ULTRASTRUCTURE OF DEVELOPING MYOCARDIUM OF RAT EMBRYOS AND CYTOCHEMICAL LOCALIZATION OF NUCLEOSIDE PHOSPHATASE (ATPase) ACTIVITY

by

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To
The Memory of My Parents

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INTRODUCTION

The morphogenesis and cyto-differentiation of striated muscle has long been an area of active investigation. The process of differentiation of myoblasts to form functional striated muscle involves the early synthesis, spatial arrangement and ordering of essentially two types of myofilaments into myofibrils. This aspect of the cytodifferentiation of muscle has received a great deal of attention in recent years. Van Breeman (1952), Hay (1963), Dessouky and Hibbs (1965), Allen and Pepe (1965), and Przybylski and Blumberg (1966), among others, have described the cytology of skeletal muscle myoblasts in much detail using both light and electron microscopes. These investigators and others have established, to some extent, the salient aspects of early cytological events in the cytodifferentiation of striated muscle. Fischman (1967) more recently has described myogenesis in the chick embryo, with particular emphasis on fibrillogenesis, and Firket (1967) and Shimada et al. (1967) have carried out similar ultrastructural studies on cultured myoblasts of the chick. It should be pointed out here that most of these studies were done on tissues where fibrillogenesis has already taken place or is in an advanced stage of development.

Many of these reports regarding the development of myofibrils still appear to be contradictory and controversial. For example, many possible sequences of the formation of the thin and thick myofilaments have been reported. Allen and Pepe (1965) suggested that the thin myofilaments (actin) develop before thick myofilaments in chick skeletal muscle cells. Hay (1961), at first, described that thick myofilaments appeared before thin myofilaments in the development of myotomes of Ambystoma opacum larvae; but, subsequently, she (1963) supported the idea that the thin and thick filaments were formed simultaneously, as was suggested previously by Ferris (1959), in the development of chick skeletal muscle, and by Bergman (1962) in rat skeletal muscle. Suggestions regarding the appearance of

cross-striations of myofibrils have been in a similar state of controversy. Hibbs (1956) and Hay (1963) reported that A and I bands appear before 'Z'-lines in chick heart muscle and salamander tail, respectively. On the contrary, Siro and Ikemoto (1957) reported the appearance of 'Z'-lines before A and I bands in the earthworm muscle. Meyer and Queiroga (1959) also stated that no myofilaments were seen before the presence of Z-lines in the development of chick heart muscle in tissue culture. Schulze (1962) disagreed with both of these possibilities. He reported that 'A' and 'I' bands as well as 'Z'-lines were formed simultaneously.

The development of the myocardium has been extensively investigated with light microscopic techniques; however, relatively few electron microscopic investigations of the very early development of this tissue have been reported, (Huang, 1967; Meyer and Queiroga, 1959; Schulze, 1962). Most of these investigators were concerned with non-mammalian hearts and the embryonic mammalian heart has not been extensively studied.

Ultrastructural development of the contractile materials of cardiac muscle would not be expected to differ substantially from that of skeletal muscle. Nonetheless, the morphogenesis, histogenesis and cytogenesis of cardiac muscle present a number of fundamental differences, since cardiac muscle myoblasts do not fuse to form multi-nucleated myotubes, but give rise to differentiated cellular units held together by areas of cell-to-cell contact. These areas are said to be specialized for transmission of mechanical tension or propagation of electrical activity. Some of these special features of the ultrastructure of developing cardiac muscle were described by Hibbs (1956), Muir (1957), Wainrach and Sotelo (1961), Challice and Edwards (1961), Olivo et al. (1964) and Cedergren and Harrary (1964). Until recently, however, methods for preparation of embryonic tissue for light and electron microscopy presented several difficulties

and resulted in both distortion and loss of many fine details of the early stages of cardiac muscle differentiation.

The purpose of the present investigation is to:

1) take advantage of significant recent improvements in fixation and preparation of early embryonic tissues for ultrastructural and cytochemical studies,
2) to examine the early ultrastructural differentiation of ventricular myocardium of rat embryos of day 10, 11, 12, 13 and 14 and to compare the same with the myocardium of the newborn, and
3) to examine the cytochemical distribution of nucleoside phosphatases (ATPase enzymes) in this tissue, utilizing the substrate adenosine triphosphate.

LITERATURE REVIEW

I. Historical Perspective

Early embryologists and histologists have investigated extensively the gross morphology, histology and development of embryonic and adult hearts. Their contributions to our present knowledge of the anatomy of heart are many; however, the limitations of techniques and methods employed by them have led to inaccurate and erroneous interpretations of several details of cardiac histogenesis. Several of these will be considered in the paragraphs to follow.

There are many excellent reviews available concerning the morphogenesis of the tubular heart and its subsequent formation into an adult structure. The reader is referred to early works by Mall (1911, 1912) and Davis (1927) on the human heart and to that of Yoshinaga (1921) on mammalian hearts with special reference to guinea pigs. Attention should also be called to reviews by Patten (1960), and Dehaan (1961), on the development of vertebrate hearts. These investigators and others have provided extensive accounts of the various stages of the development of vertebrate hearts. There seems to be little or no controversy in the descriptive accounts concerned with the external form and shape, as well as the development, of the embryonic and fetal heart.

II. Histology

A shroud of controversy and confusion, however, surrounds the histogenesis of cardiac tissue. Almost all of the early histologists expressed individual interpretations of cardiac development. The main points of their observations may be summarized as follows.

The myocardium was thought to be composed of striated, seemingly syncytial muscle cells supported by connective tissue containing a capillary network. The muscle fibers of the heart consisted of cells containing, on or near the surface, parallel, longitudinally coursing myofibrils that apparently passed through

several nuclear fields. Each myofibril exhibited transverse bands ("disks") that differed in refractivity and staining properties. An anisotropic, dark-staining "Q" band (A band) alternated with an isotropic colorless "J" band (I band) of much the same width. The alignment of A and I bands with corresponding bands in contiguous myofibrils imparted a cross-striated appearance to each cell. Equidistant from either end of every I band was the dark, narrow "Z" band, which traversed not only the myofibrils but also the sarcoplasm between them and, thus, united the myofibrils. A distinguishing feature of cardiac muscle was the presence of the so-called intercalated discs, which were said to be short bands, scattered at intervals. These discs were transverse to the myofibrils and they seemed to gather the myofibrils together tightly.

III. Origin, Differentiation and Structure of Myofibrils

The origin and significance of myofibrils is a subject which has perplexed histologists and resulted in conflicting theories regarding the genesis of this cell organelle.

According to Kurkiewiez (1910), Bruno (1918), Lewis (1922) and Olivo (1925), isolated myofibrils began to appear at the 9-10 somite stage in chick embryos. Lewis (1919, 1926), however, questioned the very existence of myofibrils in living cells. He reported that they were fixation artifacts. Renyi and Hogue (1938) reported that the effect of fixation would probably bring the individual fibrils closer together. These authors also suggested that myofibrils represented only a highly specialized form of the contractile substance of the cell.

Many other investigators held the view that myofibrils originated from mitochondria, other intracellular granules, or were formed under the influence of mitochondria. Schockaert (1909) proposed that small mitochondrial granules aligned themselves in rows and became transformed into homogeneous filaments which then underwent elongation and, finally, differentiated as striated fibrils.

Meves (1908) and Duesberg (1910) postulated that fibrils evolved from chondriosomes, filamentous bodies homologous to mitochondria. According to Bruno (1918), these filaments were abundant in the chick heart until the 15-somite stage, but disappeared by the 21-somite stage, when myofibrils became fairly numerous. The appearance of striated myofibrils coincident with the disappearance of mitochondrial filaments was also reported by Stilwell (1938). From observations of heart muscle differentiation in culture, Stilwell concluded that mitochondria were present in the non-differentiated myocardial cell in the form of both granules and short rods, and that it was the latter which gave rise to the filaments, hence to the myofibrils. It is also of interest to note that Levi (1919, 1923) and Olivo (1923, 1925, 1926, 1929) reported that the cells proliferated by cultured explants of the chick embryo heart eventually dedifferentiated, or returned to an indifferent state, and that during the course of dedifferentiation, the myofibrils were replaced by long filaments which in turn gave way to short rods.

Other workers, although not admitting a direct transformation of mitochondria into myofibrils, believed that the mitochondria played some part in myofibril formation. Regaud (1909, 1911) stated that mitochondria initiated the production of myofibrils from other cytoplasmic components. Cowdry (1918, 1926) postulated that differentiation occurred as a result of surface film reactions taking place at the interface between the mitochondria and the cytoplasm. Goss (1933), on the basis of his studies on cultured explants from 16-day rat embryo hearts, maintained that mitochondria were involved in the production of A bands.

A few other investigators, who did not limit themselves to mitochondrial staining techniques, found small cytoplasmic granules lined up in rows parallel to the long axis of the cell, and believed that these granules fused to form myofibrils (Godlewski, 1902; McGill, 1907; Weed, 1936). Heidenhain (1911) was unable to see a transformation of any pre-existing cytoplasmic elements into

myofibrils, but observed fine longitudinal filaments, which he postulated were primitive myofibrils, forming from submicroscopic elements, "the smallest living particles."

Lewis (1926) and Stilwell (1938), on the other hand, reported that tension and mechanical stress favored the formation of fibrils in living cultures of chick embryo hearts, and, hence, by inference, in vivo also. However, when myocardial cells containing a few fibrils were stretched with microneedles for 5 to 8 minutes, additional fibrils did not form (Renyi and Hogue, 1938).

MacCallum (1897), who studied the origin of heart muscle fibers in pigs, reported the presence of spindle-shaped cells composed of nuclei and fibrils which were divided into discs in cross sections of 10mm. embryos. He described five developmental stages. At the 13 to 15mm. stage, there were occasional fibrils scattered through the cells. By the time the 20mm. stage was reached, most of the cells contained them, and at the 35mm. stage all cells had the fibrils. At 55mm., when the cells were elongated, in cross sections, they assumed the appearance of adult fibrils. By the 72mm. stage, the general form of adult structure was attained. The fully differentiated cells were to be found predominantly at the periphery of the heart muscle mass, and the less differentiated cells close to the interior.

Witte (1919) corroborated the description of MacCallum. In her view, spindle cells appeared in embryos of 25mm. At 38mm. cross-striations were to be seen at the periphery, and at 76mm. no longer were there spindle cells, but a definitely developed striated syncytium, formed by the fusion of the spindle cells. At the point of fusion or union there were lines of demarcation, and these were considered to be protoplasmic bridges between cells.

In young beef hearts, Jordan and Banks (1917) described a process similar to that seen by MacCallum in pigs. At three months, post-partum, the ventricular muscle passed through a stage in which the cellular elements closely

resembled smooth muscle, in that they were closely packed, slender and fusiform. In earlier stages cardiac muscle, as well as smooth muscle, was made up of stellate and irregular cells which anastomosed to form a syncytium. According to these authors, smooth muscle, cardiac and "striped" (striated) muscle all passed through similar stages in the course of development.

In the opinion of Godlewski (1902), who proposed a different theory, the fibers in the heart developed granules which arranged themselves into rows and then fused to form homogeneous fibrils, later acquiring striations. Fibrils increased by longitudinal division.

Marceau (1904) gave yet another account. In his description, fibrils appeared first, then differentiated into granules which were primarily single, but later formed pairs. The paired granules were equivalent to what became in the adult a single Q disc (A band).

According to Heidenhain (1911), heart muscle fibrils were laid down as ultra-microscopic elements (metafibrils), which he called protomeres. These gradually increased in diameter until the fibril attained microscopic visibility and were then called myofibrils, having a transverse dimension of 0.2 micron. Groups of myofibrils formed muscle bundles and these in turn formed fibers. The fibers increased in number by division at the myofibril stage. Heidenhain proposed no theory regarding the mechanism of formation of "metafibrils." The original threads from which protomeres developed, in his view, as in that of Meves, might have been present in the fertilized ovum. The multiplication of fibers provided for lateral growth.

It had been frequently found by many early histologists that cell boundaries disappeared during the period when myofibrils began to differentiate. Many authors have noted that the cells of the epimyocardial mantle in the chick embryo were fused into a syncytium at a very early period (Rouget, 1863 ; Wagener, 1872;

Schlater, 1906; Kurkiewiez, 1910; Duesberg, 1910; Congdon, 1918; Levi, 1919; Lewis, 1919). Heidenhain (1899) depicted the myocardium of the 3-day duck (Anas platyrhynchos) embryo heart as a syncytium traversed by long fibrils. On the other hand, some observers have reported that the embryonic chick heart muscle was truly syncytial (Wieman, 1907; Schockaert, 1909; Lewis, 1926). The appearance and independent contractions of embryonic myocardial cells in culture led Lewis (1926) to the conclusion that the chick's cardiac muscle was a reticulum of anastomosed cells rather than a syncytium. Schockaert (1909) reported that cellular individuality was masked at the 4 to 6-day stage by the continuity of fibrils from cell to cell but became apparent during mitosis when the cell membrane was clearly visible.

IV. Structure of Myocardium During Onset of Contraction

Information is sketchy in older literature regarding the onset of contraction in relation to the structure of myocardium at that stage. Cohn (1932) quoted Heidenhain to refer to the works of Eckhard (1866) and Kurkiewiez (1909), who both reported that the heart began to beat before fibrils were visible in chick embryos. Marceau (1903) reported a similar situation in blind worm. Heidenhain (1899), on the other hand, claimed to have observed both homogeneous and striated fibrils in 3-day old duck embryos.

Burrows (1912) was the first to show that isolated cardiac cells could beat in a culture medium. Lake (1916) and Shipley (1916) confirmed these observations. M.R. Lewis (1920) reported that at the onset of contraction, no characteristic structure was to be found in the cells, but that the tissue exhibited a high degree of refraction. Based on investigations utilizing cultured cardiac tissue, W. H. Lewis (1923) reported that individual cells did pulsate in tissue culture. These cells migrated and formed a reticulum, but not a syncytium. Myofibrils were not seen in the living cells, but they could be detected after fixation. Some cells

contained striated fibrils and others contained homogeneous fibrils. Both types of cells contracted rhythmically. The fibrils were not seen to extend from cell to cell. According to this author, in living animals, both embryonic and adult, fibrils were probably not to be found, and the living heart muscle was probably composed of individual cells without fibrils.

Among the more recent observations on living myocardial cells were those of Rumery and Blandau (1961), who reported that, within 48 hours after culturing chick embryo hearts, striated myofibrils could be seen. These myofibrils were located along the cell borders in areas of greatest tension. Although non-striated myofibrils were visible in these cells, only those with bands were contracting. These workers concluded from this observation that the formation of striations in myofibrils preceeded contractions. They further stated that well-differentiated living myofibrils were similar in appearance to those which have been described in well-fixed and stained preparations in that they possessed well-delineated A bands with Hensen's disks in the center, and I bands with prominent Z bands. The myofibrils appeared to increase in width by a process of gradual fusion of differentiated myofibrils lying in the same plane. The length of the myofibrils varied, sometimes spanning the length of a cell, and in other instances coursing through several connected cells.

V. Structure and Origin of Cross-Striations

There is almost as much disagreement in the literature on the origin and time of appearance of the striations as there is on the origin of the myofibrils. The following paragraphs contain a summary or brief description of the light microscopy findings of early investigators. Details of ultrastructural studies will be considered elsewhere.

The various theories which have arisen could be divided very roughly into three categories: 1) the striations were formed in the cytoplasm before the

appearance of myofibrils (Luna, 1913; Cameron, 1917; Lewis, 1919; Naville, 1922); 2) the myofibrils and striations were formed concurrently, striations being an integral part of the myofibrils (MacCallum, 1898; Wieman, 1907); and, 3) the myofibril was first formed as a homogeneous structure, and the striations differentiated later (Bardeen, 1900; Asai, 1914; Schmidt, 1927; Weed, 1936).

Bowman (1840) was the first to publish significant observations on this subject. He reported that muscle was joined together in discs and that there was an end-to-end fibrillary union. Dobie (1849) gave a fair description of the Z line and at the same time reported structures which were later referred to as Q and J discs. Krause (1868) described the Z line and laid the foundation for a systematic study of muscle fibrils on the basis of compartments. Merkel (1872) and Hensen (1868) both found a line traversing the Q disc, but Merkel's chief contribution was his investigations of morphologic alterations in striated fibers associated with contraction. These changes in fiber morphology were considered to be related to movement of anisotropic substances. Engelman later (1873), in addition to adopting the idea of the partition of fibers into compartments by the Z line (intermediate discs or "Zwischenscheiben"), saw that the compartment was made up of layers arranged longitudinally, each having a different refractive power. He (1878) likewise studied the changes which took place during the course of contraction.

The next advance was made by Retzius (1881) when she demonstrated the existence of the reticular nature of certain components of muscle when treated with gold chloride. Rollet's work (1885, 1886) called attention to the existence of fibrils in muscle, which, according to him, represented the contractile substance. Since then, there had been a division of opinion among histologists; there were those who regarded the fibrils as homogeneous and supported by an intracellular reticulum, the appearance of cross-striations being formed by the

arrangement of this reticulum; those who regarded cross-striations as a property of the fibrils themselves; and, those who, like the first, believed the muscle fibrils to be homogeneous and the granules, closely applied to the fibrils, to have contributed to the cross-striated appearance. Among those who subscribed to the idea that the granules contributed to the formation of striations was Holmgren (1907, 1910). As to the reticulum, authors' interpretations differed as to its precise nature. Some, like MacDougall (1897) reported that the fibrils were traversed by membranes at the levels of the Z and M lines, while others like Marcus (1925) viewed these lines as bands which surrounded the fibrillary substance.

Many authors stated that the A and I bands were the first to become visible, but the time of their appearance has been placed at various stages and times in chick embryos: e.g., 36 to 41 hours or 10 somites (Rouget, 1863; Kurkiewiez, 1910; Bruno, 1910); 4 to 6 days (Schockaert, 1909); 5.5 to 6 days (Wieman, 1907); and, 7 days (Schlater, 1906). Stilwell (1938) noted the initial development of the Z band at the 8-day stage. Heidenhain (1899), however, had claimed to have seen the Z band, as well as the A and I bands, in the heart of a 3-day duck embryo, Anas platyrhynchos. The presence of these three bands in the myocardium of the 60-hour chick embryo was observed by Duesberg (1910), who remarked that the A bands appeared first as swellings spaced regularly along homogeneous fibrils, and that the Z bands developed almost simultaneously in the spaces midway between the A bands. Lewis (1919) reported to have seen A, I and Z bands (but no fibrils) in both fixed and living cardiac tissue from 10-somite chick embryos.

It has also been proposed that the axes of myofibrils arose from a cytoplasmic reticulum (Weiman, 1907), or from a system of intersecting planes demarcating hexahedral compartments in the cytoplasm (Congdon, 1918).

The cross-striations were supposedly produced by the deposition of mitochondrial or other granules at the point of intersection of the meshes or planes.

Baud and Haenni (1952) claimed that the first indication of transverse striations in chick embryo appeared at the 12-somite stage.

VI. Intercalated Disc

Early histologists pointed out the intercalated disc as one of the chief distinguishing characteristics of heart muscle, the other being, according to them, the syncytial nature of myocardium. Eberth (1866), to whom the first detailed description of the intercalated disc is usually attributed, regarded them as intercellular structures, both because of their histologic appearance and because of their strong reaction to silver nitrate. Cohn (1909) observed that they were not to be found in fetal hearts or in the hearts of children. He stated that the intercalated discs were composed of parallel rods separated by a light staining substance and that they were continuous with the fibrils.

Schweigger-Seidel (1870), Eberth (1866), Zimmerman (1910), Von Palczewska (1910) and Werner (1910) all believed that intercalated discs represented true cell boundaries. Many of their contemporaries did not, however, subscribe to this idea. Hoche (1897) conceded that they were indeed present in living cells and not necessarily fixation artifacts. He mentioned, nonetheless, that they were not complete barriers and that sarcoplasm was able to pass through them from one cell to the next one. Marceau (1904) observed their absence in newborn and stated that they developed, with advancing years, from I discs. He, as well as Renaut and Mollard (1905), reported that intercalated discs were of a tendinous nature. Dietrich (1910) also concurred with Marceau in that intercalated discs developed late in histogenesis, that is to say, it was an adult structure.

Hoche believed that the function of intercalated discs was to equalize

uneven contraction in the heart in the sense that active and non-active portions might be seen to be separated by them. Heidenhain, on the other hand, believed they were growth centers.

There is substantial disagreement among early workers concerning the time of appearance of intercalated discs. Witte (1919) saw them in the myocardium of fetal pigs as early as 89mm. stage. Jordan and Steel (1912) reported their presence in young beef hearts four months after birth and stated that they were numerous at seven months. They also observed intercalated discs in guinea pigs during the last week of gestation. Jordan and Banks (1917) in a later study, pointed out that the intercalated discs made their appearance in young beef hearts during the second to third month after birth. According to them, at two months post-partum, the cells were plain and fusiform. These cells began to coalesce and the intercalated discs arose at the lines of fusion and assumed a position at right angles to them. At these locations a realignment of myofibrils took place and, according to them, "the stresses involved may effect the multiplication of myofibrils which constitute the intercalated discs."

One can find many diverging theories in the literature concerning the origin of intercalated discs. Briefly, however, it is sufficient to say that most of the early histologists subscribed to the view that intercalated discs were structures which were dependent for their development on mechanical stress and strain to which the organ was subject.

VII. Ultrastructural Review

The light microscope has been, as seen in the previous pages, the main research tool of early histologists for the study of cardiac muscle. With the advent of the electron microscope, many workers began to use this tool for the investigation of myocardial structure. Most of these investigators used adult tissue for this purpose (Beams et al, 1949; Van Breeman, 1953; Weinstein,

1954; Sjostrand and Anderson et al, 1954, 1958; Hodge et al, 1954; Price et al, 1955; Kisch, 1954, 1956; Muir, 1957; Moore and Ruska, 1957; Porter and Palade, 1957; Edwards and Chalice, 1958; Fawcett, 1958; and others).

The embryonic heart, however, has received very little or no attention from these and other electron microscopists. The apparent reason for this lack of activity in this field was due to and continues to be the obvious technical difficulties and time consuming procedures associated with the collection, preparation and fixation of embryonic tissues for ultrastructural studies. An intensive search of the literature revealed less than a dozen papers dealing with the fine structure of embryonic myocardium. Of these, one was concerned with the mammalian embryonic heart (Muir, 1957), another with heart of frog embryo (Huang, 1967), and the remainder with chick embryo hearts (Hibbs, 1956; Lindner, 1957; Lanzavecchia, 1957; Wainrach and Sotelo, 1961; Manasek, 1969). Morever, each of these studies was oriented towards the investigation or elucidation of one specific aspect of the development of embryonic cardiac tissue. No exhaustive study encompassing different developmental stages of embryonic heart is to be found in the literature. It is not possible, based on these studies, to make meaningful or coherent interpretations of the fine structure of the embryonic myocardium and much remains to be elucidated.

Hibbs (1956) was among the first to apply the electron microscope to the study of embryonic hearts. He examined the hearts of chick embryos and observed that myofibril bundles could be detected after 36 hours of incubation and that the Z bands appeared soon thereafter. He believed that the "A substance" was laid down only after 60 hours incubation. At 72 hours of incubation, striated myofibrillar substance of adult appearance was to be found.

Lindner (1957) studied hearts of chick embryos of 40 hour stage and observed that myoblasts were loosely arranged with large intercellular spaces.

He reported that mitochondria lacked well-developed cristae and that myoblasts at this stage possessed a large amount of endoplasmic reticulum.

Muir (1957) was the only one, as far as can be ascertained from the literature, to study a mammalian embryonic heart with electron microscope. His investigation was solely concerned with the development of intercalated discs in embryonic rabbit hearts. In a very short paper, he reported the early existence of intercalated discs "wherever a myofibrillar axis crosses a cellular boundary."

Lanzavecchia (1957), who studied the fine structure of hearts of 72 to 120 hour chick embryos remarked that the sarcoplasmic reticulum was not yet fully developed at this period.

Wainrach and Sotelo (1961), reporting on their studies of embryonic chick hearts, observed that some myofibrillar bundles seemed to arise from Z bands and belonged to a thicker bundle. They did not find relationship between desmosomes and intercalated discs, nor between desmosomes and myofilaments.

Huang (1967) examined hearts of frog embryos (Rana pipiens) and reported that the formation of thin and thick filaments preceded that of Z lines.

Manasek (1969) investigated embryonic chick myocardium and observed the presence of large amounts of free ribosomes and particulate glycogen in embryonic myocardial cells. He also pointed out the extensive granular reticulum in these cells.

VIII. Cytochemical Review

The literature abounds with reports by biochemists, chemical embryologists and similar researchers regarding various enzymes, their distribution and existence in both embryonic and adult tissues and organs. Many of these enzymes have been implicated in the differentiation and functional development of these tissues and organs. Enzyme patterns may suggest where critical changes are occuring in the vital activities of the developing organs and tissues, and by

pursuing such leads it may be possible to increase the knowledge and understanding of the physiology of the growing embryos and developmental processes. Since the developing organ reveals the transition from non-functional to functional states, such studies may also contribute to the understanding of the relation between enzyme action and tissue function in adult life. From the excellent works of early biochemists and chemical embryologists, it can be concluded with a reasonable amount of confidence that in those instances where an enzyme is known to be involved in the function of an adult organ or tissue, then the differentiation of that enzyme is also related to or associated with the differentiation of function.

Several biochemical studies can be cited from the literature to show the correlation between enzyme differentiation and organ function. Each enzyme tends to concentrate preferentially in some tissues or organs.

Even at two days, in chick embryos, tissues differed markedly in their alkaline and acid glycero-phosphatase activity (Moog, 1944) and at three days, the epithelium was substantially richer in dipeptidase than was the surrounding mesenchyme (Palmer and Levy, 1940). A striking non-uniformity of the distribution of alkaline phosphatase in chick embryos was pointed out by Moog (1944). Up to the middle of second day, all ectodermal and mesodermal materials were moderately rich in phosphatase, with the endoderm somewhat less so. The myocardium was alkaline phosphatase negative as soon as it differentiated; and, the collecting ducts of mesonephros, the liver and skeletal musculature also became negative by the end of the first week. During the same period, however, the endothelium, the brush borders of kidney tubules, the sites of bone formation and other loci became far richer in the enzyme than undifferentiated tissue. Acid phosphatase showed a similar pattern of changing distribution with continuing development (Moog, 1944).

The increase or decrease in phosphatase activity was related to the

functional activity of the differentiated organ. In sites where the enzyme was known to be concentrated in mature tissue, it became concentrated just as function began. Conversely, in tissues from which the enzyme was absent, or almost so, in mature stages, the enzyme disappeared as soon as functional differentiation occurred. Furthermore, where tissues remained in a primitive state of differentiation, the level of phosphatase activity also remained unchanged. The mesonephros provided a good example for these situations. During the first half of the fifth day, when tubular secretion was just beginning (Gersh, 1937), the enzyme became highly concentrated in the newly differentiated brush borders and, then suddenly, fell to a very low level in the cells of the tubular walls. The undifferentiated mesonephros tissue, meanwhile, retained the moderate level of phosphatase activity characteristic of the whole organ at earlier stages.

Similar data are also available to show the relationship between enzyme activity and differentiation in brain, skeletal muscle, liver and many other tissues in chick embryos. In the above organs, Moog (1947) investigated the distribution of ATPase (apyrase), and Nachmansohn (1939) investigated the activity of cholinesterase. The pattern of differentiation of adenyl pyrophosphatase, the ATP-splitting enzyme, was peculiar to each of the three organs. Nachmansohn and Machado (1943) suggested that the role of ATPase in the brain might be that ATP served as an energy-donor in the acetylation of choline. In the spinal cord, cholinesterase was shown to undergo a five-fold increase in the period between five to ten days of incubation when reflex activity and spontaneous muscular movements were said to be appearing (Wenger, 1951). In the skeletal muscle, where ATPase is known to be an important functional component, the enzyme reached its maximum level on the day when the muscles were called upon to work in the hatching process (Moog, 1947).

In 1939, Engelhardt and Ljubimowa made the important observation that

there was a very close association between ATPase and myosin, based on their studies of adult tissues, and this report has been repeatedly confirmed. Subsequently, several workers have undertaken biochemical studies of ATPase activity in embryonic tissues, namely the chick embryo. They have reported that ATPase activity in the chick increased before the contractile proteins increased in amount (Moog, 1944, 1947; Csapo and Herrmann, 1951; Robinson, 1952; De Villafranca, 1954). Similarly, Herrmann and Nicholas (1948) demonstrated that in rat, there was an increase in ATPase activity prior to a significant change in the contractile proteins.

From these and many other similar biochemical studies it is now established that there is a direct and definite correlation between the enzyme, ATPase, and muscle differentiation, as well as muscle activity, in both the embryo and adult. It is evident, however, that all these studies were based on biochemical techniques, and were primarily concerned with the total amount of enzyme in the whole tissue or organ. Practically no attempt was made to localize these enzymes in a tissue or on a cellular level. It is more recently that newly available techniques of ultrastructural localization of enzymes were applied to the study of enzyme activities in muscle tissues. It is not surprising that in most experiments the investigators chose adult tissue for this purpose instead of embryonic tissue with its inherent problems of preparation and fixation (Maruyama, 1954; Tice and Barrnett, 1962; Engel, 1963; Essner and Novikoff et al, 1963, 1965; Tice and Smith, 1965; and others). The literature, as far as it can be determined, is devoid of cytochemical studies of nucleoside phosphatases (ATPase) in either embryonic heart or developing skeletal muscle. In view of this dearth of information, as well as the fact that there is a definite correlation between the enzyme ATPase, protein synthesis and muscle function, it was considered desirable to investigate the cytochemical localization of nucleoside phosphatases (ATPase) in the developing cardiac tissue concomitant with the proposed ultrastructural studies.

MATERIALS AND METHODS

I. Animals and Mating Procedure

Rats of the Sprague-Dawley strain, kept in an inverted light cycle room and fed Purina rat chow and water, ad libitum, were used for this investigation. Vaginal smears were taken regularly, and animals in proestrous and early estrous were weighed and allowed to mate with healthy, experienced males. The females were examined every two hours for the presence of sperm or vaginal plug. By this method, the time of mating could be determined within 1 hour. The day sperm or a vaginal plug was detected was counted as day zero and twenty-four hours after the detection of sperm or vaginal plug was counted as day one. Day one in this method is comparable to day two in practice followed by some investigators. After mating, the animals were weighed periodically to check for an increase in weight and the progress of pregnancy.

II. Preparation of Tissues

Pregnant rats were anesthetized by intra-peritoneal injection of Sodium Nembutal (50 mg/Kgm body weight) and were autopsied on days 10, 11, 11 1/2, 12, 13, 14 and on the last day of gestation. Each conceptus was surgically removed starting from the anterior end of each uterine horn, i.e., the conceptus closest to the ovary was removed first. Care was taken in this procedure to see that there was a minimum amount of bleeding so that the rest of the conceptuses were left undisturbed as far as possible. After rinsing briefly in ice-cold normal physiological saline and in 0.1M sodium cacodylate containing 0.22M sucrose (the buffer) to wash off tissue fluids and blood, the isolated conceptus was put in the fixative (glutaraldehyde) and further dissection was carried out in it. This procedure made it possible for the fixative to come in direct and immediate contact with the embryo proper as soon as it was exposed. The heart

was dissected loose from the embryo while it was still in the fixative. In those cases where the tissues were to be fixed directly in Osmium without pre-fixation in glutaraldehyde, the embryos were first dissected out in the buffer and immediately transferred to the fixative.

III. Fixation Procedure

A. Ultrastructural Studies

1. Pre-fixation in Glutaraldehyde

Tissues were fixed in 3.25% glutaraldehyde (Ladd Research Industries, Inc.) buffered to pH 7.4 with 0.1M sodium cacodylate buffer for 60-90 minutes at 3-4° C. (The solutions were iced.). After fixation, to remove excess glutaraldehyde, the tissues were routinely washed in several changes of ice-cold 0.1M sodium cacodylate containing 0.22M sucrose.

2. Post-fixation in Osmium Tetroxide

Following rinsing and washing in the buffer, tissues were post-fixed for one hour in ice-cold 1% Osmium tetroxide buffered to pH 7.4 with 0.1M sodium cacodylate containing 0.22M sucrose. After post-fixation in Osmium for one hour, tissues were dehydrated and embedded in Epon as described elsewhere in this section.

3. Direct Fixation in Osmium

Following the excision of the embryo from the isolated conceptus in the buffer, it was transferred to another vial of buffer. The heart was then dissected out and immediately transferred to ice-cold 1% Osmium tetroxide solution buffered to pH 7.4 with 0.1M sodium cacodylate containing 0.22M sucrose. After one hour fixation, tissues were dehydrated and embedded in Epon.

4. Dehydration and Embedding

After fixation in Osmium (either direct or post-fixation), tissues were dehydrated in graded ice-cold ethanol as follows:

50% ethanol	10 minutes
70% ethanol	10 minutes
95% ethanol	5 minutes
95% ethanol	5 minutes
100% ethanol	5 minutes
100% ethanol	5 minutes
propylene oxide	10 minutes
propylene oxide	10 minutes

Following dehydration, tissues were infiltrated with a mixture containing equal amounts of propylene oxide and Epon embedding mixture for 2-3 hours. The tissues were then transferred to plastic capsules filled with epoxy resin mixture and allowed to polymerize in the oven as indicated: 12 hours at 37°C., 12 hours at 45°C. and 12-16 hours at 60°C. The epoxy embedding mixture was prepared as follows:

Epon 812	22ml.
DDSA (Dodecenyl succinic anhydride)	14ml.
NMA (Nadic Methyl anhydride) . .	10ml.
DMP (2, 4, 6, -tri (dimethylamino-methyl) phenol) (Used as an accelerator) . .	1.5%

This mixture was mixed well for at least one half hour in an electric mixer before use.

B. Cytochemistry

1. Pre-fixation in Glutaraldehyde

Tissues were pre-fixed in ice-cold 3.25% glutaraldehyde buffered to pH 7.4 with 0.1M sodium cacodylate for 20-40 minutes, instead of 60-90 minutes for ultrastructural studies. This short fixation time was used in order to

preserve the maximum enzyme activity. Following pre-fixation in glutaraldehyde tissues were rinsed in the buffer and processed for incubation for the localization of enzymes. To do this, specimens were placed on the platform of a Smith-Farquhar Tissue Chopper in a drop or two of neutral agar solution. When the solution solidified (about 30 seconds or so), sections were cut at 25 micron and 50 micron thick, and the cut sections were transferred by a brush to the buffer prior to incubation.

2. Incubation

Wachstein and Meisel method (1957) for the demonstration of phosphatase was used for this purpose. The substrates utilized were ATP and ADP (Sigma Chemical Co., St. Louis, Mo.). Stock solutions were prepared not more than 24 hours prior to use and were as follows: 1) magnesium sulfate 0.1M ($MgSO_4 7H_2 0$); 2) lead nitrate 2%; and, 3) Tris (hydroxymethyl) aminomethane maleate (tris-maleate) buffer, pH 7.2, 0.2M. The substrate solutions were always made one or two hours before incubation. Final incubation mixture, mixed fresh just prior to incubation, consisted of the following proportion of stock solutions and one of the substrate solutions (ATP or ADP) as follows:

Respective phosphate ester 125mg/100ml.	20ml.
Tris maleate buffer, pH 7.2, 0.2M	20ml.
Magnesium sulfate, 0.1M	5ml.
Lead nitrate, 2%	3ml.
Distilled water	2ml.
Total	50ml.

Incubations were carried out at room temperature. 25 micron and 50 micron sections were incubated for 25-35 minutes and small whole hearts were incubated for 40-45 minutes.

3. Controls

Control preparations were made as follows:

1) Incubation in substrate-free media.

2) Incubation in media containing equimolar concentration of B-glycerol phosphate in place of the nucleotide phosphate esters (ATP and ADP).

3) Pre-treatment of specimens for one hour in 1% buffered Osmium at pH 7.2 to inactivate the enzymes, followed by incubation in medium containing ATP or ADP.

4. Post-Fixations in Osmium Tetroxide

Following incubation in the appropriate media, tissues that were to be osmicated were washed in the buffer and post-fixed for one hour in ice-cold 1% Osmium tetroxide solution buffered to pH 7.2 with 0.1 sodium cacodylate containing 0.22M sucrose. After post-fixation, specimens were dehydrated in graded ethanol and embedded in epoxy resin mixture as described previously.

C. Sectioning and Staining

Epon embedded tissues were sectioned in a Porter-Blum MTI Microtome (Sorvall). Thick 1 micron sections were cut with glass knives, mounted on glass slides and stained with Toluidine blue (Richardson's Stain) for light microscope orientation purposes. Thin sections about 500-600 angstroms thick (Gold sections) were cut with a Diamond Knife (Dupont) and collected on either 200 or 300 mesh uncoated copper grids. 30-50 such grids were collected from each epon block.

Sections were stained with heavy metal solutions. The staining of organelles and contrast could considerably be improved by a "Triple Stain" used by this investigator. Staining procedure was as follows:

2% lead citrate pH 12 . . .	5 minutes
Wash in distilled water . . .	several changes

Saturated uranyl acetate solution .	20 minutes
Wash in distilled water . . .	several changes
2% lead citrate	5 minutes
Wash in distilled water . . .	several changes

Drops of staining solutions were placed on dental wax plates, and the copper grids with sections on them were floated on these drops. After staining in each solution for the prescribed time, washing was accomplished by holding the grids with fine-tipped forceps and dipping the grids successively in four 100ml. beakers filled with distilled water, 15-20 dips in each beaker.

Stained sections were viewed in an RCA Model EMU 3F Electron Microscope operated at 50 K. V. and were photographed on 2x2 or 3 1/4 x 4 plates (Kodak). Kodak F-3 or polycontrast paper was used for printing. Enlargements were made on an Omega Model D-2 Enlarger using appropriate filters. Prints were developed and dried using standard procedures.

RESULTS

In this section the cyto-differentiation of embryonic myocardium and its organelles is described separately for each day examined, namely, 10, 11, 12, 13, 14 days and the newborn. In order to provide the reader with an overview of the process of the development of embryonic rat heart, a brief account is presented below.

The first elements of the primordial heart appear in the cardiogenic area, that is, in the mesoderm lying anterior to the head fold of the embryo. This mesoderm separates into two layers: one nearest the ectoderm, called the somatic mesoderm, and the other nearest the endoderm,called the splanchnic mesoderm. It is the splanchnic mesoderm that gives rise to the walls of the heart. With subsequent growth the cluster of cells which are destined to form the heart undergoes further differentiation and reorganization with the resultant formation of two endocardial tubes, which later on fuse in a cephalo-caudal direction and form a single endocardial tube. As these events occur, the mesoderm adjacent to the endocardial tube (the splanchnic mesoderm) proliferates, and by the time the tubes have fused, forms a mantle around it, called the myoepicardial or epimyocardial mantle, thus forming the tubular heart consisting of a tube-within-a tube. The endothelial tube is separated from the mantle at first by a gelatinous substance called the cardiac jelly, but later the jelly is invaded by mesenchymal cells which play a role in the formation of valves. As further development continues, the wall of the heart tube consists of three layers. From inside out, they are the endocardium, which forms the internal endothelial lining of the heart; the myocardium forming the muscular wall; and the epicardium or visceral pericardium investing the outside of the tubular heart. Finally, by a series of

bending, looping and differential growth the tubular heart transforms and molds itself into the adult form.

The description of results that appears in the accompanying pages has in it some terminology which could have differing connotations in different contexts. In order to avoid confusion, the intended use and meaning of these terms are listed.

Myofilaments ..... the contractile proteins, the actin and myosin filaments.

Thick and thin filaments... myosin and actin filaments, respectively.

Myofibril..... a collection of myofilaments organized into a functioning unit containing the A, I and Z bands.

Myofiber..... the cell or cells which contains several of these myofibrils; cell may also contain isolated myofilaments in addition to the myofibrils.

Myoblast..... a cell that produces the myofilaments.

A band..... contains myosin (thick) filaments and actin (thin) filaments.

I band..... contains actin (thin) filaments.

Z band or disc or line..... appears in the middle of I band.

H zone or band or line..... appears in the middle of A bands and contains parts of thick filaments.

M line or band..... appears in the middle of H zone or line.

Sarcomere..... the distance between one Z disc and the next; the structural and functional unit of myofibril.

Sarcoplasm..... the cytoplasm of muscle cell.

Sarcolemma..... the plasmalemma of muscle cell.

Preceding the ultrastructural description of the results, a table listing the gross morphological features of rat embryos at various stages of development is presented.

Table 1. Gestational Age, Somite Number, Stage of Development and External Features of Rat Embryos.

Day	Somites	Stage*	Identifiable Morphological Features
10	6-10	17-18	Delimited otic (4th) rhombomere and post-otic sulcus. Neural canal closed from the level of 2nd to 6th somite. Heart-tube forms a C-shaped bend towards the right, emerges from septum transversum.
11	15-24	19-20	Neural folds fused at diecephalic-mesencephalic junction. Anterior neuropore and rhombencephalon closed. Optic pit visible. Heart-tube emerges from septum transversum, becomes S-shaped, atrial dilation visible behind the bulbus. Anterior limb bud appears (22-24S).
12	26-36	21-22	Posterior neuropore and otic pit closed. Maxillary process reaches lateral nasal process. Rathke's pouch and posterior limb bud appears. Bulbus and ventricle differentiated and the heart appears four-chambered.
13	36-46	22-23	Lens vesicle closed. Primitive posterior nares visible. Rathke's pouch closed. Caudal migration of liver continues. First trace of upper eye lid appears. Ventricles and atria prominently visible.
14	48-	24-25	First vibrissary papilla appears on maxillary process. First traces of digital condensations in fore-paw. External ear flap and lower eyelid begin to form. Truncus arteriosus identifiable.
Newborn	--	--	Known features.

*Christie, G.A., (1964).

I. 10-Day Myocardium (6-10 Somites)

A. Ultrastructural Observations

1. Superficial Layer of Cells

For descriptive purposes the cells of 10-day myocardium may be grouped into two layers: a superficial layer and a deep layer of cells. One of the chief distinguishing characteristics of myocardium at this stage of development is the loose packing and assemblage of the prospective myoblast cells (Figs. 1, 2).

In the surface layer extensive and tortuous intercellular spaces are very conspicuous (Figs. 1, 2). Large nuclei with one or two prominent nucleoli occupy most of the cytoplasm. The nuclear envelopes and perinuclear cisternae are distinctly visible; however, the extension of perinuclear space into the cisternae of endoplasmic reticulum is seldom seen. Around the periphery of the nucleus small condensations of chromatin can be seen, and elsewhere the chromatin is evenly dispersed in the karyoplasm (Fig. 1). Ultrastructural details of nucleolus are distinctly discernible in these cells. The coarse dense strands of nucleolonema branch and anastomose to form an irregular tridimensional network (Figs. 1, 2).

Many of the common cell organelles in the cytoplasm are absent or inconspicuous. Those organelles which are present appear to be in a primitive or immature stage of development.

Mitochondria are not numerous and in both cross and longitudinal sections exhibit a narrow matrix with a small diameter. The outer limiting membrane appears as a solid thick line and does not exhibit the typical inner and outer leaflet with a clear space in between (Fig. 3). Cristae mitochondriales are very poorly developed, and there is no definite shape or organization (Figs. 3, 1).

In the surface layer of myocardial cells, just beneath the epicardium, the

granular endoplasmic reticulum is not distributed extensively. When present, it is frequently associated with mitochondria (Fig. 3). A widespread distribution of elaborate amounts of free ribosomes in the cytoplasm is a constant feature of these cells. Rosettes of ribosomes (polyribosomes) and individual particles of ribosomes are both easily distinguishable (Figs. 2, 3). The nuclear envelope and endoplasmic reticulum are studded with ribosomes.

Plasmalemma is not well-delineated and cannot be resolved with any appreciable clarity. Cell surfaces are thrown into irregular protoplasmic processes (Figs. 3, 1), and cell-to-cell contact is maintained by close apposition of cell surfaces without the intervention of desmosomes or similar specialized areas of cell attachments (Figs. 3, 2).

2. Deep Layer of Cells

There are several new and different features to be pointed out regarding the morphology and cytology of the cells in this layer as compared to the superficial layer of cells. The beginning of the formation of specialized areas of cell-to-cell contact is very obvious, and early stages of developing desmosomes are distinguishable at several sites. These structures begin their development as short dense strips along the plasmalemma by the laying down of a homogeneous electron-dense cytoplasmic material on the inner aspects of two apposing membranes (Figs. 5, 6). In some instances these strips are long and more dense, as shown in Figs. 11, 12. These primitive desmosomes, or "protosomes," appear to serve as one of the chief cementing regions between the cells of the deep layer in 10-day myocardium. Cell surfaces are seen to be coated with a filamentous, matty material, and when two cells are in apposition this filamentous material on the cell surfaces appears to stick together, as shown in Figs. 5, 3. This provides another possible method of cementing cells together in the

rat embryonic myocardium.

The plasmalemma cannot be resolved very adequately, and cell surfaces are characterized by intense activity in that they are thrown into several folds and possess long slender processes. The ectoplasm extends into these folds and processes (Figs. 12, 11). Constrictions are observed at the bases of many of them and appear to pinch off as free-floating cytoplasmic bodies into the region of cardiac jelly which is located between the epimyocardial mantle and the endocardium. The above mentioned cells and processes border the cardiac jelly on the myocardial side and the ultrastructural evidence for intense secretory activity in these cells is very apparent.

The nuclei are proportionately smaller than those of the superficial cells. Both the inner and outer membranes of the nuclear envelope are studded with ribosomal particles, which is true of the superficial cells as well. It should also be pointed out that frequently the ribosomal particles on the inner membrane of the nuclear envelope may be obscured from view by the peripheral distribution of chromatin material (Figs. 2, 11). Nucleoli are prominent and details of the nucleolemma are distinctly visible.

The cytoplasm of the deep layer of cells undergoes considerable change in that more organelles are discernible than in the superficial cells, although mitochondria are still in a primitive stage of development with poorly developed and organized cristae. The outer and inner membranes cannot be discerned.

The granular endoplasmic reticulum occurs in greater abundance, and the close association of mitochondria and granular endoplasmic reticulum is apparent. The cisternae are distended and contain an amorphous or flocculent material indicative of secretory activity (Figs. 11, 8). In addition to being attached to the membranes of nuclear envelope and endoplasmic reticulum, the

ribosomes occur as free particles in large quantities in the cytoplasm.

Lipid droplets of varying diameters are regularly seen, most of which occur as single droplets (Figs. 11, 12).

In some cells of the deeper layer of the 10-day (8-10 somites) myocardium of the beating heart the presence of myofilaments can be detected for the first time. These filaments are not restricted to any one site or locus and are dispersed at random throughout the cytoplasm (Figs. 4, 5). Many filaments are obscured from clear view by the over-crowding of ribosomal particles (Figs. 5, 6). Thin filaments (actin), if present, are not easily resolvable in these micrographs owing to the over-crowding of ribosomal particles which would obscure them.

Condensations of proteinaceous material in the form of small strips of varying width and length can be observed at several locations in the cytoplasm. Myofilaments are either attached to or radiate from these condensations, which appear to be developing Z bands, forming tufts of fibrils (Figs. 4, 5). From this evidence it seems quite likely that the synthesis of morphologically identifiable Z bands and myofilaments, probably thick filaments, occurs simultaneously. Examples where several myofilaments are intersected by a Z line are illustrated in Figs. 6, 4. It is noteworthy, however, that no structure resembling an I band is visible on either side of the Z line. This again suggests that the synthesis of thin filaments may follow the synthesis of Z and A bands.

In some cells myofilaments are organized in a very irregular and loose manner between two or three developing Z bands (Figs. 7, 6). This structure, comprised of loosely arranged myofilaments and Z bands, is the early stage of formation of a myofibril and is herein named the "protofibril." In a protofibril, there are at least two Z bands or Z band condensations with a few myofilaments

attached between them. The filaments are invariably covered with clusters of free ribosomes, and the I band is not recognizable in the protofibril. Branching may be witnessed in some protofibrils.

One of the most noticeable features of the 10-day myocardial cells containing myofilaments is the peculiar appearance and structure of mitochondria. They are long and filamentous; the middle segment is very narrow or sometimes thread-like; and the end portions are swollen or ballooned out (Figs. 4, 7). In some instances the middle segment is also blown out into vacuolar enlargement (Fig. 9). Mitochondria appear as a solid dark mass of material interspersed with light areas or bands suggestive of some sort of compartmentalization (Figs. 4, 7). Distinct and separate membranous elements are not recognizable, nor can they be resolved. Close association of clusters of free ribosomes and the developing mitochondria is also very apparent.

The presence of extensive areas of Golgi bodies is another prominent feature of these cells, and many different stages of development of the Golgi regions can be recognized (Figs. 9, 11). These organelles begin to appear as curved arrays of parallel membranes enclosing narrow cisternae. At times they display an oblong whorled structure as illustrated in Fig. 8. The cisternae in many instances contain a flocculent material. Small vesicles and vacuoles are present near the assemblage of the cisternae. Some of the vesicles are enlarged and contain an amorphous product and are probably intermediate stages in the formation of secretory granules (Figs. 8, 9).

Numerous examples of mitotic cells are readily visible in myocardium (Fig. 10). In the cytoplasm of these same cells numerous sites of formation of myofilaments are easily distinguishable. Several such cells displaying simultaneous mitotic activity and myofilament synthesis are observed throughout the

developmental period covered in this investigation.

In non-dividing cells the chromatin material is most often distributed in clumps not restricted to the periphery of the nuclear envelope (Fig. 8) and nucleoli are very prominent. A single centriole is occasionally discernible in myocardial cells (Fig. 8).

The granular endoplasmic reticulum becomes more abundant in cells which contain myofilaments. These cells exhibit large and distended cisternae containing some flocculent material. Ribosomes are prominently and linearly arranged on both membranes of the reticulum. The cytoplasm contains a great quantity of free ribosomes (Fig. 5). The plasmalemma in cells containing myofilaments is not well defined and does not differ from that of other cells of the myocardium in this respect. Desmosomes are rare; however, a few are distinguishable in this early phase of development (Figs. 7, 5). Intercalated discs are not recognizable at this stage of development.

