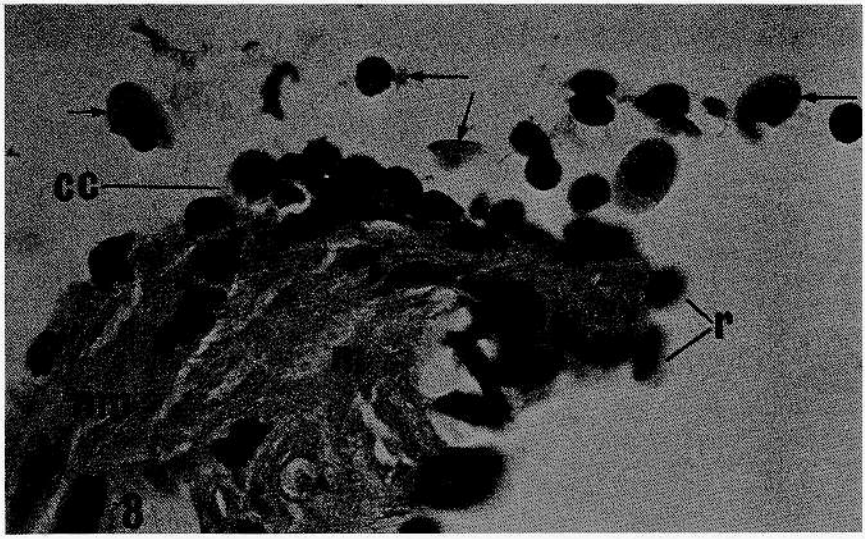

Figure 19-8. Newt ventricle (three hours postsurgery). A muscle trabeculum *(m)* is shown, not part of the wound surface, with ejected red cell nuclei clustered on and also within the trabeculum. At *(cc)*, part of what appears to be a cytoplasmic continuum when viewed under oil immersion is seen. At *(r)* two intact dedifferentiating red cells are present. *Arrows* point to mature red cells that have ejected or are in the process of ejecting their nuclei. A myocyte nucleus *(mn)* is also present in this field. H&E stain; ×400.

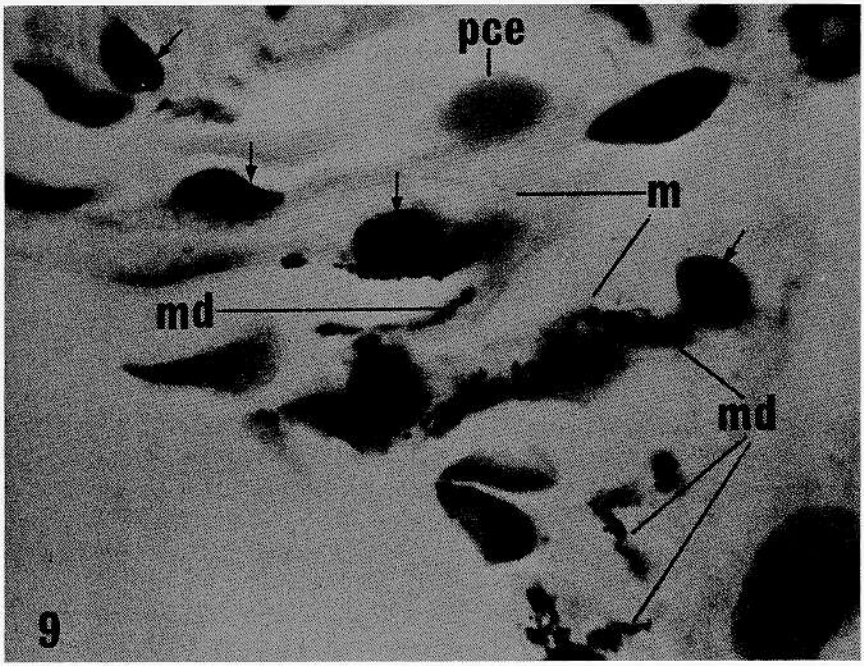

Figure 19-9. Newt ventricle (three hours postsurgery). There is part of a muscle trabeculum *(m)* lightly stained and slightly out of focus. Within and on this trabeculum, there are ejected red cell nuclei *(arrows)*. A dedifferentiating red cell resembling a polychromatophilic erythroblast is shown at *(pce)*. Dendritic processes from melanocytes in the vicinity *(md)* are also seen. ×640.