B. Cytochemistry

Very intense enzymatic activity is observed after incubation of 10-day hearts with ATP as a substrate. The final reaction product is deposited either as small granules or as clusters of granules on plasmamembranes and cell processes. Heavy deposits of reaction product are especially conspicuous at regions where the cell membrane is thrown into folds and villus-like projections and processes (Figs. 11, 12). The ectoplasm can be observed extending into these processes. Many of them pinch off and are seen in micrographs as free-floating profiles with a heavy deposit of final reaction product on their surfaces. The intercellular distribution of reaction product consists of a uniform distribution of precipitate on the adjoining cell membranes of apposing myoblasts. Intracellular localization of the enzyme is confined to pinocytotic vesicles, or

caveolae (Figs. 13, 12). A fibroblast with considerable enzymatic activity is shown in Fig. 13. It appears that the reaction product is being deposited around some of the lipid droplets (Figs. 13, 12).

II. 11-Day Myocardium (15-24 Somites)

A. Ultrastructural Observations

Loosely packed cells are still encountered in the 11-day (15-24 somites) myocardium, but not to the extent seen in the 10-day myocardium. Many of these cells are in an active stage of division (Figs. 14, 15). These cells contain individual chromosomes, mitochondria, endoplasmic reticulum and a great abundance of free ribosomes. Partial reconstruction of the nuclear envelope from the assemblage of elements of granular endoplasmic reticulum is vividly distinguishable (Fig. 15). More examples and evidence for this phenomenon will be presented elsewhere in this section.

One of the most characteristic features of the myocardium at this stage of development is the presence of long, well-organized myofibrils, as opposed to the "protofibrils" and isolated myofilaments seen in the 10-day myocardium. Each of these fibrils already displays most of the morphologic aspects of adult structure. The Z, I, A and H bands can be easily distinguished (Figs. 23, 28). Unlike in the 10-day myocardium, the evidence for the spatial organization of the filaments (actin) into I bands is unmistakable. Furthermore, thin (actin) and thick (myosin) filaments, organized into an hexagonal array, are easily discernible in cross sections of myocardial cells. The manner of arrangement of these filaments appears to be one where a central thick filament is surrounded by five-six thin filaments. Each of the thin filaments, however, is shared by two or more thick filaments (Figs. 26, 31). In longitudinal sections most of the fibrils are seen coursing through the long axis of the cell, invariably paral-

lel to the plasmalemma (Figs. 23, 28). These individual fibrils are not gathered or organized into masses or groups of fibrils in any of the cells examined at this stage of development. On the contrary, in most cases the fibrils are separated from one another by very irregular cytoplasmic spacings (Figs. 23, 28). In some instances, however, convergence and adherence of a portion of three or four fibrils into a single unit or mass is recognizable, although, the opposite ends of these fibrils remain separated (Figs. 23, 28). This suggests the possible beginning of organization of the fibrils into a fiber. While well-organized fibrils are easily identifiable in some cells, it is not uncommon to distinguish unorganized individual myofilaments in random distribution and orientation in other cells, and sometimes in the same cells (Figs. 29, 33).

Cardiac muscle is characterized by the phenomenon of branching of the fibrils. It appears that branching of the myocardium is initiated mainly at the Z disc regions (Figs. 29, 30). In some instances the branching myofibrils display a "Y" or "X" shaped configuration, and the points of intersection represent the Z disc region. An example of this is shown in Fig. 30, where five or six Z discs are involved in this process wherein the myofilaments run in different angles so as to form irregular and polygonal figures bounded by myofilaments and Z lines. Another site where myofibrils are seen branching is the region of the developing intercalated discs. Details of this will be dealt with elsewhere.

Intimately associated with the Z disc regions is the development of the T-system of microtubules. With close scrutiny, one or two circular or horizontal profiles of tubular elements can be recognized in close association with the Z lines (Figs. 28, 23). These smooth surfaced tubular elements constitute the early development of the T-system (Fig. 28). At one region two Z discs of different fibrils lying side by side are apparently connected or bridged by two

such tubular profiles lying perpendicular to the fibrils. More documentation of this system will be presented for the late periods of development to be described.

Unlike the 10-day myocardium, the plasmalemma of 11-day myocardium is well-defined and can be resolved adequately. Intense pinocytotic activity is associated with sarcolemma and numerous micropinocytotic vesicles or caveolae are discernible subsarcolemmally, as well as deep in the cytoplasm (Figs. 28, 29).

Regions of cell-to-cell contact are much more evident in these cells. Desmosomes are commonly observed (Figs. 29, 30). Another feature of 11-day myocardium is the presence of developing intercalated discs. These develop between apposing membranes of adjoining myocardial cells as small "desmosome-like" structures. In early stages of development intercalated discs assume a straight or slightly curved form. Myofilaments or myofibrils are intimately associated with the intercalated disc, even during the incipient stages of formation (Figs. 30, 20). When a myofibril terminates at an intercalated disc, the distance between that disc and the next Z disc is approximately equal to the distance between any two Z discs of that fibril (Figs. 30, 20). Myofibrils intersect the sarcolemma at the region of the intercalated discs, and the point of intersection is the area where the next Z disc would have appeared if there had been no intervention of an intercalated disc. A desmosome is invariably present in the vicinity of a developing intercalated disc. Large numbers of pinocytotic vesicles are consistently seen in and around the regions of the intercalated discs (Figs. 21, 29). As was pointed out earlier, the myofibrils may branch at the Z disc. At the locus of the intercalated disc the phenomenon of branching is also encountered (Figs. 20, 30). An example of three different

intercalated discs which are connected or bridged by myofilaments without the intervention of any Z line is shown in Fig. 20. Cases where myofibrils radiate out in different directions from the intercalated discs, thus in effect bringing about the phenomenon of branching, are very common in the developing myocardium.

One of the significant features of 11-day myocardium is the presence of glycogen particles. These particles are in intimate association with the myofibrils, and in cross sections clusters of glycogen particles can be seen to surround individual fibrils (Figs. 31, 32). It may be recalled here that no glycogen particles were recognizable in 10-day myocardium.

Golgi bodies are present in large numbers and display high secretory activity (Fig. 32). Golgi cisternae are distended and contain a dense flocculent material. A proportionately greater number of vesicles are seen in and around the Golgi regions (Figs. 22, 19), and an amorphous material is detectable in them. It appears that these vesicles are intermediate stages in the formation of secretory granules. Fully formed secretory granules are frequently seen distributed at random in the myocardium (Figs. 24, 25). All these granules are membrane-bound and contain dark, electron-dense material. And their proximity to myofibrils is also noticeable. Multivesicular bodies are also frequently observed in and around the Golgi region (Fig. 32). It should be pointed out here that unlike many mammalian cells in which the Golgi bodies occupy a supranuclear position, in the differentiating myocardium it appears that there is no such polarization of the Golgi bodies. They are, rather, distributed at random throughout the cytoplasm. The close association of Golgi bodies with granular endoplasmic reticulum, mitochondria and myofibrils is readily recognizable.

Mitochondria are quite numerous and are widely distributed. The outer

limiting membrane is separated into an inner and outer leaflet by a clear space; and the cristae mitochondriales are well-formed but loosely packed (Figs. 19, 20). Some mitochondria possess tubular cristae, many of which blindly end in the interior, while others traverse the entire width of the organelle. A suggestion of a zig-zag course of some of the cristae, characteristic of adult cardiac muscle, is detectable (Fig. 19) in some mitochondria. Filamentous and ballooned-out mitochondria, as seen in 10-day myocardium, are seldom found in these cells. The intimate association of mitochondria and developing myofibrils is very apparent (Figs 28, 20). Similar topographical proximity of mitochondria with granular endoplasmic reticulum and glycogen particles is often observed (Figs. 32, 33).

The granular endoplasmic reticulum is in an advanced state of development and is well-dispersed in the cytoplasm. This organelle appears as long tubular structures arranged in parallel stacks of tubular cisternae (Figs. 24, 34), is arranged in whorls and ring-like configurations, or exhibits a single long tubular profile (Figs. 35, 34). An amorphous material is recognizable in many of the endoplasmic cisternae, indicative of secretory activity, and highly distended cisternae are regularly visible (Figs. 34, 33). The close association of this organelle with mitochondria, Golgi bodies and myofibrils is very evident (Fig. 19).

Free ribosomes occur in great abundance and are profusely dispersed in the cytoplasm. Occasionally they are seen to be arranged into linear or zig-zag chains (Figs. 19, 28).

The nuclei become more and more longitudinally compressed and oriented parallel to the long axis of the elongating myocardial cells. Prominent nucleoli, one or two per nucleus, are still a constant feature. Chromatin material is dis-

tributed peripherally, the nuclear envelope at several places is shown to be continuous with the endoplasmic cisternae (Fig. 16), and nuclear pores are easily recognizable. Associated with the nuclear envelope and in close proximity to it are several small vesicles and vacuoles (Figs. 22, 36).

The phenomenon of myofilament synthesis and mitotic process in the same myocardial cell is regularly encountered, as previously described in the 10-day myocardium.

A single myofibril may be seen in a state of contraction while other fibrils of the same cell may remain uncontracted (Fig. 27). In this functional stage, A, Z, and H bands are visible, but the I band is not. Several vesicles and vacuoles are frequently observed close to the Z band of the contracted fibril (Fig. 27). The internal structure of some of the mitochondria displays a very peculiar arrangement and configuration in such myocardial cells where one or two fibrils are in a state of contraction. It appears as though the cristae are interconnected at numerous points so as to form a reticulum or labyrinthine system (Fig. 27).

Long, slender epicardial cells form a one cell thick mantle over the myocardium. The two are, however, separated by large intercellular spaces. One of the prominent features of epicardial cells is the arrangement and distribution of granular endoplasmic reticulum in the form of stacks of parallel cisternae. The cytoplasm abounds with profusely dispersed free ribosomes (Figs. 17, 18).

B. Cytochemistry

After incubation in a media containing ATP as the substrate, the final reaction product is seen to be deposited mainly on the cell surfaces of the myocardium (Fig. 34). Intercellularly, the reaction product is also deposited on the apposing plasmalemma of adjoining cells. Intracytoplasmic localization is most often restricted to a few pinocytotic vesicles or caveolae (Fig. 34).

Frequently, in those regions where the myofibrils are in contact with the sarcolemma, there appears to be a heavier deposit of reaction product (Fig. 37).

As a general observation, it should be pointed out that the final reaction product is deposited in smaller clusters of granules and is usually less pronounced than that found in the 10-day myocardium.

III. 12-Day Myocardium (26-36 Somites)

A. Ultrastructural Observations

By the 12th day of development the myocardial cells are arranged more compactly than previously noted (Fig. 45). The only indication of the previous state of affairs is the large intercellular spaces occasionally observed. The plasmalemma is well-defined, and desmosomes are observed frequently. Other cell-to-cell contacts are also readily recognizable (Fig. 39). Intense pinocytotic activity is associated with the sarcolemma, and the formation of vesicles derived from the sarcolemma can be visualized at several locations (Figs. 39, 42). Both smooth contoured and fuzzy or spiny vesicles are distinguishable. Some are found just beneath the cell membrane, while others lie deep in the cytoplasm. A large number of vesicular and vacuolar elements are present in the cytoplasm (Figs. 42, 39), a feature not noted in 10 and 11-day myocardium.

By the 12th day of development, all the myocardial cells examined contain either myofilaments, myofibrils or both. Some cells contain randomly distributed isolated myofilaments, while in the adjoining cells myofilaments are being organized into myofibrils (Fig. 45). This process of progressive stages of differentiation of myofilaments and fibrils in different cells of embryonic myocardium is a regular feature of all stages of development covered in this investigation.

In most cells, however, myofilaments are ordered and organized into

fibrils. In some, these fibrils appear as long, single units coursing longitudinally through the cell, separated from one another by very irregular spacings (Fig. 43). In others, several of the fibrils are grouped or organized into "sheets" or "masses" (Figs. 40, 42). In the latter, the branching fiber is readily apparent with the Z discs and intercalated discs serving as pivotal points from which fibrils and groups of myofilaments diverge at wide angles. This process of branching appears to bring about anastomoses and interconnections between various intercalated discs, Z discs and myofibrils. In some cells, individual fibrils may follow a zig-zag course, the Z discs acting as the points of reflection or bending (Fig. 43).

Free ribosomes are associated with developing filaments and fibrils. Some of these particles are seen to be organized in the form of irregular chains. It can be observed at numerous sites that two such chains will be arranged parallel to each other, and between them are located many myofilaments. It appears as though myofilaments are being laid down between these chains of ribosomes and slowly are being pushed out in length (Figs. 42, 40).

In addition to being freely distributed in the cytoplasm, ribosomes are attached to the inner and outer membranes of the nuclear envelope (Figs. 39, 44). Some areas of the outer membrane of the nuclear envelope are denuded of ribosomal particles (Figs. 40, 39). The nucleus is longitudinally compressed and contains one or two prominent nucleoli. The chromatin is uniformly distributed in the nucleoplasm.

Mitotic myocardial cells provide convincing evidence of the reconstruction of nuclear envelope from the elements of endoplasmic reticulum. In cells of late telophase, tubular elements of endoplasmic reticulum, placed end-to-end, can be observed to encircle the nuclear masses, thus forming an envelope

(Figs. 48, 47). As it can be recognized from this reconstruction process, the nuclear pores appear at the sites of the end-to-end union of the tubular elements of endoplasmic reticulum. The continuity of perinuclear cisternae into the endoplasmic reticulum, as well as their incipient stages of formation, is also discernible. An example of a cell in metaphase with a large assemblage of tubular elements of endoplasmic reticulum in the vicinity of nuclear material, presumably for the anticipated deployment for the reconstruction of a new nuclear envelope, is illustrated in Fig. 49. This myocardial cell also provides a good example of the simultaneous but independent incidence of mitosis and myofilament synthesis in the same cell. This phenomenon is observed frequently in the embryonic myocardium. A single centriole is occasionally seen in non-dividing myocardial cells (Figs. 38, 47).

The presence of intercalated discs in the 12-day myocardium is a common feature. Some of these cell attachments are in an advanced state of development; however, most remain less well-defined (Figs. 39, 38).

There is no marked advance in the development of sarcoplasmic reticulum and the T-system and tubules in 12-day myocardium. Good examples of Z disc-tubular association are, however, observable (Fig. 41). The granular endoplasmic reticulum is widely distributed, and cisternae are distended and contain a flocculent material (Figs. 46, 44).

Golgi bodies are found in greater abundance than previously noted in the earlier stages of development and are randomly distributed in the cytoplasm (Figs. 44, 46). All three elements of the Golgi bodies are recognizable, with vacuoles and vesicles predominating. Secretory activity of the Golgi bodies is much in evidence. An occasional multivesicular body is visible in the myocardial cells in the region of the Golgi bodies (Fig. 46).

Mitochondria are well-developed and are distributed extensively throughout the cytoplasm. Cristae mitochondriales are well-formed, but still loosely packed. The close topographical relationship of mitochondria, Golgi bodies, endoplasmic reticulum and myofibrils is very conspicuous (Figs. 44, 40).

Lipid droplets occur frequently in myocardial cells at this stage of development and appear as single droplets or as aggregates of droplets (Figs. 45, 38)

B. Cytochemistry

The distribution and deposition of final reaction product after incubation with ATP as the substrate remain the same as in the 11-day myocardium. Intercellular deposition of the precipitate on adjoining sarcolemma is readily discernible. Intracellular localization is not distinguishable. In comparison to 10-day myocardium, the amount and nature of the precipitate is very much reduced (Figs. 50, 51, 52).

IV. 13 Day-Myocardium (36-46 Somites)

A. Ultrastructural Observations

Myocardium of 13-day embryonic heart exhibits some features which are seen for the first time in the developmental period covered in this investigation, while others are much more clearly defined than observed in the stages described above.

An examination of cross and longitudinal sections of myocardial cells reveals an apparent increase in the amount and number of myofibrils in comparison to 10-day and 11-day myocardium (Figs. 61, 54). There appears to be more organization of the myofibrils, and many of them are gathered together into groups of fibrils. An example where branching and interconnection of myofibrils unite several of them together into what appears to be a developing myofiber is illustrated in Fig. 61. As observed in previous stages, branching

occurs mainly at two sites, namely, the regions of Z disc and intercalated disc. The process of branching is a common occurrence in 13-day myocardium (Figs. 61, 62). In the same plane of section myofibrils are seen cut in all possible angles: transverse, longitudinal, oblique, tangential, and otherwise. This arrangement is indicative of the extreme complexity and ramification of the distribution of myofibrils within each cell (Figs. 62, 54). In cross sections thick and thin filaments, arranged in an hexagonal configuration, are clearly visible. Thin filaments are much more pronounced and are easily recognizable relative to earlier stages.

Several features of the sarcolemma are of interest. At this stage of development the beginnings of a close association of sarcolemma and myofibrils are distinctly discernible. The principal contact is established at the Z disc regions by indentations or invaginations of the sarcolemma (Figs. 58, 55). Subsarcolemmal fibrils running parallel to the sarcolemma in the long axis of the cell appear to be in close contact with it.

The sarcolemma contributes the two leaflets of the intercalated discs, which have undergone further differentiation. The plicated nature of these structures is more and more apparent with advancing age. Myofibrils terminate at the intercalated discs, and sometimes it appears as though they fan out in different directions from it (Figs. 61, 59). One or two myofibrils frequently bridge two separate intercalated discs without the intervention of Z discs. Many of the structural details of intercalated discs described earlier are obvious in the 13-day myocardium. Smooth as well as fuzzy or spiny vesicles are associated with them in great numbers. The intermediate line seen in adult intercalated discs is not formed, or it is not resolvable.

A regular feature of the sarcolemma is that it is thrown into folds (Fig. 61),

or, in some cases, the sarcolemma of adjoining cells displays complementary projections and depressions so that the two cells fit together snuggly (Fig. 65).

The sarcolemma is actively involved in the phenomenon of micropinocytosis. Numerous smooth-contoured vesicles can be seen subsarcolemmally, as well as deep in the cytoplasm. In addition, several fuzzy or spiny vesicles are also discernible (Figs. 65, 55).

Evidence of the formation of the sarcoplasmic reticulum and the T-system and tubules is frequently encountered, as in earlier stages, and elements of endoplasmic reticulum are seen associated with Z regions at several locations (Figs. 65, 55).

As noted previously, several mitotic cells are usually observed in a section of the 13-day myocardium. The cytoplasm of these cells contains isolated myofilaments and myofibrils. Numerous microtubules are also in view, representing the remnants of the spindle apparatus (Fig. 60).

Mitochondria are widely distributed in larger numbers than previously reported. The cristae are well-formed and more tightly packed than in the 10, 11 or 12-day hearts, and tubular cristae are occasionally recognizable (Fig. 64). It appears that several mitochondria are in the process of multiplication by constriction or by budding (Figs. 64, 55). The topographical proximity of the organelle to Golgi bodies, endoplasmic reticulum and myofibrils is again very evident (Figs. 64, 59).

Golgi bodies are well developed, very active and extensively distributed throughout the cytoplasm. All the three elements of the Golgi apparatus, namely, cisternae, vacuoles and vesicles, are easily recognizable. Stacks or skeins of parallel cisternae are associated with numerous vacuoles containing a flocculent material (Figs. 64, 63), many of which exhibit an intermediate stage of secre-

tory granule production.

The granular endoplasmic reticulum is also well-developed, widely distributed and occurs as single tubular profiles or as skeins of parallel cisternae (Fig. 66). The cisternae are distended and contain an amorphous material (Figs. 64, 65). Some of them are seen surrounding mitochondria in a "C"-shpaed or ring-like fashion (Figs. 64, 65). Their distribution near Golgi regions and myofibrils is unmistakable.

The distribution of free ribosomes in the cytoplasm is still widespread, but less extensive than in the 10-day myocardium (Fig. 63).

The nuclei have undergone considerable longitudinal compression and are oriented in the long axes of the myocardial cells in the center of the cytoplasm, and the myofilaments are distributed around them. Prominent nucleoli are very frequently observed, and chromatin material is uniformly distributed in the karyoplasm. The nucleolonema is recognizable, but not as distinctly as in 10-day myocardium. At places the nuclear envelope is seen to be continuous with the endoplasmic reticulum.

One of the most surprising findings in the 13-day myocardium is the presence of neural elements* In cross-sections nerve fibers can be observed between myocardial cells (Figs. 54, 54A), and the close association of the neural elements with the myocardial cells is very evident. Each of the nerve fibers is enclosed in a membrane, and the neurofilaments appear as small dots distributed uniformly in the axoplasm. An example of a single nerve process located between several myoblast cells is shown in Fig. 56. The cytoplasm or axoplasm of neural cell processes contains an abundance of fine neurofilaments oriented in the longitudinal axis of the cell. The endoplasmic reticulum of neural elements is also of interest in that the cisternae are highly distended and contain a floccu-

* See appendix

lent material (Figs. 56, 53). This is a very characteristic feature of developing nerve cells. Protrusions, projections, and "bumps" appear on the surface of both the muscle and nerve cell process, and they grow out towards each other, presumably to establish neuro-muscular contact (Figs. 56, 62). In the 13-day myocardium several such contacts have already been established (Figs. 56, 62). Cell surfaces of both the neural elements and the myocardial cells are coated with a filamentous material, and this is especially apparent at those regions where both these elements come together. Nerve cell processes make deep penetrations into the extensive crevices and spaces between myocardial cells, bend on themselves, thus making a loop, and come back out again. The ascending and descending limbs are parallel and apposed to each other. This deep penetration and looping of neural processes appears to bring about contact between them and the remote cells of the myocardium (Fig. 62). The close proximity of neural and vascular elements is also readily recognizable in 13-day myocardium (Figs. 57, 53).

B. Cytochemistry

The pattern and nature of distribution of reaction product after incubation with media containing ATP as the substrate are the same in these cells as in early stages. The intensity of reaction and the amount of deposit are, however, considerably curtailed. Very fine granules of the precipitate are deposited instead of clumps of reaction product as seen in 10-day myocardium (Figs. 66, 67, 68).

V. 14-Day Myocardium (48 - Somites)

A. Ultrastructural Observations

Details of the ultrastructure of 14-day myocardium and 13-day myocardium to a large extent remain the same. Most of the observations made on 13-day

myocardium can also be applied here.

Myofibrils appear to be tightly packed, and many of them fuse to form large fibrils (Figs. 77, 75). This is indicated by the observation that in micrographs of the same magnification the Z bands in 14-day myocardium are wider and longer than those of 12 and 13-day myocardium, and the I band is very prominent. Number of myofibrils per cell is also seen to have increased (Fig. 73).

Sarcoplasmic reticulum and T-system and tubules are further differentiated and more pronounced (Figs. 74, 76). Indentations of sarcolemma make contact with the myofibrils at the Z disc regions (Figs. 76, 74).

Golgi bodies appear very active, and numerous rows of secretory granules are recognizable in the region of this organelle. The cisternae are filled with flocculent material, and the granules are formed by what appears to be a pinching off process (Fig. 77). Secretory granules are round, membrane-bound and have a spiny or fuzzy appearance.

Mitochondria, granular endoplasmic reticulum, nucleus, etc. are in a stage of development similar to that observed in the 13-day myocardium.

The presence of neural elements*in embryonic myocardium is frequently observed in the 14-day myocardium. Long nerve cell processes are located between myocardial cells (Figs. 69, 70) and contain clearly recognizable neurofilaments and free ribosomes in the axoplasm. Nerve-muscle "contact" appears to be established by the evaginations or protrusions of sarcolemma towards the neural processes (Figs. 69, 70). In the regions where neural elements are recognized, the cell surfaces of both myocardial and neural elements are heavily coated with a layer of filamentous material. In cross-sections groups of nerve cell processes can be observed in close contact with myocardial cells (Fig. 71). Cross-sectional profiles of greatly distended endoplasmic reticulum

* See appendix

are very conspicuous in these cells (Fig. 71). Structures resembling synaptic vesicles are discernible in and around myocardial cells and may indicate the locus of nerve terminals in the myocardium (Figs. 72).

B. Cytochemistry

After incubation in medium containing ATP as the substrate, the final reaction product is deposited in a manner very similar to what is seen in 13-day myocardium. Localization seems to be restricted to cell surfaces, and the intensity of reaction appears to be less than noted in 10-day and 11-day myocardium (Figs. 78, 79, 80).

VI. New Born Myocardium

A. Ultrastructural Observations

A critical examination of the ultrastructure of new born myocardium makes possible the further elucidation and clarification of several fine details of developing organelles. Some structures which were in a primitive or premature state of development have undergone further growth and maturation and have attained definitive form and shape.

The sarcoplasmic reticulum and T-system and tubules are in an advanced state of differentiation, and the intimacy of association of T-tubules with Z lines is unmistakable (Figs. 81, 90). They may appear as small circular profiles next to the Z discs, representing longitudinal tubules in cross-sections, or appear as slender tubular profiles when cut in a horizontal or transverse plane. The sarcoplasmic reticulum appears as an elaborate network of tubules and anastomosing channels which, in tangential sections, appear as circular, diagonal and various other profiles. Points of contact between the sarcolemma and Z disc regions of the myofibril are very prominent, and the sarcolemma exhibits exaggerated indentations at regular intervals (Figs. 84, 81). Associated with

the sarcolemma is the active production of numerous smooth-contoured vesicles, which pinch off and move towards the myofibrils (Fig. 84). In this respect, it should be pointed out that vacuoles and vesicles occur in much greater abundance in the new born myocardium than in earlier stages. Smooth contoured (Fig. 84) and spiny or fuzzy vesicles are equally prevalent (Fig. 81).

Unlike the earlier stages, the cytoplasm is tightly packed with a great abundance of organelles. The granular endoplasmic reticulum is well developed and widely distributed. The cisternae are dilated and contain secretory products in the form of amorphous or flocculent material. This cell organelle is arranged in tubular whorls, in parallel skeins of cisternae, in ring-like configurations around mitochondria, in single sets of tubular profiles, and in various other configurations (Figs. 81, 91). The close topographical relationships of endoplasmic reticulum with other organelles, for example, mitochondria, Golgi bodies, myofibrils, etc., are very apparent.

The quantity and distribution of free ribosomal particles are considerably curtailed in comparison to earlier stages of development. Nevertheless, some ribosomes are associated with or attached to membranes of the endoplasmic reticulum and nuclear envelope, and a few free ribosomes are interspersed among myofibrils along with glycogen particles.

The nuclei are greatly elongated and occupy a central position in the cell (Fig. 93). Associated with the nuclear envelope and the surrounding cytoplasm are numerous small vesicles and vacuoles (Figs. 85, 93). Nucleolus is still a prominent feature of the nucleus, and the chromatin is either uniformly distributed in the karyoplasm or dispersed peripherally (Figs. 93, 85).

Mitochondria are well-formed, occur in greater numbers, and the cristae are closely packed. Most cristae are oriented in a transverse direction and do

not follow the zig-zag course seen in the adult cardiac muscle. In some instances, however, longitudinal cristae are also encountered (Figs. 86, 90). Mitochondria assume a definite pattern of distribution in that they occupy an interfibrillar position, arranging themselves parallel to the course of the myofibrils in the longitudinal axis of the cell (Fig. 84). The close relationship of mitochondria with myofibrils is very evident. At places it appears as though there is a tubular connection between mitochondria and the Z bands (Figs. 91, 84). Apparent open channels of communication between mitochondria and the anastomosing conduits of sarcoplasmic reticulum are also occasionally recognizable (Figs. 86, 91).

Glycogen occurs in abundance, and much of it is localized in the interfibrillar sarcoplasm where it is associated with the sarcoplasmic reticulum. It is also associated with the filaments of the myofibrils. Glycogen particles can be recognized in the regions of intercalated discs as well.

In the new-born, myofibrils are more organized, and, as a rule, they are closely packed into compact masses of fibrils. The fibrils are usually arranged parallel to one another in the longitudinal axis of the cell. Interfibrillary spaces are occupied by rows of mitochondria oriented in the same direction as the fibrils (Fig. 84). All the bands, with the exception of the M bands, namely, Z, I, A and H bands, are clearly delineated and well-organized; M bands are not discernible in any of the stages examined. The phenomenon of branching of myofibrils is still encountered. As in the earlier stages, branching occurs at the Z disc and intercalated disc regions (Figs. 88, 92).

The intercalated disc has undergone much growth and maturation. It has attained the highly plicated and interlocking nature of its membranes as seen in adult cardiac muscle. All the recognized components of adult intercalated

disc are easily distinguishable, namely, macula adherens, macula occludens and fascia adherens (Figs. 89, 90); fascia occludens, called nexus in smooth muscle, is also discernible (Figs. 90, 88). The presence of an intermediary line gives a trilaminar appearance to the intercalated disc (Fig. 87). It may be recalled here that this intemediary line was not recognizable in earlier stages.

Often times myofibrils are seen attached to two facing or opposing intercalated discs with the intervention of a single Z disc (Fig. 90). Normally, the regions where the next Z discs would have appeared are occupied by the intercalated discs. Further, it is the I band (actin filaments), and not the A band (myosin filaments), that is attached to these intercalated discs. This is analogous to the location of I bands next to Z discs. Occasionally, when an intercalated disc is located more than one, but less than two, sarcomere lengths away from the closest Z disc, another Z disc intervenes. In such a situation, however, the Z disc is so close to the intercalated disc that only an I band intervenes between the two; that is to say, the bands follow this sequence: Z band-I band-intercalated disc (Fig. 90). In other words, it can be visualized that myofibrils are attached to the intercalated discs by way of I bands or actin filaments.

Numerous small vesicles and vacuoles are consistently found to be associated with areas of intercalated discs, some of which are smooth contoured, some others are fuzzy or spiny, while yet others contain some sort of granular material in them, representing secretory vesicles (Fig. 87). Membranes of the intercalated disc produce some of these vesicles, and stages of formation of these from the membranes are easily recognizable (Figs. 94, 90). Existence of one or more desmosomes near and around the region of an intercalated disc is a constant feature (Fig. 88). Occasionally it appears as though there is a

protoplasmic continuity between myocardial cells through isolated gaps in the intercalated disc (Figs. 87, 94). At a site where there is an apparent opening or break in the intercalated disc, a structure resembling a micro-tubule is seen stretched across two cells (Fig. 87).

The new born myocardium is well-vascularized. Capillaries are freely interspersed between myocardial cells, and there is a very close proximity of myofibrils and capillary endothelial cells (Figs. 82, 83). The endothelial cell cytoplasm is characterized by an abundance of smooth contoured vesicles, and these vesicles appear in various states of formation from the endothelial membranes. The capillary lumen is usually filled with red blood cells or a flocculent material. Endothelial cells exhibit desmosomes at points of cell membrane contact.

Fibroblasts and connective tissue elements not frequently seen in the earlier stages can now be recognized in the myocardium of newborn. Long bundles of collagen fibers contained in fibroblasts are interposed between myocardial cells, and periodicity of collagen fibers is well-preserved in glutaraldehyde fixed material (Figs. 95, 96).

In some contracting regions of the new born myocardium, two types of cells can be recognized in that one stains more darkly than the other (Figs. 97, 98). The sarcolemma and intercalated disc form an abrupt and sharp line of demarcation between the two types of cells. The myofibrils of dark-staining cells are in an intense state of contraction, whereas the contraction in the light-staining cells is much less pronounced (Figs. 97, 98). The outer limiting membranes of mitochondria in these cells possess thick, dark, solid walls, and appear to fuse with one another and with the myofibrils, thus suggesting a structural continuity between individual mitochondria on one hand and between mitochondria

and myofibrils on the other. This sort of structural fusion or "amalgamation" is seen to a lesser degree in the light-staining cells (Figs. 97, 98).

B. Cytochemistry

Localization of ATPase is mainly restricted to membranes. The site of deposition of reaction product continues to be cell surfaces of myocardial cells, intercellular areas and small pinocytotic vesicles (caveolae)(Figs. 99, 100). The reaction product is also observed on red blood cells and fibroblasts (Fig. 101). Compared with 13 and 14-day myocardium, there appears to be a slight increase in the amount of deposit judging from the granule size and distribution.

Correlative Summary

To provide an overview of the results presented above, a brief account of the general characteristics and gross morphological aspects of the embryonic myocardium is presented here. This short account is followed by a table in which a comparative summary of the development of the cell organelles of the myocardium is presented for each day of gestation examined in this investigation.

The 10-day myocardium is composed of loosely packed mononucleated cells without any apparent specialized areas of cell-to-cell contacts. Three layers of cells can be recognized: a superficial layer close to the pericardium, a middle layer where the myofilaments become first identifiable, and a deep layer, bearing numerous microvilli, which borders the cardiac jelly. The cytoplasm of 10-day myocardium is characterized by a dearth of organelles. Large round nuclei with prominent nucleolei occupy most of the cytoplasm in which large amounts of ribosomes are widely dispersed. By the 11th day of gestation and progressively during the subsequent days of development there is a rapid proliferation and differentiation of most of the organelles . The myofilaments become organized into fully formed striated fibrils, mitochondria increase in number and contain well-delineated cristae, Golgi elements and endoplasmic reticulum exhibit perceptible morphological changes indicative of secretion, intercalated discs develop, desmosomes form, the distribution of ribosomes is progressively reduced, and by the time the newborn stage is reached the myocardium displays most of the features characteristic of adult myocardium.

Table of summary follows.

Table 2. A REFERENCE TABLE OF CORRELATED DEVELOPMENT OF RAT MYOCARDIUM (I)

Organelles	Day: 10 Somites: 6-10 Stage: 17-18	Day: 11 Somites: 15-24 Stage: 19-20	Day: 12 Somites: 26-36 Stage: 21-22	Day: 13 Somites: 36-46 Stage: 22-23	Day: 14 Somites: 48- Stage: 24-25	Newborn
Nucleus	Round, large, occupies most of the cytoplasm. One or two nucleolei. Nucleolonema conspicuous. Uniform or peripheral distribution of chromatin.	Slightly elongate. One or two prominent nucleolei. Uniform distribution of chromatin. Pericisternal space continuous with ER.	Longitudinally compressed. Otherwise, same as day-11.	Long and narrow. Parallel to the long axis, and centrally placed in the cell. Several vacuoles associated with nuclear envelope.	Same as day-13.	Same as day-13.
Cell membrane (Sarcolemma)	Not easily resolvable. "Matty material" coats the surface (glycocalyx, or external lamina). Microvilli on cells bordering cardiac jelly. Micropinocytosis - moderate.	Resolvable. Several invaginations and evaginations. Micropinocytosis-moderate.	Several folds and interlocking with adjoining cells. Scallops and makes contact with Z disc regions. Micropinocytosis-abundant.	Same as day-12.	Same as day-12.	Same as day-12.
Cell-to-cell contacts (Desmosomes)	Loosely packed cells, junctional complexes and tight junctions recognizable. Desmosomes not visible.	Intercellular spaces greatly reduced. Tighter packing of cells. Desmosomes begin to appear, but poorly differentiated.	Closely packed cells. Desmosomes are numerous and more differentiated.	Well developed desmosomes. Some are always found near intercalated discs.	Same as day-13.	Adult features of desmosomes readily visible.
Intercalated discs	Not yet differentiated. Not recognizable.	Recognizable. Straight or slightly wavy outline, not plicated. Myofibrils inserted at these regions. Micropinocytotic vesicles seen in the vicinity.	Easily discernible. Number slightly increased. Not fully developed. Trilaminar structure not visible. Slightly plicated. Many vesicles and vacuoles in the vicinity.	Same as day-12. Both fuzzy and smooth vesicles visible in the vicinity.	Longer, more plicated. Otherwise, same as day-13.	Highly plicated. Very long. Trilaminar structure and other adult features readily visible.
Myofilaments	Being synthesized. Isolated thick filaments (myosin) and occasional thin filaments (actin) present.	Filaments organized into fibrils. Isolated filaments present in large numbers.	Filaments organized into fibrils. Isolated filaments also present.	Filaments organized into fibrils. Isolated filaments seen occasionally.	Filaments mostly organized into fibrils. Isolated filaments still visible.	Mostly fibrils. Isolated filaments still seen.

Table 2. A REFERENCE TABLE OF CORRELATED DEVELOPMENT OF RAT MYOCARDIUM (II)

Organelles	Day: 10 Somites: 6-10 Stage: 17-18	Day: 11 Somites: 15-24 Stage: 19-20	Day: 12 Somites: 26-36 Stage: 21-22	Day: 13 Somites: 36-46 Stage: 22-23	Day: 14 Somites: 48- Stage: 24-25	Newborn
Myofibrils	Tufts of fibrils-"protofibrils," "non-striated" fibrils.	Long, well organized, in the long axis of the cell. Branching somewhat recognizable.	Well organized and fully formed. Several fibrils grouped together. Branching discernible.	Well organized and fully formed. Number of fibrils increased. Groups of fibrils appear. Branching discernible.	Well organized "sheets" or "masses" of fibrils. Myofibrillar number increased. Branching discernible.	Well organized "sheets" of fibrils. Number considerably increased. Branching very pronounced.
Striations A, I, Z, H and M bands	Developing Z band material recognizable. Filaments may or may not be attached to these Z bands. No other bands visible.	Banding pattern is readily recognizable. All bands except M-line visible.	Except M-line, all bands discernible.	M-line still not discernible.	Same as day-13.	All bands recognizable. M-line not visible.
Ribosomes	Both free & attached. Dispersed in great quantities, associated with the myofilaments. Chains of helical polysomes visible.	Abundant distribution. Associated with myofibrils. Polysomes discernible.	Same as day-11, but reduced in quantity.	Same as day-11, but reduced in quantity.	Same as day-13.	Same as day-13. Polysomes visible.
Sarcoplasmic reticulum and T-tubules	Not recognizable.	Small tubules associated with Z discs mark the beginning of T-tubules. SR barely discernible.	Z disc-T-tubule association very evident. SR elements become more apparent.	Same as day-12. SR more evident.	Same as day-13.	SR well developed and forms anastomosing channels between fibrils. Z disc-T-system association very conspicuous.
Mitochondria	Few in number. Poorly differentiated. Cristae not delineated. Assume filamentous form. Sparse distribution.	Rapid increase in number. Well formed. Cristae delineated, but loosely packed. Tubular and longitudinal cristae recognizable.	Same as day-11. Close association with myofibrils, ER, Golgi bodies recognizable. Increase in number.	Same as day-12. Cristae more closely packed.	Distributed in greater number in interfibrillary position.	Oriented parallel to the fibrils in the long axis of the cell. Many cristae & well-packed. Zig-zag cristae visible occasionally.

Table 2. A REFERENCE TABLE OF CORRELATED DEVELOPMENT OF RAT MYOCARDIUM (III)

Organelles	Day: 10 Somites: 6-10 Stage: 17-18	Day: 11 Somites: 15-24 Stage: 19-20	Day: 12 Somites: 26-36 Stage: 21-22	Day: 13 Somites: 36-46 Stage: 22-23	Day: 14 Somites: 48- Stage: 24-25	Newborn
Endoplasmic reticulum.	Sparse distribution. Present as individual elements. Stacks of cisternae not recognizable.	Much developed. Whorls and skeins of cisternae discernible. Wide distribution. Contains floculent material.	Wide distribution. Well developed. Contains amorphous material. Closely associated with the developing organelles in the cell.	Same as day-12.	Same as day-12.	Same as day-12.
Golgi elements	Poorly developed. Flattened cisternae. Few vesicles and vacuoles: sparse distribution.	Much developed. Random distribution. Distended cisternae. Many secretory vacuoles and vesicles. Secretory granules in the vicinity.	Large assemblage of cisternae, vacuoles, and vesicles. Random distribution. Well developed & very active.	Same as day-12.	Same as day-12.	Same as day-12.
Glycogen	Not recognizable.	Present in single particles and aggregates. Associated with myofibrils.	Same as day-11.	Same as day-11.	Same as day-11.	Associated with myofibrils and elements of SR.
Neural elements.	Not recognizable.	Not recognizable.	Not recognizable.	Long nerve processes containing neurofilaments readily identifiable.	Same as day-13.	Present. Newborn myocardium is innervated.
Vascular elements	Not visible	Not visible	Not visible.	Present	Present	Present
Connective tissue elements (fibroblasts, collagen)	Not visible.	A few collagen fibers visible.	Same as day-11.	More collagen fibers visible.	Same as day-13.	Bundles of collagen fibers & fibroblasts interposed between myocardial cells.
Nucleoside phosphatase (ATPase) activity.	++++ (Intense reaction)	+++	++	++	++	++-

DISCUSSION

The fine structure of the differentiating myocardium and its organelles of rat embryonic hearts of gestational age 10, 11, 12, 13, 14 days and the newborn has been studied for the purposes of examining the morphological aspects of cyto-differentiation and development. In the preceding section entitled "Results," the ultrastructural morphology of the developing rat myocardium is presented separately for each stage of development examined. The following discussion, however, is subdivided on the basis of cell organelles, not stages of development. This arrangement provides for greater clarity and conciseness when comparing the significance of the observations presented here with those presented in the pertinent literature.

I. Early Myocardial Cells

In the early 10-day embryos (6-10 somites, stage 17), the developing heart is a C-shaped tubular structure with no circulation of blood or fluids. The heart at this stage exhibits pulsatory movements, but regular rhythmic beating has not been established. The myocardium at this point is characterized by loosely packed cells held together by lateral cell-to-cell contact and developing junctional complexes; desmosomes are not yet visible. Towards the end of the 10th day of development (10-12 somites), the loosely packed cells arrange themselves into three layers: an inner layer bordering the cardiac jelly and bearing microvilli; a middle layer where, for the first time, morphologically identifiable myofilaments become recognizable; and a non-differentiated superficial layer close to the epicardium or pericardium. This layer of the heart becomes differentiated in subsequent stages of development, and in earlier stages of development does not uniformly invest the developing myocardium

(Manasek, 1969).

II. Cardiac Jelly

Of these three layers of the 10-day myocardium, it is in the middle layer that the contractile proteins first appear. The layer bordering the cardiac jelly is characterized by numerous cellular projections and processes or microvilli. Similar observations have been made by light microscopists (Davis, 1924; Barry, 1948; Patten et al., 1948). In the present study, however, these cellular processes appear to pinch off as free-floating bodies in the regions of the cardiac jelly. Based on this observation, this author suggests that the myocardial cells, at least in part, contribute to the production of the cardiac jelly.

The gelatinous material occupying the space between the endocardium and myocardium of developing hearts was first called cardiac jelly by Davis (1924). Patten, Kramer and Barry (1948) reported that cells from both the endocardial and myocardial layers migrate into this mucoprotein matrix to establish the loose reticulum of stellate cells characteristic of the early tubular heart. Numerous functions have been attributed to this material, among which is its valvular action in developing hearts (Barry, 1948). Cardiac jelly has been shown to be a sulfated acid mucopolysaccharide (Ortiz, 1958; Gessner and Bostrom, 1965; and others), and to lose its metachromatic staining properties after treatment with hyaluronidase (Barry, 1951). It has also been shown that in the early embryo the cardiac region is an active site of incorporation of inorganic sulfate (Johnston and Comar, 1957). The site of sulfation of the product of secretion is the Golgi body region in many types of cells (Berlin, 1967; Lane et al., 1964; Godman and Lane, 1964; Neutra and Leblong, 1966), and probably the same is true of myocardial cells. The results of radioautographic studies of Manasek

(1968) indicate that the embryonic myocardial cells actively incorporate labeled sulfate. On the basis of this evidence and the cytological evidence obtained in the present study, it can be inferred that one of the functions of embryonic myocardium is secretory, and that one product of this secretion is a component of cardiac jelly. Manasek (1969) has proposed a similar function in chick embryonic hearts.

III. Early Myofilaments

As mentioned earlier, the myofilaments are recognizable for the first time in the pulsating, but not beating, hearts of the 10-day embryos (6-10 somites, stage 17). The earliest identifiable filaments are the thick (myosin) filaments. This does not, however, preclude the possibility of the presence of thin (actin) filaments, which are not easily detectable because of the dense concentration and wide distribution of free ribosomes which could easily obscure their visualization. Consequently, when examining electronmicrographs, it is difficult to determine if the synthesis of thin filaments follows that of thick filaments or vice versa. It should be emphasized, however, that the thick filaments are easily recognized even in early stages of development.

There is considerable disagreement as to the temporal sequence in the synthesis and appearance of thick and thin myofilaments. Allen and Pepe (1965) reported that the thin filaments develop before thick filaments in chick somite cells, and that the thin and thick filaments observed in embryonic muscle are morphologically indistinguishable from those observed in adult muscle. Hay (1961) described the appearance of thick filaments before the appearance of thin ones in the development of myotomes of Ambystoma opacum larvae. She (1963) subsequently described two types of filaments in the myoblasts of developing salamander tails. Thick filaments in her material are easily identifiable, but

the thin filaments are difficult to recognize. Based on these later observations, she supported the idea that thick and thin filaments are formed simultaneously. The concept of the simultaneous formation of both filaments is also supported by the works of Ferris (1959) in the development of chick skeletal muscle and of Bergman (1962) in rat skeletal muscle. Przybylski and Blumberg (1966), from their investigation of myogenesis in chick branchial somites, reported the simultaneous production of both types of filaments at stage 15-16. In the development of heart muscle in the frog, Rana pipiens, Huang (1967) reported that the thick and thin filaments form at the same time. A similar situation is reported in somitic muscle of chick embryos (Dessouky and Hibbs, 1965) and in embryonic chick myocardial cells (Manasek, 1969). In regenerating muscle of rats (Price et al., 1964) the order of appearance differs from the above. In this system the thin filaments are observed in the mononuclear myoblasts, with thick ones appearing only after binuclearity has been obtained. Fischman (1967) reported that there is no clear-cut time interval separating the synthesis of thin from that of thick filaments. This observation is in agreement with the results obtained in this investigation and those reported by Hay (1963) and others, but in disagreement with the reports of Ogawa (1962), Allen and Pepe (1965), and others.

Experiments utilizing fluorescent antimyosin antibodies have shown that mononuclear skeletal muscle cells, which are synthesizing DNA, do not contain antigenic myosin. Myotubes also yield negative reactions (Stockdale and Holtzer, 1961). On the basis of precipitin reactions with extracts of whole chick embryos, Ogawa (1962) reported the presence of actin at 72 hours of development and myosin at 96 hours. Holtzer et al. (1957) have also utilized fluorescent antimyosin antibodies in glycerol-extracted chick embryos to demonstrate the presence of

contractile proteins in the development of branchial somites. Positive staining of cytoplasmic myofibrils in the mononuclear cells is evident just after cell elongation (50-60 hours) and is not detectable in the cytoplasm of non-elongate myoblasts (Holtzer et al., 1957, 1958). Therefore, it is plausible that either the filaments dispersed in the cytoplasm do not contain antigenic sites to anti-myosin antibodies, or that their concentration is too low for detection, or that glycerination of the tissue removes the filaments and/or deposits them at the periphery. For all practical purposes, however, one can assume that the filaments seen in electron micrographs are present at the same time as antigenically responsive proteins. One may also infer from these results of fluorescent antibody studies and electronmicrograph studies that chemodifferentiation probably coincides with morphodifferentiation.

It has been demonstrated in many studies quoted above that the number of thin filaments far exceeds that of thick filaments. It is conceivable that some free thick filaments were not visualized in the preparations of Allen and Pepe (1965), thus explaining why these authors reached different conclusions regarding the preferential or sequential appearance of thin and thick filaments. It can be argued, also, that the immunological method used by Ogawa (1962) may not have been sensitive enough to detect small concentrations of myosin in a large excess of actin. It would be somewhat premature to conclude from the published data that the synthesis of the two contractile proteins is initiated sequentially. The present study lends support to the idea of simultaneous production of thin and thick filaments.

IV. Structure of Myofilaments

Huxley (1963) has examined the structure of thin and thick filaments by mechanically disrupting isolated myofibrils and comparing, by negative staining,

the natural filaments with synthetic filaments prepared from pure protein solutions. The naturally occuring thick filaments, which are formed when the ionic strength of solutions of pure myosin is lowered, are so similar that one is inclined to conclude that the structure of the filament is a reflection of the properties of the myosin molecule itself. Both the natural and synthetic myosin filaments bear numerous regularly spaced, short, lateral projections, except in the central (H-zone) regions of the filaments. The development of the myosin filaments in solution can be deduced by comparing progressively larger aggregates. The smallest aggregates consist of two to eight myosin molecules in which aggregation has occured "tail-to-tail." Myosin is composed of one molecule of L-meromyosin and one of H-meromyosin. L-meromyosin is a rod-shaped particle and attached to it is a heavy-meromyosin molecule with a globular end (Zobel and Carlson, 1963; Huxley, 1963). It is this "tail-to-tail" aggregation which establishes the central region free of lateral projections, i. e., the globular heads. As the aggregate grows in both directions from this central region, molecules are added with each tail region overlapping the tail of the preceding molecule having its globular head projecting laterally from the axis of the filament. It is these laterally projecting globular heads which form the reversible cross-linkages between thick and thin filaments. Cross-linkages can, in fact, be demonstrated to occur in solution between thin filaments and myosin or H-meromyosin (Huxley, 1963).

Synthetic polymers of myosin (Huxley, 1963) and actin (Hanson and Lowry, 1963; Huxley, 1963) closely resemble their counterparts present in the A and I bands, respectively, of skeletal muscles, as can be seen in electronmicrographs of intact glycerinated muscle and the isolated fragments. The only difference is that the reconstituted actin occurs in varied lengths. Heavy-meromyosin has

been shown to contain the actin-binding and adenosine triphosphatase activity (Szent-Gyorgyi, 1953).

In the adult muscle, both cardiac and skeletal, there is a numerically greater abundance of thin filaments than thick filaments. The embryonic myocardium is no different in this respect. For example, in 11-day rat embryo, more thin filaments in the myocardium are recognizable than the thick type, although some of the thin filaments are overshadowed by a dense concentration of free ribosomes. The findings of many other workers in developing skeletal and cardiac muscle are in agreement with this observation (Hay, 1963; Allen and Pepe, 1965; Przybyloski and Blumberg, 1966; Fischman, 1967; Huang, 1967; Manasek, 1969; and others). Fischman (1967) reported that there is a greater than 7:1 ratio of thin to thick filaments in embryonic chick skeletal muscle.

V. Myofibril Orientation

After the myofilaments are formed, they are organized into a myofibril, which when fully formed exhibits the characteristic striations and sarcomeric subdivisions. A single sarcomere, which is the unit of length between successive Z lines, is constructed of an ordered series of thin filaments attached to each lattice-like Z band. The distal portion of both groups of thin filaments interdigitate with a single array of thick filaments in the middle of the sarcomere. Huxley and Hanson (1960) have proposed that contraction is effected by the thin filaments sliding along the thick filaments, thereby drawing the Z bands closer together. It has also been established that actin is restricted to the I band region, which contains the thin filaments, and myosin to the A band containing the thick filaments (Huxley and Hanson, 1957; Holtzer et al., 1957). In cross sections, when viewed at higher magnifications, myofibrils are con-

sistently and characteristically seen to be composed of hexagonally packed thick and thin filaments. Considering that the thin filaments interact with the cross bridges of the thick filaments (Huxley, 1958), a hexagonal array of thin filaments around each thick filament seems to be a most stable configuration.

It is seen in the rat embryonic myocardium that the myofilaments, once synthesized, are oriented lengthwise and are then organized into a sort of primitive fibril, named in this study the "protofibril." The protofibril may appear as a tuft of fibrils, that is to say, a few filaments being attached to a developing Z disc region and extending in both directions. In other areas the filaments are seen to be organized in the form of "non-striated" fibrils where they are very loosely held between two or three successive Z bands. A and I bands are not distinguishable in these fibrils, and they are characteristically found in 10-day (8-10 somites) myocardium. By the 11th day (15-17 somites), well-organized fibrils exhibiting the adult banding pattern, with the exception of M bands, are easily recognizable. Allen and Pepe (1965) reported also that in embryonic chick skeletal muscle small aggregates of thick and thin filaments become aligned and form what appears to be loosely organized, non-striated myofibrils, but they make no reference to Z bands in these fibrils. The fact that non-striated fibrils appear before striated ones is in agreement with many early workers in light microscopy (Godlewski, 1902; Duesberg, 1910; Haggquist, 1920; Weed, 1936; Holtzer et al., 1957; Holtzer, 1961). It has been proposed by Holtzer (1961) that there is a period of several hours in the history of every myoblast in which it contains fine non-striated longitudinal myofibrils. He suggested further that the new non-striated sarcomeres are added meristematically at the tips of already cross-striated myofibrils. Allen and Pepe are in agreement with this idea. The present study can neither confirm or deny the concept of meristematic growth.

Starting with the 11th day of development (15-24 somites), however, the myocardial cells contain long, fully formed, striated myofibrils, frequently oriented in the long axis of the cell. Nonetheless, it should be pointed out that at any given time of development some cells are much more advanced than others. Thus, it is obvious that the histo- and cytodifferentiation of the heart are not at all consistent throughout the organ. Even as late as 12 days in the chick embryos (Weissenfels, 1962) or 18 days in rabbit (Muir, 1957), poorly differentiated myoblasts are still present with primitive myofibrillar bundles indistinguishable from those in early tubular hearts. This certainly is the situation in the embryonic rat myocardium, as revealed by the present investigation.

VI. Precursors of Myofibrils

In the orientation and organization of the filaments into fibrils no visible physical transformation can be detected, in the present investigation, between the filaments and any intracellular organelles. Suggestions and proposals to the contrary cannot be substantiated or sustained in the light of evidence presented in this study. The filaments do not develop from any preformed organelles. The mitochondria do not transform directly or indirectly into myofibrils, as proposed by Duesberg (1910), Meves (1909), Naville (1922), and others; nor do they act as template for the condensation of cytoplasmic particulates into fibrils as envisioned by Cowdry (1926) and Regaud (1909). A reticular network of the cytoplasm seen in embryonic cells has been suggested to have formed the fibrils, or to have elaborated a precursor for their formation (MacCallum, 1898). Numerous cytoplasmic granules seen in the myoblast were thought to align and fuse into fibrils (Echlin, 1965; McGill, 1910; Moscona, 1955; Weed, 1936). The interested reader is referred to the "Introduction" for details of other theories of early workers on this subject. The smooth surfaced membranes of the sarcoplasmic

reticulum are not present or are not present in sufficient quantity to be a significant factor in the synthesis, alignment or aggregation of myofilaments.

In the rat embryonic myocardial cells the filaments and fibrils are scattered throughout the cytoplasm, and no selective subsarcolemmal orientation or proliferation is noticeable. On the other hand, in embryonic skeletal muscle, filament synthesis and orientation are most pronounced in the subsarcolemmal regions of the cells where myofibrils first develop (Fischman, 1967; Przyblyski and Blumberg, 1966; and others), and microtubules are said to be most numerous in these regions. In the myocardial cells of the rat embryo, microtubules are seen only in mitotic cells, and there is no subsarcolemmal restriction to their distribution. It has been reported that microtubules may be involved in cytoplasmic streaming, intracellular fluid movements, and cellular elongation (Slautherback, 1963; Behnke, 1964; Tilney and Porter, 1965; Tilney, 1968). The experimental studies on the axopodia of Actinosphaerium nucleofilum, a protozoan, furnish very pertinent data on this subject (Tilney et al., 1966). If microtubules play a similar role in developing muscles, then alignment of myofilaments might be expected in the vicinity of microtubules, provided the cytoplasmic streams set up by the microtubules had produced a sufficient velocity gradient to orient these highly asymetric filaments. This may or may not be the case in the embryonic skeletal muscle where subsarcolemmal and peripheral distribution of myofilaments and fibrils is a regular feature. No such phenomenon is observed in embryonic myocardium. Further, even in embryonic skeletal muscles, myofibrils in early stages of formation and aggregates of myofibrils can be seen deep within many cells, where the presence of microtubules is not observed as regularly as seen subsarcolemmally (Fischman, 1967; and others). Cedergren and Harary (1964) observed, in some cultured beating heart cells of rat, developing myofibrils

near the periphery where "dark thickenings" of the plasmalemma were said to occur at regular intervals, and they speculated that the first filaments may develop from the plasma membrane. The "dark thickenings" described by these authors probably represent the developing intercalated discs with the attached myofilaments; this might have given these investigators the impression that myofilaments arose out of the plasmalemma, which could explain the basis of their speculation.

VII. Origin of Striations

The origin and detection of striations in the myofibrils have been variously described by the early workers. Some of the authors reported that striations are formed in the cytoplasm before the appearance of myofibrils (Luna, 1913; Cameron, 1917; Lewis, 1919; Naville, 1922), while others speculated that myofibrils and striations are formed concurrently, striations being an integral part of the myofibrils (MacCallum, 1898; Wieman, 1907). Still others postulated that the myofibrils are first formed as a homogeneous structure, and that the striations differentiate later (Bardeen, 1900; Asai, 1914; Schmidt, 1927; Weed, 1936).

Many light microscopists have stated that the A and I bands are the first to become visible, but the time of their appearance has been placed at various stages and times in chick embryos, for example, 36 to 41 hours or 10 somites (Rouget, 1863; Kurkiewicz, 1910; Bruno, 1910); 4 to 6 days (Schockaert, 1909); 5.5 to 6 days (Wieman, 1907); and 7 days (Schlater, 1906). Heidenhain (1899) reported the presence of Z bands, as well as A and I bands, in the heart of 3-day duck embryos. Duesberg (1910) observed these three bands in the 60-hour chick embryos and, according to him, A bands appear first as swellings spaced regularly along homogeneous fibrils, and Z bands develop almost simultaneously

midway between the A bands. Lewis (1919) reported seeing A, I and Z bands, but no fibrils, in both fixed and living cardiac tissue from 10-somite chick embryos.

The observations of this investigation reveal that condensations of the Z band material are the first banding pattern to be observed in the 6-8 somite, 10-day myocardium. They are detectable as free, amorphous, electron-dense areas with or without attached myofilaments, and it is inferred here that the Z band material is produced concomitantly with the production of the myofilaments or immediately thereafter. The findings of this study are in agreement with many workers; however, the time of appearance of the Z band is usually placed later in development by many investigators (Weed, 1936; Van Breeman, 1952; Hibbs, 1956; Wainrach and Sotelo, 1961; Hay, 1963; Shafiq, 1963; and Huang, 1967).

Van Breeman (1952), who performed the first studies of skeletal myogenesis utilizing electron microscopy, stated that a constriction at the site of the future Z line is the first banding pattern. Hibbs (1956), from electron microscopic studies of developing cardiac muscle of chick, concluded that the Z bands appear concurrently with or shortly after the first orientation of myofilaments into myofibrils. In the ultrastructural studies of regenerating flight muscles, Shafiq (1963) could find at no time during development myofibrils which do not contain a Z band. Wainrach and Sotelo (1961) and Hay (1963) have postulated that Z bands first appear as amorphous, densely staining bodies to which myofilaments become secondarily attached in embryonic chick hearts and skeletal muscle, respectively. In the fine structure studies of developing chick skeletal muscle, Allen and Pepe (1965) noticed tubules at regular intervals along the early myofibrils and claimed that this tubular system is the first indication of a banding pattern (stage 20). By stage 24 a dense, osmophilic substance (Z line) is some-

times found associated with the myofibrils at the level of these tubules and, according to these authors, the I bands are visible only after the Z lines are present. Przybylski and Blumberg (1966), based on their ultrastructural investigation of skeletal myogenesis in chick embryo, reported that by stage 16-17 various foci in the sarcoplasm showed aggregates of filaments forming sarcomeres with recognizable A and I band striations and with electron-dense Z band material or smooth membranous tubules bisecting I band filaments. Electron microscopic studies of frog hearts led Huang (1967) to report that A and I bands are formed simultaneously and that M lines and H zones appear to develop during the formation of A and I bands. He observed that the development of Z lines is a much slower process than the development of A and I bands in the frog embryonic hearts.

Heuson-Steinnon (1965) speculated that the Z band material is derived from a pinching off of invaginations of the plasma membrane. He may have arrived at this conclusion from the fact that the sarcolemma scallops and makes connections with the Z discs of some of the subsarcolemmal myofibrils. In this study, however, this phenomenon is seen only in cells of advanced embryonic development where well-formed striated fibrils are already discernible throughout the cytoplasm. Further, Z band regions with or without attached myofilaments are distinguishable deep in the cytoplasm of 10-day rat embryonic myocardium, and they are seldom seen next to the plasmalemma. Also, no connection between the developing Z disc regions and the plasmalemma is detectable at the early stages of myocardial development in the rat embryo. Heuson-Steinnon further suggested, in addition to his previously discussed proposal regarding the source of Z band material, that the Z band densities are spaced at regular intervals within the cytoplasm prior to the appearance of myo-

fibrils and speculated that the sarcomere spacing is regulated by the prior spatial deposition of Z band material on to which thin filaments are then attached. The evidence from the present investigation does not substantiate such a suggestion or speculation.

The observation that Z bands may be seen as centers from which myofilaments arise in irregular directions has suggested to several authors that the Z bands may be the site of growth or deposition of myofibrillar material (Challice and Edwards, 1961; Fawcett and Selby, 1958; Meyer and Queiroga, 1961; Wainrach and Sotelo, 1961). Grimley and Edwards (1960) have commented upon the structural continuity and similarity between Z bands and intercalated discs, suggesting that these structures may be involved in sarcomere function. This idea was supported by Franzini-Armstrong and Porter (1964), who postulate that the Z disc is a modified membrane structure, homologous to the desmosome and intercalated disc. In the present study, however, structural continuity or a similarity of these two structures is not obvious, and there is no evidence to support the claim that the Z disc is a membrane structure. It should also be noted that, biochemically, the Z band has been shown to be composed of the protein, tropomyosin (Reedy, 1964).

VIII. Mechanism of Myofibril Orientation

There are several theories and models proposed for the eventual alignment and orientation of the myofilaments into the adult myofibril. Three such hypothetical models may be considered here.

One such theory envisages a model consisting of thick filaments packed in an hexagonal array, spaced and held in position by the cross-bridges between thick filaments in the M band (Franzini-Armstrong and Porter, 1964; Page, 1965). Thin filaments either attached or unattached to Z band material could then be

aligned with already organized thick filament lattices. A second model, proposed by Fischman (1967), postulates that thick and thin filaments are packed in an hexagonal pattern solely as a consequence of the cross-bridges linking the two sets of filaments. A Z-band lattice would form at the free ends of thin filaments which already contain Z band material at these ends. And the third proposed model postulates the formation of a Z band lattice (Knappies and Carlson, 1962; Reedy, 1964), presumably composed of tropomyosin, which provides an attachment site upon which thin filaments can be spatially positioned. Thick filaments are spaced secondarily by cross-bridge attachments to the already positioned thin filaments. In this model, Z band lattice formation is a necessary prerequisite for myofibril formation. The first and second models would not necessitate the prior synthesis of Z band material for the hexagonal placement of the myofilaments. All of these models, however, would require Z band material for the longitudinal myofibrillar growth, for it is assumed that this material is always interposed between thin filaments in back-to-back sarcomeres.

No one theory or model proposed to date could satisfactorily explain such a complex and complicated process as the alignment and orientation of filaments into a well-organized striated myofibril. The present investigation lends support to the third and second models. Z band material is shown to be present in the myocardium with or without attached filaments. Further, Knappies and Carlson (1962) and Reedy (1964) have demonstrated that thin filaments are branched at their Z band insertion. This is in agreement with the branching at Z band regions observed in the present study.

IX. Ribosome and Its Role

Large quantities of free ribosomes and polysomes are found widely distri-

buted in the developing myocardial cell of rat embryos. As more and more filaments are formed and organized into fibrils, there is a corresponding decrease in the amount and distribution of ribosomes in the cytoplasm. Throughout development of the myocardium, ribosomes are found associated with the differentiating fibrils. A similar phenomenon is noted in embryonic chick heart (Manasek, 1969) and in developing skeletal muscles (Przybylski and Blumberg, 1966; Allen and Pepe, 1965; and others). It has been suggested that this temporal relationship may reflect the first gene-initiated activity of a ribosome-messenger RNA complex involved in the synthesis of contractile proteins (myosin and actin) or some other protein synthetic event of differentiating muscle cells. In the myocardial cells of rat embryos, the ribosomes are very closely associated with the non-aggregated myofilaments. These ribosomes form long zig-zag chains probably representing helically arranged particles, and isolated strands of filaments may be observed being "pushed out" from these chains. Such a close spatial relationship may facilitate the immediate organization of the rapidly synthesized monomers of filaments into polymers. Herrmann (1952) has shown that actomyosin does accumulate at an exponential rate during the latter half of embryogenesis. The ribosomes in sarcomeres would also explain the presence of ribonucleic acid in myofibrils isolated by cell fractionation and centrifugation (Perry et al., 1959; Winnick and Winnick, 1960).

Since the first demonstration of the existence of the multiple ribosomal structure, the polysome, and its role in protein synthesis (Warner et al., 1962, 1963; Slayter et al., 1963; Wettstein et al., 1963), several investigators, including this author, have reported the presence of structures suggestive of polyribosomes in developing muscle. Waddington and Perry (1963) reported the presence of helically arranged ribosomes in the tail somites of Rana pipiens embryos.

Heuson-Steinnon (1964) has described similar helically arranged ribosomes, apparently held together by a filament. These chains in the embryonic myocardium of rat are associated in some cases with the endoplasmic reticulum, with myofibrils or are free in the cytoplasm.

The significance of these multiple ribosomal structures observed with the electron microscope can be understood only by reference to studies in which the ribosomal monomers have been demonstrated to be both held together by RNA and to be active in amino acid incorporation (Warner et al., 1963). Breuer, Davies and Florini (1964) have examined such RNA-ribosomal correlations in cell-free preparations of adult rat skeletal muscle. Their isolated ribosome fractions contain a spectrum of multiple ribosome structures in which the most rapidly sedimenting fractions have the highest specific activity after labeling in an in vitro protein-synthesizing system. Using appropriate enzymatic treatments, they demonstrated that the integrity of the polysomal structure is maintained by RNA and not by DNA, protein or lipid, and they examined the treated preparations by both sucrose density and electronmicrographic techniques. Their most striking finding is the extremely long chain lengths of some of these polysomes (60-100 monomers per chain). Based on alternative assumptions of the structure, that is, extended or coiled, of the associated messenger RNA, they speculated that a protein of the size of the myosin subunit could be accommodated by a m-RNA molecule large enough to form a polysome containing 60-70 monomers.

In the present study individual ribosomes and chains of ribosomes (polysomes) are observed to be present even in early developmental stages examined (10-day myocardium, 6-8 somites), whether or not myofilaments are identifiable. Allen and Pepe (1965) claimed that in chick skeletal muscles, the thin filaments

are formed first, but that thick filaments and polysomes occur later and simultaneously. Przybylski and Blumberg (1966), on the other hand, reported that thin filaments and polyribosomes are recognizable at the same time. They stated, however, that free ribosomes and helical aggregates of ribosomes are seen in the cytoplasm of all stages of embryonic development from the onset of myogenesis to hatching, but not before myogenesis. Huang (1967) reported that, in the development of frog myocardium, ribosomes, particularly polysomes, are seen to associate very closely with developing myofilaments, and many of these ribosomes are arranged in a helical fashion. In chick embryonic skeletal muscle, myofilaments become visible in the electron microscope in elongated cells containing abundant quantities of free RNP granules (Fischman, 1967). In cultured beating heart cells of rat, Cedergren and Harary (1964) observed helices and chains of polysomes parallel to the developing myofilaments. These cell organelles are most abundant where the filaments are seen undergoing elongation. This agrees, to a large extent, with the findings of the present investigation.

It has been repeatedly stated by many workers that ribosomes are involved in the process of muscle protein synthesis in developing cells, e. g., in the rat fetus by Behnke (1963), in Rana pipiens by Waddington (1963), in flight muscle by Shafiq (1963), to mention a few. It has been also suggested that aggregates of ribosomes are the structures actively involved in protein synthesis (Warner, 1963). Evidence has been put forward to show that the polysome structure is the result of attachment of ribosomal particles to messenger RNA (Slayter et al., 1963). They have reported that isolated polysomes show a thread-like connection from ribosome to ribosome, which is sensitive to ribonuclease. If the muscle polysome is a helix, the most likely possibility is that the ribosomes are attached in a spiral arrangement to an extended messenger-

RNA. This would allow a close packing of ribosomes along the RNA molecule and a more efficient utilization of the coding information contained in the polynucleotide arrangement in the messenger RNA. One concept put forth (Warner et al., 1963) for the mechanism of polysomal activity is that each ribosome attaches to the beginning of the messenger-RNA, travels along the polynucleotide synthesizing the polypeptide, and comes off the end of the RNA with the completed protein. Thus, a chain of ribosomes would contain various stages of the completed protein. What is most suggestive in this regard in the present study is the parallel arrangement of the polysomal chain to the myofilaments and the location of these chains in areas of what appears to be active myofilament synthesis. It appears that the polysomal chains are specifically localized and oriented for the synthesis of myofilament proteins in the region of myofilament formation. In one of two models proposed for myofilament synthesis (Cedergren and Harary, 1964) the ribosomes are attached along the nucleotide, winding around it helically until the protein is completed. Once completed, the filament protein molecule detaches and is available for filament assembly. In the second model the messenger-RNA itself may be arranged helically with the ribosomes attached. Since polysomes 6000 A in length have been observed, this would mean that the helical messenger-RNA, where extended, would be extremely long. The length of the linear polysome isolated from reticulocytes has been reported to be 1500 A (Slayter et al., 1963). This has been correlated with the messenger-RNA necessary for the synthesis of a hemoglobin chain with a molecular weight of 16,000 containing about 150 amino acids. The extreme length of the polysomal helices in the muscle may be related to the length of the messenger-RNA necessary to synthesize such a large molecule as myosin. The molecular weight of dog cardiac myosin has been reported to be about

240,000 (Ellenbogen et al., 1960), and skeletal myosin about 500,000 (Von Hippel, 1958).

X. Granular Endoplasmic Reticulum

The presence of large amounts of granular endoplasmic reticulum in the developing myocardial cells of the rat is a significant observation. In this respect, the myocardial cells differ from the developing skeletal muscle cells where very limited amounts and sparse distribution of granular endoplasmic reticulum are a regular feature (Przybylski and Blumberg, 1966; and many others). Very perceptible changes are noticeable in the amount and state of activity of the endoplasmic reticulum during the period of embryonic development covered in this investigation. 10-day myocardial cells contain relatively small quantities of granular endoplasmic reticulum in the form of irregular and short tubules; cisternae and vesicles are not very apparent. By the 11th and subsequent days of development, there is a rapid proliferation and increased distribution of this organelle throughout the cytoplasm. They assume varied configurations and are observed closely associated with other developing organelles, such as myofibrils, mitochondria, Golgi bodies, etc. By this time the cisternae and vesicles are very prominent and contain a flocculent material, presumably representing some sort of secretory product.

The degree of development of the granular reticulum and the relative proportions of its tubular, cisternal and vesicular elements vary greatly in different cells and in different phases of physiological activity, but the reticulum reaches its greatest development in glandular cells elaborating a protein-rich secretory product. Granular endoplasmic reticulum is characteristic of cells with secretory functions, whereas large amounts of free ribosomes are generally correlated with production of intracellular protein The close association of

ribosomes with the membranes of the reticulum appears to be necessary only when the product of protein synthesis is to be exported from the cell as a secretion. The elaborate system of intercommunicating channels comprising the reticulum provides an intracellular compartment for segregation of the product and a system of pathways for its transport to the Golgi region, where it is concentrated and packaged in the form of secretory droplets or granules and eventually released.

XI. Golgi Complex

Golgi complex undergoes a pattern of development similar to that of the granular endoplasmic reticulum in the developing myocardial cells. Whereas in 10-day myocardium sparse amounts of Golgi elements are recognizable in the form of small assemblage of membranes with no discernible cisternae nor any appreciable amount of small vesicles and vacuoles, by the 11th day (15-24 somites) and during subsequent development there is a sudden increase in the amount and distribution of Golgi elements. Hypertrophied large assemblage of multilayered arrays of cisternae, and a substantial number of vesicles and vacuoles are easily distinguishable in this organelle. An amorphous material is frequently discernible in the cisternae and vesicles, and large numbers of membrane-bound, electron-dense secretory granules are visible in the Golgi region and vicinity. A distinct feature of Golgi complex of the rat embryonic myocardium is its non-polarity and random distribution in the cytoplasm.

XII. Secretory Function of Myocardium

The presence of large quantities of free ribosomes and the rapid proliferation of granular endoplasmic reticulum and hypertrophied Golgi elements and secretory granules strongly suggest that the developing myocardial cells, unlike the skeletal muscle cells, may be synthesizing not only enormous amounts of

intracellular proteins in the form of myofilaments, but also synthesizing large quantities of proteinaceous material for secretion. Since developing skeletal muscle contains very little or no granular endoplasmic reticulum, this organelle may not have a direct role to play in the production and elaboration of myofilaments and fibrils. These observations lead one to infer that the developing myocardial cells start secreting material at the same time as recognizable myofilaments become evident and, as is seen in this study, continue to do so through myofibril synthesis. Membrane-bound secretory granules are frequently seen in rat embryonic myocardium. As far as is known to this author, the presence of secretory granules in embryonic myocardium has not been previously reported. The presence of these granules again lends support to the secretory activity of the myocardial cells. Jamieson and Palade (1964) have demonstrated the presence of granules in adult atrial cells. Further, the secretion of a steroid, physiologically similar to aldosterone, by mature cat myocardium is described by Lockett (1967). These observations lead one to conclude that the adult myocardium, like the embryonic myocardium, also has a secretory function in addition to the function of contraction.

XIII. Nuclear Envelope

The granular endoplasmic reticulum is reported to be continuous with the outer membrane of the nuclear envelope in a variety of cells. Embryonic myocardium provides a good source to study the reconstruction of the nuclear envelope by the coalescence of isolated tubular elements of granular endoplasmic reticulum around the nuclear mass in telophase. Consequently, one would expect the outer and inner membranes of the nuclear envelope, which is made up of and is a part of the granular endoplasmic reticulum, to bear ribosomes. And this is exactly the case in the cells of rat embryonic myocardium. It is sur-

prising, however, that all the references in literature indicate that only the outer membranes bear ribosomes (Watson, 1959; Moses, 1960; Fawcett, 1966; and others); no reference is made to the fact that the ribosomes are also attached to the inner membrane of the nuclear envelope. This may be due to the fact that invariably the ribosomes on the inner membrane are obscured from view by the dense concentration and peripheral distribution of chromatin material, a condition not so pronounced in the early embryonic rat myocardium. The incidence of nuclear pores should be a natural and expected phenomenon considering the way in which the nuclear envelope is reconstructed in telophase by the end-to-end apposition and coalescence of tubular portions of endoplasmic reticulum. The pores could occur at the sites of coalescence. Following the same line of reasoning, the continuity of the outer membrane of the nuclear envelope with granular endoplasmic reticulum is also a logical relationship which would be expected.

XIV. Mitochondria

The manner of development of mitochondria in the rat myocardial cells follows a pattern similar to those of Golgi elements and granular endoplasmic reticulum in that there is a progressive and rapid increase in the proliferation and differentiation directly correlated with the age of the embryonic myocardium. In the 10-day myocardium, for example, the cristae and outer limiting membrane are very poorly formed, and the mitochondrial distribution is very sparse. During the 11th day and subsequent stages of development, however, the number of mitochondria is substantially increased, and the cristae are numerous, closely packed and distributed widely throughout the cytoplasm and also in close proximity to myofibrils. Both tubular and longitudinal cristae are present, and the zig-zag cristae seen in adult cardiac muscle are occasionally noticeable

in the newborn, but mitochondrial granules are seldom observed. The cristae do not appear to be arranged in a spiral or concentric pattern as previously reported by Moore and Ruska (1957) in adult rat myocardium. Interfibrillar arrangement of the mitochondria parallel to the long axis of the cell and parallel to the myofibrils, is partially completed by the time of birth. Even in the new born myocardium, however, mitochondria are still distributed throughout the sarcoplasm, and no distinct overall pattern is visible as has been reported in the development of skeletal muscle (Frost, 1954; Allbrook, 1962; Dessouky and Hibbs, 1965; and others). An arrangement of one mitochondria per sarcomere of skeletal muscle as described by Hodges et al. (1954) and Porter and Palade (1957) is seldom noticed in the rat embryonic and fetal myocardium. In developing skeletal muscle Przybylski and Blumberg (1966) have reported that with the appearance of myofilaments the matrix density increases markedly, vacuolated areas are no longer obvious, and intercristal space seems enlarged. These features are, however, fixative sensitive. It has been shown that antimyosin A destroys myotubes in skeletal muscle, probably through its binding with a mitochondrial component, since it acts between cytochrome b and c. Herold and Borel (1963) have demonstrated the progressive changes in the composition of the cytochrome system in honey bee wing muscle during development. Cytochrome b_5 and $a+a_3$ are the only detectable cytochromes during the pupal period, whereas cytochrome b_5 abruptly becomes undetectable when myofibril synthesis stops. The suggestion is made that its initial presence may coincide with the onset of fibrillogenesis.

The increase in the number of myocardial mitochondria and their proximity to myofilaments, directly correlated with embryonic age, suggest an increase in cardiac dependence on oxidative metabolism for energy with advancing develop-

ment. This interpretation is supported by a recent report by Cox (1970) which indicates that isolated 11-day (16 somites) rat hearts can maintain cardiac contractile rates at normal levels in the absence of O_2, whereas the 12 and 13 day hearts exhibit progressively reduced heart rates in an anaerobic environment

XV. Sarcoplasmic Reticulum and T-System

The interfibrillar spaces of cardiac and skeletal muscle fibers in adult vertebrates contain two distinct membranous systems: the T-system and the sarcoplasmic reticulum. The T-system consists of T-tubules, oriented transversely between the fibrils. The sarcoplasmic reticulum is oriented longitudinally between fibrils, and in the cardiac muscle it is a system of anastomosing membrane-limited channels composed predominately of smooth surfaced or agranular membranes. The terminal segments of the sarcoplasmic reticulum face the T-system and show transverse orientations at this level. Two terminal segments of sarcoplasmic reticulum and a tubular element of the T-system between these segments have been called a triad (Porter and Palade, 1957). A triad is characteristically found near each junction of the A and I bands in reptiles, birds and mammals.

Examination of the developing rat myocardium affords an opportunity to trace the development of the T-system. In the 10-day myocardium, where there are no organized, fully-formed myofibrils, no trace of this system can be detected. In the 11th and subsequent days of development, where the cells contain increasingly large numbers of myofibrils, for the first time the beginnings of the formation of the T-system can be visualized in the form of small infoldings, small vesicles and tubules disposed in close association with the Z band regions. The tubular elements are oriented transversly, and when two or three Z discs of

adjacent myofibrils line up end-to-end, that is, are in register, these tubules can be seen stretched across from one Z disc to the next. The anastomosing network of membrane-limited channels of sarcoplasmic reticulum is not discernible until the 13th and 14th day of development, and in the newborn these channels are very prominent. Triads are not recognizable at any stage of development examined in this study. The literature contains, to this author's knowledge, no recorded observations of T-system at such an early stage of development as reported here. Bergman (1962), in his electron microscope study of the morphogenesis of rat skeletal muscle, observed channels of sarcoplasmic reticulum in 17-20 day rat fetuses, but no mention is made of T-tubules and triads. Walker and Schrodt (1968) studied muscle fibers of 19-day fetal rats and reported that there is an extreme sparsity of triads at this stage of development. This is emphasized by the fact they reported that "...in about 400 electron microscopic examinations of longitudinal sections and cross sections of gastrocnemius and psoas muscle fibers, structures identified as triads were seen in only 45 sections." These triads are usually oriented longitudinally and rarely transversely, which is not the usual orientation in the adult. This may cast some doubt as to whether these structures are really triads at all. These authors further state that, "It is reasonable to assume that these dense structures might represent partially formed triads." It is clear that no definitive triads have been reported in fetal tissues and probably develop much later in development. Schiebler and Wolff (1966) examined rat fetal hearts from the 14th day of gestation to the 45th day after birth and reported that, in the histogenesis of cardiac muscle of rat, the sarcoplasmic reticulum acquires its characteristic differentiation after the 24th post-natal day. According to these authors, by the 31st day of life (post-natal) the trans-

verse system (T-system) shows its typical structure, although from a quantitative point of view it is not fully developed. In skeletal muscle the terminal cisternae of the sarcoplasmic reticulum are said to be connected to the nuclear envelope (Peachey, 1965; and others), but no such connection is observed in rat embryonic myocardium. The findings of the present study show that the formation of myofibrils preceeds that of the sarcoplasmic reticulum and T-system.

In order to put in perspective the significance and possible roles of these organelles, it is desirable to consider their functions in the adult tissue, where exhaustive biochemical, physiological and ultrastructural studies have been made for the elucidation of these functions. For example, Porter and Palade (1957), in their description of adult sarcoplasmic reticulum, proposed two major functions for this system. One of the functions is that the channels may serve as conduits for the transport of metabolites to and from the interior of the cell, and the second function is that the limiting membrane may be important in the conduction of an electrical impulse into the interior of the cell, that is, it is involved in the excitation-contraction coupling. This second function has been reinforced by the works of Huxley and Taylor (1958). The sarcoplasmic reticulum has also been suggested as the intracellular site of relaxing factor synthesis or storage (Huxley, 1960).

The role of the transverse tubular system in excitation-contraction coupling is discussed at length in recent literature (Dreifuss, 1966; Forssman, 1966, 1970; Huxley, 1964; Katz, 1967; and many others). The suggestion is made that in skeletal muscle the T-system synchronizes the contraction of the myofibrils through the entire cross section of fast muscle fibers. The T-tubules perfect this feature by providing a passageway for the inward spread of excitation, the precise mechanism of which still remains to be resolved . Some authors

reason in terms of an electrotonic depolarization of the T-tubular membrane (Sandow, 1965; and others), while others favor the hypothetical shunting of part of the action current toward certain strategic loci of the contractile machinery through points of the tubular membrane that display particular permeability features (Girardier, 1965; Girardier et al., 1963; Reuben et al., 1967).

In the adult myocardium the problem is more complex than in skeletal muscle due to the plexiform arrangement and small size of the cells. Nelson and Benson (1963) argued that the very size of the myocardial cells makes unnecessary a specialized structure insuring the inward spread of excitation, since diffusion of an activator, for example, calcium ions, from the excited plasma membrane to the contractile machinery would be fast enough to afford synchronization of contraction. But, the injection of calcium through a micropipette directly into striated muscle fibers of crayfish, having small cells like the myocardium, triggers contraction of only a sharply circumscribed region in the immediate vicinity of the tip of the micropipette (Reuben et al., 1968). Consequently, it may be inferred that, in spite of the relatively small size of myocardial cells, a T-system may be necessary to assure synchronous contraction of the whole population of myofibrils in one cell.

A reversible swelling of the T-tubules can be produced by one immersion of fragments of ventricular tissue in hypertonic solution (Dreifuss, 1966; Girardier, 1964, 1965) or by perfusion of the heart with such solutions. This swelling is associated with an equally reversible drop in conduction velocity, which suggests that the T-system does shunt an appreciable fraction of the action current (Dreifuss, 1966; Forssman, 1970). Similarly, Corabeuf and Denoil (1966) have suggested that the low conduction velocity of guinea pig heart muscle, compared with that of rat heart, is due to the larger diameter of the

T-tubules in the guinea pig. Another indication of the possible involvement of the T-system in excitation-contraction coupling can be found in the investigation of Muller (1966). This investigator reported that the myocardium does not respond to a local stimulus by contracting locally, as has been shown by Huxley and co-workers (1959-1964) in different types of skeletal muscles; in the myocardium the contraction spreads over at least a few sarcomeres. The peculiar response of cardiac muscle to local stimulation may possibly be due to the longitudinal branching of the T-system (Forssman, 1970).

Unfortunately, there is a complete lack of experiments and studies in the embryonic myocardium which could be used to elucidate the role and function of T-tubules. This lack of investigation is understandable, however, in the light of the fact that even descriptions of their presence in early stages of myocardial differentiation have not been previously reported. Until such studies are carried out, one can only infer the functions of T-tubules in the embryonic myocardium from what is known in the adult tissues. T-tubules become recognizable in the 11-day (15-24 somites) myocardium, where fully-formed myofibrils are discernible and the heart is functional. In view of this, the question may be asked whether the T-tubules play a similar role in the embryonic myocardium as they do in the adult. And are they essential for the normal functioning of the embryonic heart? These questions and others can be answered only after satisfactory physiological and biochemical studies are carried out in living cells. The finding that the tubules are present in the beating embryonic hearts capable of maintaining blood circulation, and not before, is a significant observation and is worth further study.

XVI. Cell-to-Cell Contact and Intercalated Discs

The 10-day myocardium is characterized by loosely packed cells held to-

gether by cell-to-cell contacts. An amorphous, matty layer termed glycocalyx (Bennett, 1963) or external lamina (Fawcett, 1966) surrounds each of these cells and is probably involved in the process of cell adhesion. Although desmosomes are not present, tight junctions develop between contiguous cells. These are specialized areas of cell-to-cell contact and are implicated in the phenomenon of electrical coupling. Sheridan (1966) has studied the electrical coupling of special connections between cells in the early chick embryo and pointed out the significant electrophysiological property of these junctions in relation to passage of ions.

In early stages of development many myofilaments are seen attached to these tight junctions, which may thus serve as starting points for the organization of cardiac myofibrillar network. The regions where the filaments and fibrils are inserted are characterized by a dense material along the cytoplasmic aspect of the plasma membrane. These tight junctions with the attached myofilaments are considered by this investigator to be the incipient stages of formation of intercalated discs. Grimley and Edwards (1960) postulated that intercalated discs develop from the expansion of peripheral Z band material. The present study cannot support such a contention.

By the 11th, 12th and subsequent days of development there is a rapid proliferation and structural differentiation of the intercalated disc , and by the time of birth the adult form is approached in that the discs now possess a large number of prominent interdigitating rugose or mammilate projections. Myofibrils are inserted into the discs by way of an I band, and the discs are characteristically located one sarcomere length away from the preceeding Z band.

The intercalated discs serve as cell boundaries, and the cardiac muscle is divided into discrete cell territories. The electron microscopic studies of

Van Breeman (1953), Sjostrand et al. (1954, 1958), Price et al. (1955) and Muir (1957) show that the adult intercalated disc is the site of junction of neighboring myocardial cells. This is in agreement with the early light microscopic studies of Werner (1910), Von Palezewska (1910), Schafer (1910) and others. Lewis (1926), employing tissue cultures of cardiac muscle in which individual cells contracted at different rates, concluded that the cells are physiologically and probably structurally independent. The tendency for cardiac muscle to segment at the sites of the discs, as reported by Saphir and Karsner (1924) and Beams et al. (1949), seems to support the cellular concept, although these authors, who favored the concept of syncytial structure, interpreted their findings as a demonstration of the fragility of myofibrils at these points. The microdissection experiments of de Renyi (1945) in which he noticed that greater pressure is needed to force the fluid through the disc than through the sarcoplasm elsewhere, and observations of D'Ancona (1929), on the difference in the state of contraction on either side of the disc, also support the idea that intercalated discs represent cell boundaries. A difference in the state of contraction in some cells of the new born myocardium is noticed in the present study. The early electron microscopic studies of Beams et al. (1949) and Berrian (1953) did not show the double membrane in the substance of the discs, and these authors consequently supported the then generally accepted view that cardiac muscle is syncytial. The interested reader is referred to the appropriate sections of the "Introduction" for a detailed account of the works of early investigators.

Numerous functions have been attributed to the intercalated discs. These include uninterrupted transmission of mechanical forces between the fibrils of adjacent cells (Fawcett and Selby, 1958); intracellular cohesion (Van Breeman, 1953); participation in fibrillar growth (Heidenhain, 1911); and transmission of

electrical impulses between cells (Bourne, 1953; Muir, 1957; and Karrer, 1960). Bourne's work (1953) in localizing dephosphorylating enzyme activity at the adult mammalian disc may be important evidence of the role of the disc in the orientation of myofilaments and fibrillar growth and also in the transmission of impulses.

XVII. Mitosis in Myocardial Cells

A unique observation in the developing myocardium is the detection of the simultaneous occurrence of mitosis and synthesis of contractile proteins in the same cell. Mitotic cells containing sites of myofilament formation are discernible throughout the gestational period covered in this investigation, including the new born myocardium. In spite of this intense mitotic activity, myocardial cells are characterized by mononuclearity, and, unlike skeletal muscle, cell fusion is not distinguishable in the embryonic cardiac tissue. Stockdale and Holtzer (1961) proposed that in skeletal muscle the accumulation of detectable quantities of muscle-specific protein occurs only after cell division ceases. The rat embryonic myocardial cells are strikingly different in this respect in that the onset of fibrillogenesis is not correlated with the cessation of mitotic activity.

Olivo and Slavich (1930) have studied the growth rate of heart in chick embryos throughout the course of development and showed that the mitotic index, that is, the number of mitotic figures per 100 cells counted, is high in the tubular heart on the second day of incubation, but this value gradually decreases before hatching. According to these authors, there is no tangible evidence of mitosis in the heart 10 days after hatching. DeHaan (1965) has suggested that the gradual reduction in the mitotic index is probably due to the progressively imposed mitotic restraints or controls on the myocardial cells; however, the mechanism of mitotic control remains unresolved. The idea that mitosis is nonexistent in the

adult cardiac tissue is supported by the observation that the fully-formed heart is composed of a population of stable, nondividing cells (Leblond et al., 1959; Sparagen et al., 1962; and Pelc, 1964). The post-embryonic heart is said to grow by an increase in fiber diameter (Shipley et al., 1937). According to Richter and Kellner (1963), even hypertrophied hearts show no evidence of cell division nor an increase in the number of fibers. Such hearts apparently undergo hypertrophy by an increase in the diameter of fibers and synthesis of new myofibrils within each cell.

XVIII. Neural Elements*

The adult heart and myocardium receive an ample supply of nerves, although the rhythmic activity of myocardium is not dependent upon the nervous system. The sympathetic (via cervical cardiac branches) and the parasympathetic (via vagus) divisions of the autonomic nervous system form extensive plexuses around the heart. The vagal fibers are cardio-inhibitory, whereas the sympathetic fibers are cardio-acceleratory . It is commonly assumed that the autonomic system acts indirectly upon the myocardium by modifying the inherent rhythm of the pacemaker cells. In the adult heart, in addition to the unmyelinated nerve fibers found close to the specialized cells of the conduction system, a large number of other unmyelinated fibers are also shown to be present in close relation to the normal atrial and ventricular cardiac fibers (Fawcett, 1968).

The innervation of the embryonic heart is a subject seldom discussed in the literature. The author could not find a reference regarding the innervation of myocardium in any of the light or electron microscopic studies dealing with the development of heart. In this investigation, neural elements are observed to be present in the 13th and 14th day myocardium. They can be visualized in the form of long slender processes located between myocardial cells. The axoplasm

* See appendix

of these cells is characterized by fine neurofilaments, mitochondria and distended granular endoplasmic reticulum. At places structures strongly resembling synaptic vesicles are discernible in cross sections in close juxtaposition to myocardial cells already containing a full complement of fully formed myofibrils. It is difficult to say from the examination of electronmicrographs whether or not these fibers are sympathetic or parasympathetic, since there is no established criteria in electron microscopy to distinguish between these two sets of fibers. A light microscopic examination of a collection of human embryo materials, maintained by Dr. A. A. Pearson, Professor and Chairman of the Department of Anatomy, University of Oregon Medical School, reveals that the vagus nerve (N. X) reaches the cardiac region in the 4-6 mm (C-R length) embryos. This gestation age of human embryos approximately corresponds to 13 to 14 days of development in rat embryos. Consequently, it is possible that the nerve fibers seen in this study are the end branches of vagus nerve, and, hence belong to the parasympathetic division of the autonomic nervous system. This does not and should not rule out the existence of the sympathetic fibers. It can be concluded that the embryonic myocardium contains elements of the autonomic nervous system, at least by the 13th day of gestation. In the electron microscopic study, it is rather difficult to determine the presence or absence of neural elements in earlier stages unless a complete serial study is undertaken coupled with the application of histochemical techniques. Recent electron microscopic studies (De Robertis et al., 1963; and others) have shown that acetylcholinesterase, the enzyme which inactivates acetylcholine, is associated with plasma membranes of axons and muscle fibers as well as with synaptic vesicles. A similar study to localize the distribution of cholinesterase in the developing myocardium would be highly beneficial, not only to demonstrate the presence of neural elements, but

also to establish the t[illegible]uring which the neural elements begin to appear in the cardiac region of the embryos.

XIX. Cytochemistry

For the purpose of demonstrating the general localization of nucleoside phosphatases (ATPase), embryonic tissues pre-fixed in glutaraldehyde were incubated in a standard Wachstein-Meisel (1957) incubating medium containing ATP as the substrate, followed by post-fixation in osmium. For control purposes the tissues were incubated without the substrate; or, the substrate was substituted with either ADP or sodium B-glycerophosphate; or, the tissues were pretreated with osmium prior to incubation. Cloudy control experiments in which the tissues were incubated with lead phosphate instead of the substrate were also carried out.

None of the tissues incubated in the control media show any final reaction product. On the other hand, following incubation with ATP as the substrate, granular deposits of reaction product are discernible on the cell membranes of the embryonic myocardium. There is a noticeable decrease in the intensity of reaction product as visualized in the electronmicrographs from the 10th day of gestation to the 14th day, and in the newborn there is a slight increase relative to the 14th day, but still less intense than in 10-day myocardium. No granular reaction products are recognizable in myofibrils, mitochondria or other organelles in the cytoplasm. It appears that there is a selective deposition of the reaction product on the cell membrane or structures derived from it.

The first attempt at histochemical localization of ATPase activity was reported by Glick and Fischer (1945, 1946), who employed the Gomori-Takamatsu principle using acetone-fixed paraffin sections and frozen sections. Subsequently, various authors sugg[illegible]sted procedures to improve on the specificity of the reaction

(Moog and Steinbach, 1946; Maengwyn-Davies et al., 1952; Morell et al., 1951; Wade and Morgan, 1954). Padykula and Herman (1955) have proposed a revised technique for better localization and specificity. These authors claimed that higher phosphatase activity could be demonstrated with ATP and other substrates at an alkaline pH when thin, unfixed frozen sections were incubated in the medium described by Gomori (1941) than when incubated in the medium employed by Maengwyn-Davies et al. (1952). This increase in activity was attributed by these authors to the low salt content in their media.

Wachstein and Meisel (1957) introduced the now widely used lead precipitation techniques using unfixed and cold formalin-fixed frozen sections incubated at pH 7.2. Novikoff and co-workers (1958), Pearse (1960), Barka and Anderson (1963), Tice and Barnett (1962), Tice and Smith (1965) and many others have strongly endorsed the usefulness of this technique for the localization of ATPase and other phosphatases. Sabatini et al. (1963) have introduced glutaraldehyde fixation for electron microscopic cytochemistry, which greatly improves the ultrastructural preservation. Numerous reports have appeared in recent years in the literature utilizing the Wachstein-Meisel technique for the localization of ATPase activity in a variety of tissues. Since the author undertook this study some papers critical of Wachstein-Meisel techniques have appeared (Moses and Rosenthal, 1967; Rosenthal and Moses, 1969), primarily from one group of investigators. Novikoff (1967) and many others have defended the Wachstein-Meisel techniques and objected to these criticisms. It would not serve the purpose of this thesis to extensively discuss the afore-mentioned reports. The reader interested in this lively debate is referred to the above mentioned authors for more details.

Engelhardt and Ljubimowa (1939) first made the observation that a close

association exists between the enzyme ATPase and the myofibrillar protein, myosin, and they speculated that the enzyme might, in fact, form part of the myosin molecule itself. Similar associations between the enzyme and myosin in the developing tissues are not readily demonstrated. The ATPase activity of chick (Moog, 1947; Robinson, 1952) increases before the contractile proteins increase in quantity (Csapo and Herrmann, 1951; Robinson, 1952). Similarly, Herrmann and Nicholas (1948) find in the rat an increase in ATPase activity precedent to a significant change in contractile proteins. Fractionation of the homogenate by these workers (Herrmann et al., 1949) resulted in a scattering of ATPase activity among the fractions, with no definitive correlation of ATPase activity and myosin evidenced. This may be due to the existence of a non-myosin ATPase associated with the cell particulate bodies rather than with the myofibrils, as has been shown by Kielley and Meyerhof (1948) in rat muscle. A similar ATPase has been demonstrated in rabbit muscle by Perry (1952). Both of these groups of investigators observed a Mg-activated ATPase associated with the particulate matter of the cell. On the other hand, the ATPase associated with the myosin is strongly activated by calcium ions (Banga and Szent-Gyorgyi, 1943; Mommaerts and Seraidarian, 1947). De Villafranca (1954) extracted and isolated two ATPases, myosin ATPase and non-myosin ATPase, from developing rat skeletal muscles and suggested that both of these enzymes are involved in the differentiation of contractile mechanism as a contributing factor in its formation. These enzymes showed an initial increase in activity at approximately the onset of contractility. In the present study the most intense activity is noted in the 10-day myocardium where the myofibrils are not yet formed and cardiac function not yet established. By the 11th day onwards there is a decrease in the activity when the heart is functional, and fully formed myofibrils are recognizable.

Whether these findings can be correlated with those of De Villafranca and other workers quoted above is problematical.

Robinson (1952) applied biochemical procedures to the study of ATPase activity in developing chick muscle in terms of the activity of proteins associated with the myofibrils and in terms of the sarcoplasmic proteins. In the earlier developmental stages studied almost all of the ATPase remains in the supernatant solution (sarcoplasmic fraction), and as growth continues there is a progressive decrease in the proportion of ATPase in this fraction and a corresponding increase in the proportion associated with the precipitated myofibrillar protein. When the activity of the myofibrillar fraction is expressed in terms of its protein content, it rises to a peak prior to hatching. A similar value is reported by Herrmann et al. (1949) in developing rat muscle. Robinson (1952) proposed that a mechanism exists whereby the ATPase activity passes into the myofibrillar fraction as development proceeds; that is, a water soluble ATPase becomes associated with the myofibril and gives rise in the adult to an ATPase firmly combined with the myosin proper.

Herrman and Nicholas (1948) studied the Ca^{++} activated apyrase activity of developing rat muscle and found that after the 16th day of gestation the total activity, expressed in terms of dry weight of the muscle, begins to rise from a low level, and about three weeks after birth reaches an approximately constant value. Later studies (Herrmann et al., 1949) of particulate protein fractions showed that at earlier developmental stages only a small amount of the total apyrase activity was contributed by the myofibrillar fraction.

The above two biochemical studies are in remarkable agreement with the observations of the present electron cytochemical study. In the embryonic rat myocardium the ATPase activity is primarily or exclusively associated with sarcolemma and other membranous elements and may be construed as analagous

to the sarcoplasmic ATPase reported by the above investigators. There is a gradual reduction of this membrane-bound activity as development proceeds, a situation very similar to that described by Robinson (1952) and Herrmann et al. (1949) in chick and rat muscles, respectively. A possible explanation for this apparent decrease in enzyme activity in the embryonic rat myocardium can be obtained by relating it to the biochemical studies cited above. It should be pointed out that the technique employed in the present investigation is not sensitive enough to localize the enzymes in the myofibril itself. This should explain why no activity is discernible in the myofibrils, although a reduction in the activity associated with the sarcolemma is noticeable in later stages of development.

ATPase activity in the sarcotubular fraction of skeletal muscle has been reported by several authors (Ebashi and Lipmann, 1962; Muscatello et al., 1961). These authors concluded that the SR-ATPase corresponds to the ATPase found by Kielley and Meyerhof (1948) in the "granular fraction." Girardier et al. (1963) have demonstrated in crayfish that current can flow between the interior of the muscle fiber and the periphery through the T-system, and that the T-system membranes adjacent to the SR contain a chloride battery capable of locally accumulating cations, such as calcium, which could initiate excitation-contraction coupling. It has been postulated that the elements of SR contain a pump mechanism moving calcium ions to and from the contractile elements (Ca-pump) and the energy for the pump is derived from ATP dephosphorylation catalyzed by the enzymes of the reticulum (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1962; Martonosi and Feretos, 1964; Nagai and Makinose, 1960; Weber et al., 1963). It has been demonstrated that the sarcoplasmic reticulum plays essentially a similar role in cardiac muscle (Berne, 1962; Fanburg et al., 1964; Weber et al., 1963). These studies thus show the role

of non-myosin ATPase in cation transport, excitation-contraction coupling and maintenance of calcium pump. Since the enzyme activity in the present study seems to be primarily associated with membranous elements, inferentially it may be argued that these enzymes are involved in a similar process in the rat embryonic myocardium. It should be pointed out here that the findings of the present study are not by any means conclusive. To assign any one specific function to the ATP-splitting enzymes in the cell membrane of the rat embryonic myocardium at this time would be premature. In addition, the present study was intended only to define the general distribution of ATP-splitting enzymes on the ultrastructural level, and no attempt was made to focus attention on the enzymes associated with any particular organelle or cytoplasmic constituent. Because of the fixation and incubation procedures employed in this investigation, only relatively resistant enzymes could be expected to be demonstrated. Consequently non-localization of final reaction products on the mitochondria or myofibrils or other intra-cytoplasmic constituents cannot be construed as evidence for the non-existence of these enzymes in these organelles. For these and other reasons further study is certainly needed to come to any definitive and meaningful conclusion regarding the significance and role of nucleoside phosphatases in the differentiating myocardium of the rat.