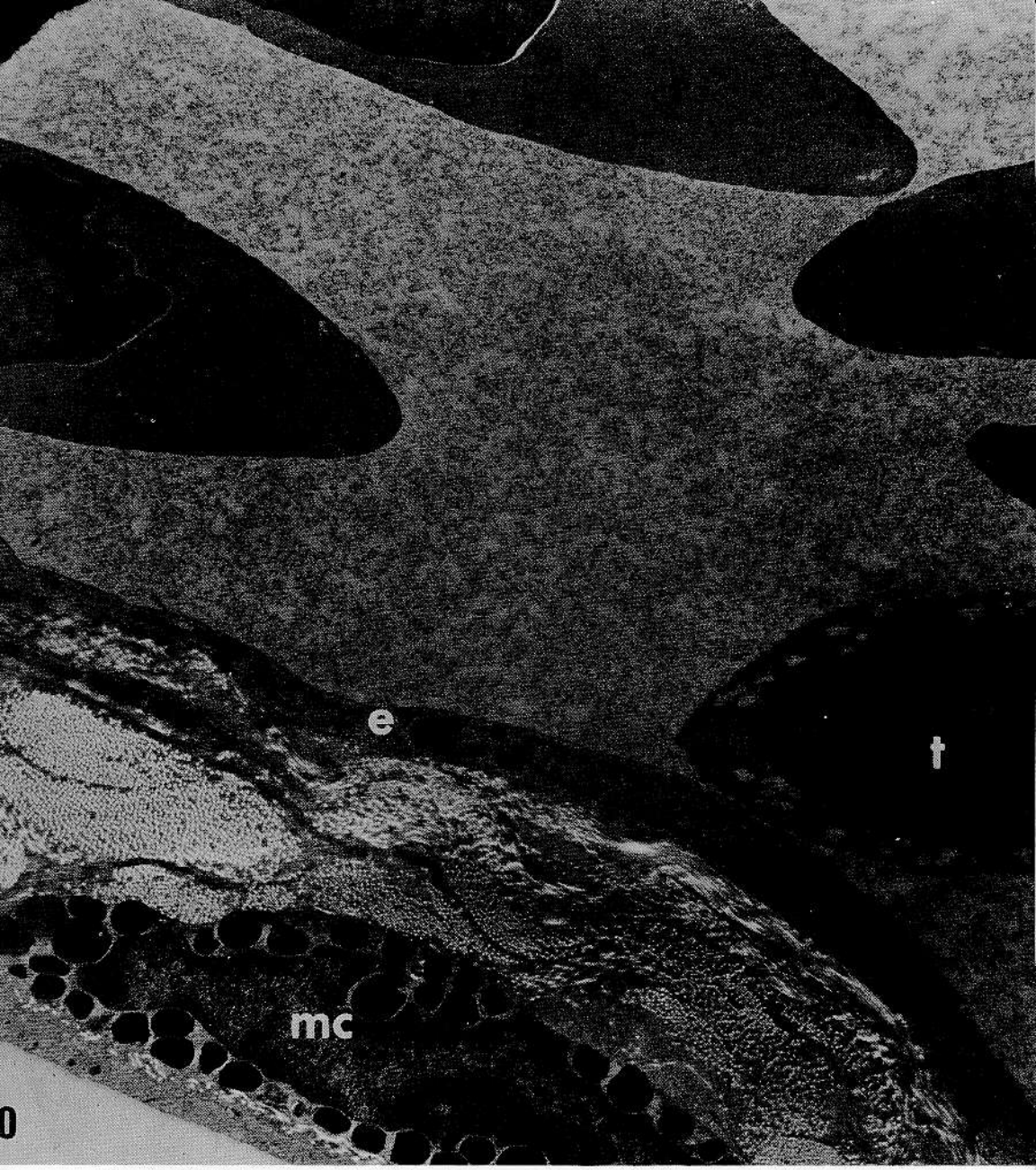

Figure 19-10. Newt ventricle. The characteristic discoid appearance of the mature nucleated red cell (*r*) is shown. The cytoplasm appears relatively homogeneous and lacks organelles. The nuclear outline is relatively smooth. A thromboblast (*t*), endothelial cell (*e*) and melanocyte (*mc*) are also present. ×8,000.

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Figure 19-11. Newt ventricle wound edge (2 hours postsurgery). A dedifferentiating red cell (*r*) has assumed an amoeboid form and is adhering to the wound surface where parts of three myocytes (m_1-m_3) are present. Tight junctions (*tj*) are present in the interface between red cell and wound surface, whose outermost layer is a cell process the origin of which cannot be determined from this section. Mitochondrial profiles (*mi*), vesicles (*v*), and channels (*cc*) are now evident within the altering red cell cytoplasm, and the nuclear outline has become irregular and indented. The myocytes are each surrounded by a distinct basal

lamina (*bl*). They contain bundles of myofilaments (*my*) and electron-dense bodies (*eb*). A desmosome-like structure (*d*) is present between cells m_1 and m_2, and there is material resembling z-band (*z*) in cell m_2. ×14,000.

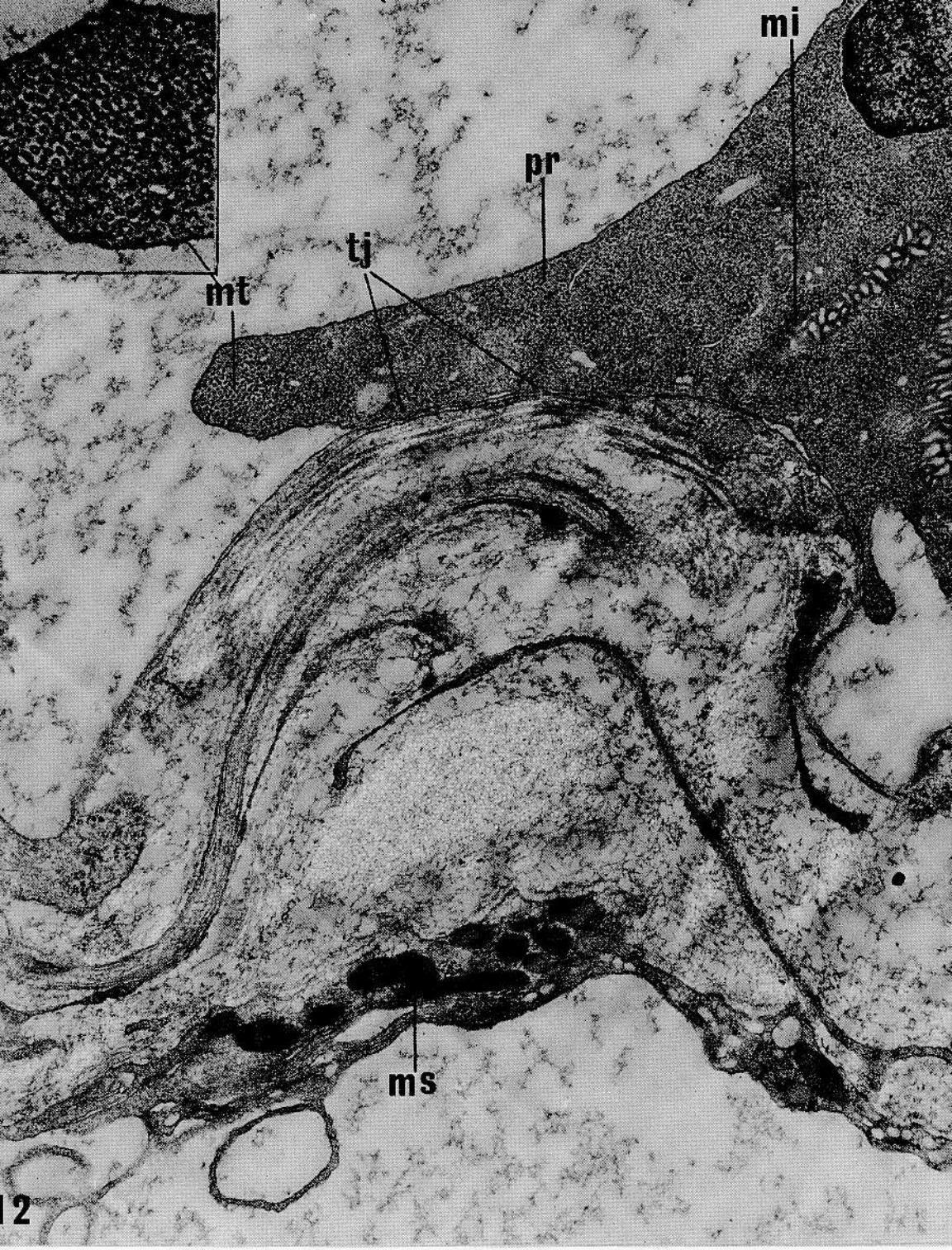

Figure 19-12. Newt ventricle wound edge (three hours postsurgery). A dedifferentiating amoeboid red cell attached to wound surface contains enlarged mitochondrial profiles (*mi*) and a profusion of randomly dispersed polyribosomes (*pr*). At the interface between the amoeboid cell and wound surface cell processes, there are tight junctions (*tj*). Microtubules (*mt*) of the marginal band system, although present but not detectable in the mature red cell, have been unmasked and are now detectable as a result of the decrease in hemoglobin