SUMMARY

The early ultrastructural differentiation of the myocardium of rat embryos of 10, 11, 12, 13 and 14 days of gestation is investigated and compared with the fine structural details of the myocardium of newborn. The cytochemical distribution of nucleoside phosphatases (ATPase) in the myocardium is also investigated utilizing ATP as the substrate. The course of ultrastructural differentiation of some major organelles of the developing myocardium is presented. These organelles include myofilaments, myofibrils, intercalated discs, mitochondria, granular endoplasmic reticulum, Golgi bodies, ribosomes and others.

a. Myofilaments

Both thick (myosin) and thin (actin) filaments become identifiable for the first time in 10-day myocardium where the heart is contracting but the circulation of blood is not established. The synthesis of these filaments is observed to continue throughout the period covered in this investigation. Isolated filaments and sites of filament synthesis are identifiable in all embryos and fetuses examined, including the myocardium of the newborn. Concomitant with the appearance of the myofilaments is the synthesis of Z band material in the 10-day myocardium. Z bands are implicated in the myofibril assembly.

b. Myofibrils

The myofilaments are organized into fully formed striated fibrils by the 11th day of gestation. A, I, H and Z bands are present and the fibrils are frequently oriented in the long axis of the cell separated by large irregular cytoplasmic spacings. In subsequent days of development the fibrils become grouped together with considerable reduction in the interfibrillar spacings. The phenom-

enon of branching of the fibrils can be traced to the Z disc regions, and these "branches" interconnect with adjoining fibrils, thus contributing to the fusion or anastomosis of fibrils. The branching is also recognizable at the intercalated discs.

c. Intercalated discs

This structure is not discernible in the 10-day myocardium. The intercalated discs begin their development as small wavy lines on the 11th day and become progressively plicated and, in the myocardium of the newborn, well developed discs with numerous mammilate projections are identifiable. These structures frequently occur at one sarcomere length away from the preceeding Z lines and serve as points of attachment for myofilaments and fibrils and as distinct cell boundaries, thus indicating that embryonic myocardium is not a syncytium.

d. Mitochondria

Mitochondria appear immature in the 10-day myocardium, the cristae are not recognizable and the bileaflet membrane cannot be resolved. There is a rapid and perceptible change in the number and morphology of mitochondria by the 11th day and during the subsequent days of gestation when the heart becomes functional. In later stages of development the loosely packed cristae of 11-day myocardium become tightly packed, the number of mitochondria increases and they progressively assume an interfibrillary arrangement parallel to the myofibrils.

e. Granular endoplasmic reticulum and Golgi bodies

There is a rapid proliferation and differentiation of these organelles from the 10th to the 11th and later stages of development. The cisternae of these structures and the Golgi vacuoles frequently become filled with an amorphous material, and numerous membrane-bound secretory granules become identi-

fiable. The presence of large amounts of distended endoplasmic reticulum, extensive regions of hypertrophied Golgi bodies and several secretory granules suggests that the developing myocardium has a secretory function in addition to its contractile function. It is proposed here that the myocardial cell secretions contribute to the production of cardiac jelly.

f. Ribosomes

Large quantities of free ribosomes are widely dispersed in the cytoplasm, the greatest concentration of which is seen in the 10-day myocardium. After the 10th day of gestation there is a gradual and progressive reduction in the amount of free ribosomes. They occur as free particles, as clusters of rosettes and as helically arranged chains of polysomes. An intimate association of ribosomes and polysomes with the developing myofilaments and fibrils is discernible.

g. Other organelles and points of interest

The presence of neural tissue, probably elements of vagal fibers, in the 13 and 14-day myocardium is reported here for the first time in an early mammalian embryo. Structures closely resembling synaptic vesicles are recognizable within the 13 and 14-day myocardium. Vascular elements become identifiable in 13-day myocardium and are associated with neural processes.

The T-system and sarcoplasmic reticulum begin to appear in 11-day myocardium, and from the very beginning the elements of developing T-system are associated with the Z disc regions. Also associated with the Z disc regions are the infoldings of the sarcolemma which is implicated in the formation and function of sarcoplasmic reticulum and T-system.

The embryonic myocardium displays intense mitotic activity throughout its development. A unique feature of embryonic myocardial cells is the simultaneous occurrence of myofilament synthesis and mitotic activity within the same cells.

Nucleoside phosphatases (ATPase) exhibit maximum activity in the 10-day myocardium, presumably coincident with the beginning of myofilament synthesis, followed by an apparent progressive reduction in activity during the later stages of development.

Theories concerning origin of fibrils, origin of striations, mechanisms of assembly of myofilaments into fibrils, role of ribosomes in filament synthesis, formation and functions of intercalated discs, functions of T-system and sarcoplasmic reticulum, the secretory function of myocardium and other related topics are discussed.

There is no end. Every end is but a beginning.

The Author

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ABBREVIATIONS USED IN THE LEGENDS AND MICROGRAPHS

A-A band
C-centriole
Cap L-capillary lumen
Chr-chromatin, chromosome
Co-collagen fibers
D-desmosomes
EL-external lamina or glycocalyx
EN-endothelial nucleus
Enc-endothelial cell or cytoplasm
EPC-epicardial cell
ER-granular endoplasmic reticulum
FA-fascia adherens
FB-fibroblast
Ffb-free floating body
FRP-final reaction product
Fv-fuzzy or spiny vesicles
G-Golgi body, Golgi elements
Gv-Golgi vesicles and vacuoles
Gy-glycogen
H-H band or line
I-I band
ICS-intercellular space
ID-intercalated disc
Li-lipid droplets
M, m-mitochondria
MA-macula adherens
MF-myofibril
Mfl-myofilaments
MO-macula occludens
Mt-microtubules
MV, Mv-microvilli
Mvb-multivesicular body
Myc-myocardial cell
N-nucleus
NE-nuclear envelope
NF-nerve fiber
Nfl-neurofilaments
Nlm-nucleolonema
NP-nerve process
NT-neural tissue
Nu-nucleolus
PFR-"protofibril"
PRi-polyribosome
Pv-pinocytotic vesicles, pinocytosis
RBC-erythrocyte, red blood cell
Ri-ribosome
SGr-secretory granules

Sl, Slm-sarcolemma
SR-sarcoplasmic reticulum
T-thick (myosin) filament
t-thin (actin) filament
Ts-T-system
Tt-T-tubules
V, v-vacuoles, vesicles
Z-Z band

Note On Micrographs

All micrographs are from thin sections of tissue fixed in 3.25% glutaraldehyde and post-fixed in 1% osmium tetroxide and viewed with an RCA Model EMU3F electron microscope. For cytochemical localization of enzymes, the tissues were incubated in the appropriate media prior to post-fixation in osmium tetroxide. All the electron micrographs, except the ones in the original copy of the thesis, have been photographically reproduced. Magnification values given refer to the final micrographs. For details of tissue preparation, fixation, incubation procedures, etc., "Methods and Materials" section should be consulted.

Figure 1. 10-Day Myocardium

Superficial layer of myocardium showing the loosely packed cells with large intercellular spaces (ICS). Nuclei (N) are large and round and occupy most of the cytoplasm. Prominent nucleolei (Nu) with tridimensional configuration of nucleolonema are clearly discernible. Mitochondria (m) are in an immature state of differentiation. Large amounts of free ribosomes (Ri) are dispersed in the cytoplasm, which is characterized by a dearth of organelles.
N-nucleus; Nu-nucleolus; NE-nuclear envelope; M-mitochondria; Ri-ribosomes; ICS-intercellular space.

X 11,000

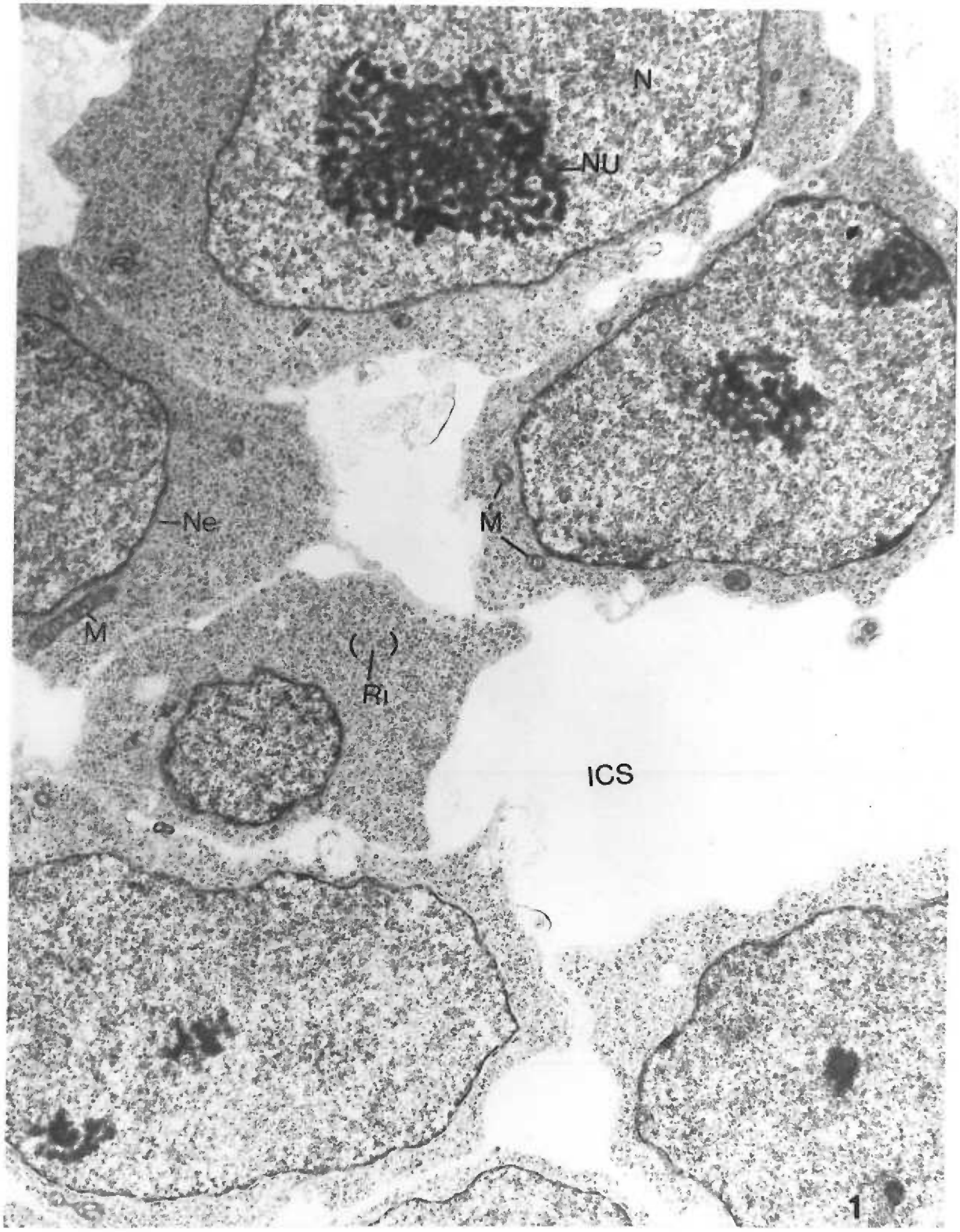

N
NU
Ne
M
M
RI
ICS
1

Figure 2. 10-Day Myocardium

Superficial layer of cells with large intercellular spaces (ICS). The cell membrane is in an immature state of differentiation and is not readily resolvable. Cell-to-cell contacts (arrows) are maintained by close apposition of cell surfaces without any specialized areas of cell contacts. Desmosomes are not recognizable. Ribosomes are seen attached to inner leaflet of the nuclear envelope, in addition to the outer membrane (arrows). Mitochondria (m) are poorly developed.

N-nucleus; Nlm-nucleolonema; M-mitochondria; ICS-intercellular space.

X 15,975

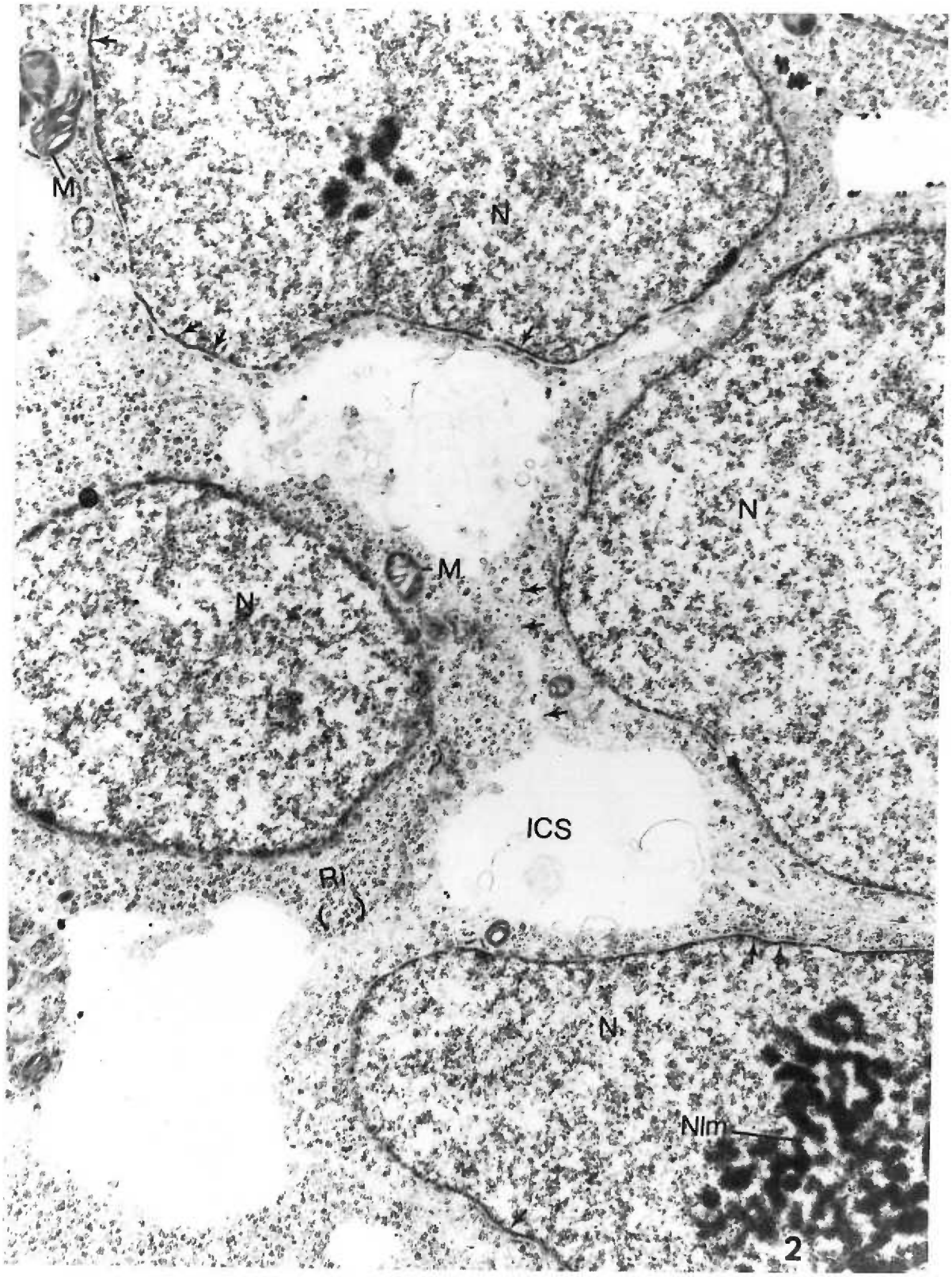

M
N
N
M
N
ICS
Ri
N
Nlm
2

Figure 3. 10-Day Myocardium

Superficial layer of cells, showing the poorly developed mitochondria (M). The outer limiting membrane of mitochondria appears as a solid thick line and does not exhibit the typical inner and outer leaflets. Cristae mitochondriales are in a state of formation and are not well-delineated. Granular endoplasmic reticulum (ER) is not extensively distributed, but cisternae are distended and contain an amorphous material (arrows). Rosettes of ribosomes (Ri) are clearly discernible. Cell surface is thrown into irregular folds and processes. ICS-intercellular space; ER-endoplasmic reticulum; M-mitochondria; N-nucleus; Ri-ribosomes.

X 22,350

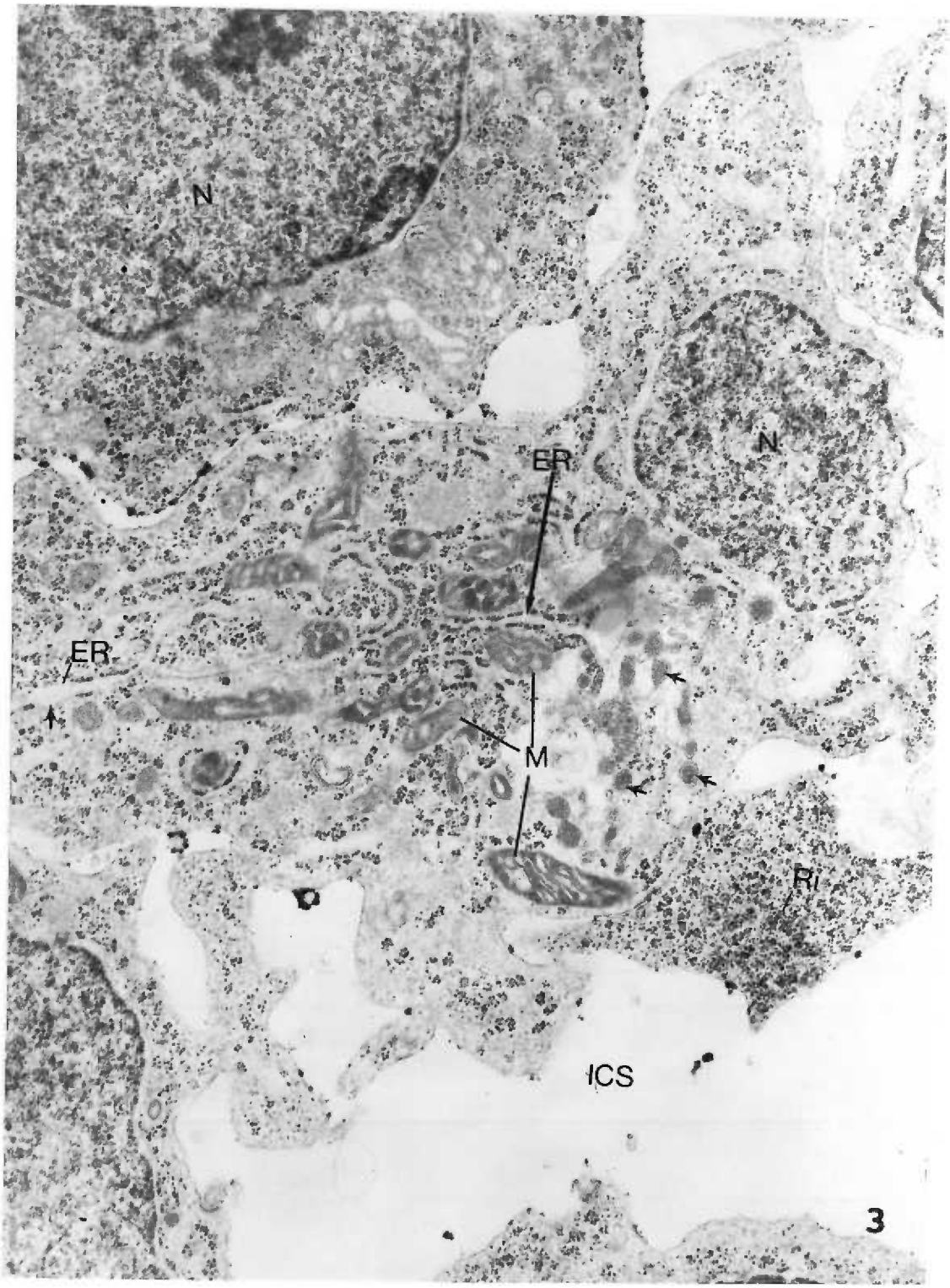

N
N
ER
ER
M
RI
ICS
3

Figure 4. 10-Day Myocardium

Deep layer of cells showing myofilaments (Mfl) for the first time. These filaments are distributed at random throughout the cytoplasm. Developing Z band regions (Z) can be visualized in the form of electron dense, amorphous material (Z and arrows). These regions are distributed in the course of the myofilament. Mitochondria (M) are long with thread-like mid-segments and swollen end portions. Granular endoplasmic reticulum is recognizable (ER).

Chr-chromatin material; ER-endoplasmic reticulum; M-mitochondria; Mfl-myofilaments; Z and arrows-developing Z band regions; N-nucleus.

X 22,350

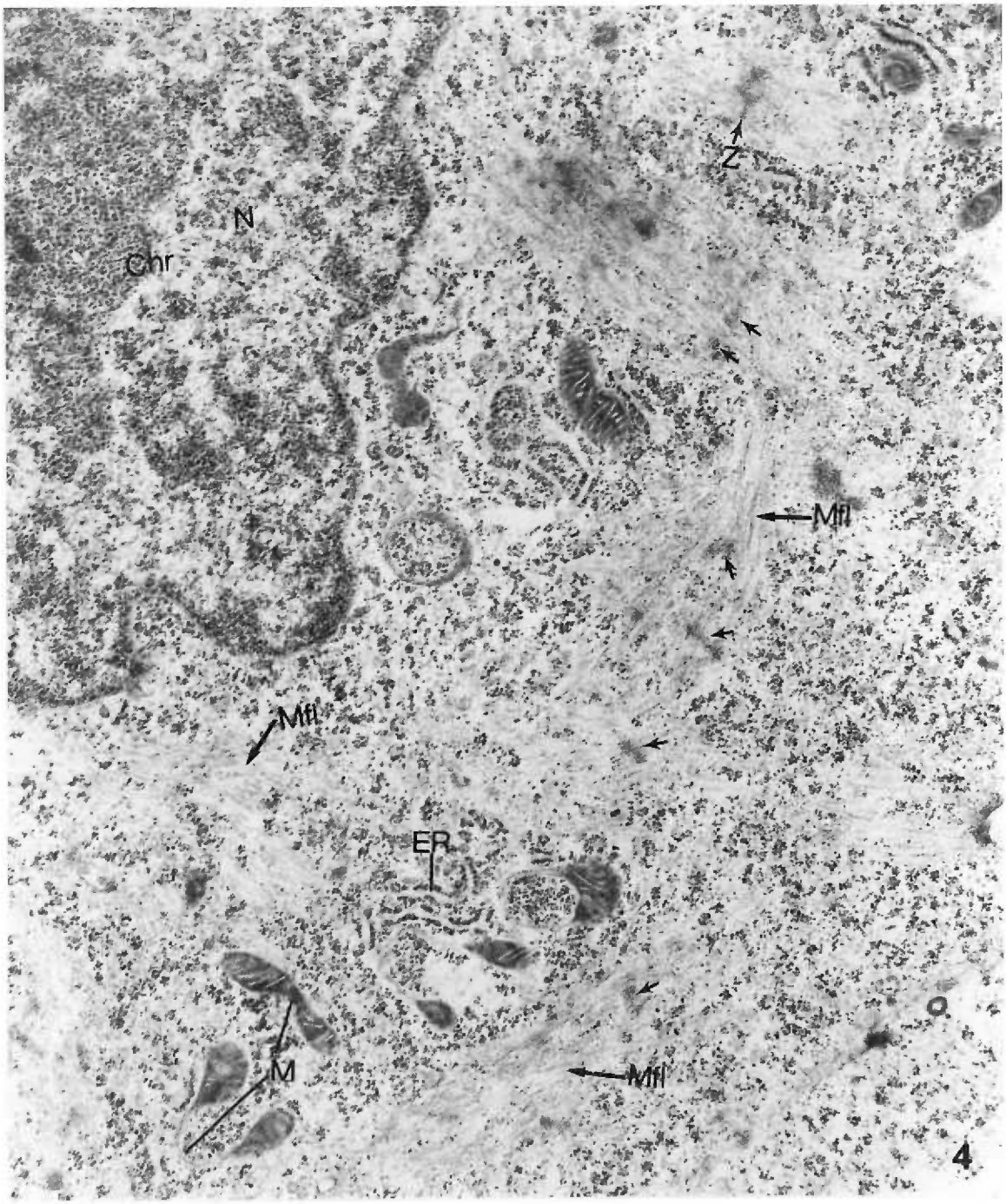

Z
N
Chr
Mfl
Mfl
ER
M
Mfl
4

Figure 5. 10-Day Myocardium

Deep layer of cells. Condensations of electron dense material are seen in the cytoplasm. These are the developing Z bands (Z). Myofilaments (Mfl) are either attached to or radiate from these condensations. Many myofilaments are obscured from view by the dense concentration of ribosomes (Ri), which may occur as single particles, as rosettes or as chains of polyribosomes (PRi). Two developing desmosomes (D) are shown. Mitochondria (M) are poorly developed and are filamentous.

Er-granular endoplasmic reticulum; ICS-intercellular space; Mfl-myofilaments; M-mitochondria; Ri-ribosomes; PRi-polyribosomes; D-desmosomes; Z-Z band regions.

X 15,975

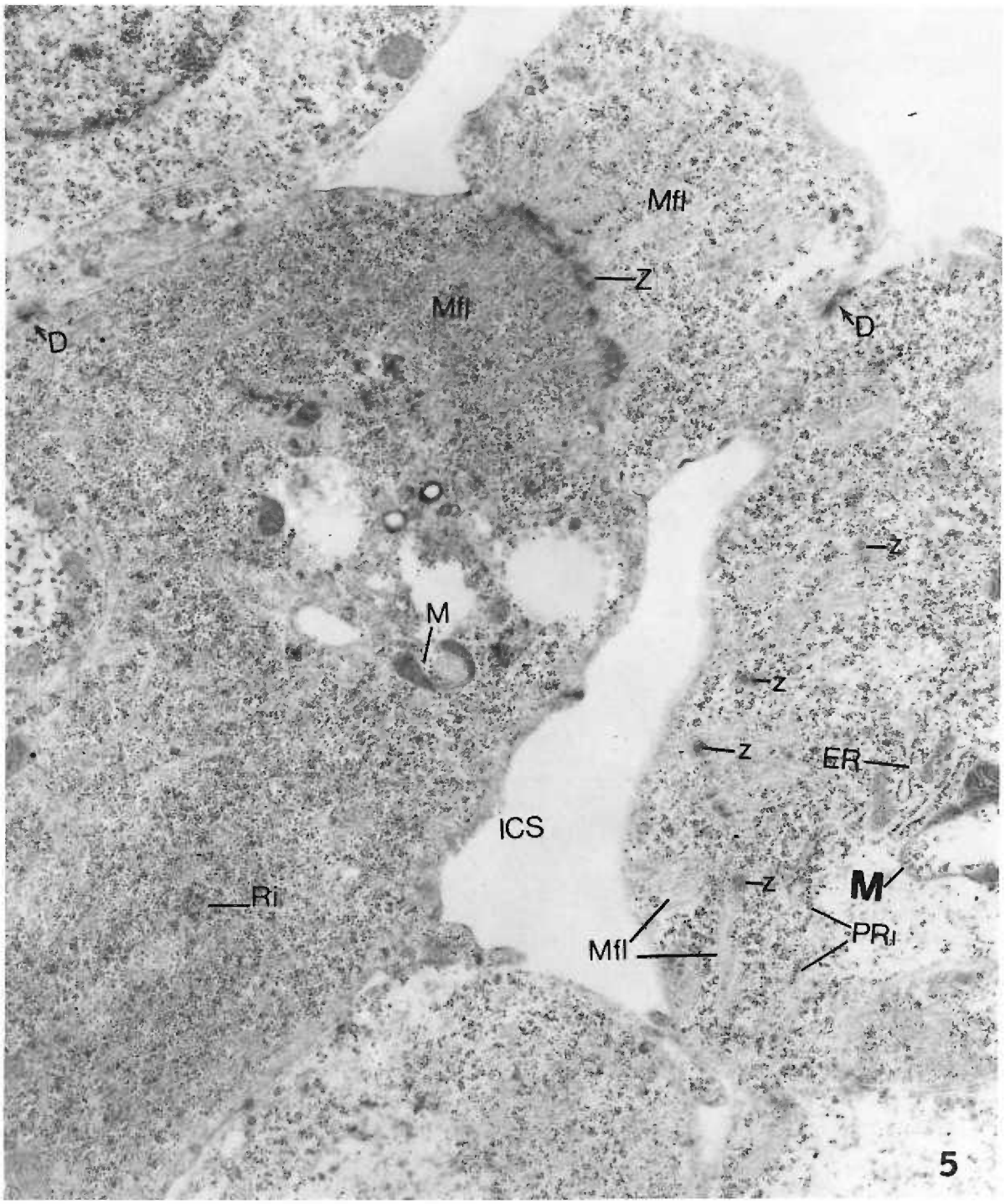
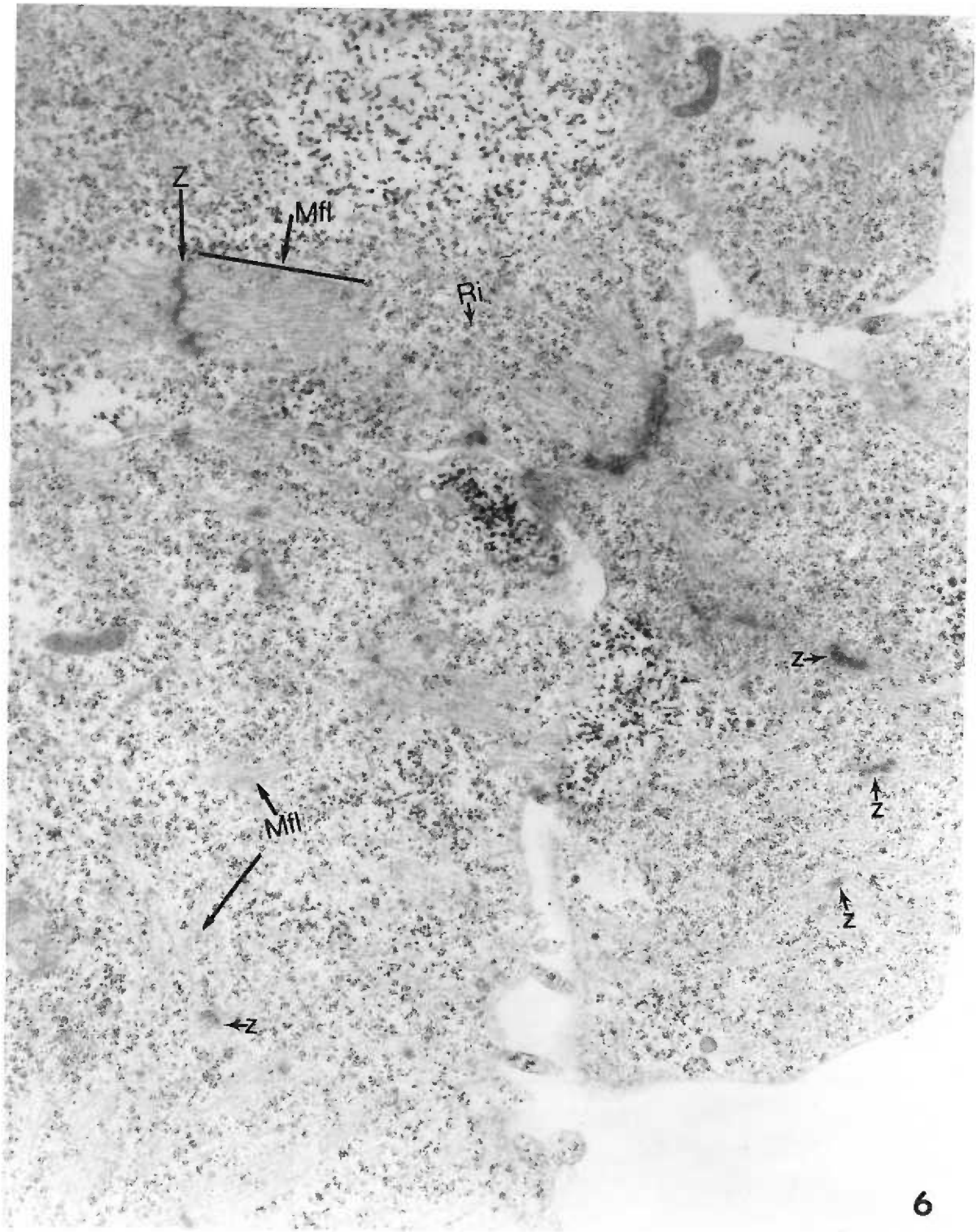

Mfl
Z
Mfl
D
D
Z
M
Z
Z
ER
ICS
Ri
Z
M
PRi
Mfl
5

Figure 6. 10-Day Myocardium

Deep layer of cells. Several developing Z regions can be recognized (Z). An example where a group of myofilaments is intersected by a Z band region is shown in the top left of the picture (Z-Mfl). Myofilaments (Mfl) are scattered throughout the cytoplasm and some are obscured from view by the dense concentration of ribosomes (Ri).

Mfl-myofilaments; Z-Z band regions; Ri-ribosomes.

X 15,975



6

Figure 7. 10-Day Myocardium

Deep layer of cells. The formation of the first myofibrils is indicated in that the myofilaments are very loosely organized between two or three Z bands. This fibril is termed the protofibril (PFR). In the protofibrils the filaments **are invariably** covered with clusters of free ribosomes. The A and I bands **are not** recognizable as separate entities.

M-mitochondria; D-developing desmosome; ICS-intercellular space; ER-endoplasmic reticulum; Ri-ribosomes; PFR-protofibril; Z-Z disc regions.

X 22,350

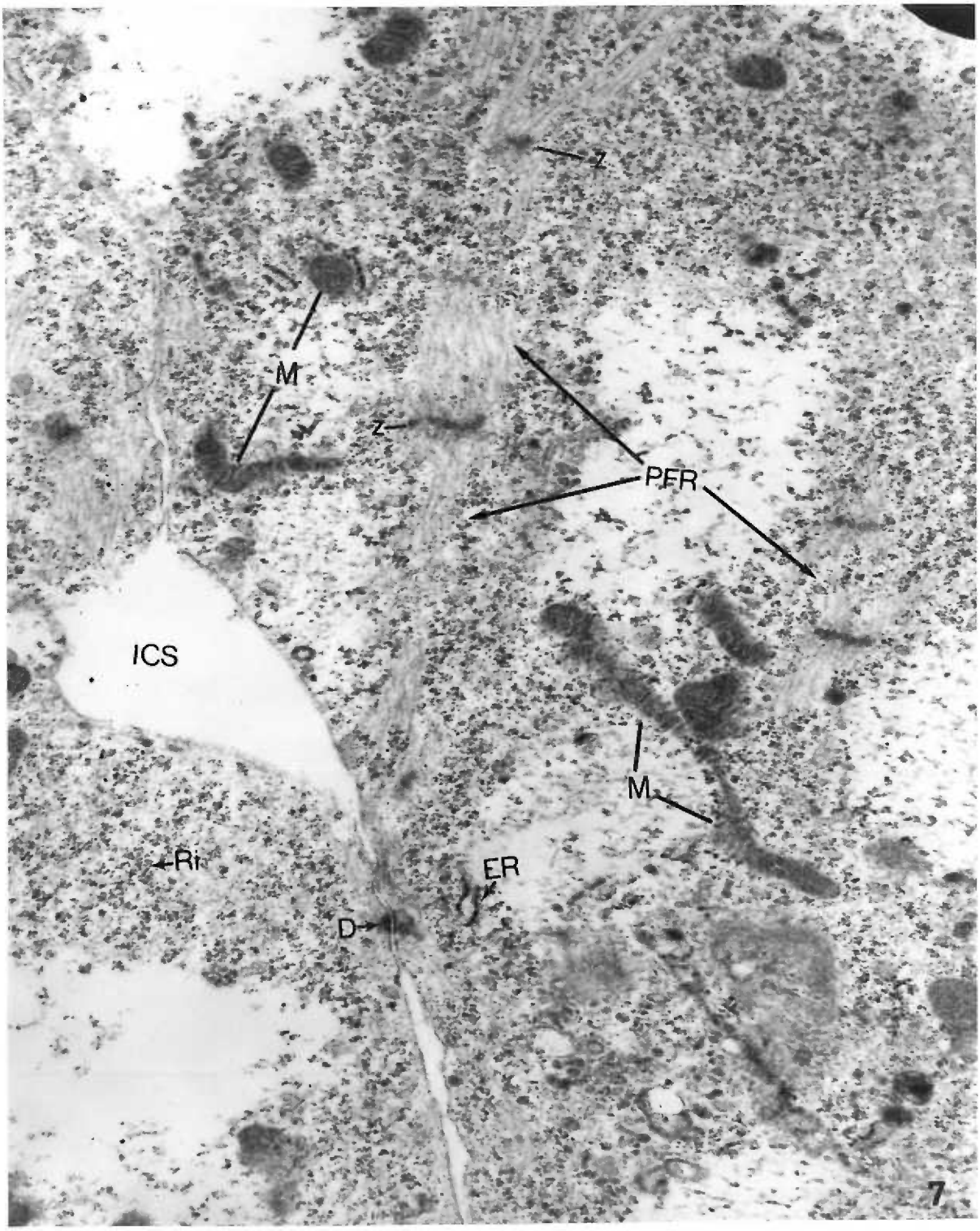

Z
M
Z
PER
ICS
M
Ri
ER
D
7

Figure 8. 10-Day Myocardium

Deep layer of cells. The presence of extensive regions of Golgi elements is noticeable (G). They begin to appear in the embryonic myocardium as curved arrays of parallel membranes and sometimes display a whorled structure. The cisternae are seen to contain a flocculent material. A single centriole (C) is often discernible in the non-dividing myocardial cells.

M-mitochondria; ER-endoplasmic reticulum; N-nucleus; Chr-chromatin material; C-centriole; Mfl-myofilaments; G-Golgi bodies; Z-Z band regions.

X 22,350

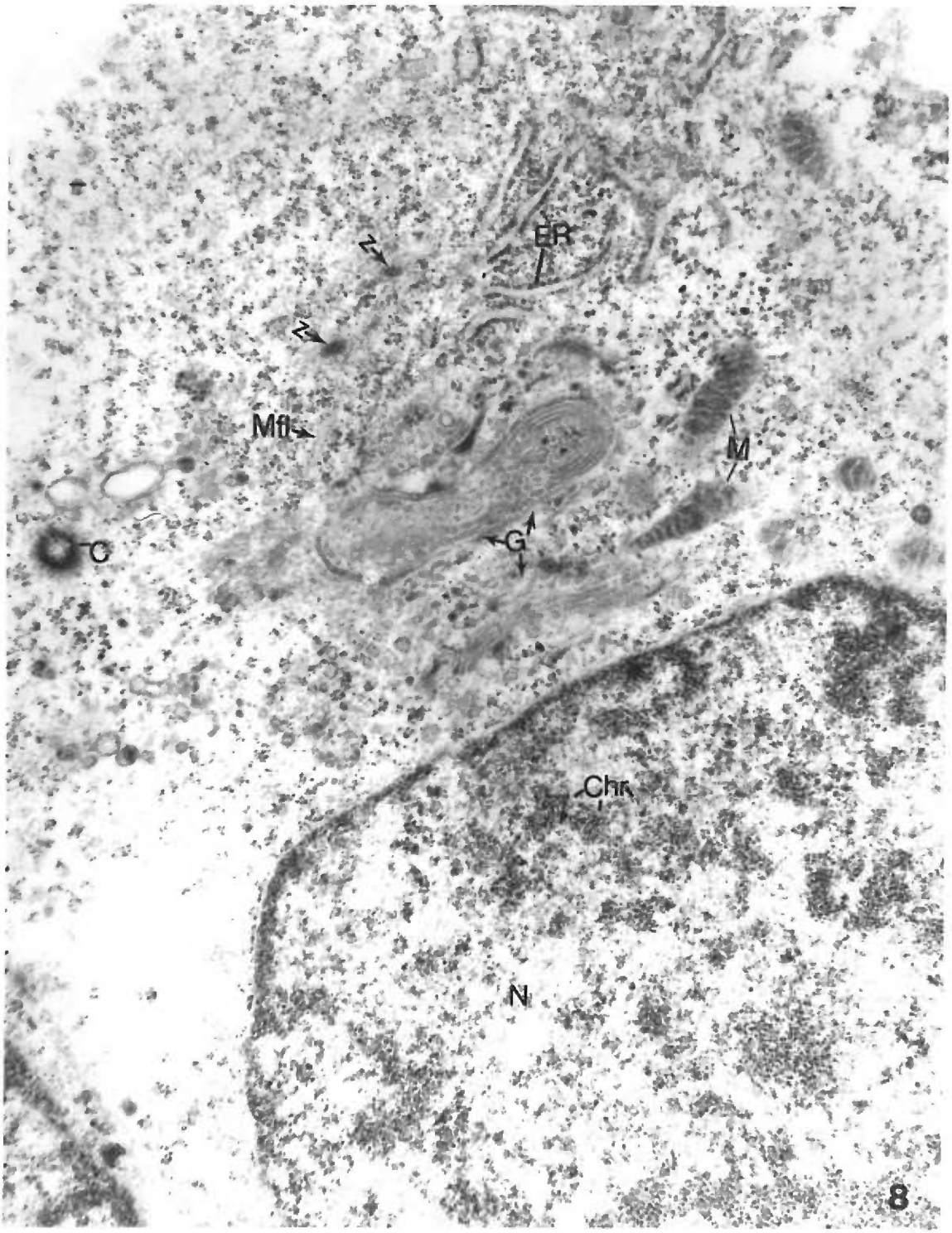

ER
Mfl
M
C
G
Chr
N
8

Figure 9. 10-Day Myocardium

Deep layer of cells showing extensive areas of Golgi bodies (G). The Golgi vacuoles contain an amorphous material. Swollen or ballooned out mitochondria are discernible (M). At the lower right-hand corner is shown a protofibril (PFR). Myofilaments are scattered throughout the cytoplasm (Mfl). ER-endoplasmic reticulum; Ri-ribosomes; M-mitochondria; Mfl-myofilaments; PFR-protofibril; G-Golgi bodies.

X 22,350

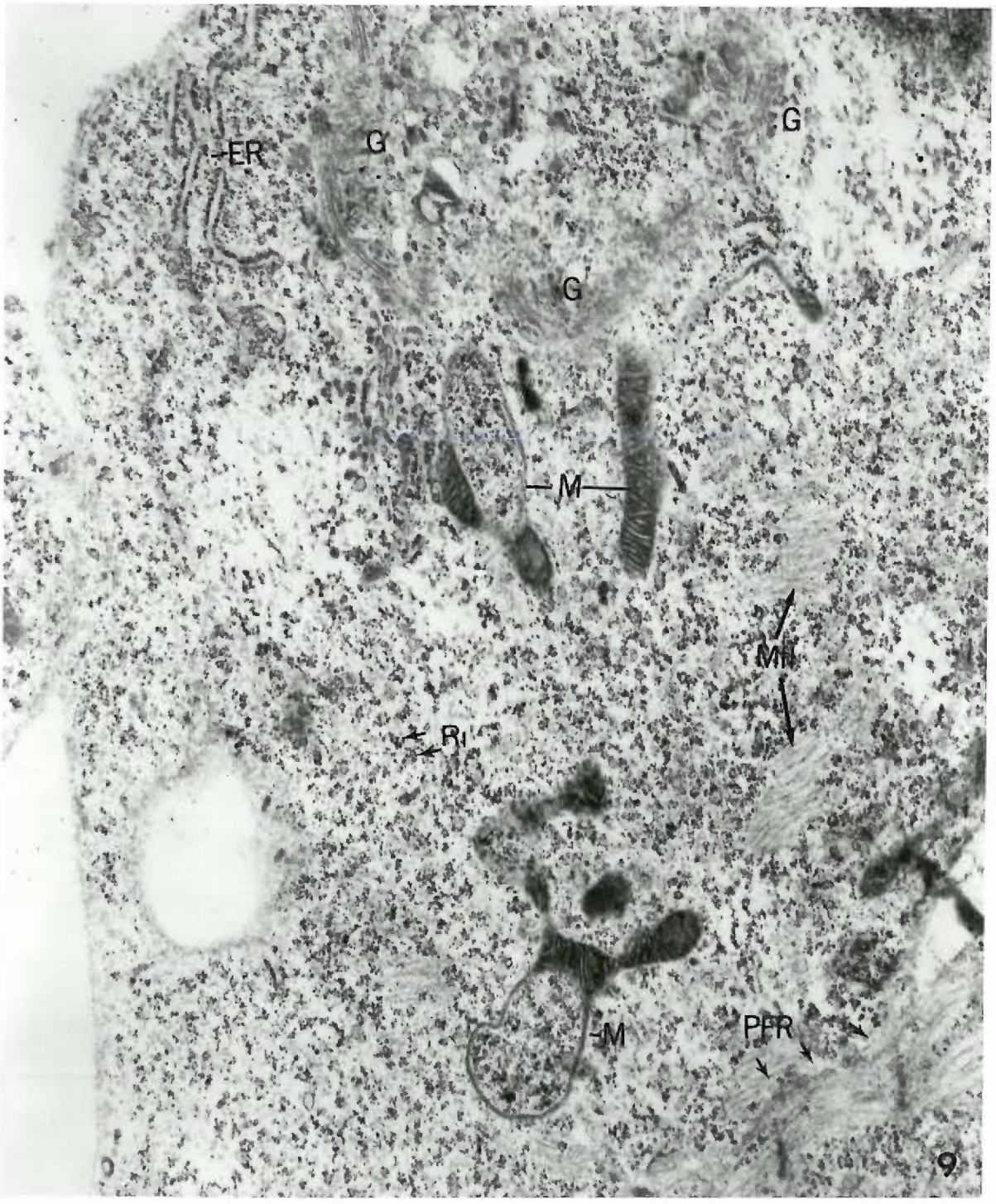

ER
G
G
G
M
MI
RI
M
PFR
9

Figure 10. 10-Day Myocardium

Deep layer of cells. A mitotic cell is shown in the upper left corner. In this mitotic cell several loci of myofilament synthesis are observable (Mfl and arrows). This phenomenon of simultaneous mitotic activity and myofilament synthesis is a regular feature in the embryonic myocardium of the rat. Extensive Golgi body regions are indicated (G). Mitochondria are in a poor state of development.

ER-endoplasmic reticulum; M-mitochondria; G-Golgi bodies; Chr-chromatin material; Mfl and arrows-areas of isolated myofilaments.