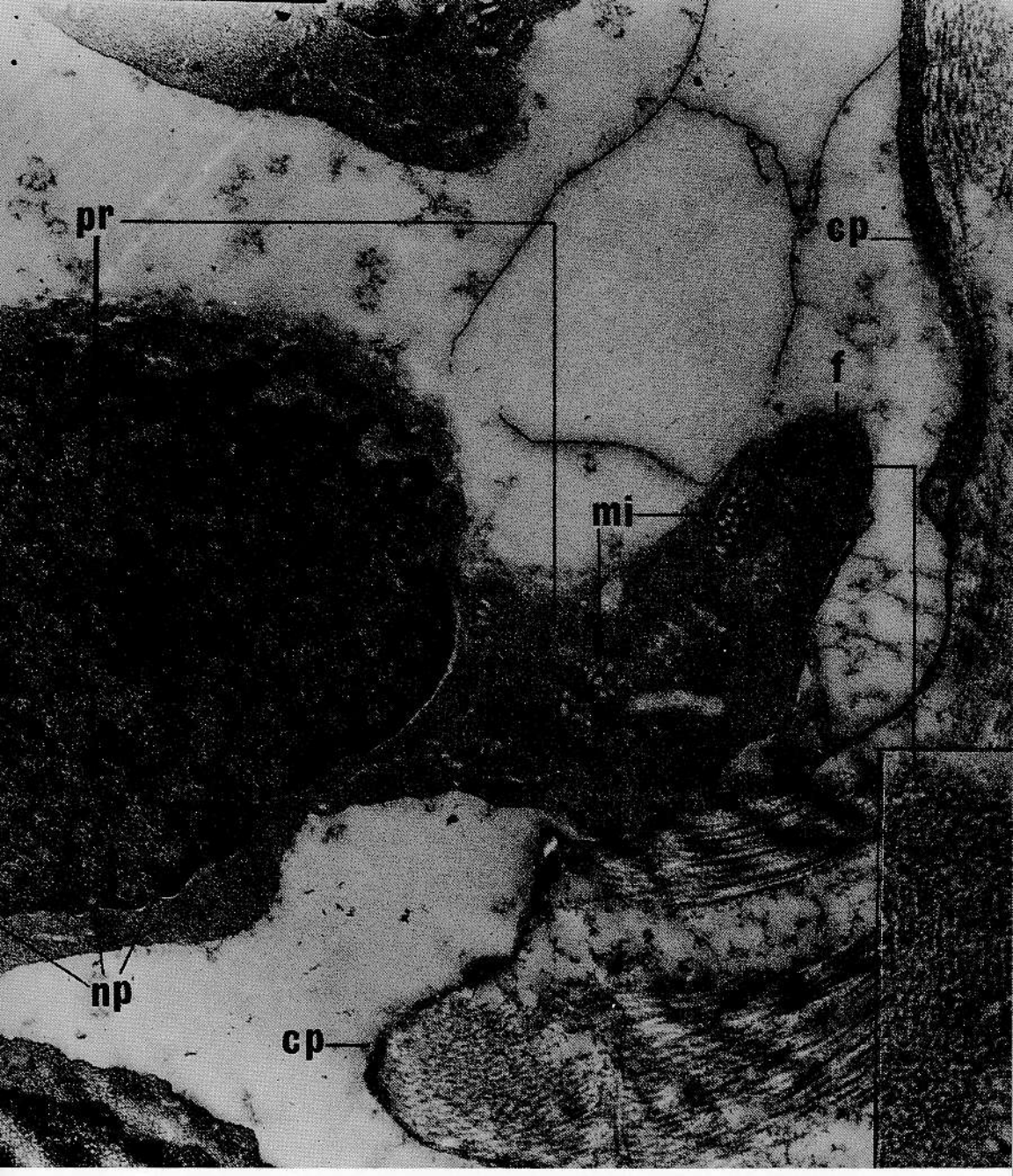

Figure 19-13. Newt ventricle wound edge (three hours postsurgery). Another altering amoeboid red cell adjacent to a wound surface covered by cell processes (*cp*) is shown. The amoeboid cell cytoplasm contains prominent mitochondrial profiles (*mi*) and is full of randomly dispersed polyribosomes (*pr*). There are also linear aggregates (f) that are not microtubules (see insert). The nuclear surface has many irregularities, and the interchromatin regions are filled with randomly distributed polyribosomes (*pr*). Nuclear pores (*np*) are also present. ×25,650; insert ×59,000.

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concentration of this dedifferentiating red cell. *Insert:* higher magnification of microtubules of marginal band system. Melanosomes (*ms*) from a nearby melanocyte are present in the lower portion of the picture. ×25,650; insert ×59,000.

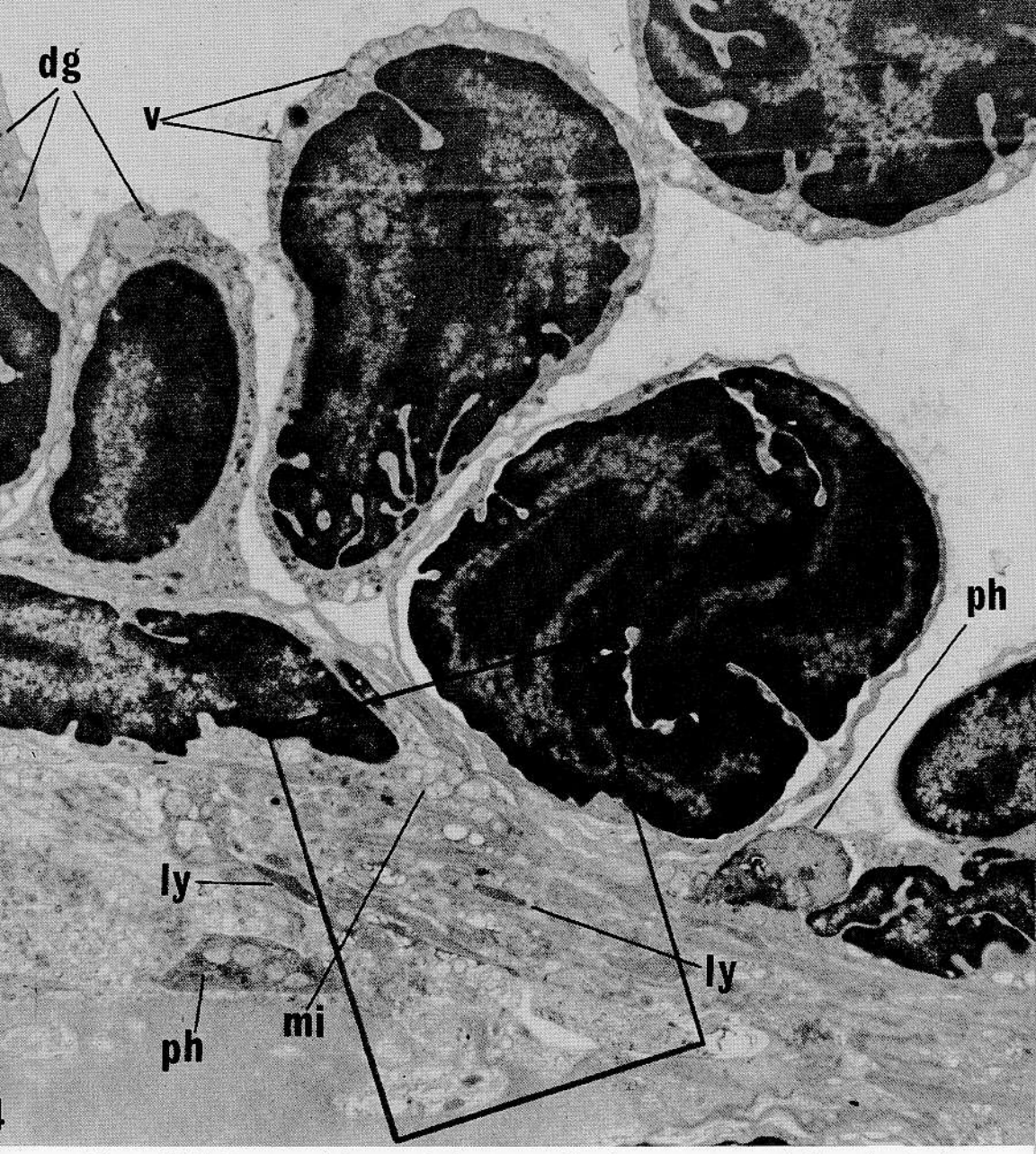

Figure 19-14. Newt ventricle wound edge (twenty-four hours postsurgery). Ejected red cell nuclei surrounded by variable thicknesses of cytoplasm are aggregated on a cut myocardial surface. Cytoplasmic contents include mitochondria (*mi*), dense granules (*dg*), and vesicles (*v*). The underlying myocardium contains phagosome-like (*ph*) and lysosome-like (*ly*) bodies. ×8,000.

dial cells (m_1, m_2, m_3), each surrounded by a distinct basal lamina (bl). The cytoplasm of these cells contains bundles of myofilaments (my), mitochondria (mi), electron-dense bodies (eb), and segments of z-band-like material (z). A desmosome-like structure (d) is present between cells m_1 and m_2.

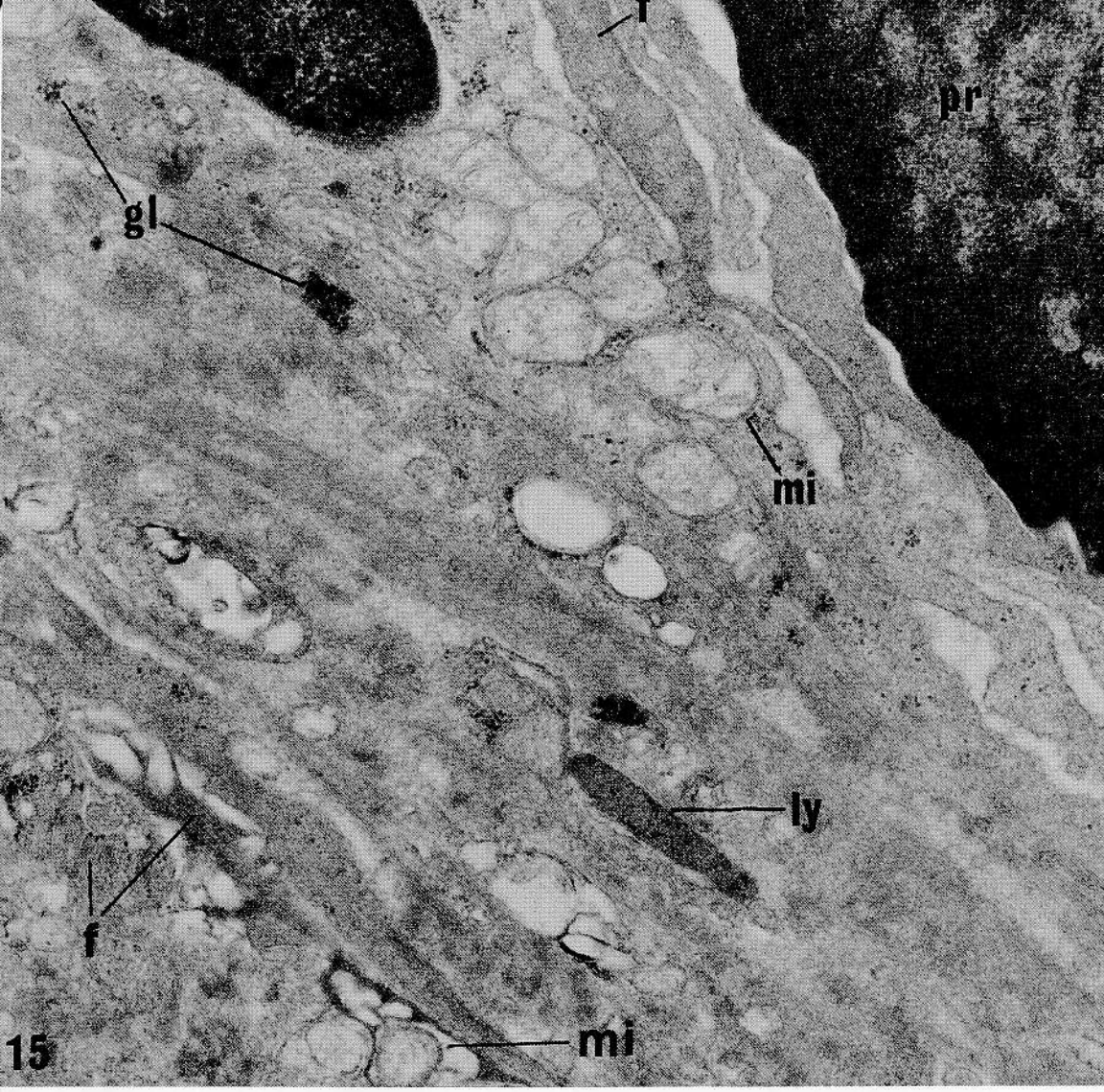

Figure 19-15. Higher magnification (×31,250) of rectangular-marked area in Figure 19-14. The interchromatin regions of the ejected nuclei are filled with polyribosomes (*pr*). Their cytoplasm contains mitochondria (*mi*), glycogen (*gl*), and linear aggregates (f). The underlying myocardium is a dense meshwork of interpenetrating degenerating and synthetic elements such as lysosomes (*ly*), glycogen (*gl*), and filamentous linear aggregates (f).

Oberpriller and Oberpriller (1974) reported desmosomes between new myocytes with tritiated thymidine-labeled nuclei and nonlabeled myocytes twenty days after newt ventricular excision.

Figures 19-12 and 19-13 (three hours postsurgery) also show amoeboid red cells on wound surfaces. There are increases in size and number of mitochondrial profiles (mi), changes in nuclear outline and the appearance of nuclear pores (np, Fig. 19-13). Cytoplasm and interchromatin regions are filled with polyribosomes (pr). At the wound surface the