X 15,975

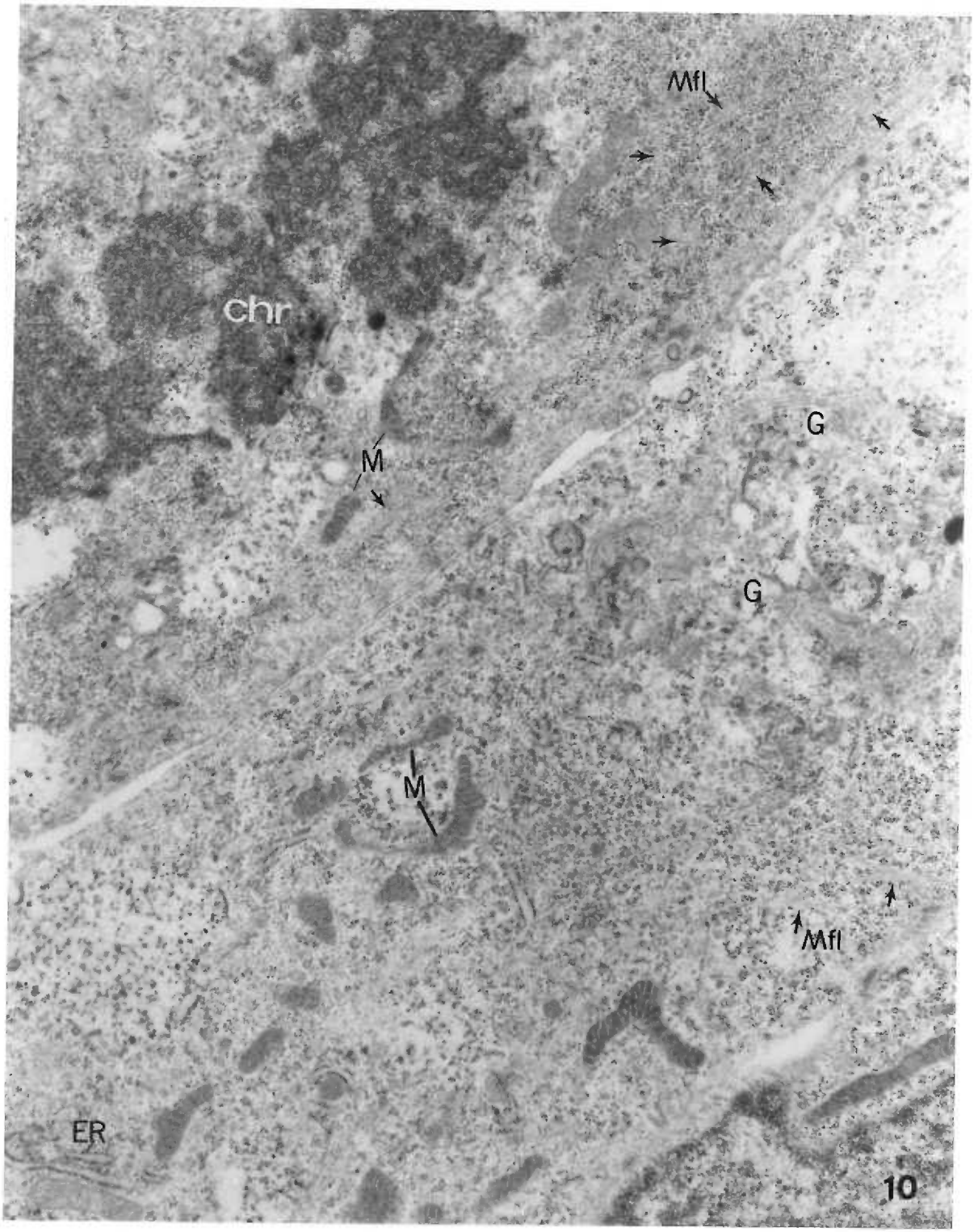
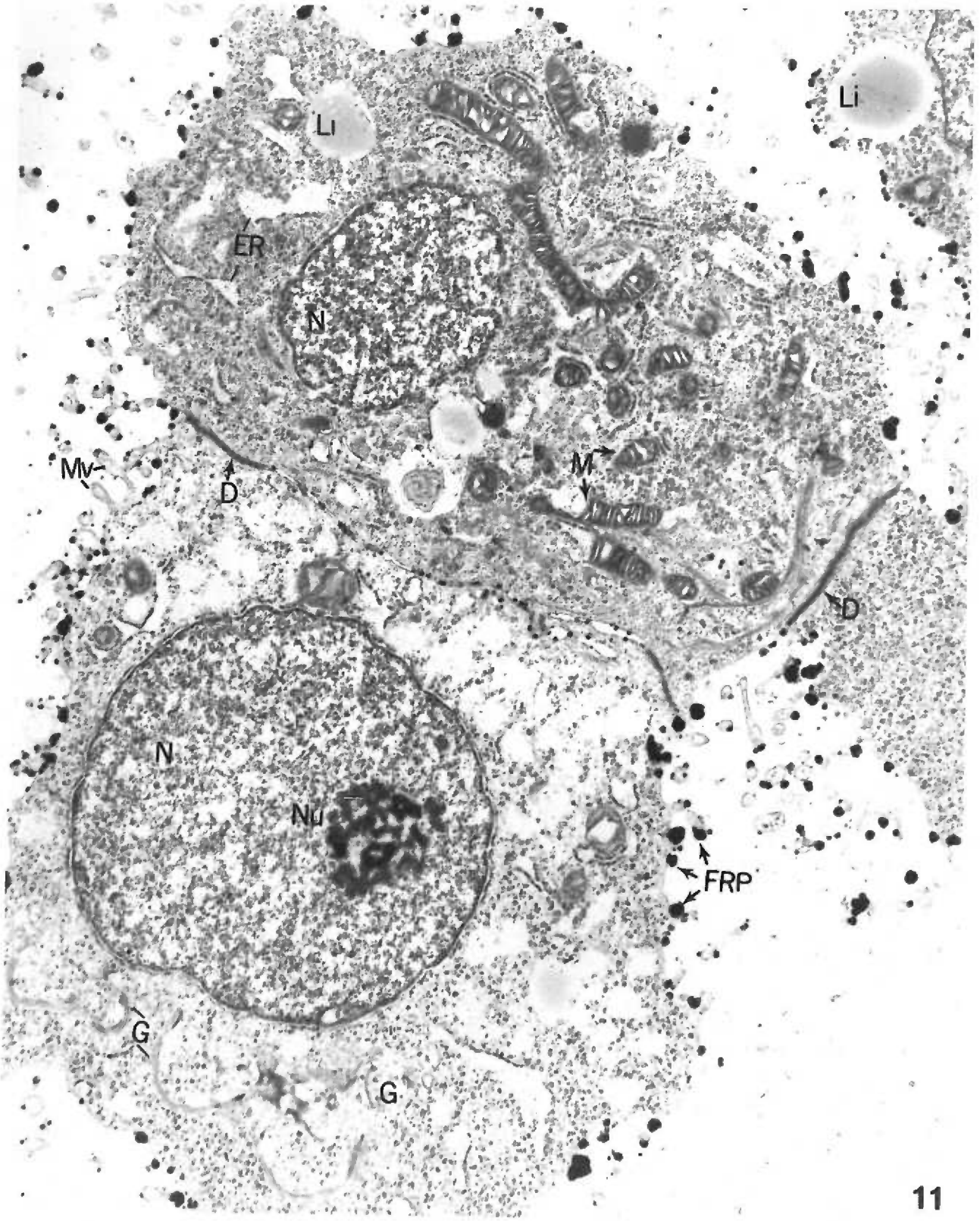

Mfl
chr
G
M
G
M
Mfl
ER
10

Figure 11. 10-Day Myocardium

Tissue incubated in Wachstein-Meisel medium containing ATP as the substrate. Final reaction product (FRP) is deposited on the microvilli (MV) and intercellularly. Distended cisternae of endoplasmic reticulum (ER) containing amorphous material are visible. These cells border the cardiac jelly. FRP-final reaction product; G-Golgi body; D-developing desmosome; Li-lipid; M-mitochondria; N-nucleus; Nu-nucleolus and nucleolonema; Mv-microvilli.

X 11,000



11

Figure 12. 10-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. Final reaction product (FRP) is observable as black precipitate. Several microvilli are noticeable (Mv). These cells border the cardiac jelly.

FRP-final reaction product; M-mitochondria; Mv-microvilli; Li-lipid; N-nucleus.

X 11,000

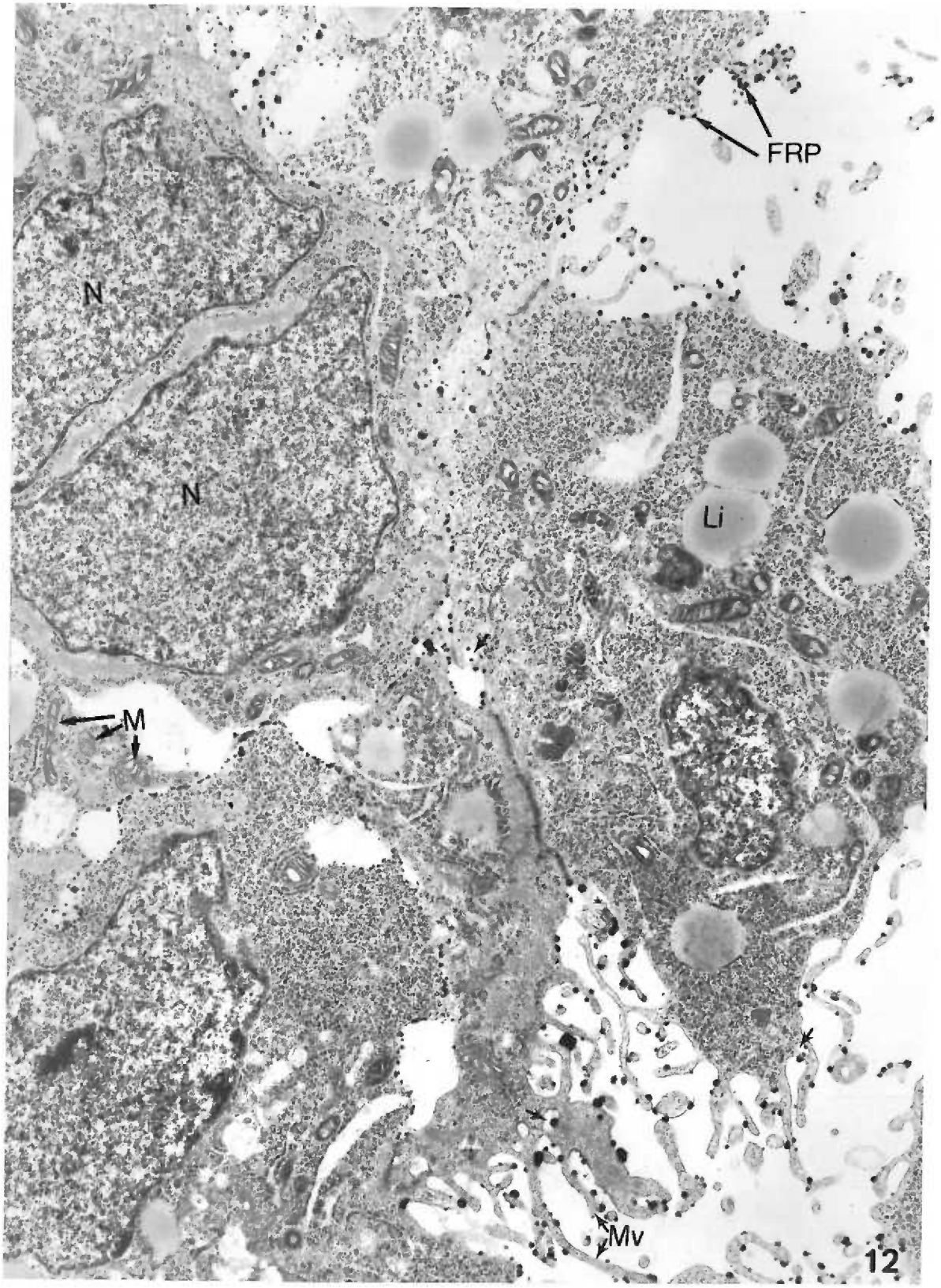
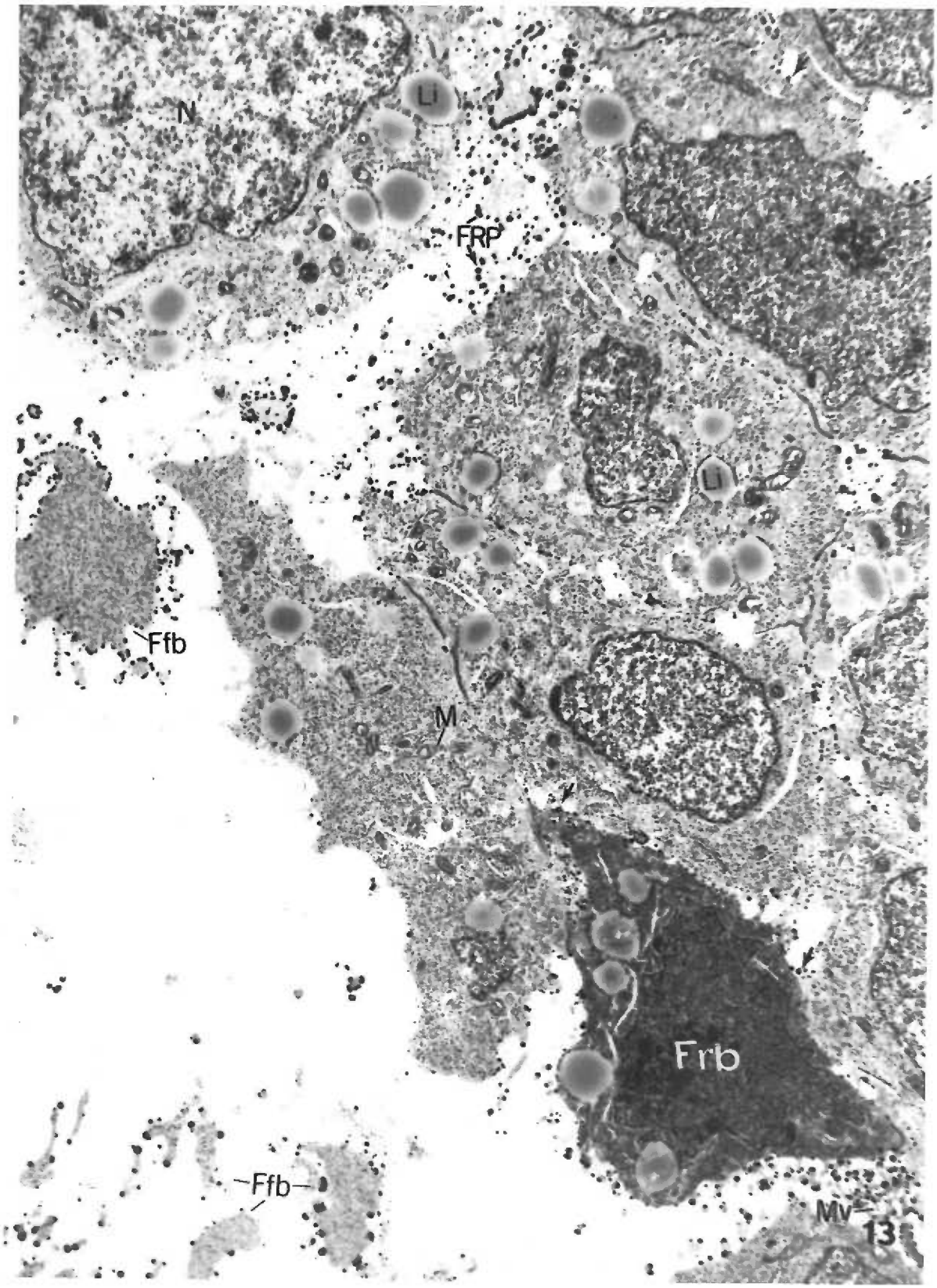

FRP
N
N
Li
M
Mv
12

Figure 13. 10-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. Final reaction product (FRP) is seen as black deposits. There is a suggestion of the product being deposited around lipid droplets (Li).

Ffb-free floating bodies; Frb-fibroblast with reaction product; M-mitochondria; arrows-reaction product in pinocytotic vesicles or caveolae; Mv-microvilli.

X 6,850



13

Figure 14. 11-Day Myocardium

Some loosely packed cells are still encountered in 11-day myocardium. A cell in mitosis is shown in the left of the picture. At the arrows (top right) the perinuclear space is continuous with the endoplasmic reticulum. Chr-chromosomes; N-nucleus; Nu-nucleolus; M-mitochondria; D-developing desmosome; ICS-intercellular space; ER-endoplasmic reticulum.

X 6,900

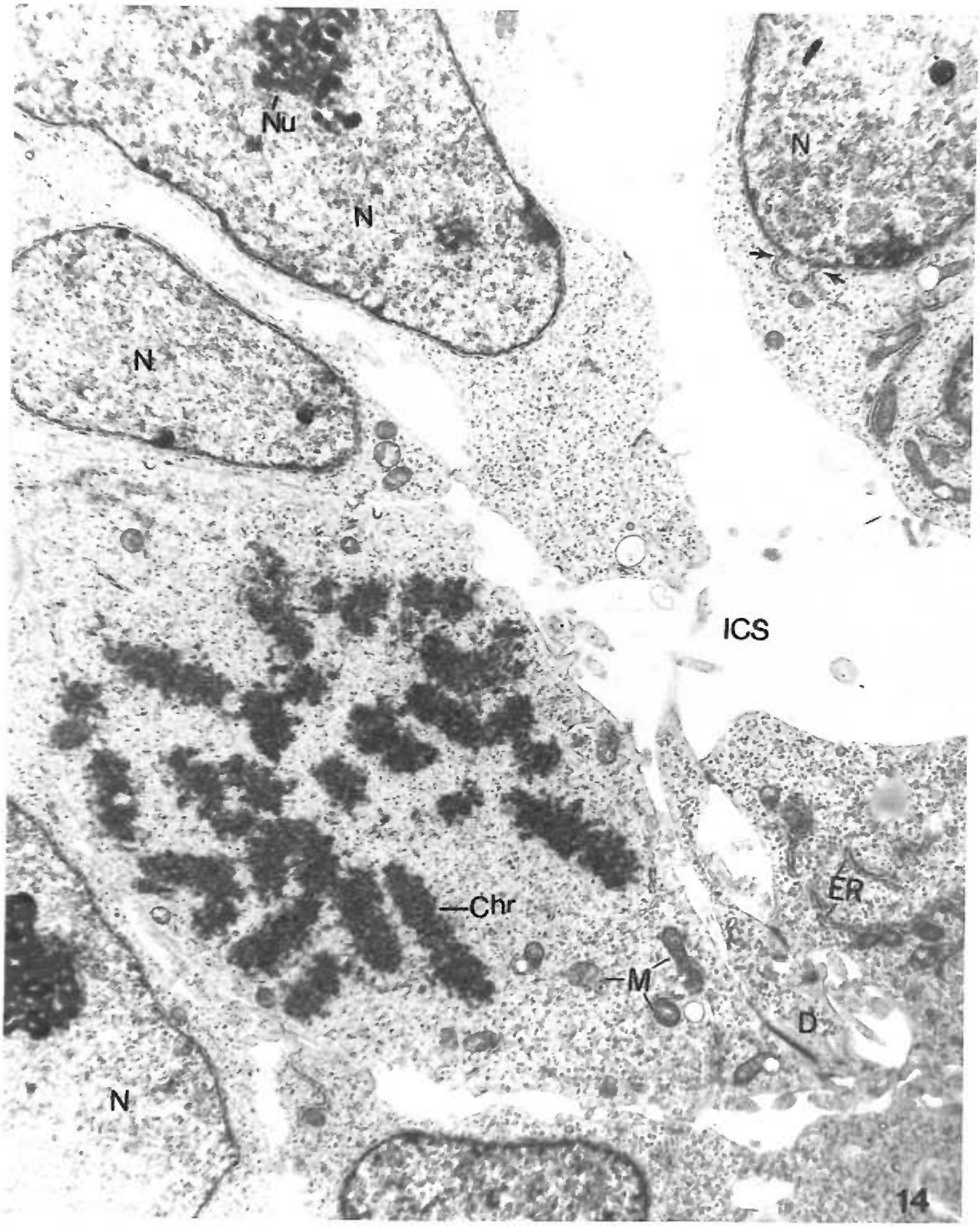

Nu
N
N
N
ICS
Chr
M
ER
D
N
14

Figure 15. 11-Day Myocardium

Two mitotic cells in telophase are shown. Partial reconstruction of the nuclear envelope (NE) from the elements of endoplasmic reticulum is clearly discernible in the lower cell (arrows).

M-mitochondria; Ri-ribosomes; ER-endoplasmic reticulum showing the distended cisternae; Chr-clumps of chromatin material; NE-developing nuclear envelope.

X 18,000

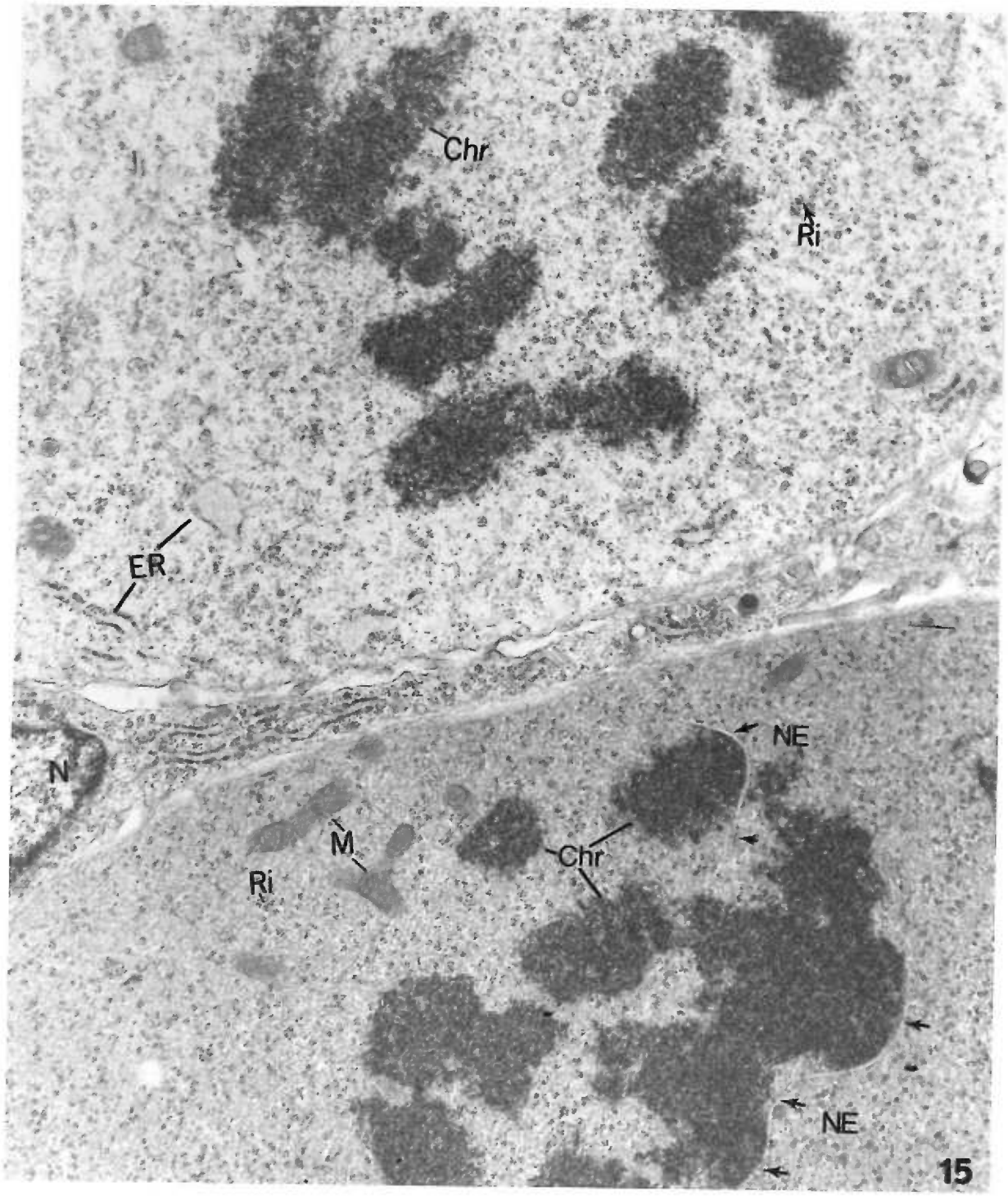

Chr
Ri
ER
NE
N
M
Ri
Chr
NE
15

Figure 16. 11-Day Myocardium

Continuation of the pericisternal space with the endoplasmic reticulum is very apparent (arrows). Developing desmosomes are indicated.

M-mitochondria; N-nucleus; Nu-nucleolus; ICS-intercellular space; MV-microvilli; ER-endoplasmic reticulum with distended cisternae; D-developing desmosome.

X 6,590

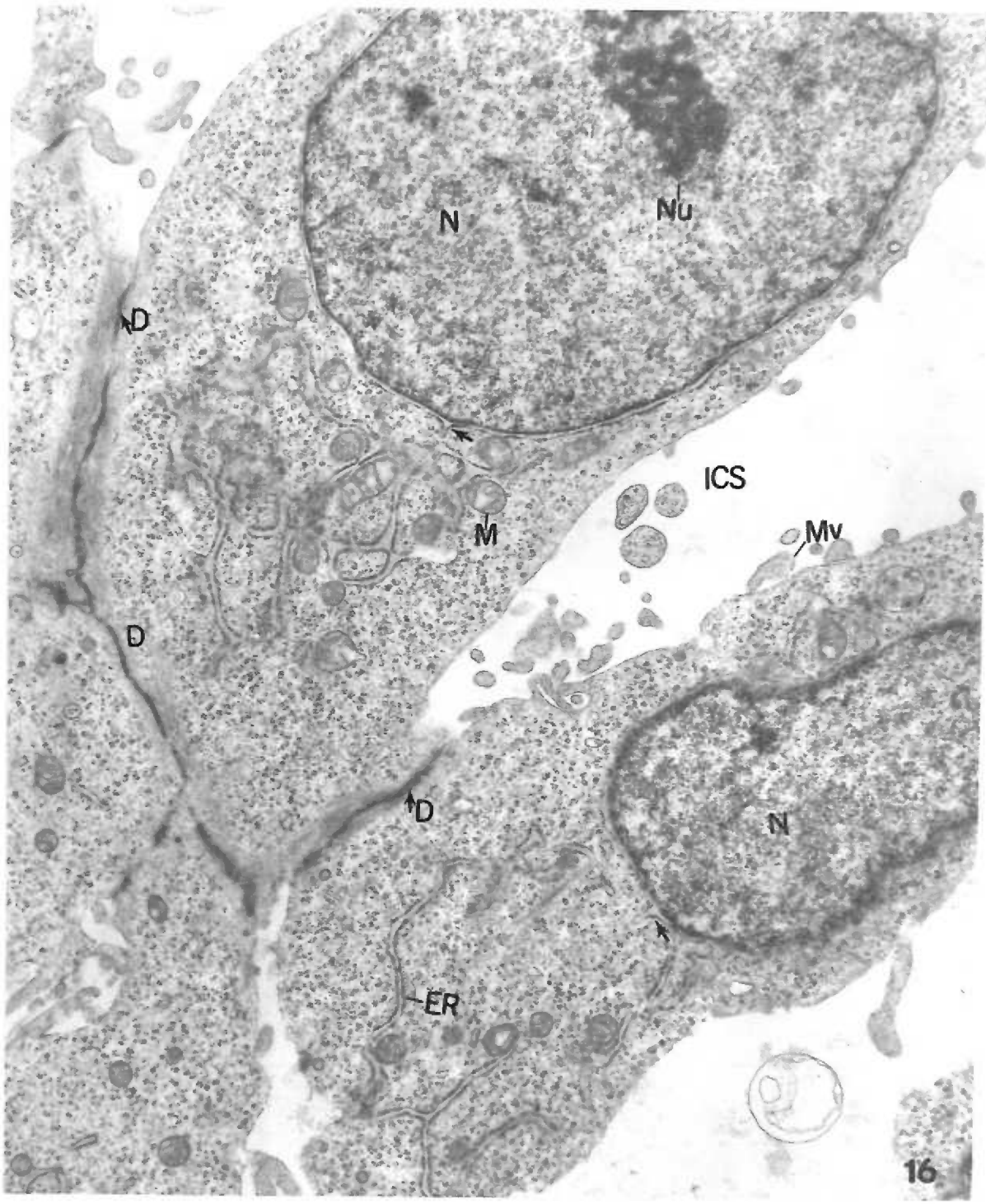

N
Nu
D
ICS
M
Mv
D
D
N
ER
16

Figure 17. 11-Day Myocardium

Epicardial cell (EPC) on the left. Myocardial cells (Myc) on the right. The epicardial cell is characterized by large quantities of endoplasmic reticulum (ER) arrayed in parallel lamallae.

MF-myofibril; M-mitochondria; N-nucleus; EPC-epicardial cell; Myc-myocardial cell.

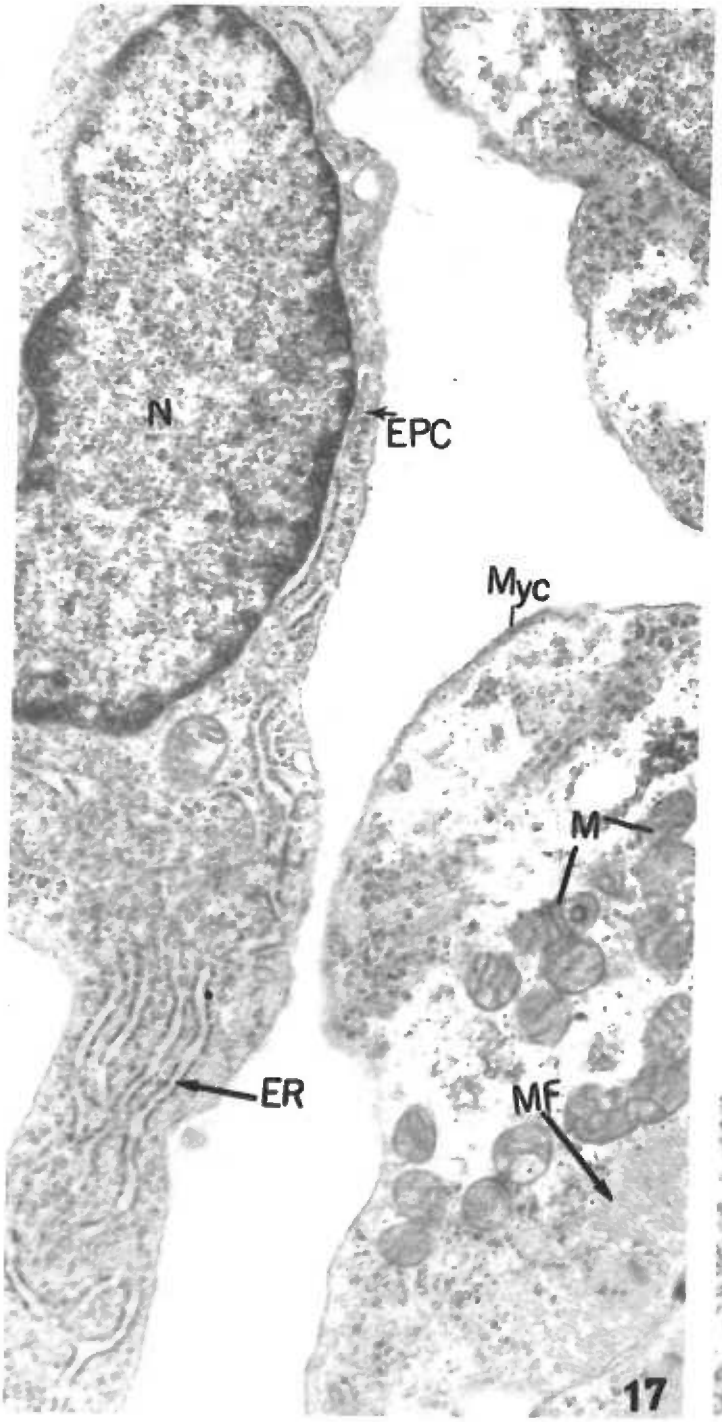
X 18,000

Figure 18. 11-Day Myocardium

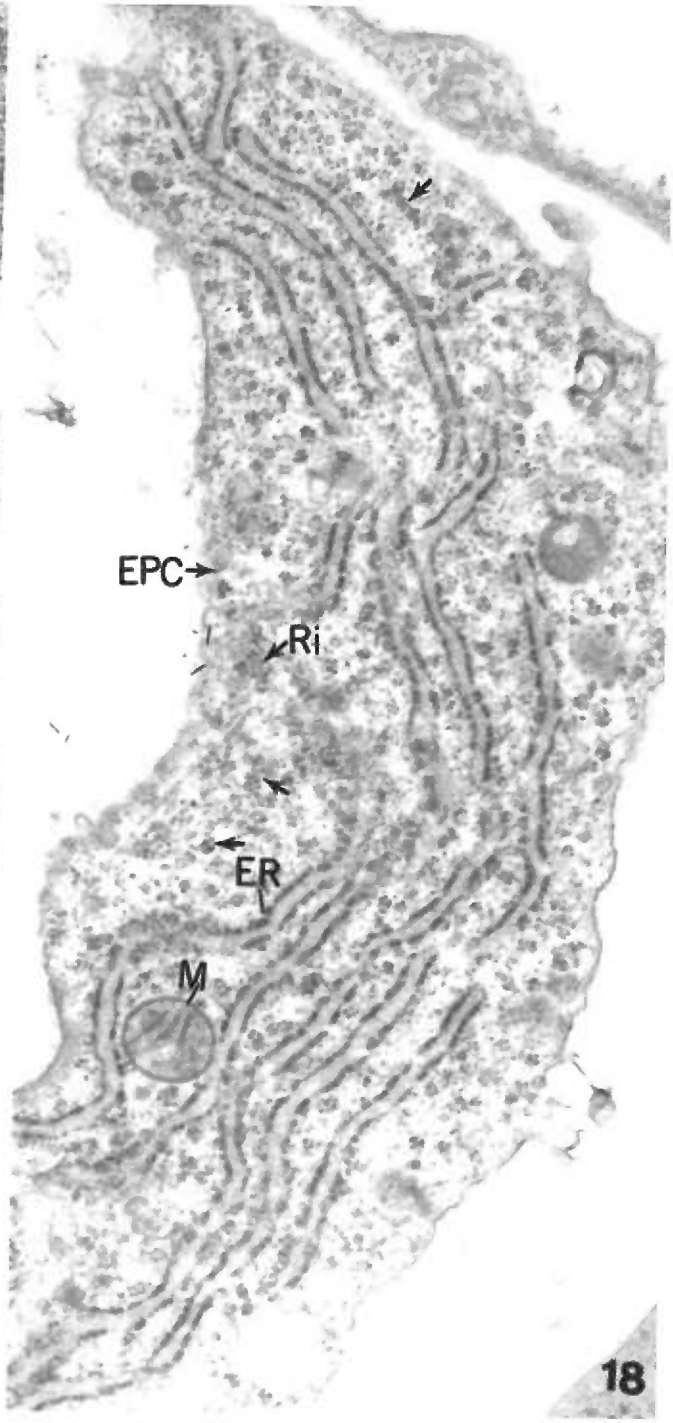
An enlarged view of an epicardial cell. Parallel skeins of endoplasmic cisternae are observable (ER). Large quantities of clusters of ribosomes are noticeable (arrows). Myofilaments or fibrils are not present in epicardium.

M-mitochondria; ER-endoplasmic reticulum; EPC-epicardial cell; Ri and arrows-ribosomes.

X 26,000



17



18

Figure 19. 11-Day Myocardium

Large numbers of Golgi vesicles (Gv) are discernible in the Golgi region(G). Mitochondria (M) show further development, and cristae are well-formed. A suggestion of a zig-zag course of some of the cristae (M and arrows), characteristic of adult cardiac muscle, is detectable. The close proximity of myofibrils (MF), mitochondria (M), endoplasmic reticulum (ER) and Golgi regions (G and Gv) is very apparent. A chain of polyribosomes (PRi) and clusters of ribosomes (Ri) are noticeable.

D-developing desmosome; N-nucleus; ICS-intercellular space; G-Golgi bodies; Gv-Golgi vesicles; PRi-polyribosomes; MF-myofibrils; ER-endoplasmic reticulum.

X 18,000

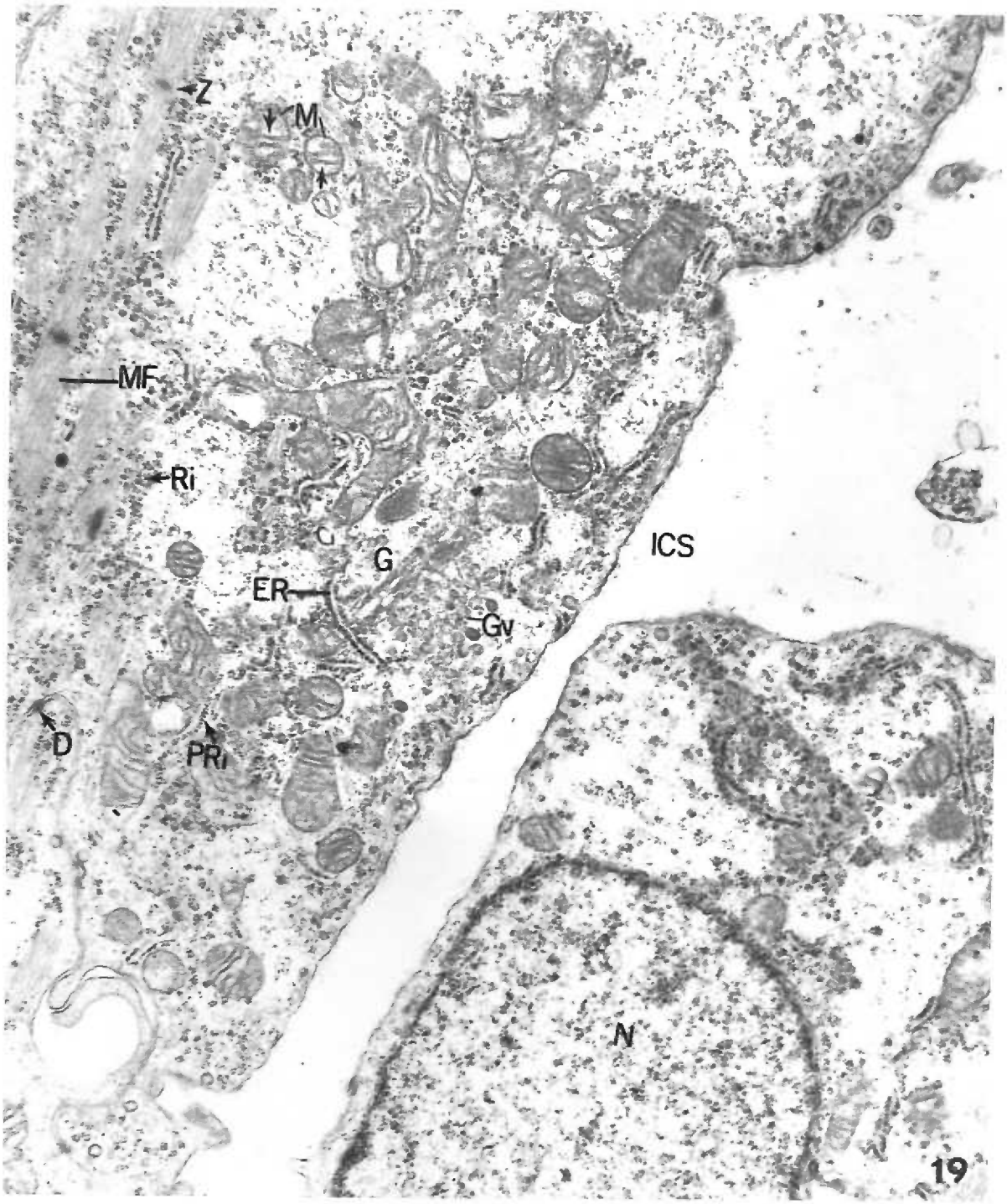

Z
M
MF
Ri
G
ER
Gv
ICS
D
PRi
N
19

Figure 20. 11-Day Myocardium

Several developing intercalated discs with attached myofilaments are discernible (ID). Myofilaments are attached to the intercalated disc at different angles so as to give the indication of branching of fibrils (arrows). Desmosomes (D) are frequently associated with intercalated discs. Myofibrils cut in many planes are visible in the cytoplasm.

M-mitochondria; x-cross section of myofibrils; Z-Z disc; Ri-ribosomes; ID-intercalated discs.

X 18,000

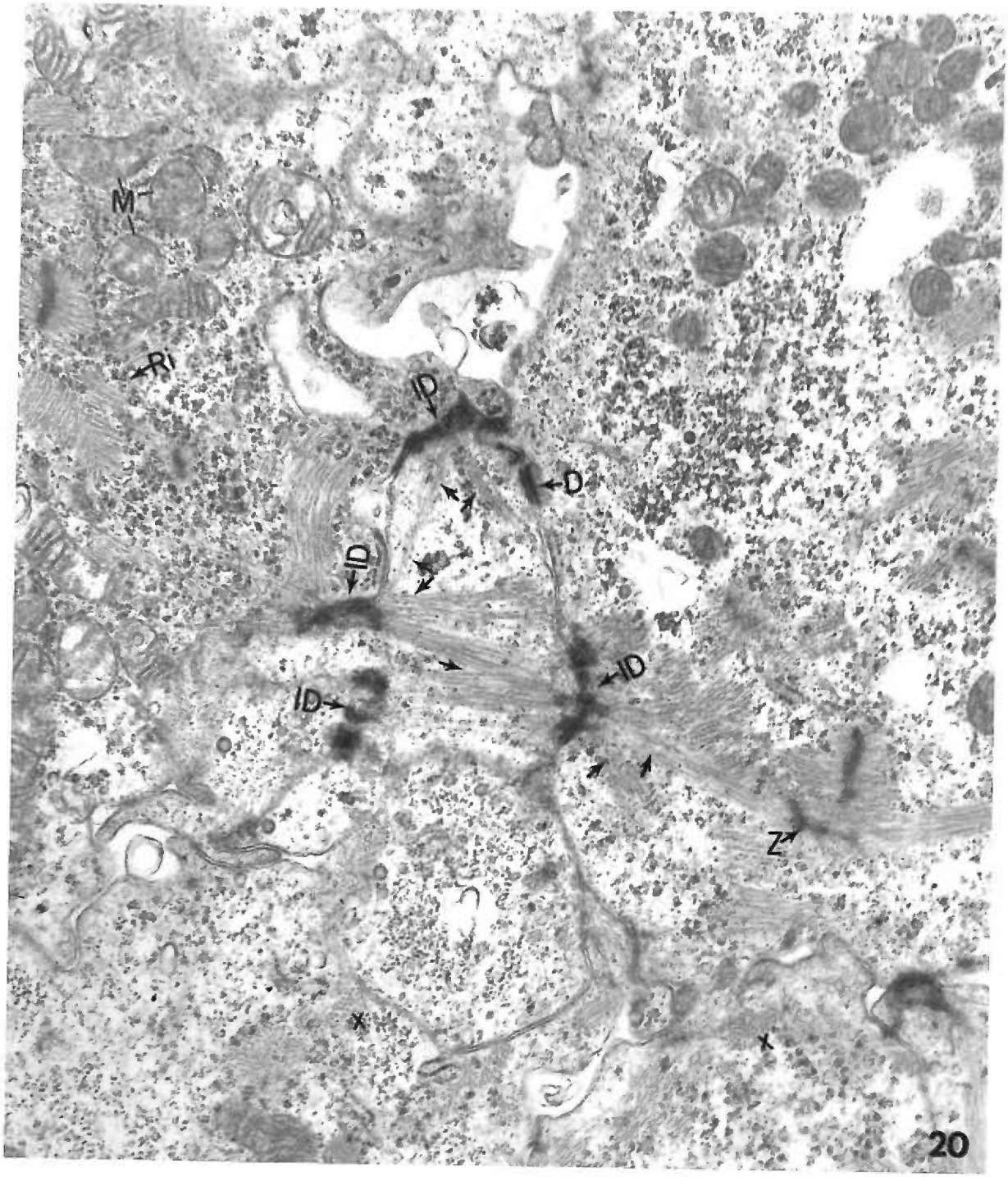

M
Ri
ID
D
ID
ID
ID
Z
X
X
20

Figure 21. 11-Day Myocardium

Large numbers of pinocytotic vesicles (Pv) are recognizable in the region of the intercalated disc (ID). Myofibrils are attached to the intercalated disc at different angles.

M-mitochondria; N-nucleus; Pv-pinocytotic vesicles; ID-intercalated disc.

X 18,000

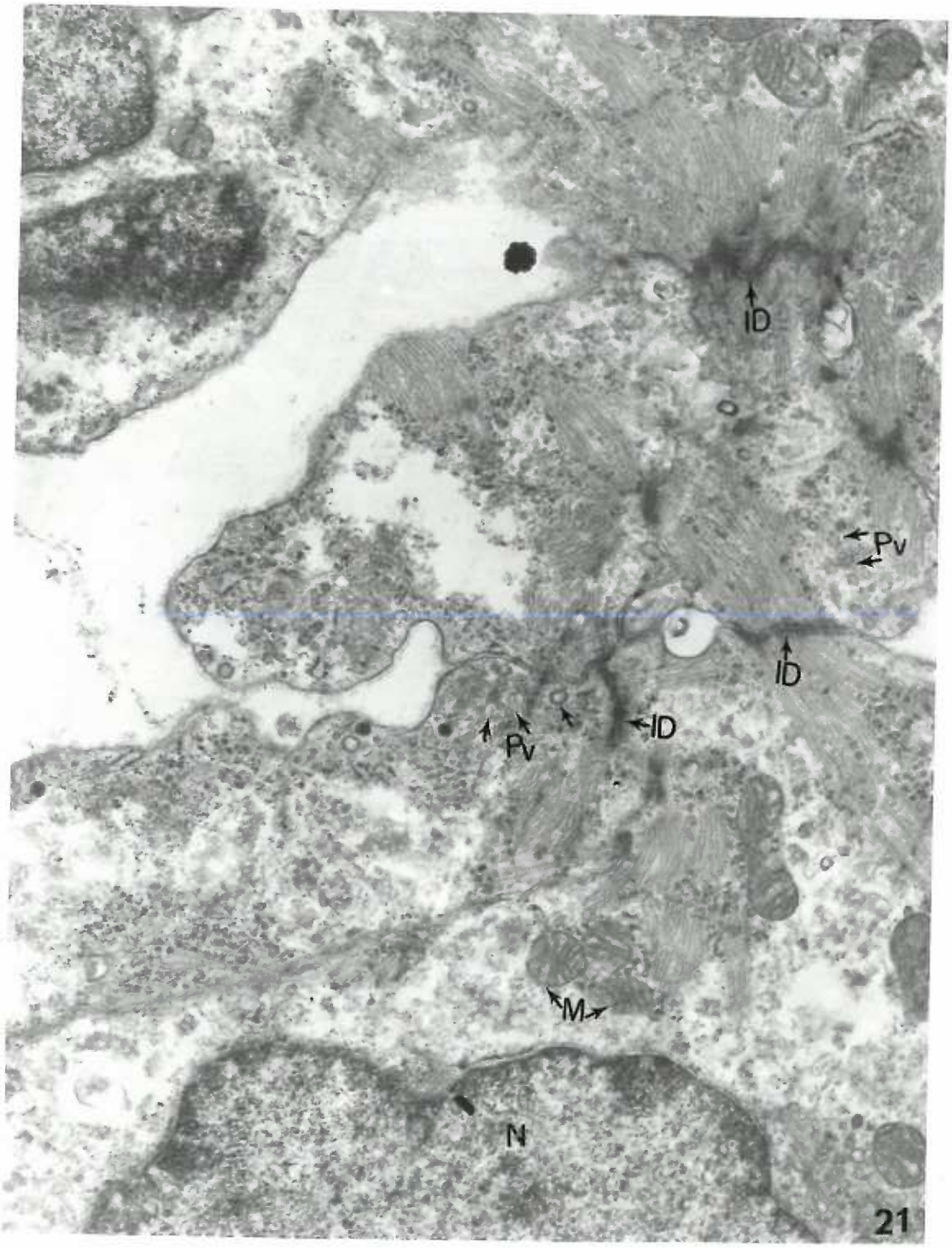

ID
Pv
ID
Pv
ID
M
N
21

Figure 22. 11-Day Myocardium

A large number of Golgi vesicles and vacuoles (Gv) are discernible in the Golgi body region (G) adjacent to the nucleus. Thin and thick filaments can be observed in the cross section of the myofibril (x).

ER-endoplasmic reticulum; MF-myofibril; Z-Z disc; N-nucleus; G-Golgi bodies; Gv-Golgi vesicles and vacuoles; x-cross section of myofibril.

X 18,000

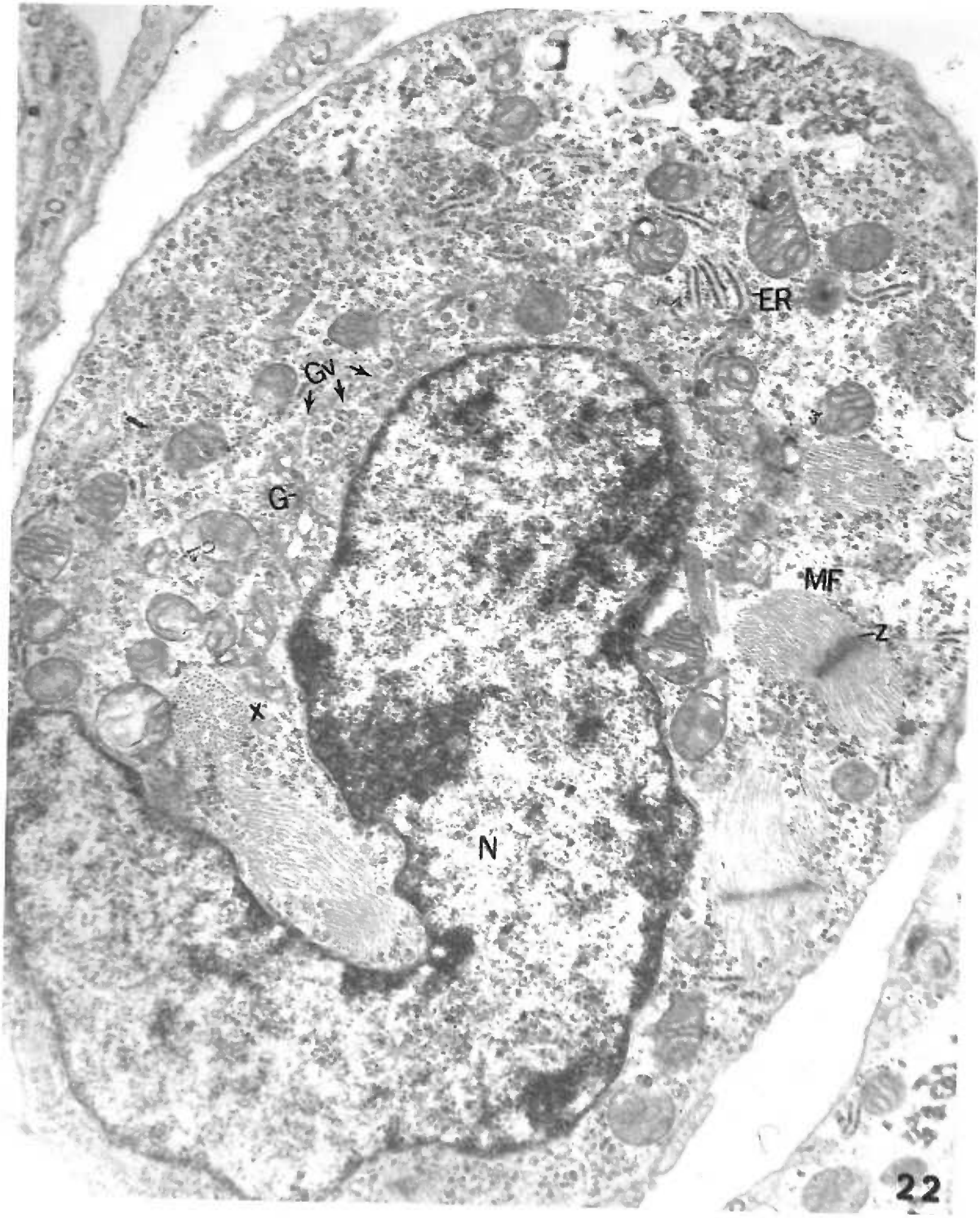

ER
GV
G
MF
Z
X
N
22

Figure 23. 11-Day Myocardium

Fully formed fibrils with striations (Z, I, A and H bands) ca be observed in the longitudinal axis of the cell, parallel to the sarcolemma. Several fibrils are seen grouped together. Intimately associated with the Z disc regions are the elements of developing T-system (Ts and arrows). Tubular cristae are recognizable in some mitochondria (M).

N-nucleus; D-developing desmosome; Ri-ribosomes; A, I, Z and H-the respective bands; Ts-developing T-system; M-mitochondria.