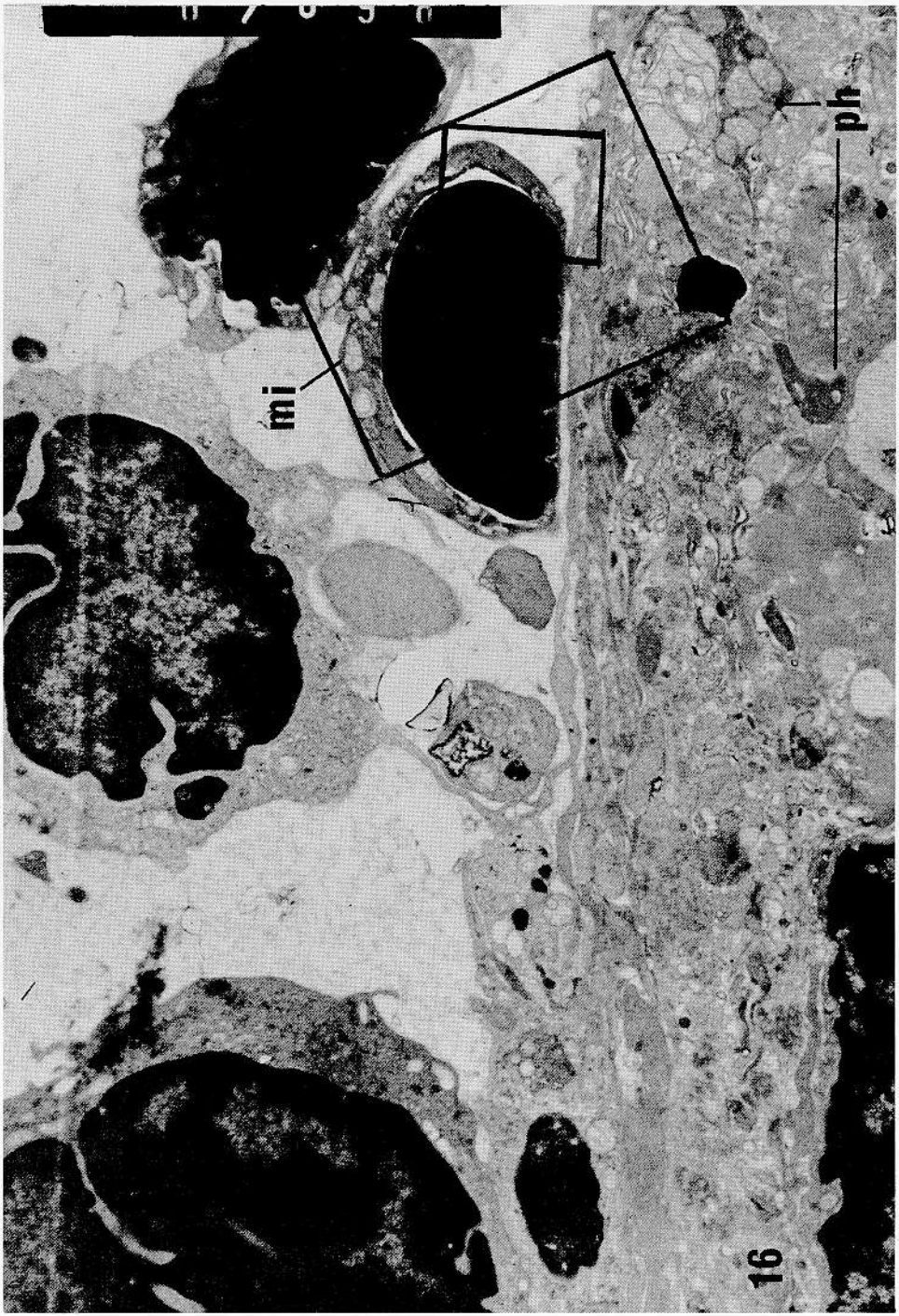

Figure 19-16. Newt ventricle wound surface (twenty-four hours postsurgery). An area adjacent to that shown in Figure 19-14. A dedifferentiating red cell that has retained its nucleus is in the center of the illustration and two ejected nuclei to the right. Evidence of side-by-side synthetic and phagocytic activity similar to that seen in Figures 19-14 and 19-15 are also present here: mitochondria (*mi*), phagosome (*ph*). ×8,000.