X 17,440

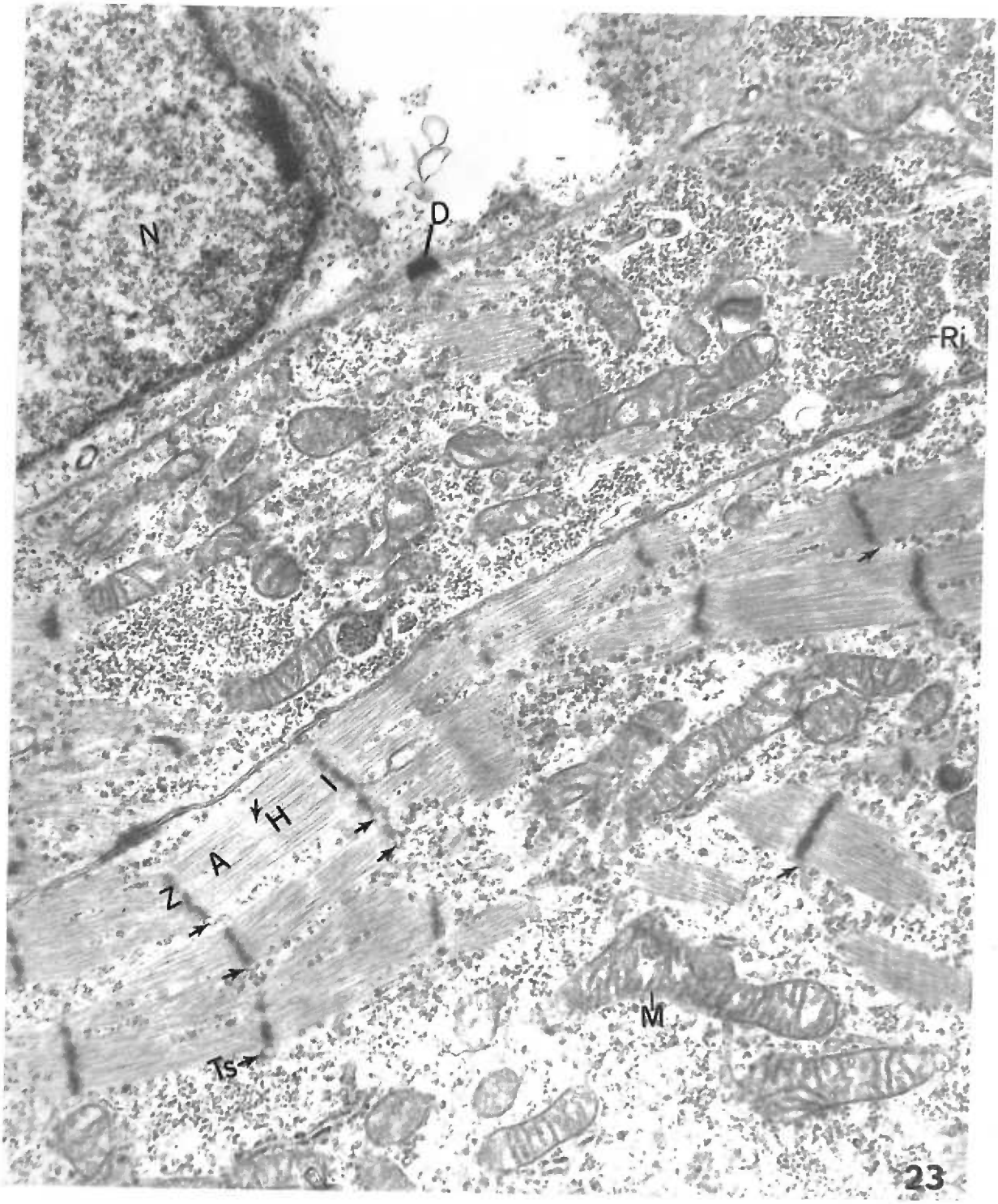

N
D
Ri
O
I
H
A
Z
M
Ts
23

Figure 24. 11-Day Myocardium

Several membrane-bound secretory granules (SGr) are discernible in the region of the Golgi body. Large numbers of Golgi vesicles and vacuoles (Gv) are also recognizable, some of which contain a flocculent material. Parallel assemblage of endoplasmic reticulum (ER) is observable at top right. N-nucleus; M-mitochondria; SGr-secretory granules; Gv-Golgi vesicles and vacuoles; ER-endoplasmic reticulum.

X 18,000

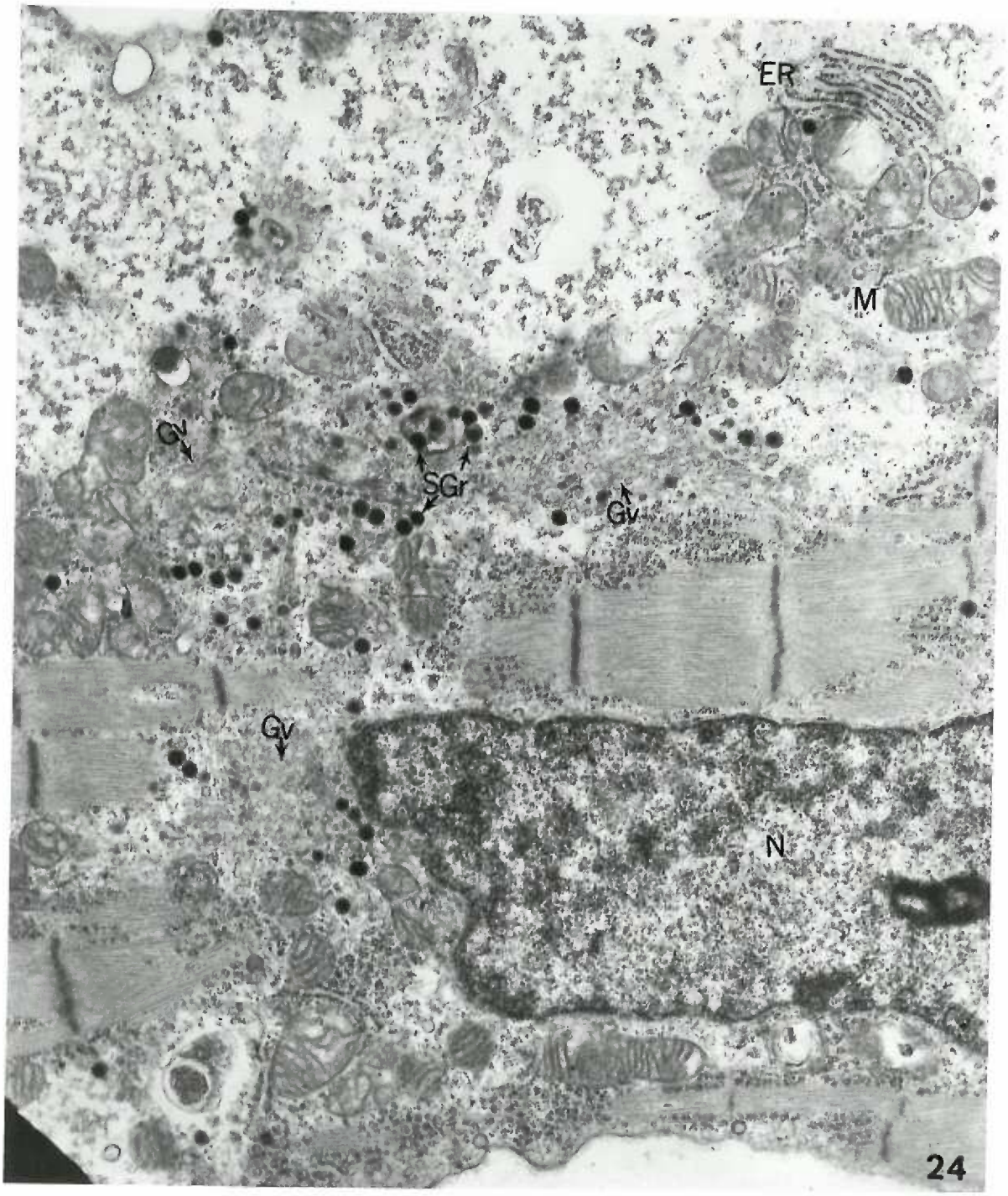

ER
M
Gv
SGr
Gv
Gv
N
24

Figure 25. 11-Day Myocardium

A high magnification view of secretory granules. These membrane-bound granules (SGr) are frequently observed in the Golgi region (G). Several vacuoles (V) containing an amorphous material are readily recognizable. Cristae mitochondriales give an impression of a zig-zag arrangement, characteristic of adult cardiac mitochondria (M, arrow).

SGr-secretory granules; V-vacuoles; M-mitochondria; N-nucleus.

X 50,000

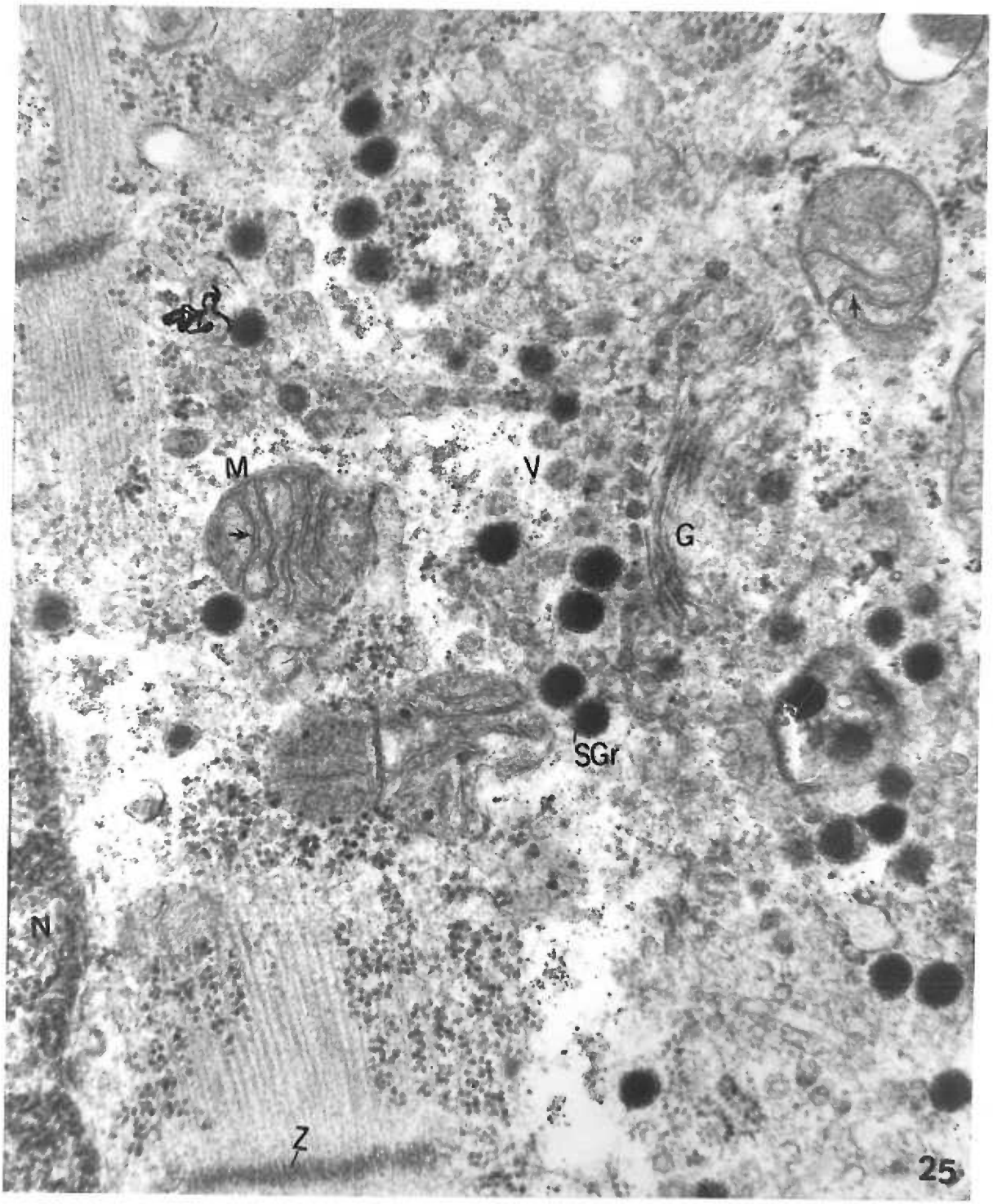

M
V
G
SGr
N
Z
25

Figure 26. 11-Day Myocardium

Cross section of a myocardial cell showing the hexagonal configuration of thick (T) and thin(t) filaments.

D-desmosomes; arrows-the filamentous material which invests the myocardial cells, called the external lamina or glycocalyx; M-mitochondria; T-thick filament; t-thin filament.

X 50,000

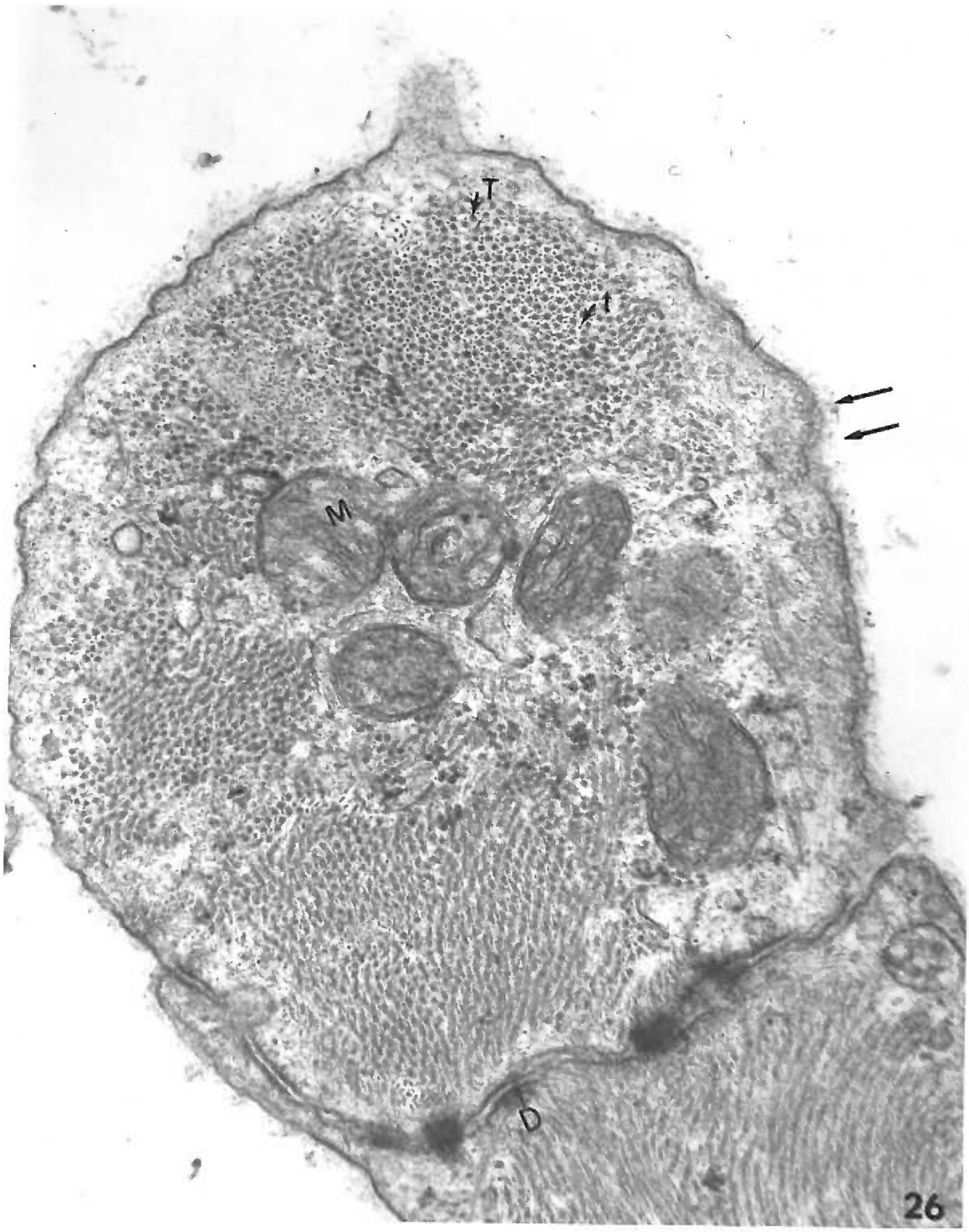

T
t
M
D
26

Figure 27. 11-Day Myocardium

A single fibril in a state of contraction is discernible. Z and A bands are visible, but I bands are not. The mitochondria (m) display a peculiar arrangement in this cell in that the cristae are interconnected at numerous points (m), so as to form a reticulum or labyrinthine system.

Z, A-respective bands; m-mitochondria.

X 26,000

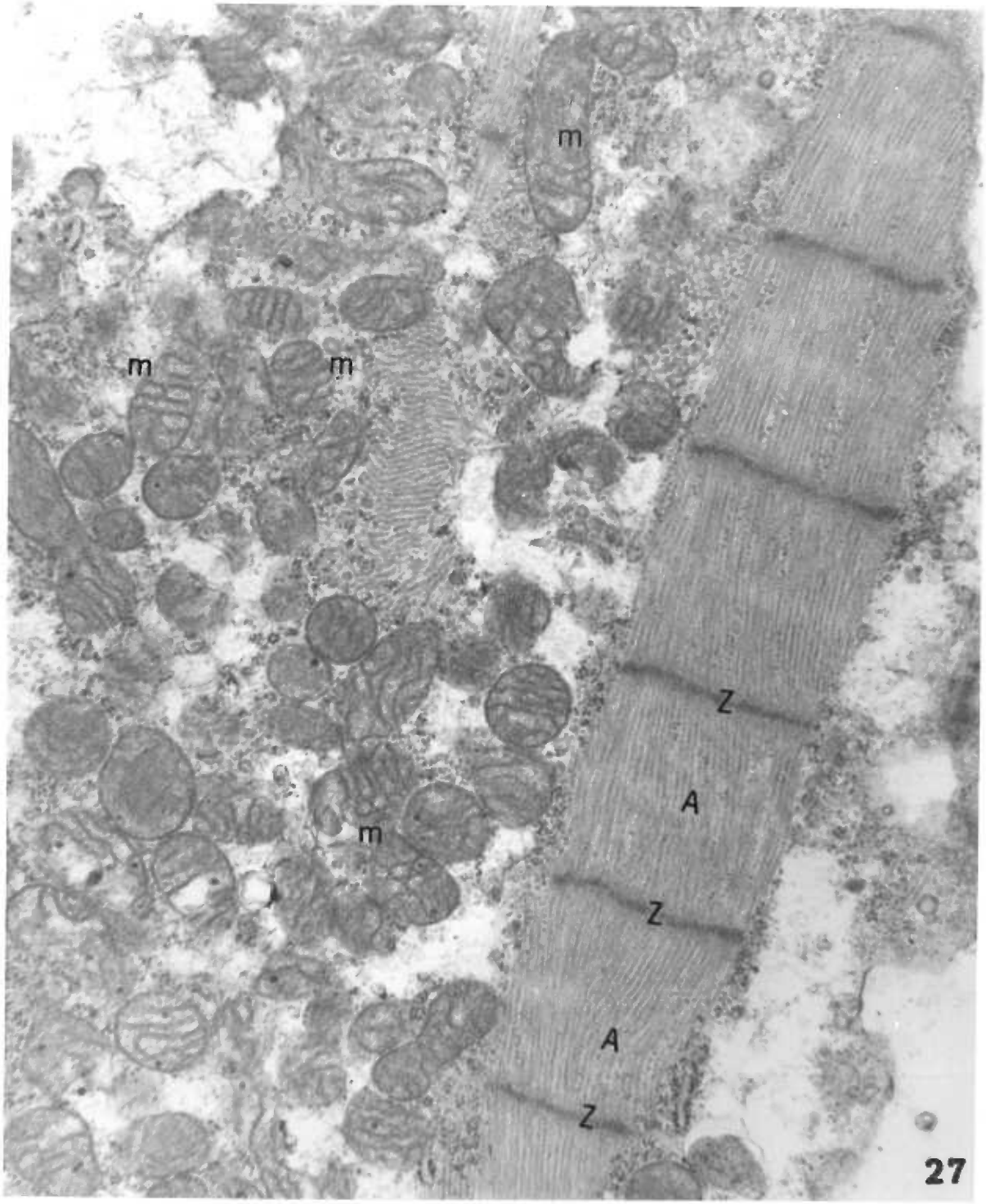

m
m
m
m
Z
A
Z
A
Z
27

Figure 28. 11-Day Myocardium

Several myofibrils (MF) coursing in the long axes of the cells are discernible. most of these fibrils are separated from one another by large interfibrillar spaces. The close association of developing T-system and Z-disc regions can be observed at several points (Ts and small arrows). The sarcolemma is actively involved in the process of pinocytosis (large arrows, bottom left). Tubular cristae are recognizable in some mitochondria (m, arrows). MF-myofibrils; m-mitochondria; Ts-developing T-system; N-nucleus; long arrows-pinocytotic vesicles.

X 17,440

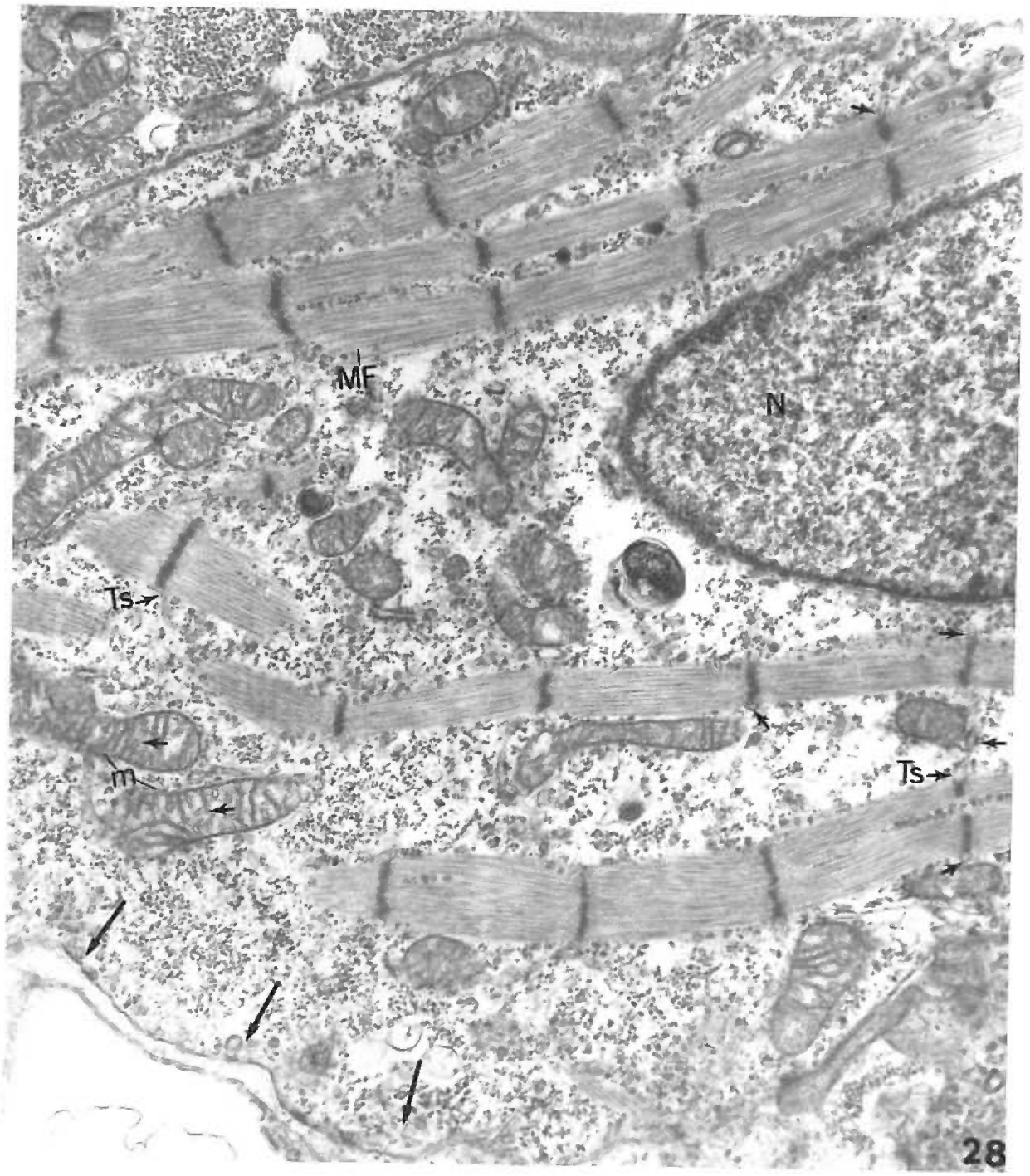

MF
N
Ts
Ts
m
28

Figure 29. 11-Day Myocardium

Two adjoining myocardial cells showing well-organized fibrils (MF) in one of them (top), while isolated myofilaments in random distribution are found in the other (Mfl). Developing intercalated discs (ID) and desmosomes (D) are discernible. Numerous pinocytotic vesicles (Pv) are apparent in these cells. The phenomenon of branching of the fibrils can be observed at two sites (x). A chain of polyribosomes is indicated (PRi).

Mfl-myofilaments; MF-myofibrils; m-mitochondria; PRi-chain of polyribosomes; Pv-pinocytotic vesicles; D-desmosome; ID-intercalated disc; x-branching fibril.

X 17,440

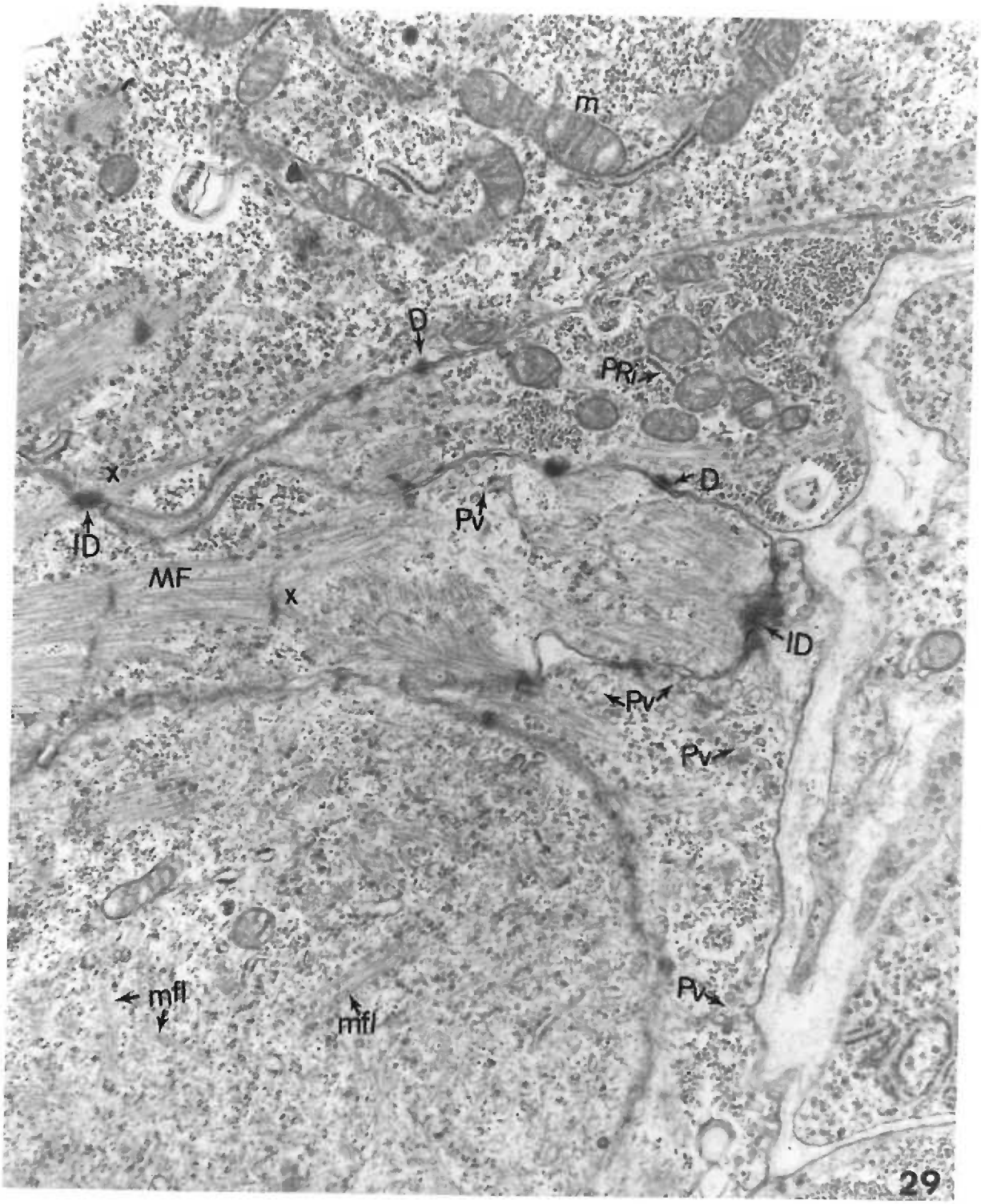

m
D
PRl
x
D
ID
Pv
MF
x
ID
Pv
Pv
mfl
mfl
Pv
29

Figure 30. 11-Day Myocardium

The phenomenon of branching of the fibrils is recognizable. Five to six (1, 2, 3, 4, 5, 6) Z disc regions are involved in this process wherein the myofilaments run in different angles so as to form irregular and polygonal figures bounded by myofilaments and Z discs. This process apparently brings about the fusion or anastomosis of several fibrils.

ID-intercalated disc; SGr-secretory granules; 1 to 5-Z disc regions; N-nucleus.

X 18,000

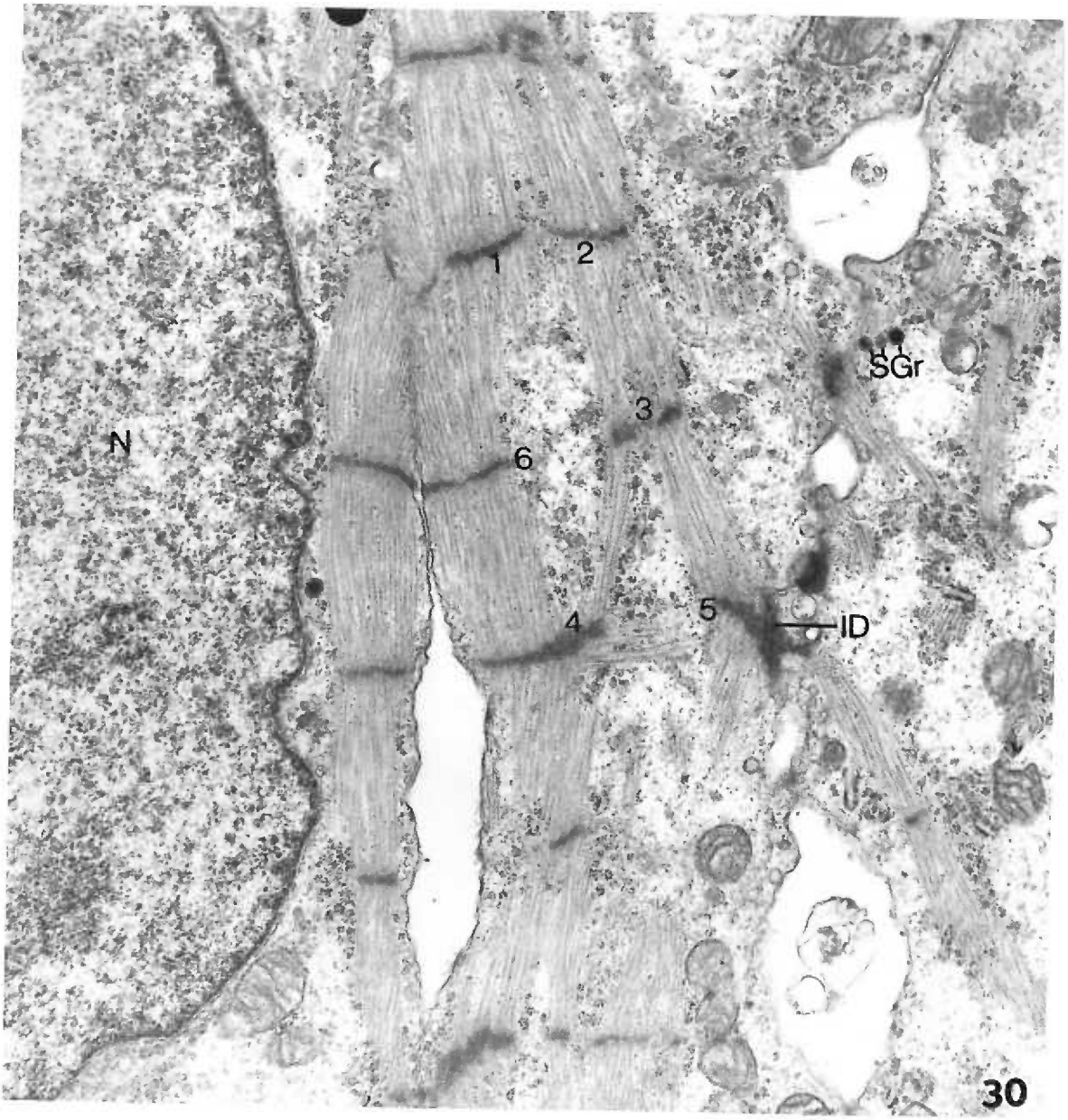

1
2
3
4
5
6
N
SGr
ID
30

Figure 31. 11-Day Myocardium

Cross sections of several myofibrils (MF) are shown. The hexagonal arrangement of thick (T) and thin (t) filaments is very apparent. The intimacy of association of glycogen particles (Gy) and myofibrils is readily recognizable. Gy-glycogen; MF-myofibrils; T-thick filaments; t-thin filaments; m-mitochondria.

X 26,000

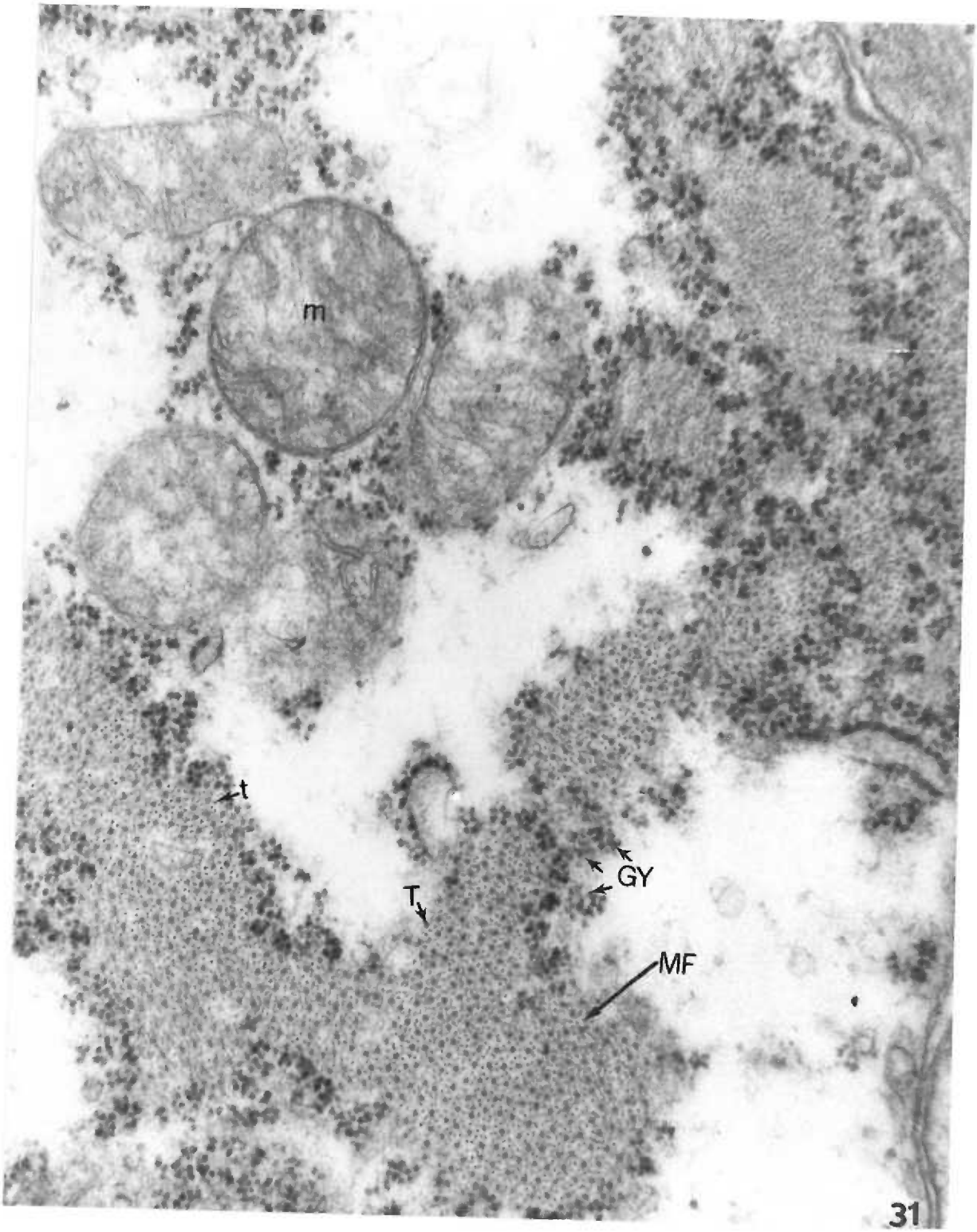

m
t
GY
MF
31

Figure 32. 11-Day Myocardium

Extensive distribution of Golgi bodies (G) is discernible. The cisternae are distended and contain a flocculent material. Several secretory vacuoles (V) are recognizable. Multivesicular bodies (Mvb) are frequently associated with Golgi regions. Clusters of glycogen particles (Gy) can be observed in close contact with myofibrils (MF).

MF-myofibrils; G-Golgi bodies; Mvb-multivesicular bodies; D-desmosome; m-mitochondria; Gy-glycogen; V-secretory vacuole.

X 26,000

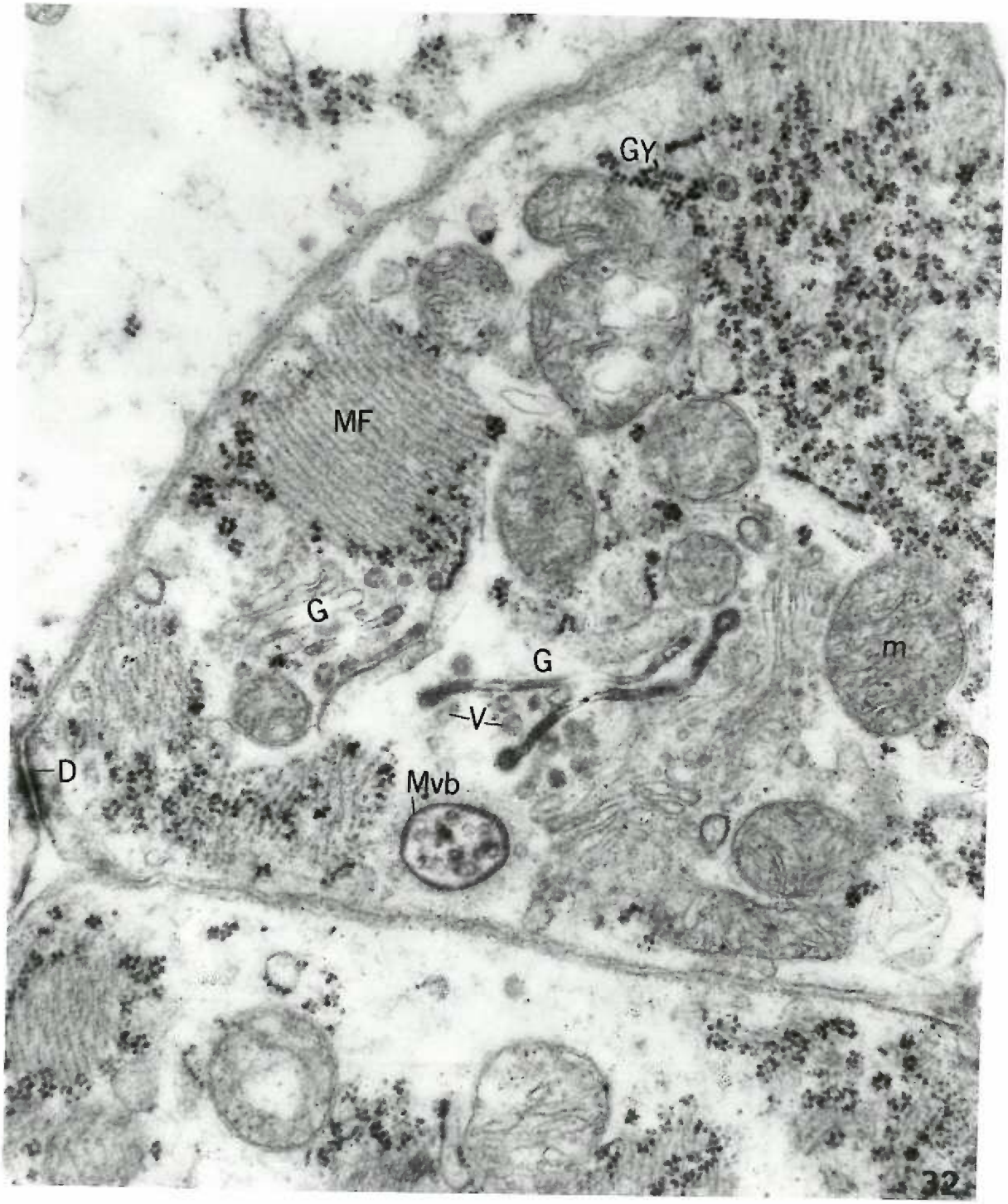
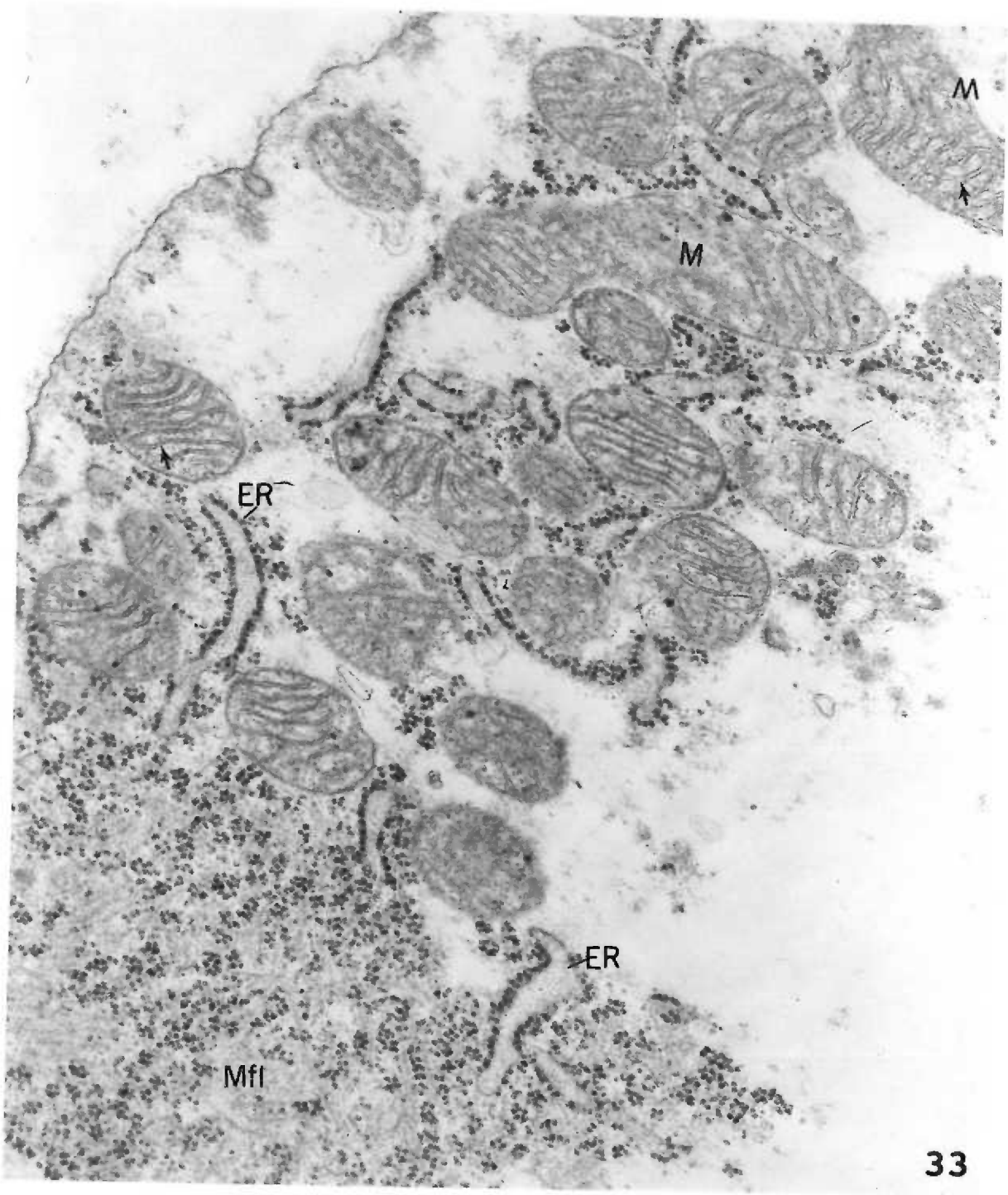

GY
MF
G
G
V
m
D
Mvb
32

Figure 33. 11-Day Myocardium

Greatly distended cisternae of endoplasmic reticulum (ER) containing an amorphous material are discernible. Isolated myofilaments (Mfl) distributed at random can be seen at the bottom left of the micrograph. Zig-zag cristae are recognizable in some mitochondria (M, arrows).

ER-endoplasmic reticulum; Mfl-myofilaments; M-mitochondria; M, arrows-zig-zag cristae.

X 26,000



33

Figure 34. 11-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. Final reaction product (FRP and arrows) is deposited as granules on the sarcolemma. Skeins of cisternae of endoplasmic reticulum (ER) containing an amorphous material are noticeable.

FRP and arrows-final reaction product; ICS-intercellular space; ER-endoplasmic reticulum.

X 17,440

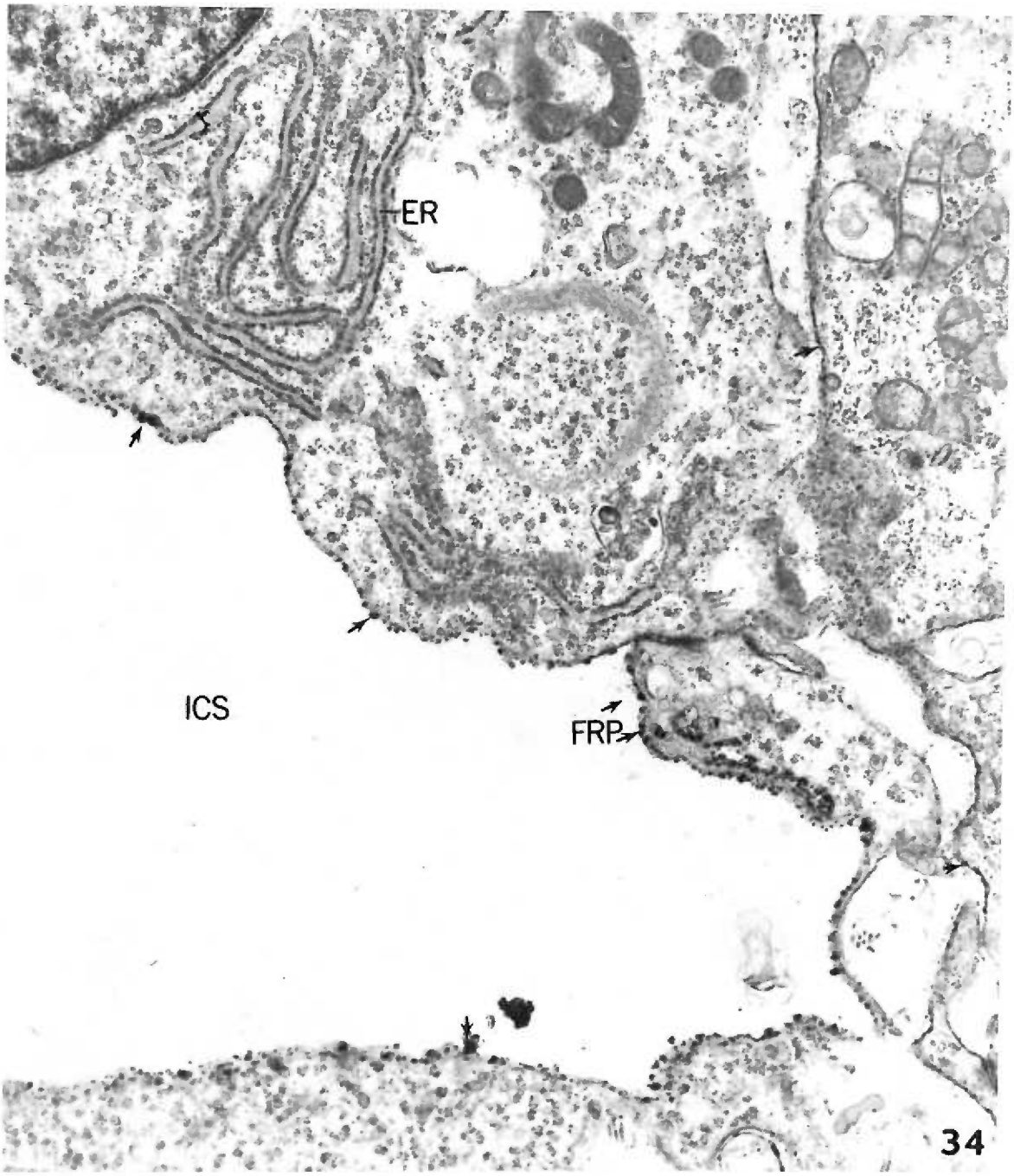

ER
ICS
FRP
34

Figure 35. 11-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. Final reaction product (FRP and arrows) is deposited as granules on the sarcolemma. The intensity of reaction is somewhat less than that of 10-day myocardium. Endoplasmic reticulum (ER) is arranged in skeins of cisternae or in single tubular cisternae. The close proximity of mitochondria (m) and endoplasmic reticulum is very apparent.

FRP and arrows-final reaction product; MF-myofibrils; Mvb-multivesicular body; ER-endoplasmic reticulum; m-mitochondria.