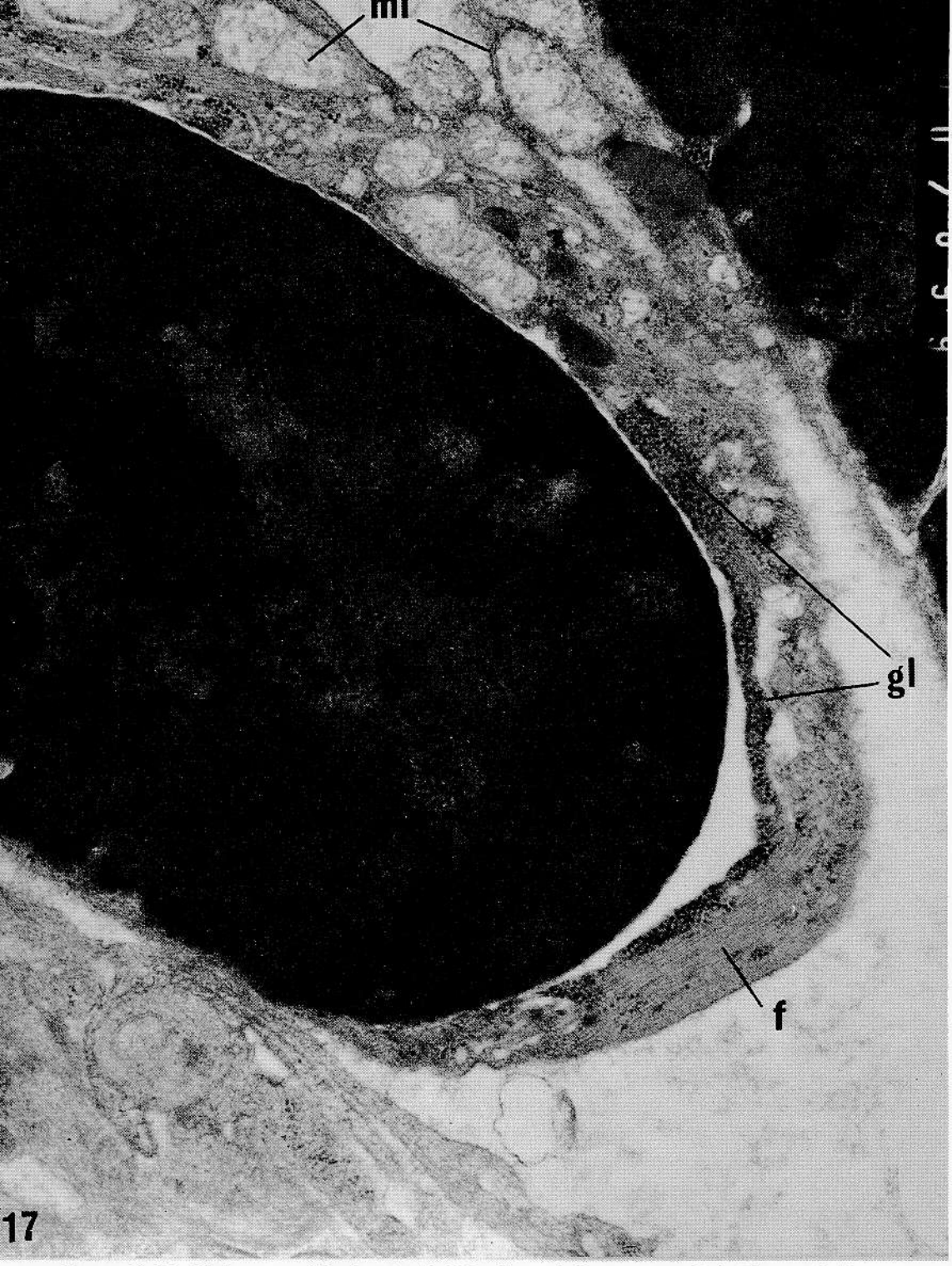

Figure 19-17. Higher magnification (×40,000) of area in large rectangle in Figure 19-16, showing presence of mitochondria (*mi*), glycogen (*gl*), and filamentous linear aggregates (f) in cytoplasm surrounding ejected red cell nucleus.

interface between the red cells and cut myocardium has tight junctions (tj, Fig. 19-12) between dedifferentiating red cell outer membrane and outer membrane of the narrow processes from other cells in the vicinity. The insert in Figure 19-12 shows, at higher magnification, microtubules (mt) of the marginal band apparatus of the red cell. Microtubules, although present, are not detectable in electronmicrographs of mature red cells because the dense hemoglobin stain masks them. However, when cytoplasmic hemoglobin content decreases (in this case as part of the dedifferentiation process) the marginal band apparatus is unmasked and becomes visible in the electronmicrograph (Cohen, 1978). In Figure 19-13, the insert shows at higher magnification linear filamentous cytoplasmic aggregates, not microtubules (f).

Figure 19-14 (twenty-four hour post-surgery) shows a field in which ejected red cell nuclei are aggregated on a cut myocardial surface. Surrounding cytoplasm of varying thickness contains mitochondria (mi), dense granules (dg), and vesicles (v). (In the light microscope, at three hours post surgery, the cytoplasmic surround of the ejected nuclei was barely visible.) The cut myocardial substrate upon which the dedifferentiating red cells rest contains phagosome-like (ph) bodies and electron-dense bodies resembling lysosomes (ly). At higher magnification (Fig. 19-15) mitochondrial profiles (mi) within a dedifferentiating red cell are visible, as are abundant nuclear polyribosomes (pr). Beneath the changing red cells is a dense meshwork of interpenetrating cell processes and cell debris and residues. Within this meshwork are lysosome-like (ly) bodies, mitochondria (mi), glycogen (gl), and filamentous structures (f). It is apparent that structures serving phagocytic, other catabolic, and also anabolic processes are present in this field.

Figure 19-16 depicts an area adjacent and similar to that shown in Figure 19-14. Two ejected red cell nuclei surrounded by cytoplasm are on the right side of the illustration, and a dedifferentiating red cell that has retained its nucleus is slightly above and to the left of them. From the cell on the left, processes extend to the myocardial surface. Evidences of phagocytic activity similar to those encountered in Figures 19-14 and 19-15 are present. In addition, however, and shown in Figures 19-17 and 19-18 (which are higher magnifications of the rectangular marked areas in Figure 19-16), the cytoplasm surrounding one of the ejected nuclei contains linear aggregates ranging between 100 and 120 A in diameter and resembling intermediate filaments (f) in appearance. Again, as in Figures 19-14 and 19-15, there is evidence of simultaneous phagocytic, other catabolic, and synthetic activity occurring side by side.

Discussion

The sequence of events following excision of one-third to one-half of the adult newt myocardium involves dramatic changes in the nucleated red