X 17,440

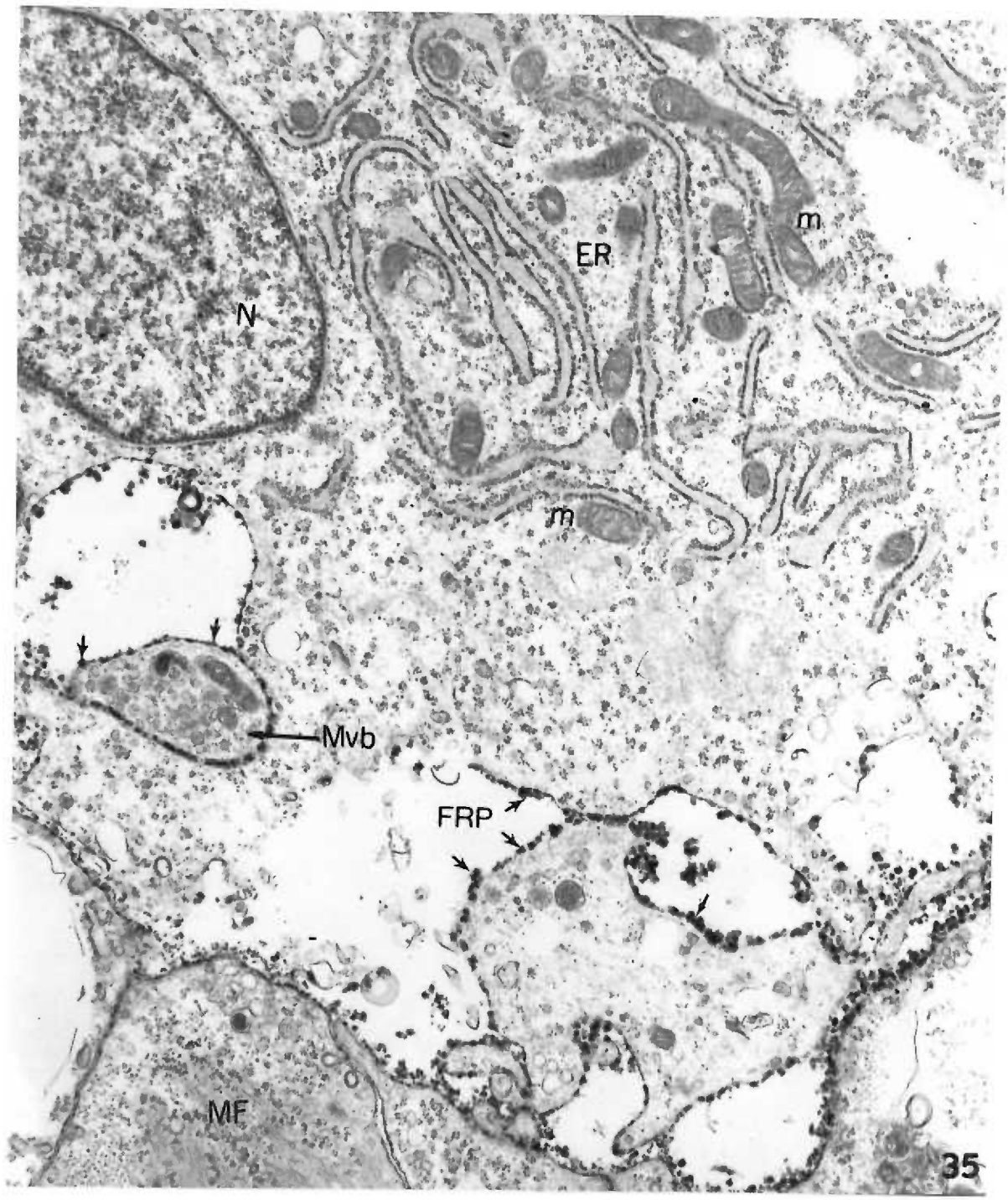

N
ER
m
m
Mvb
FRP
MF
35

Figure 36. 11-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. Final reaction product (FRP) is deposited as granules on the sarcolemma. Large vacuoles are associated with the nuclear envelope (V). A few collagen fibers (Co) are discernible.

FRP-reaction product; Co-collagen fibers; V-vacuoles; m-mitochondria; N-nucleus.

X 17,440

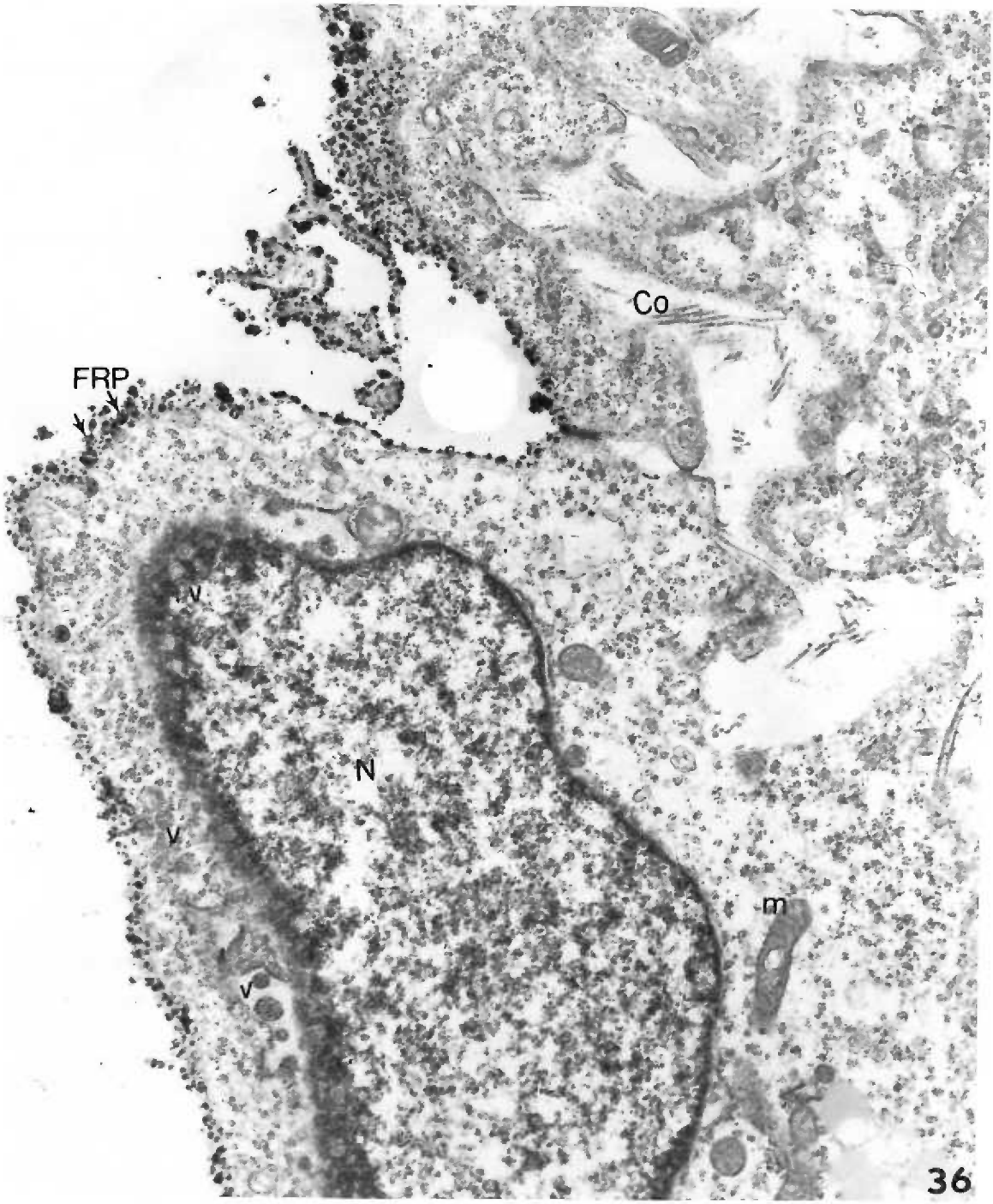
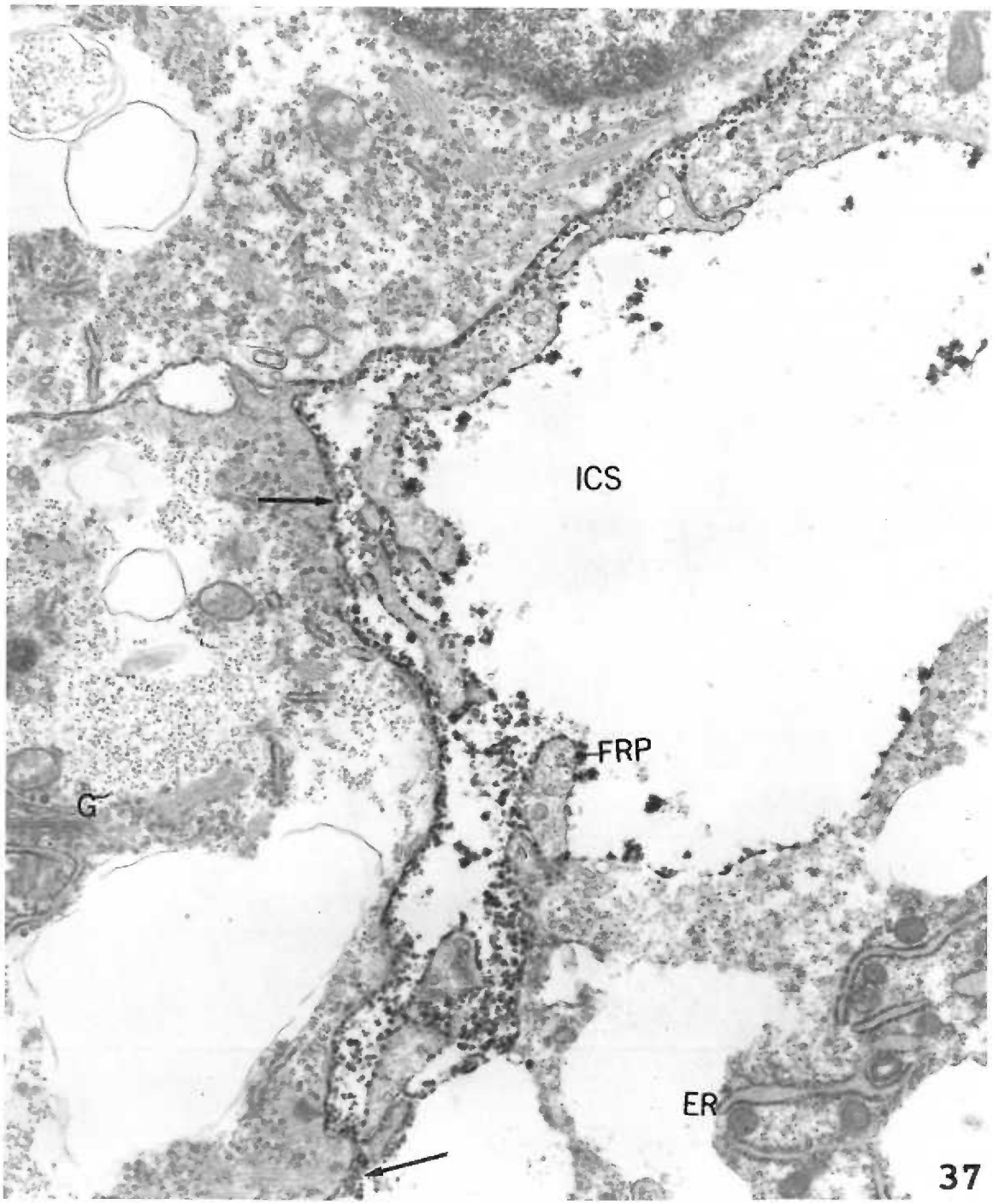

Co
FRP
v
N
v
m
v
36

Figure 37. 11-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. Final reaction product (FRP) is deposited in granular form on the sarcolemma. There seems to be a heavier deposit of reaction product where the myofibrils are in contact with the sarcolemma (long arrows).

ER-endoplasmic reticulum; ICS-intercellular space; FRP-final reaction product.

X 17,440



37

Figure 38. 12-Day Myocardium

Regions of Golgi cisternae and vesicles (G) are recognizable. Ribosomes are attached to the inner membrane of the nuclear envelope (arrows inside nucleus) in addition to the outer membrane. Frequently, ribosomes on the inner membrane are obscured from view by the peripheral distribution of chromatin. Some regions of the outer nuclear membrane are devoid of ribosomes (arrows). Tubular cristae are recognizable in some mitochondria (m, arrows). The presence of a single centriole (C) is a frequent feature in myocardial cells.

Li-lipid; ID-intercalated disc; MF-myofibrils; C-centriole; m-mitochondria; G-Golgi elements.

X 22,350

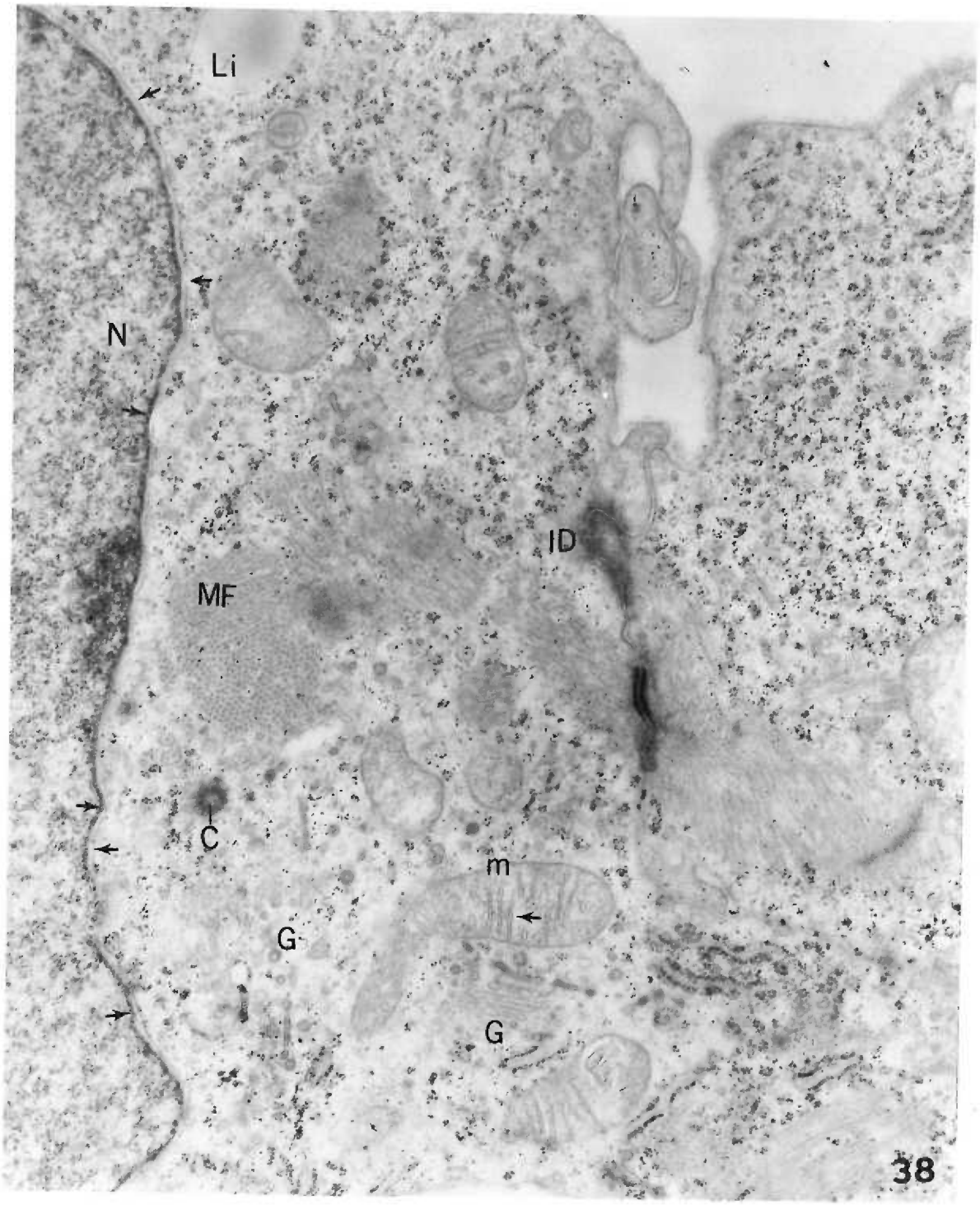

Li
N
ID
MF
C
m
G
G
38

Figure 39. 12-Day Myocardium

These cells are characterized by intense pinocytotic activity. A large group of pinocytotic vesicles (Pv) can be observed at the left of the micrograph. Several vesicles in early stages of formation can be visualized close to the sarcolemma (arrows). Intercalated discs (ID) are numerous, but still remain small and not well-defined. Ribosomes are free in the cytoplasm (Ri), and they are also attached to the inner membrane of the nuclear envelope (arrows inside nucleus) in addition to the outer membrane. Some areas of the outer membrane are occasionally denuded of ribosomes (opposite the arrows in the nucleus).

N-nucleus; D-desmosome; MF-myofibril; Z-Z disc; ID-intercalated disc; Pv-pinocytotic vesicle; Ri-ribosomes.

X 15,975

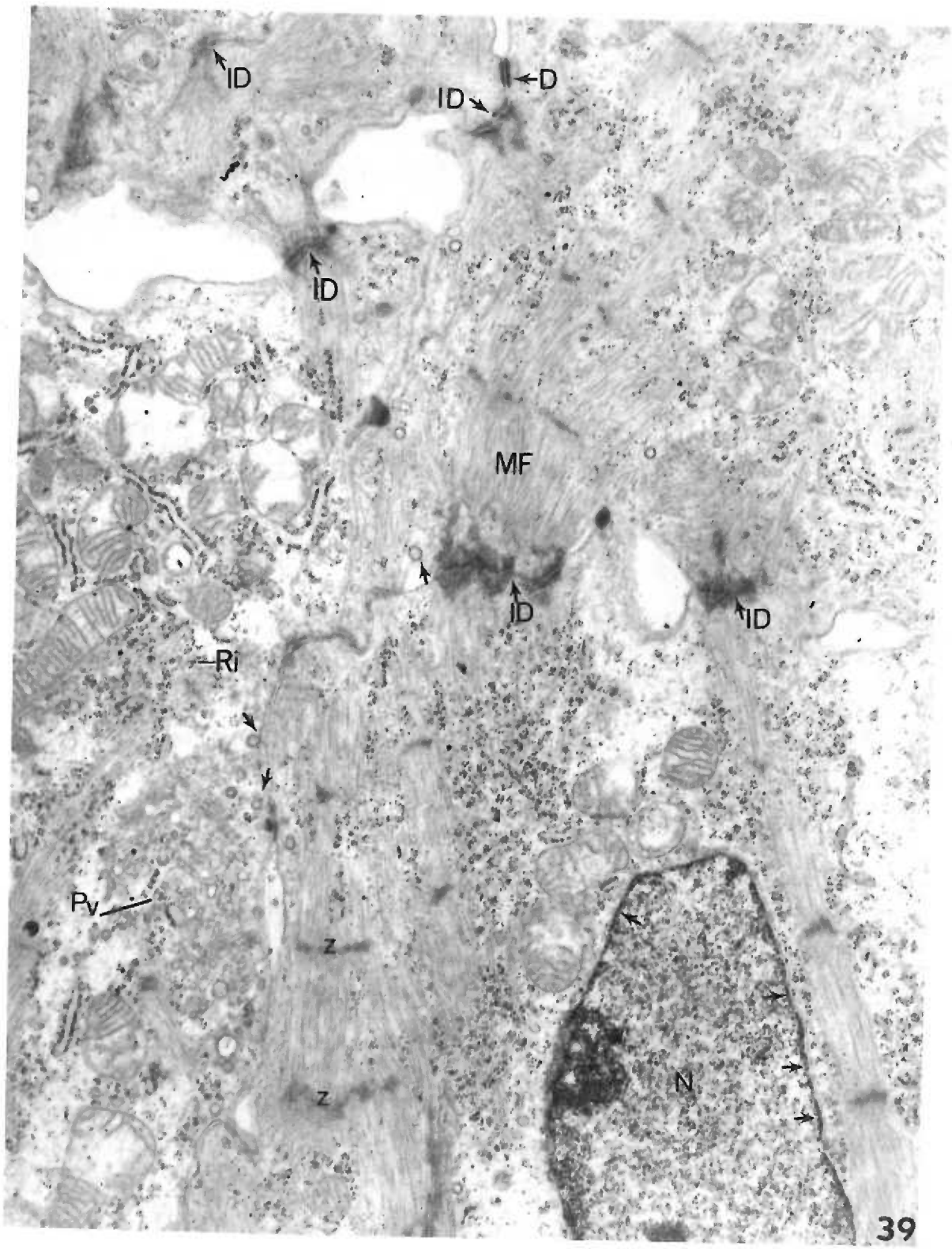

ID
D
ID
ID
MF
ID
ID
Ri
Pv
z
z
N
39

Figure 40. 12-Day Myocardium

Free ribosomes are associated with developing filaments and fibrils. Some of these are in the form of chains (arrows) oriented parallel to the myofilaments and fibrils (MF). Ribosomes can also be observed to be attached to the inner membrane of the nuclear envelope (arrows in the nucleus). Opposite these arrows, and other regions, the outer nuclear membrane is denuded of ribosomes. It appears as though several fibrils are grouped to from a "mass" or "sheet" of fibrils (MF). Branching of myofibrils is discernible at the intercalated disc (ID).

D-desmosome; m-mitochondria; N-nucleus; Z-Z disc; MF-myofibril; ID-intercalated disc.

X 15,975

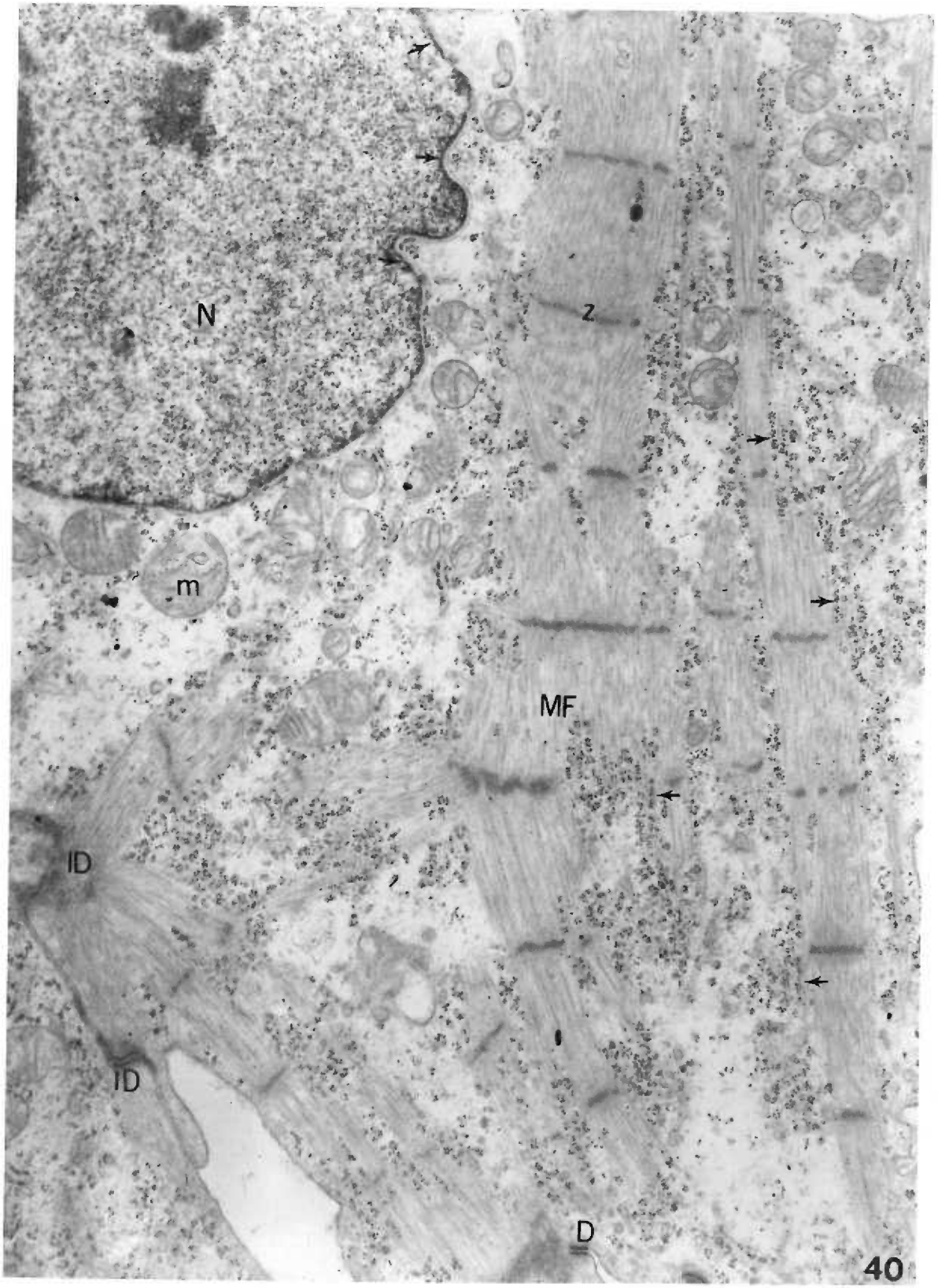
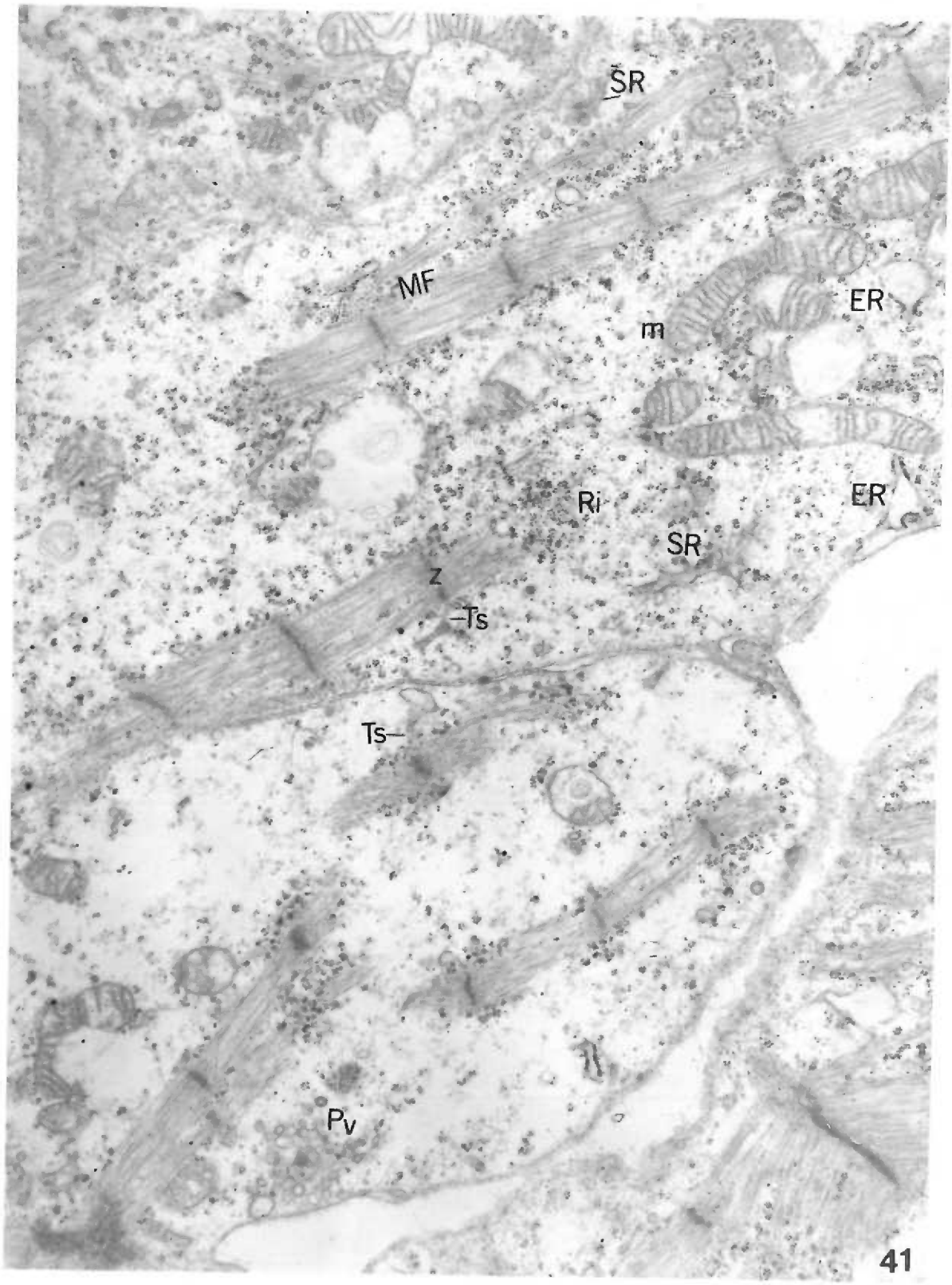

N
z
m
MF
ID
ID
D
40

Figure 41. 12-Day Myocardium

The early stage of formation of T-system (Ts) is discernible near the Z disc regions (Z). The elements of sarcoplasmic reticulum (SR) are recognizable in the interfibrillar sarcoplasm. The endoplasmic reticulum is highly distended (ER) and contains a flocculent material. Ribosomes (Ri) are closely associated with the developing myofibrils (MF), which are dispersed irregularly in the sarcoplasm with large interfibrillar spacings. Large amounts of pinocytotic vesicles (Pv) are discernible. Tubular cristae are visible in some mitochondria (m).

MF-myofibrils; ER-endoplasmic reticulum; SR-sarcoplasmic reticulum; Ts-developing T-system; m-mitochondria; Pv-pinocytotic vesicles; Ri-ribosomes; Z-disc.

X 15,975



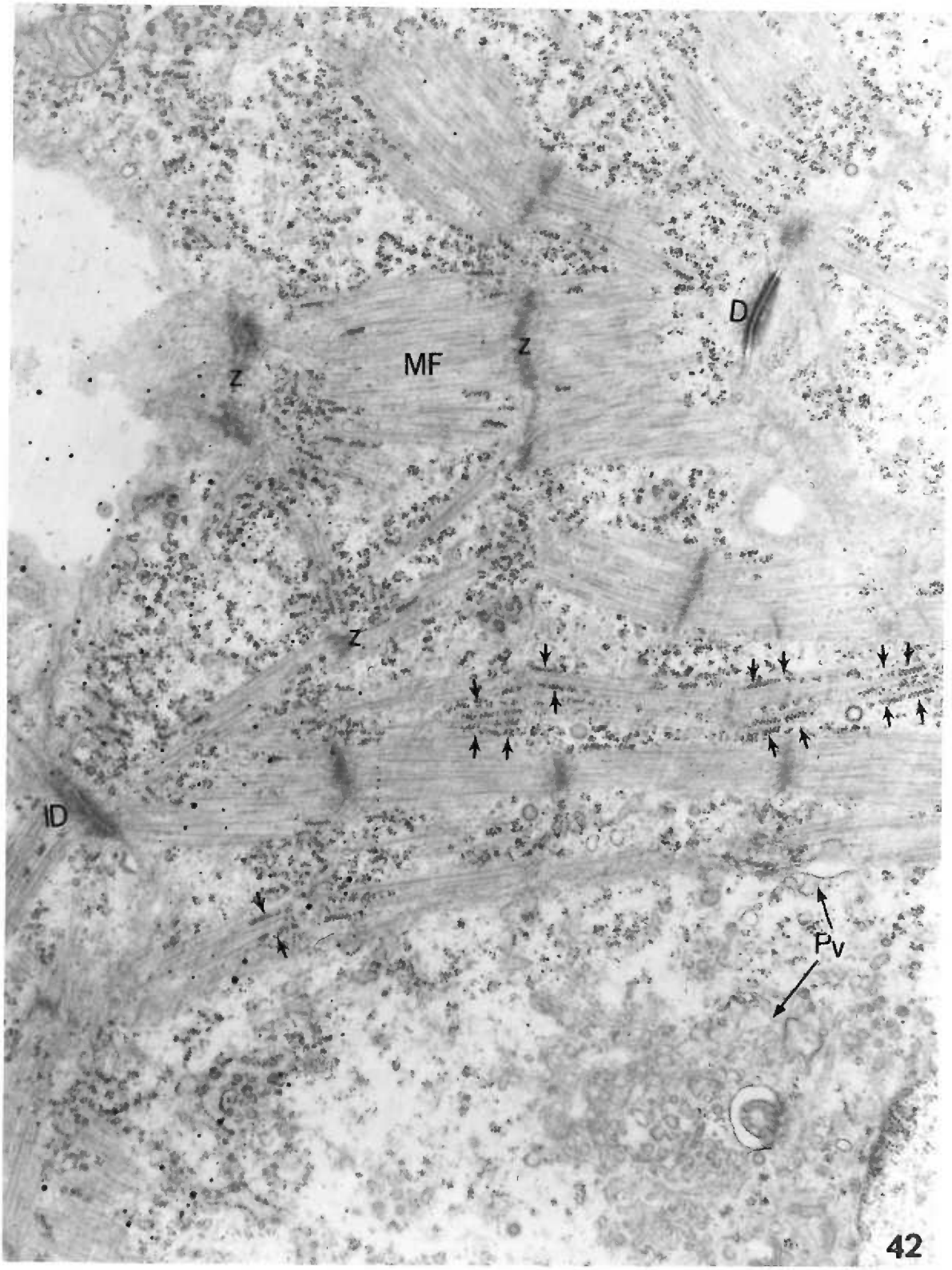
41

Figure 42. 12-Day Myocardium

Free ribosomes can be observed to be associated with developing myofilament and fibrils. Parallel chains of polyribosomes can be visualized at numerous sites around developing myofilaments (opposing sets of arrows). The phenomenon of branching of the myofibrils is recognizable at several Z band (Z) regions and at the region of intercalated discs. Large quantities of pinocytotic vesicles are seen in the cytoplasm (Pv).

ID-intercalated discs; MF-myofibril; D-desmosome; Z-Z disc; Pv-pinocytotic vesicle.

X 22,350



42

Figure 43. 12-Day Myocardium

Individual myofibrils occasionally follow a zig-zag course with sarcoplasm. The Z disc regions (Z) act as the points of reflection or bending.

m-mitochondria; ICS-intercellular space; Z-Z disc.

X 22,350

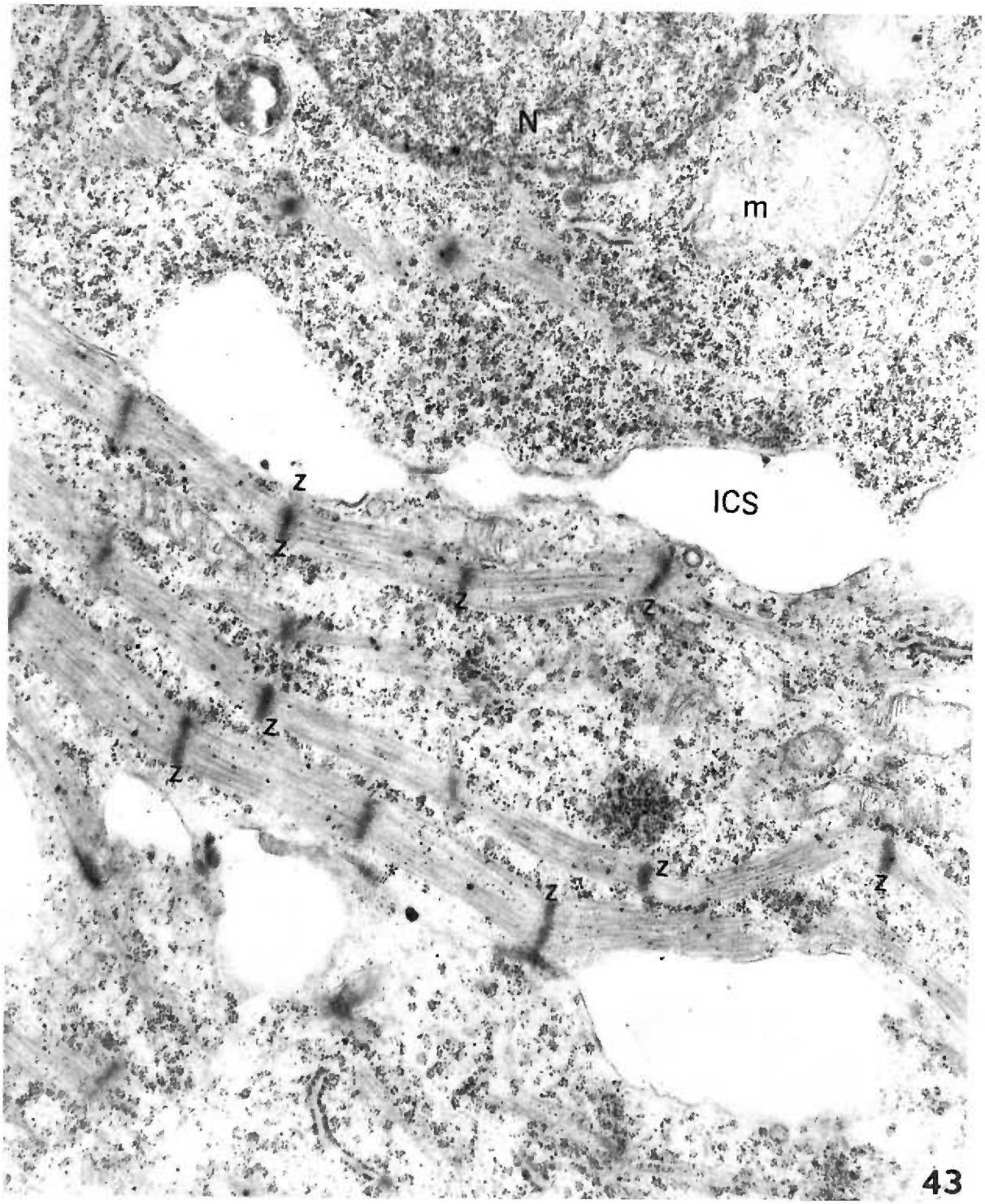

N
m
ICS
z
z
z
z
z
z
z
z
z
z
43

Figure 44. 12-Day Myocardium

Golgi bodies (G) are distributed extensively, and their close topographical relationship with myofibrils (MF), mitochondria (m) and endoplasmic reticulum (ER) is evident. Granular endoplasmic reticulum (ER) is widely distributed,and the cisternae are distended and contain a flocculent material. MF-myofibrils; ER-endoplasmic reticulum; G-Golgi elements; D-desmosome; m-mitochondria; N-nucleus.

X 15,975

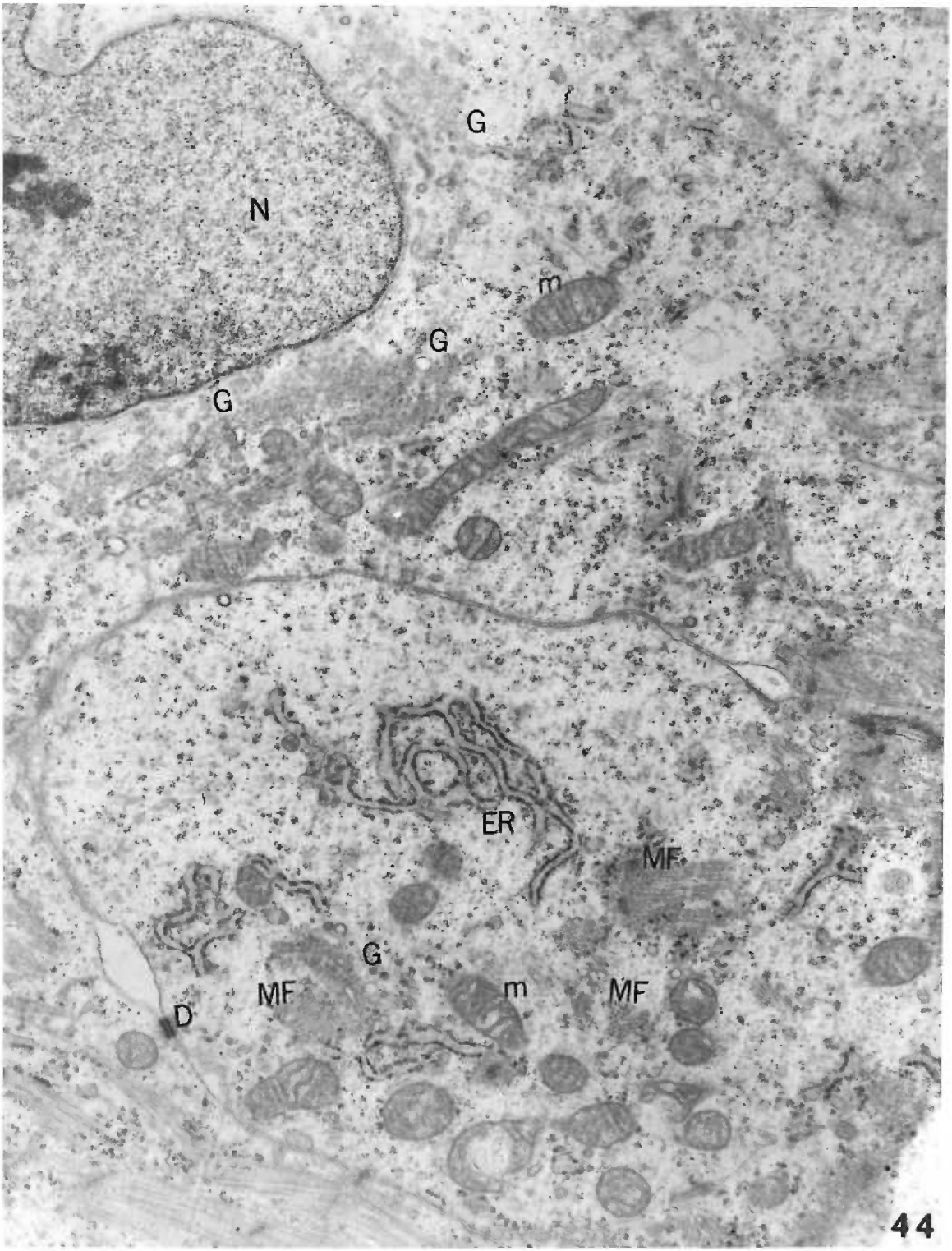

G
N
m
G
G
ER
MF
G
MF
m
MF
D
44

Figure 45. 12-Day Myocardium

The cells are more tightly packed than the 10-day myocardium. Isolated myofilaments (Mfl), Golgi regions (G), endoplasmic reticulum (ER), developing intercalated disc (ID) and desmosomes (D) are visible.

Mfl-myofilaments; ER-endoplasmic reticulum; ICS-intercellular space; D-desmosomes; G-Golgi body; N-nucleus; M-mitochondria; Li-lipid.

X 15,975

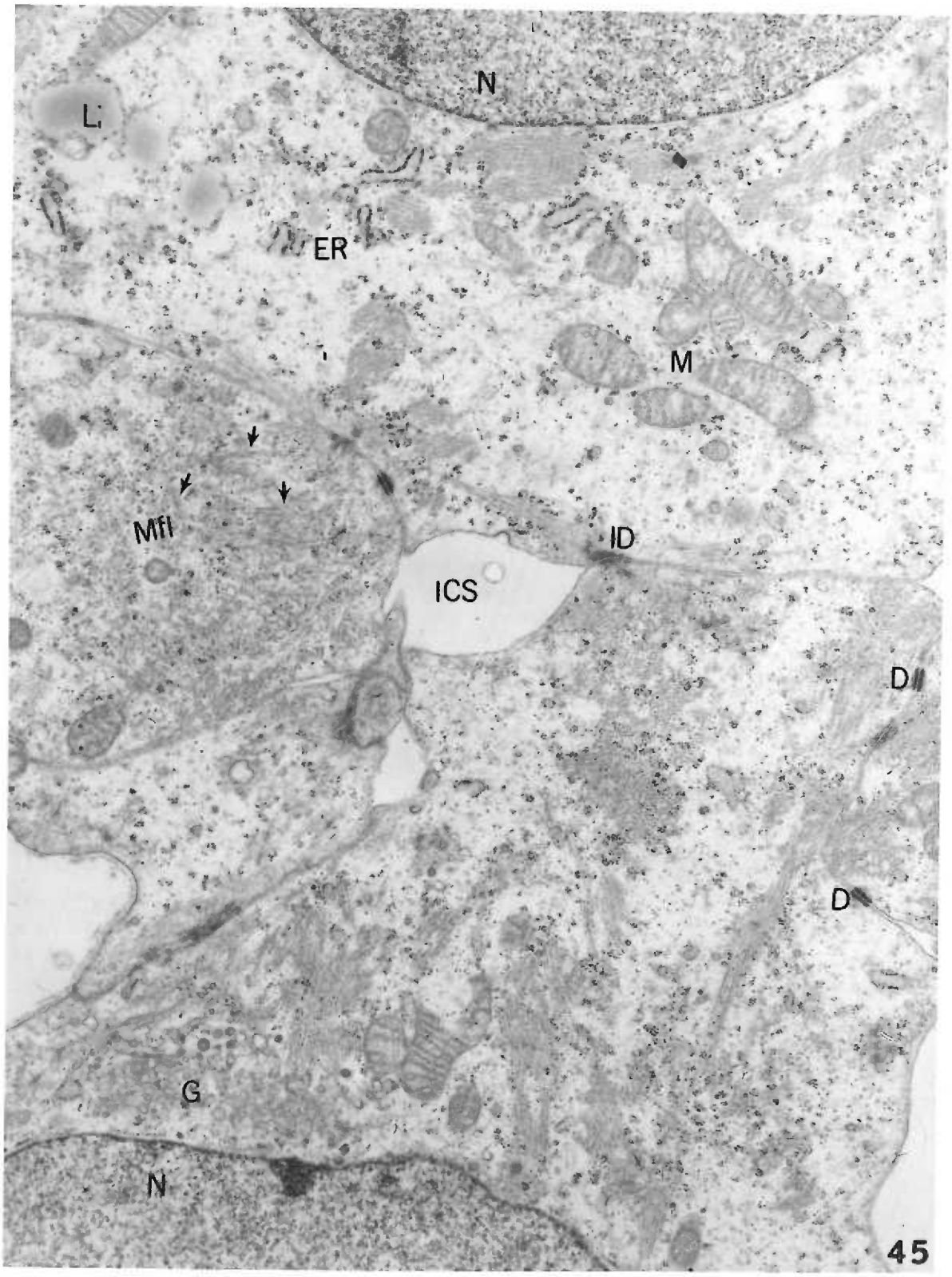
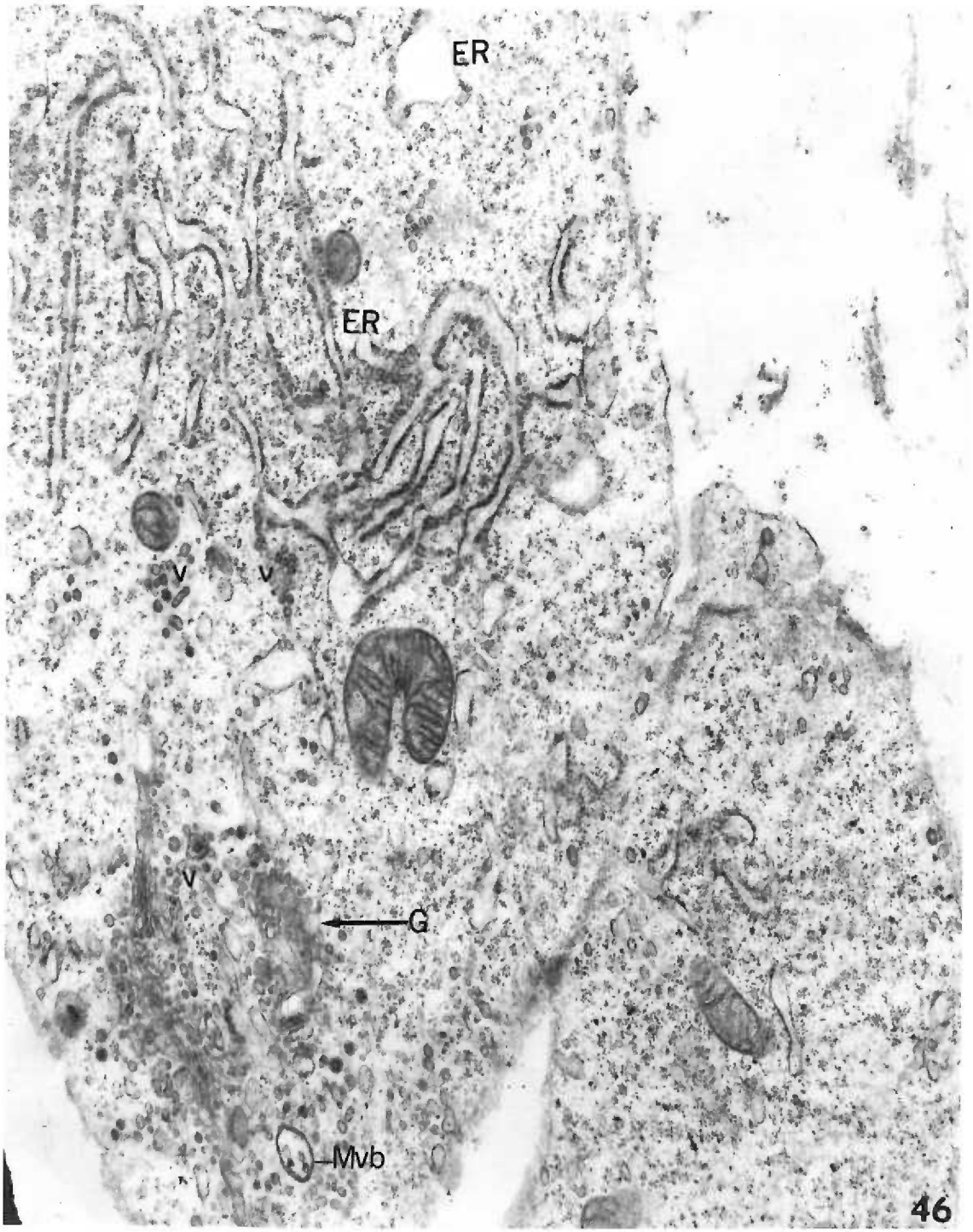

N
L
ER
M
Mfl
ID
ICS
D
D
G
N
45

Figure 46. 12-Day Myocardium

Extensive distribution of Golgi elements (G) is discernible. All the three elements of Golgi bodies, namely, cisternae, vacuoles and vesicles, are recognizable, with a predominance of vesicles and vacuoles (V). An occasional multivesicular body (Mvb) is seen in the vicinity of Golgi bodies. Granular endoplasmic reticulum (ER) is widely dispersed in the cytoplasm. The cisternae are greatly distended.

ER-endoplasmic reticulum; G-Golgi body; V-Golgi vacuoles and vesicles; Mvb-multivesicular body.