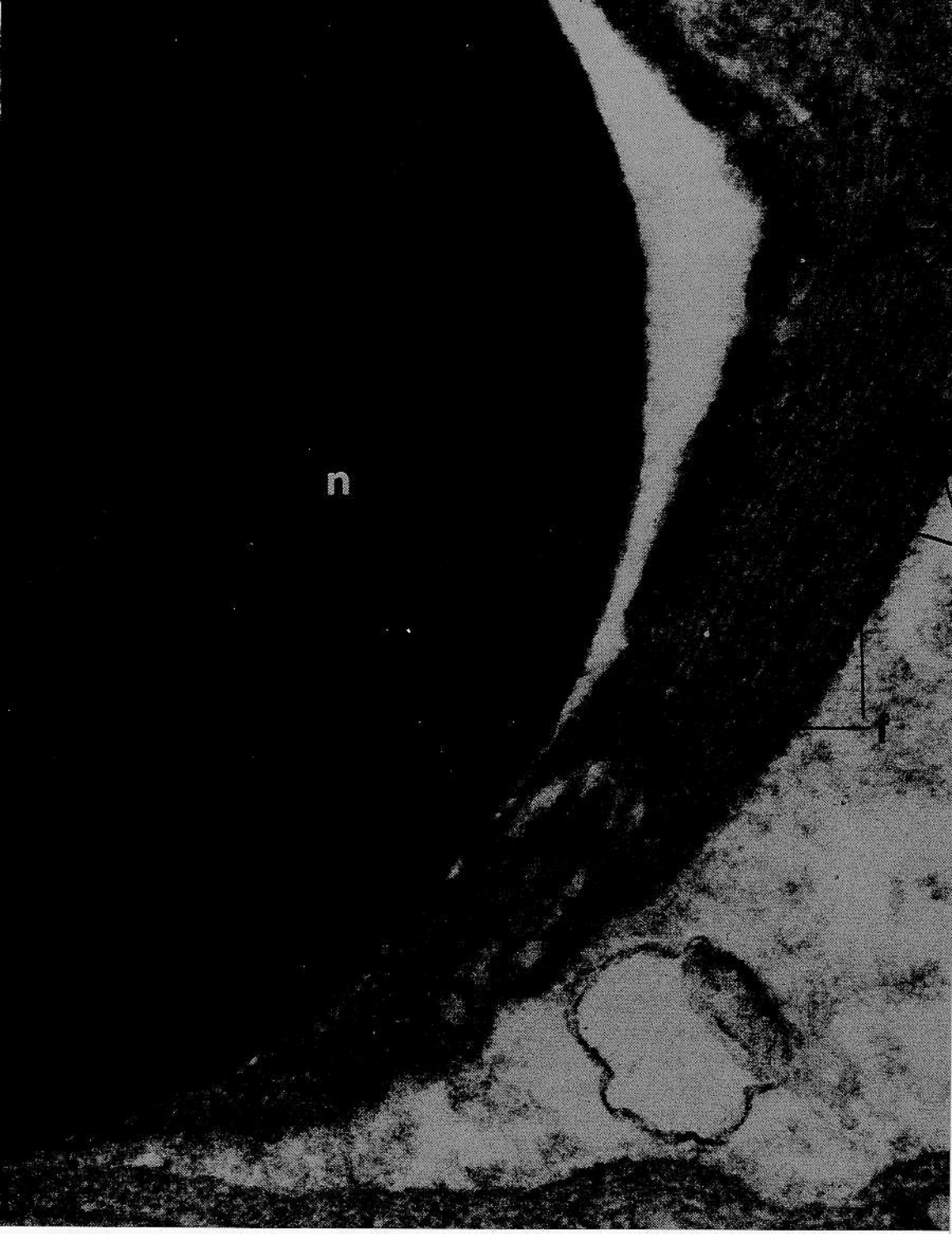

Figure 19-18. Higher magnification (×80,000) of area in small rectangle in Figure 19-16, showing nucleus (*n*), cytoplasmic filamentous aggregates (f), and glycogen (*gl*).

cells enmeshed in the inner zone of the blood clot formed on the cut myocardial surface. These changes result in the dedifferentiation of the red cells to erythroid precursor and erythroblast forms, accomplished in two ways: (1) within the inner clot, red cell nuclei and cytoplasm assume the morphologic and staining properties of the dedifferentiated states mentioned above and (2) within the inner clot, but also external to it, other red cells eject their nuclei, and the latter surrounded by a thin rim of cytoplasm also assume the characteristic appearance of the less differentiated red cell developmental stages.

The cut myocardial surface meanwhile exhibits characteristic degenerative changes, which, however, occur side by side with active synthetic and anabolic processes. Within two to three hours, the cut myocardial surface becomes covered with cell processes to which the dedifferentiating red cells attach. Among the cells involved at this interface are dedifferentiated red cell forms mentioned above, melanocytes, phagocytes, young myocytes, and other cells not yet identified. The cytoplasm of the young myocytes contains, among other organelles, mitochondria, bundles of myofilaments, and z-band-like material. Twenty-four hours after surgery (possibly earlier) the cytoplasm surrounding ejected nuclei also contains abundant mitochondria, glycogen, and filamentous structures. The nuclei and cytoplasm of the dedifferentiating red cells also contain an abundant population of randomly distributed polyribosomes.

Thus, in regard to the report by Becker, Chapin, and Sherry (1974) we have found that the newt heart does indeed regenerate within twenty-four hours (in one-third of thirty-two animals) and that the nucleated red cells of the animals dedifferentiate as described. In regard to the latter process, we may recall that Holtfreter (1947) was able to produce similar changes in amphibian red cells in vitro within 105-135 seconds, by lowering the tonicity, by raising the pH, or by changes in ion content of suspending solutions. Becker and Murray (1967) and Pilla (1974) have produced similar dedifferentiations by electrical means. The question of the origin of the young myocytes is one we cannot answer as yet. Studies of this problem are currently in progress in our laboratory.

Concerning the observations of Oberpriller and his co-workers, the apparent discrepancies between their results and those of Becker, Chapin, and Sherry and ourselves must stand for the present. Attempts to explain or reconcile these differences in the regenerative response of newt ventricle must take into account, initially, a difference in experimental design. The Oberpriller group removed only one-eighth of the ventricle as compared with removal of from one-third to one-half by the Becker group and ourselves. Perhaps the more extensive removal of ventricular tissue serves as a stronger stimulus for regeneration.

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