X 22,350



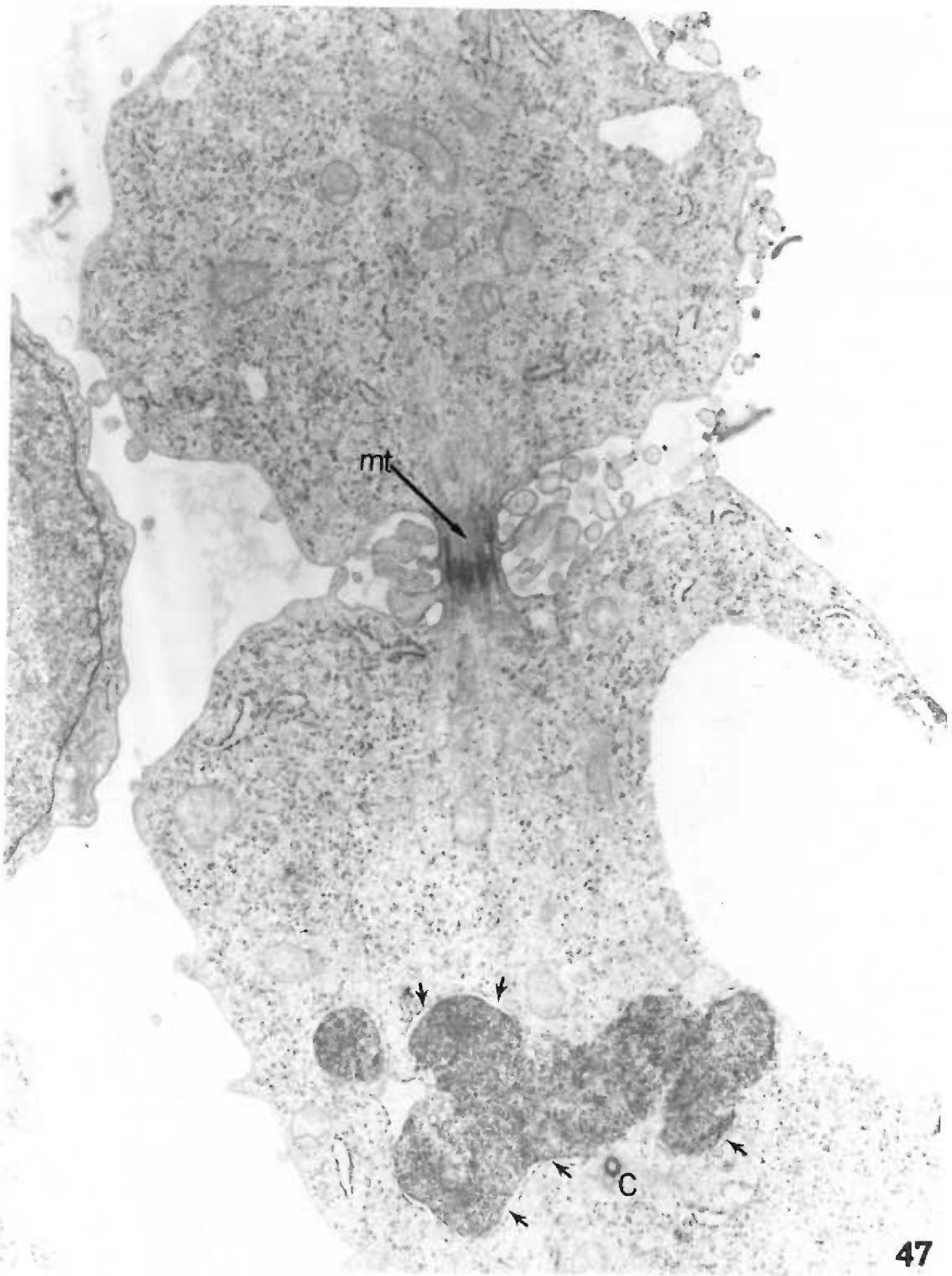
46

Figure 47. 12-Day Myocardium

Two daughter cells are connected by an isthmus called the "spindle bridge" or intermediate body. At the arrow (mt) numerous microtubules can be recognized which represent the remnants of the spindle apparatus. In the lower half of the picture, the reconstruction of the nuclear envelope by the coalescence of elements of endoplasmic reticulum (arrows) around the nuclear material can be visualized.

C-centriole; mt-microtubule.

X 11,000



47

Figure 48. 12-Day Myocardium

The formation of nuclear envelope from the elements of endoplasmic reticulum is shown. The tubular elements encircle the nuclear masses and coalesce. Nuclear pores are formed at the sites (arrows) of fusion of these tubular elements. A mitochondrion in a state of division by constriction is discernible. Numerous isolated elements of endoplasmic reticulum (x) are discernible throughout the cytoplasm.

M-mitochondria; x-isolated elements of endoplasmic reticulum.

X 11,000

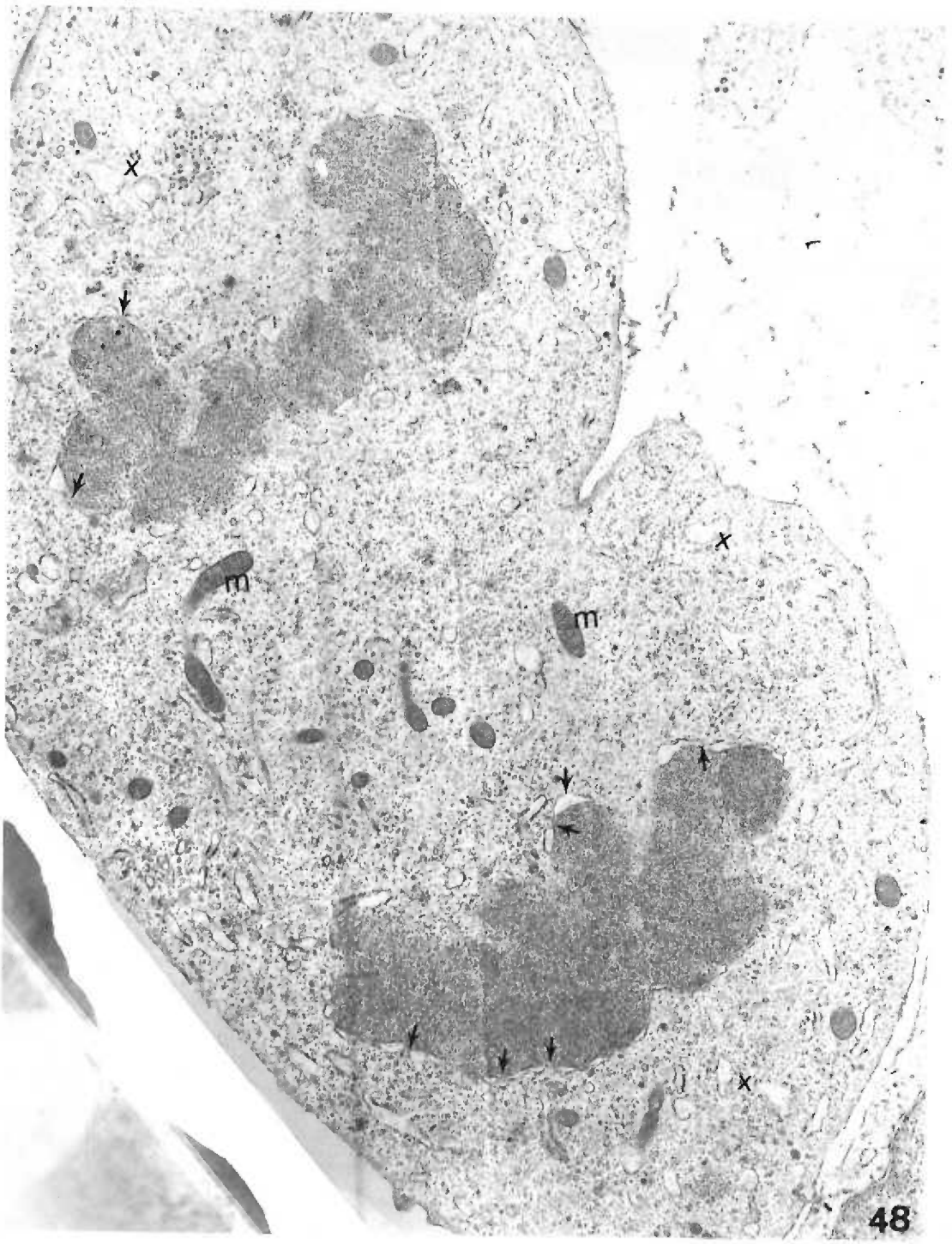

x
m
m
x
x
48

Figure 49. 12-Day Myocardium

A mitotic cell displaying sites of myofilament synthesis (Mfl) is shown. This phenomenon of simultaneous process of mitosis and myofilament synthesis in the same cells is a regular feature in the rat embryonic myocardium. A large assemblage of the elements of endoplasmic reticulum (ER) is seen next to the chromatin material. These elements encircle the nuclear material and coalesce to form the nuclear envelope (See Figs. 47, 48).

ER-endoplasmic reticulum; m-mitochondria; Chr-chromatin material; Mfl-myofilaments.

X 15,975

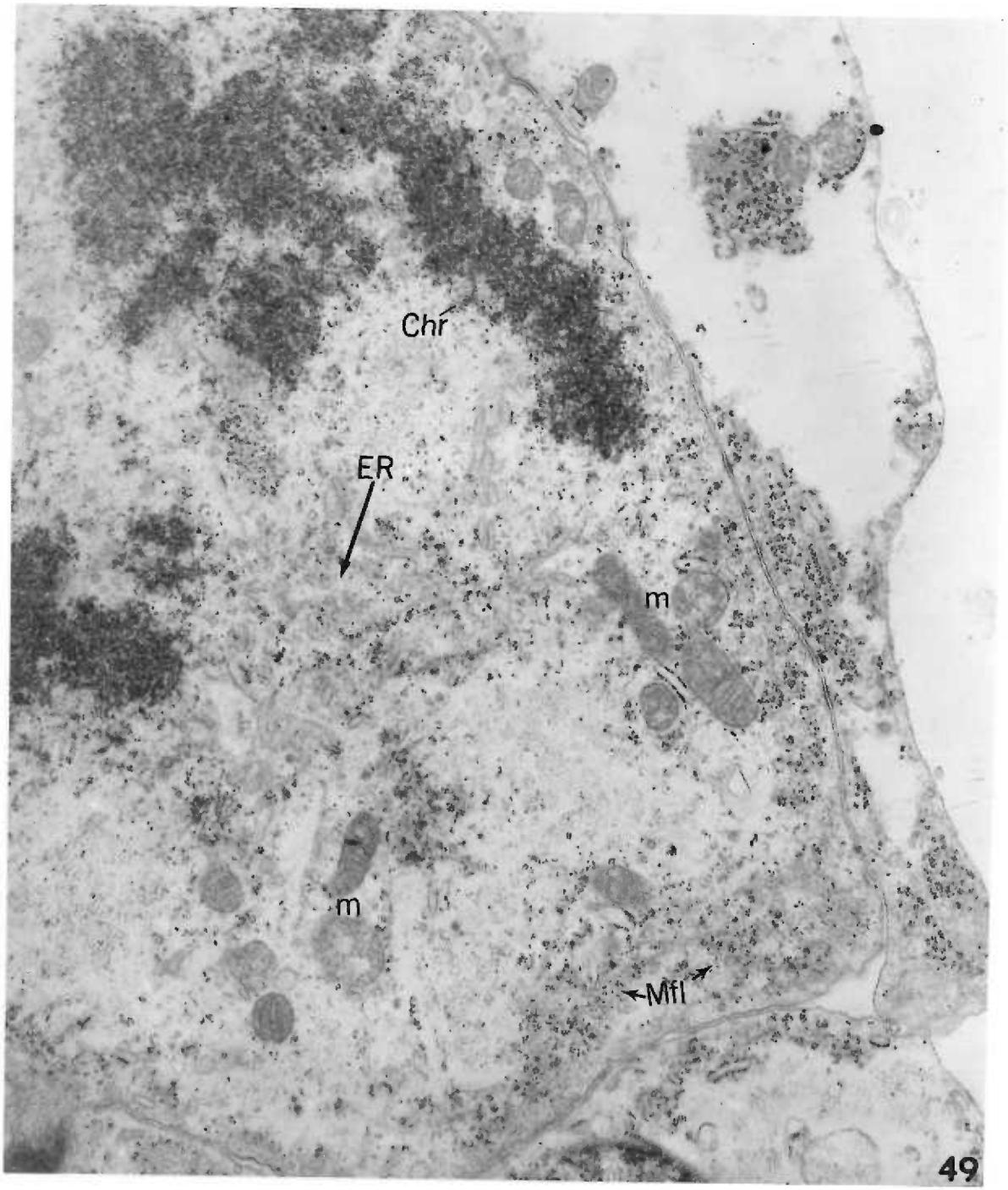

Chr
ER
m
m
Mfl
49

Figure 50. 12-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. The final reaction product is deposited as black granules (arrows) on the sarcolemma. The intensity of reaction is much less than that of 10 and 11-day myocardium.

MF-myofibrils; G-Golgi body; N-nucleus; M-mitochondria; D-desmosome.

X 11,000

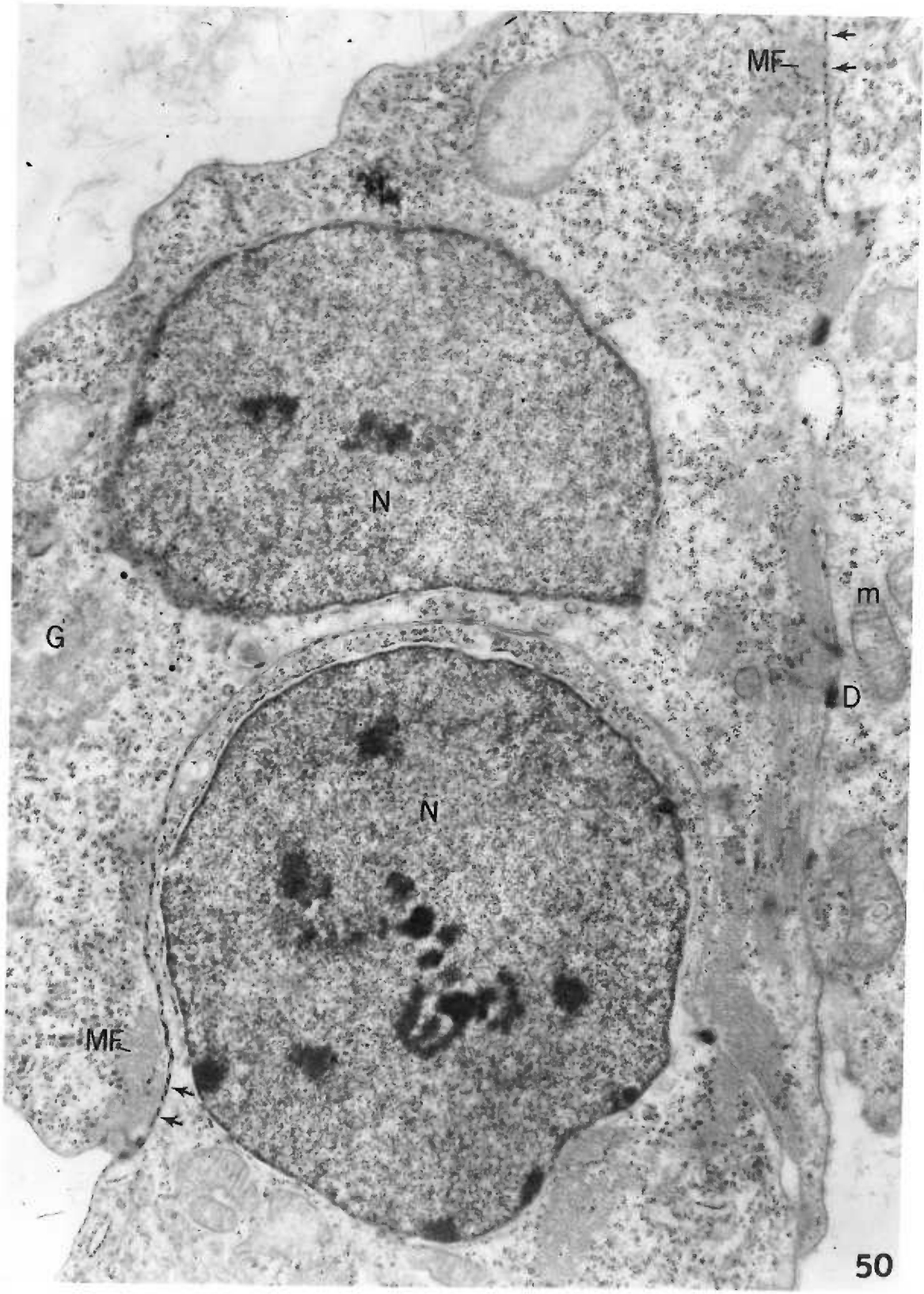

MF
N
G
m
D
N
MF
50

Figure 51. 12-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. The final reaction product is deposited as granules on the sarcolemma (arrows). The intensity of reaction is less than that of 11-day and 10-day myocardium. MF-myofibril; m-mitochondria.

X 12,780

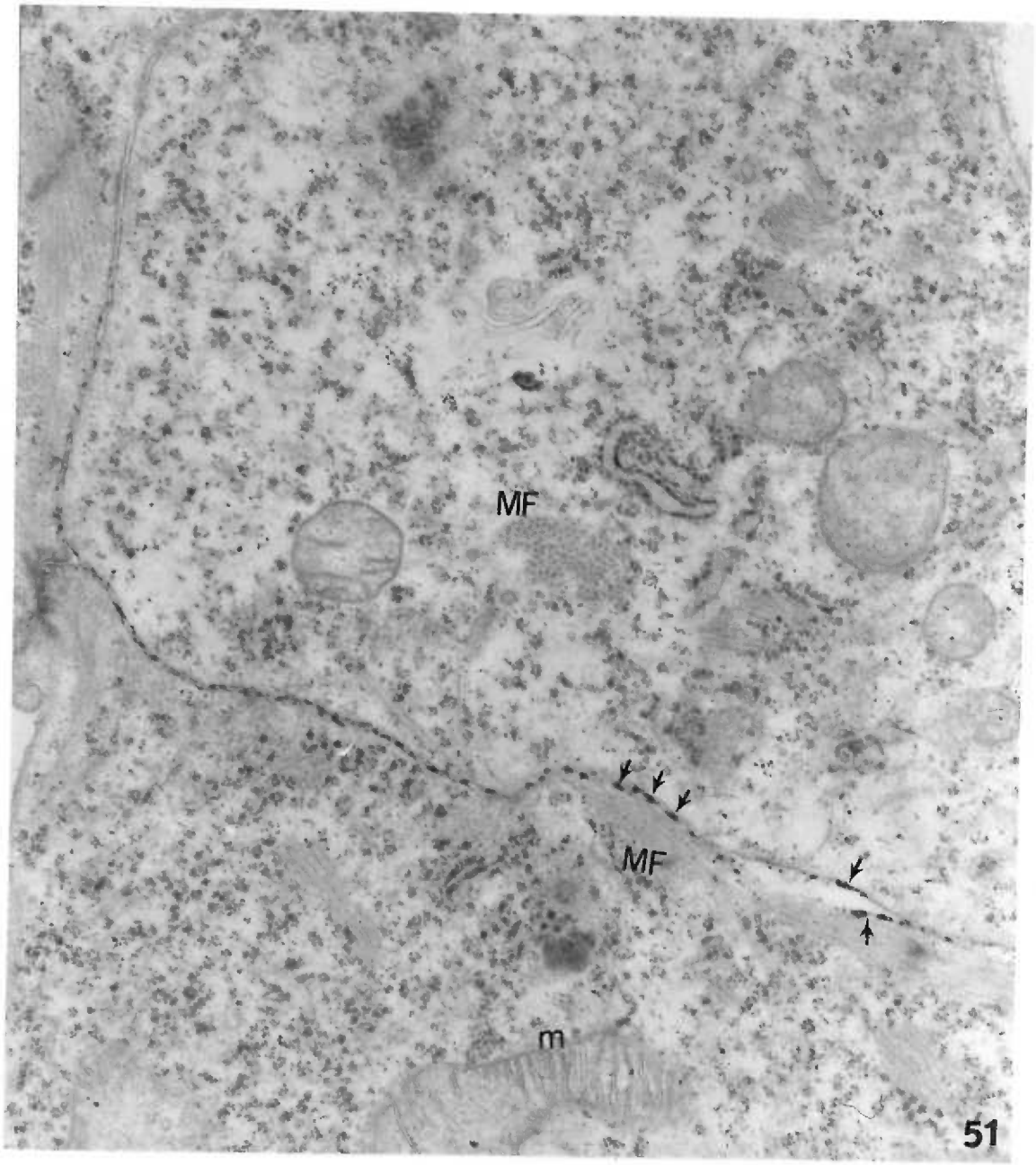

MF
MF
m
51

Figure 52. 12-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. The final reaction product is deposited as granules on the sarcolemma (arrows). The intensity of reaction is less than that of the 11-day and 10-day myocardium.

MF-myofibril; m-mitochondria; N-nucleus.

X 29,700

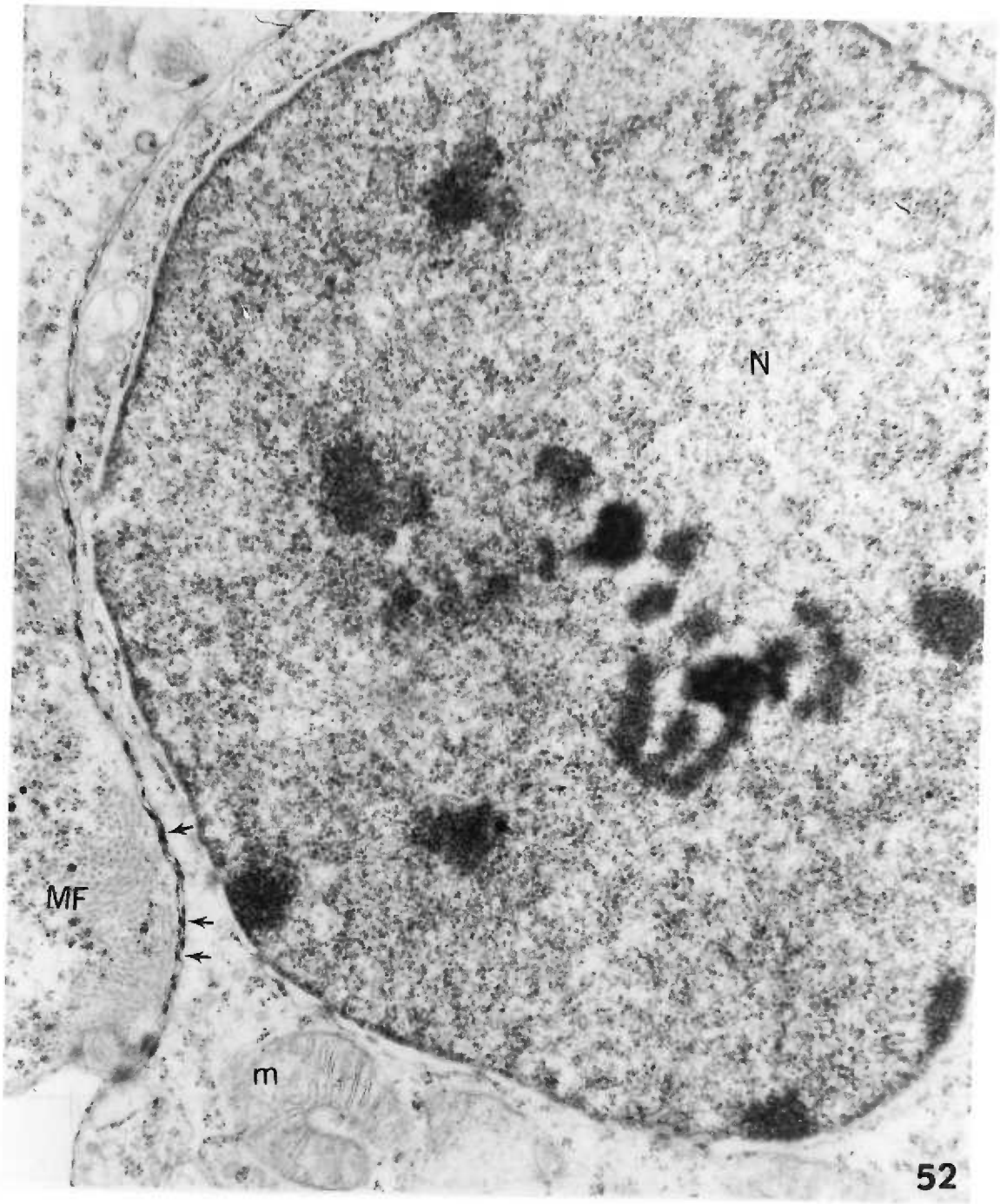

N
MF
m
52

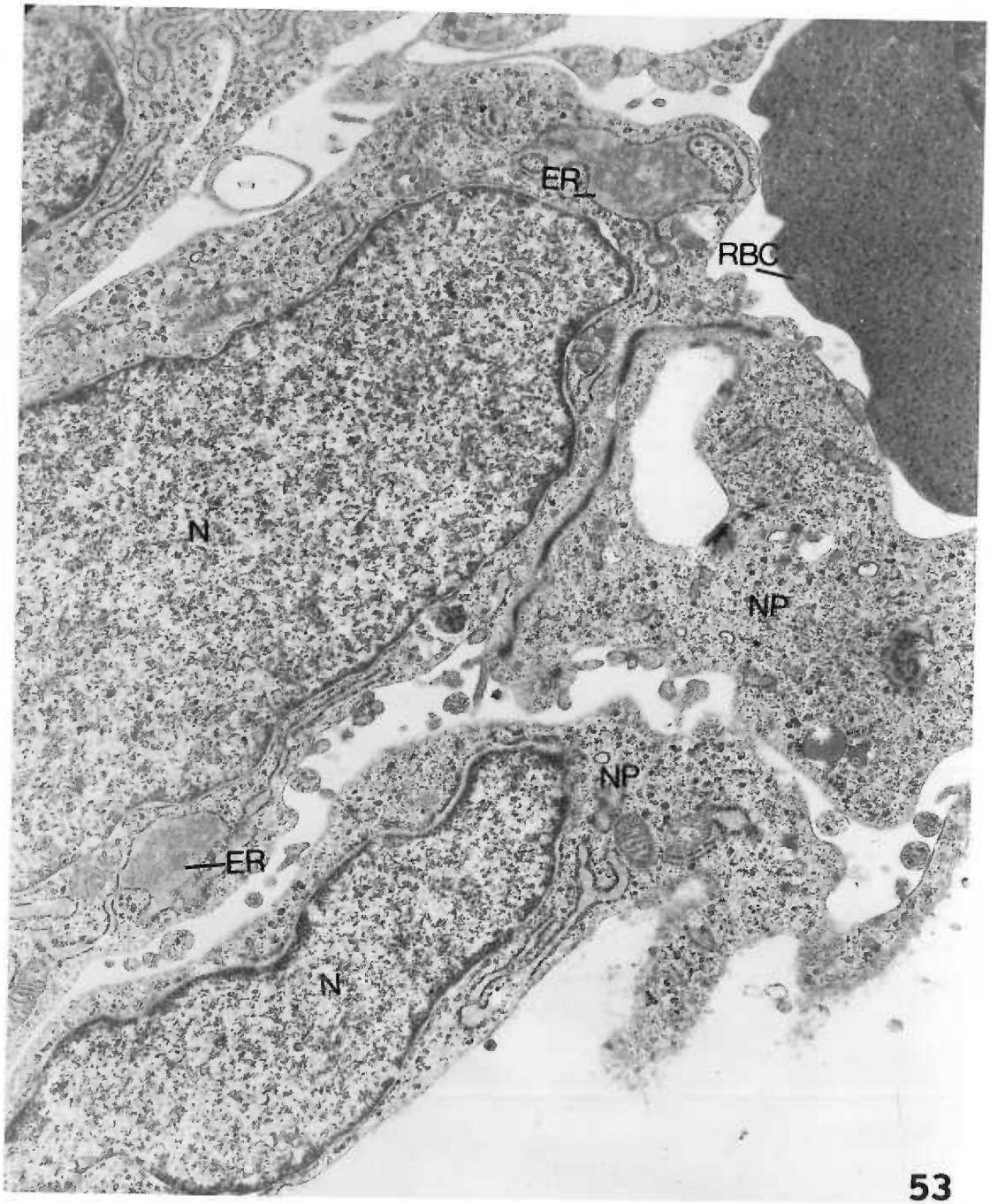
Figure 53. 13-Day Myocardium

The close proximity of vascular elements (RBC) and neural elements (NP)* is shown. The cisternae of endoplasmic reticulum of neural elements are greatly distended and contain an amorphous material.

RBC-red blood cells; ER-endoplasmic reticulum; NP-neural process; N-nucleus.

X 12,780

* See appendix



53

Figure 54. 13-Day Myocardium

There is a greater distribution of myofibrils (MF) per cell than in 12-day myocardium. The fibrils are cut in many planes indicative of the great complexity and ramification of the distribution of myofibrils. A group of nerve fibers (NF)* is shown between the myocardial cells. Each of the nerve fibers is enclosed in a membrane (arrows), and the neurofilaments appear as small dots distributed uniformly in the axoplasm. Tubular cristae are recognizable in some of the mitochondria (m, arrow).

MF-myofibril; NF-nerve fibers; m-mitochondria; ID-intercalated disc; Ri-a chain of helically arranged polyribosome; N-nucleus.

X 22,350

* See appendix

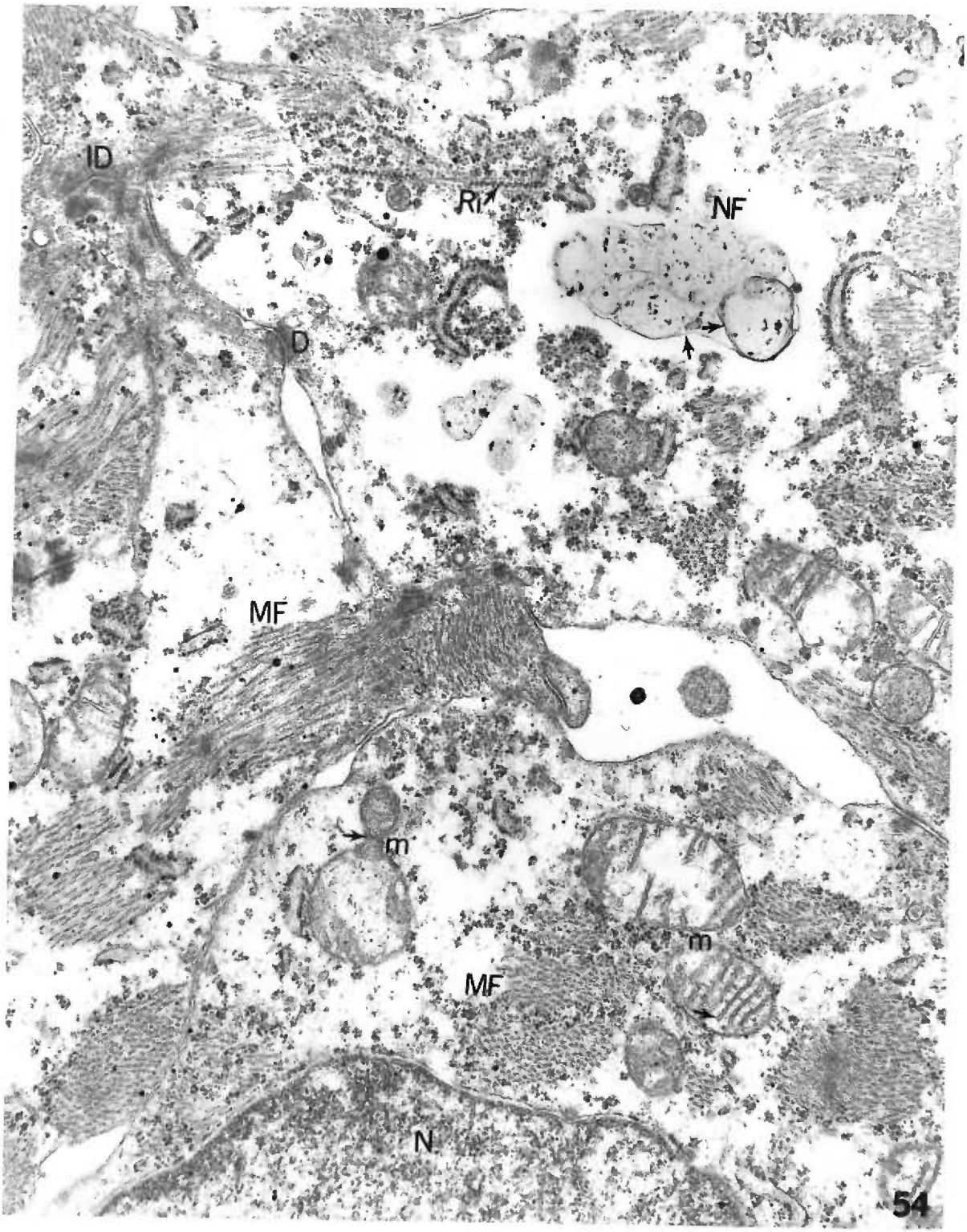

ID
RI
NF
D
MF
m
m
MF
N
54

Figure 54A. 13-Day Myocardium

An enlargement of figure 54 to show a cross section of nerve fibers (NF)* located between myocardial cells. Each fiber is enclosed in a membrane, and, in cross sections, the neurofilaments appear as small dots in the axoplasm.

NF-nerve fiber; NP-nerve process; MF-myofibrils; ER-endoplasmic reticulum; m-mitochondria.

X 40,320

* See appendix

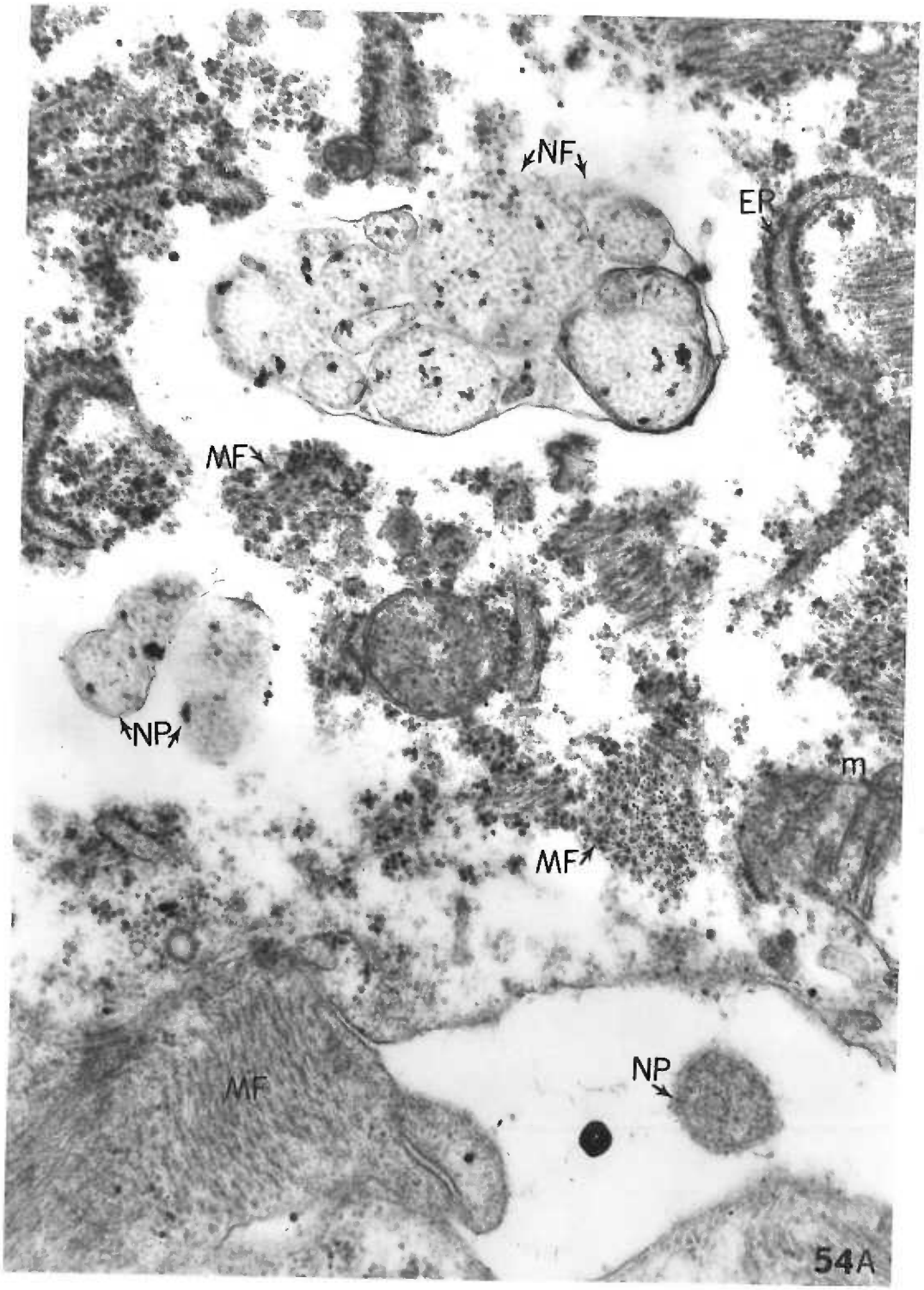

NF
ER
MF
NP
m
MF
MF
NP
54A

Figure 55. 13-Day Myocardium

The sarcolemma scallops and makes contact with the myofibrils at the Z disc regions (arrows). Several vacuoles and vesicles can be observed in the cytoplasm, a few of which are fuzzy (V) and others which are smooth contoured. A mitochondrion in the process of multiplication by constriction is indicated (m, arrow). Developing T-system is recognizable (Ts).

Mt-microtubules; MF-myofibrils and filaments; V-fuzzy or spiny vesicles; m-mitochondria; D-desmosome.

X 17,880

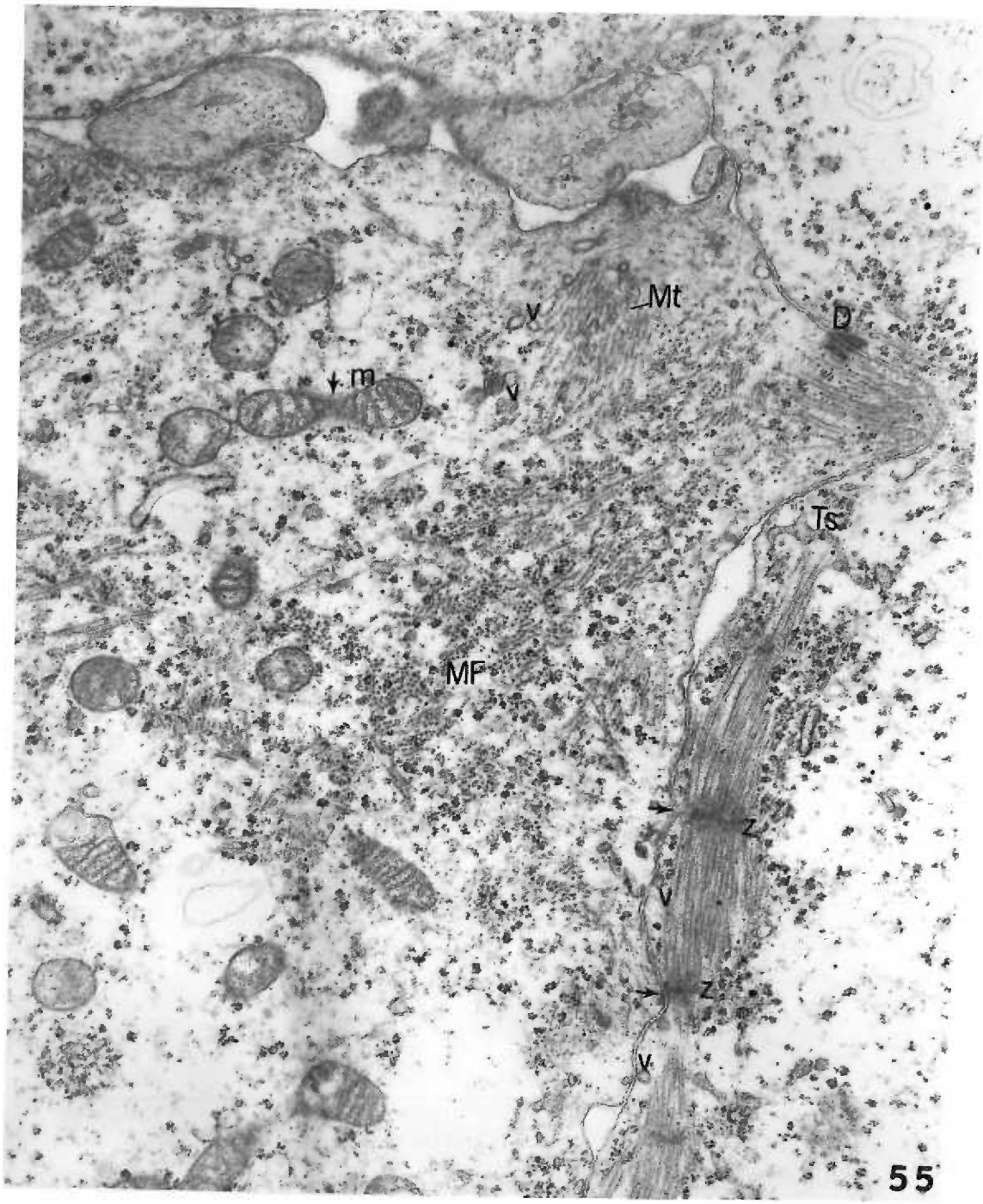

Mt
D
v
m
v
Ts
MP
z
v
z
v
55

Figure 56. 13-Day Myocardium

A long nerve process (NP)* is recognizable between several myocardial cells (Myc). Fine neurofilaments (Nfl) are distinctly discernible in the axoplasm of the nerve process. "Protrusions" and "projections" (arrows) appear on the surface of both the myocardial cells and the nerve process, and they grow out towards each other so as to make neuro-muscular contacts (arrows). Myc-myocardial cells; MF-myofibrils; Nfl-neurofilaments; NP-nerve process; N-nucleus; m-mitochondria.

X 12,780

* See appendix

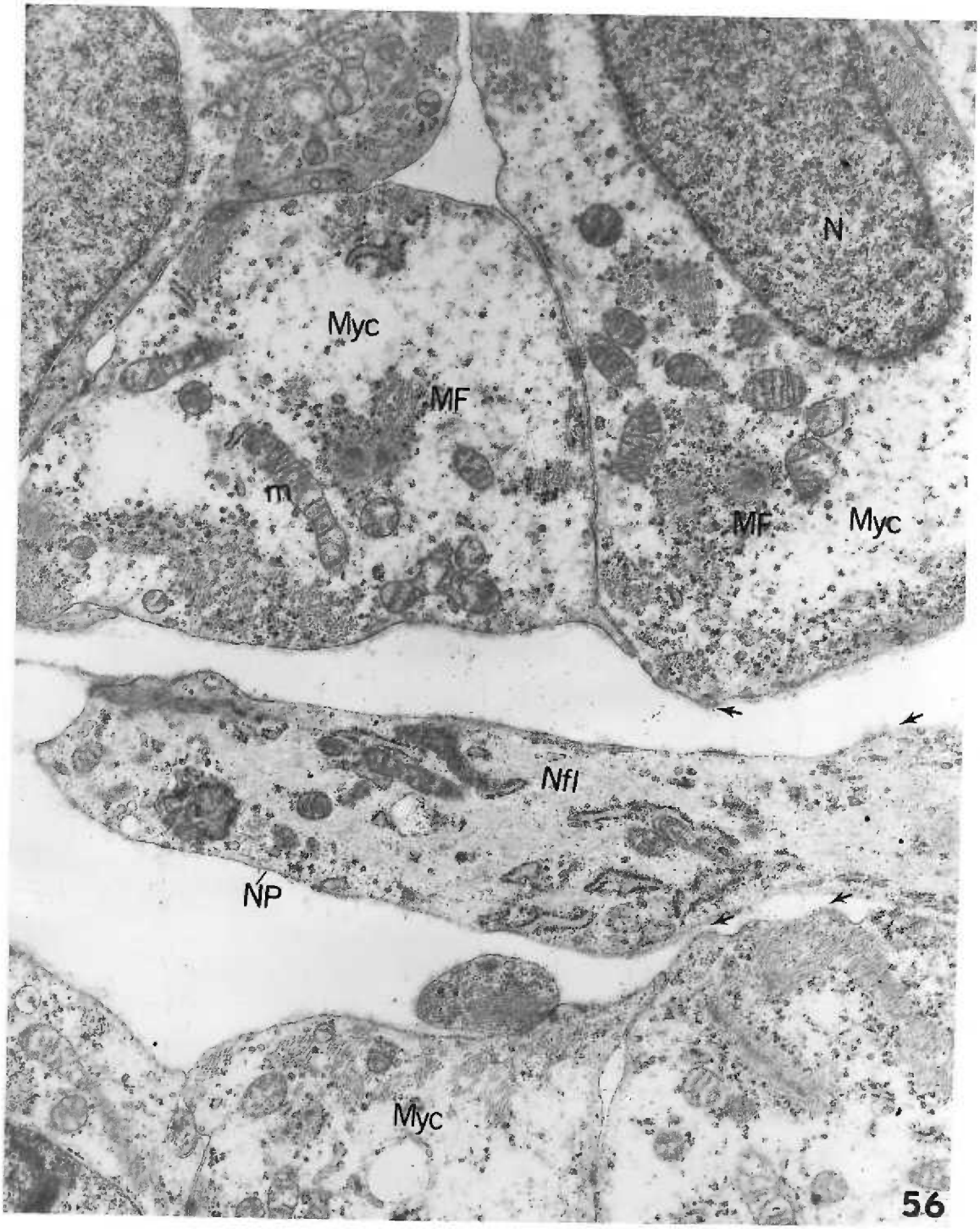

N
Myc
MF
m
MF
Myc
Nfl
NP
Myc
56

Figure 57. 13-Day Myocardium

The close proximity of neural elements (NP)*and immature erythrocytes of myocardium (RBC) (neuro-vascular association) is discernible. Fine neurofilaments (Nfl) can be observed in the axoplasm of the nerve process. A myocardial cell containing several myofibrils is seen next to the nerve process. The phenomenon of branching of the myofibrils can be recognized at the Z disc region (Z).

RBC-immature erythrocyte with nucleus; N-nucleus; NP-nerve process; MF-myofibril; m-mitochondria; D-desmosome; Z-Z band.

X 17,880

* See appendix

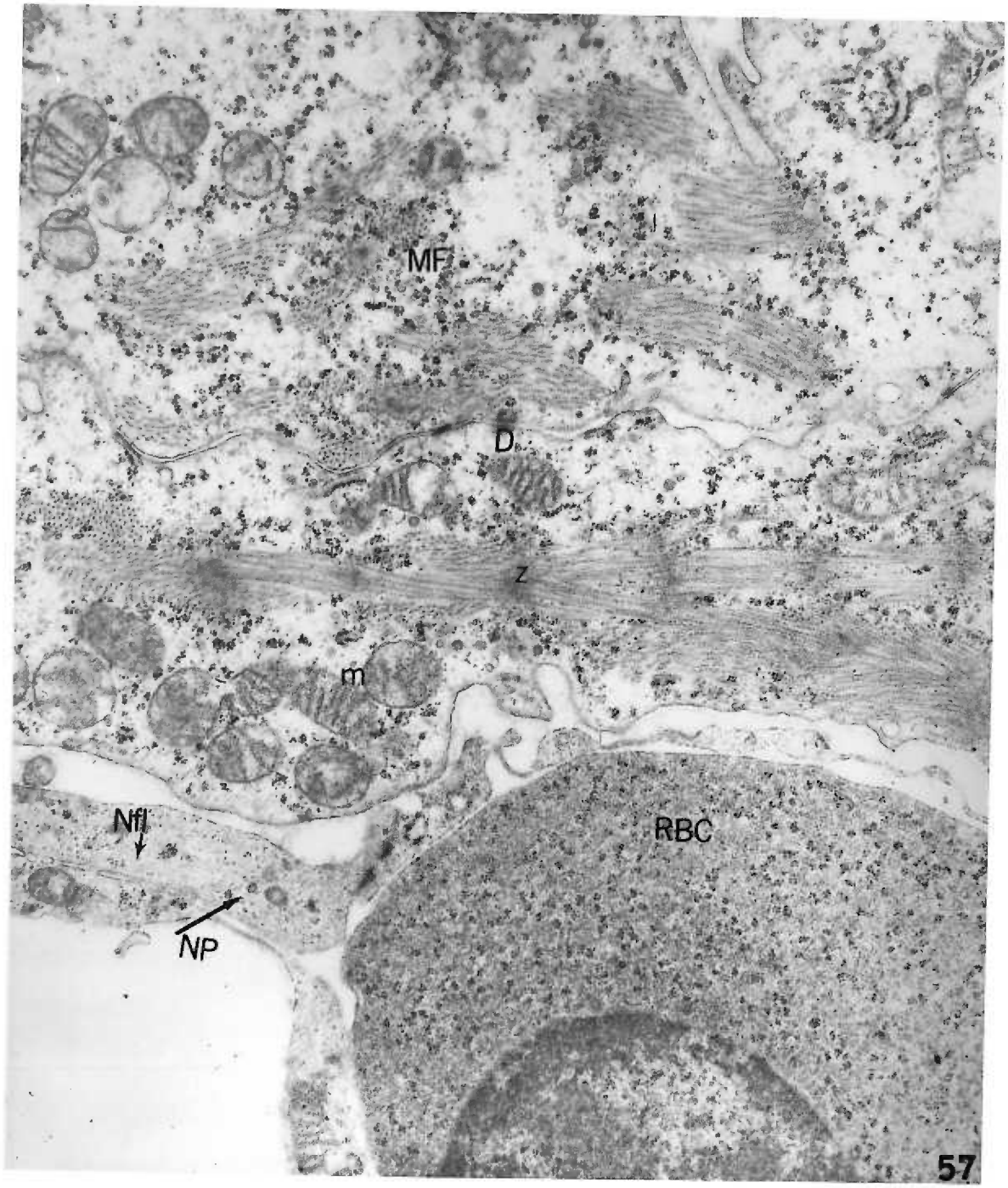
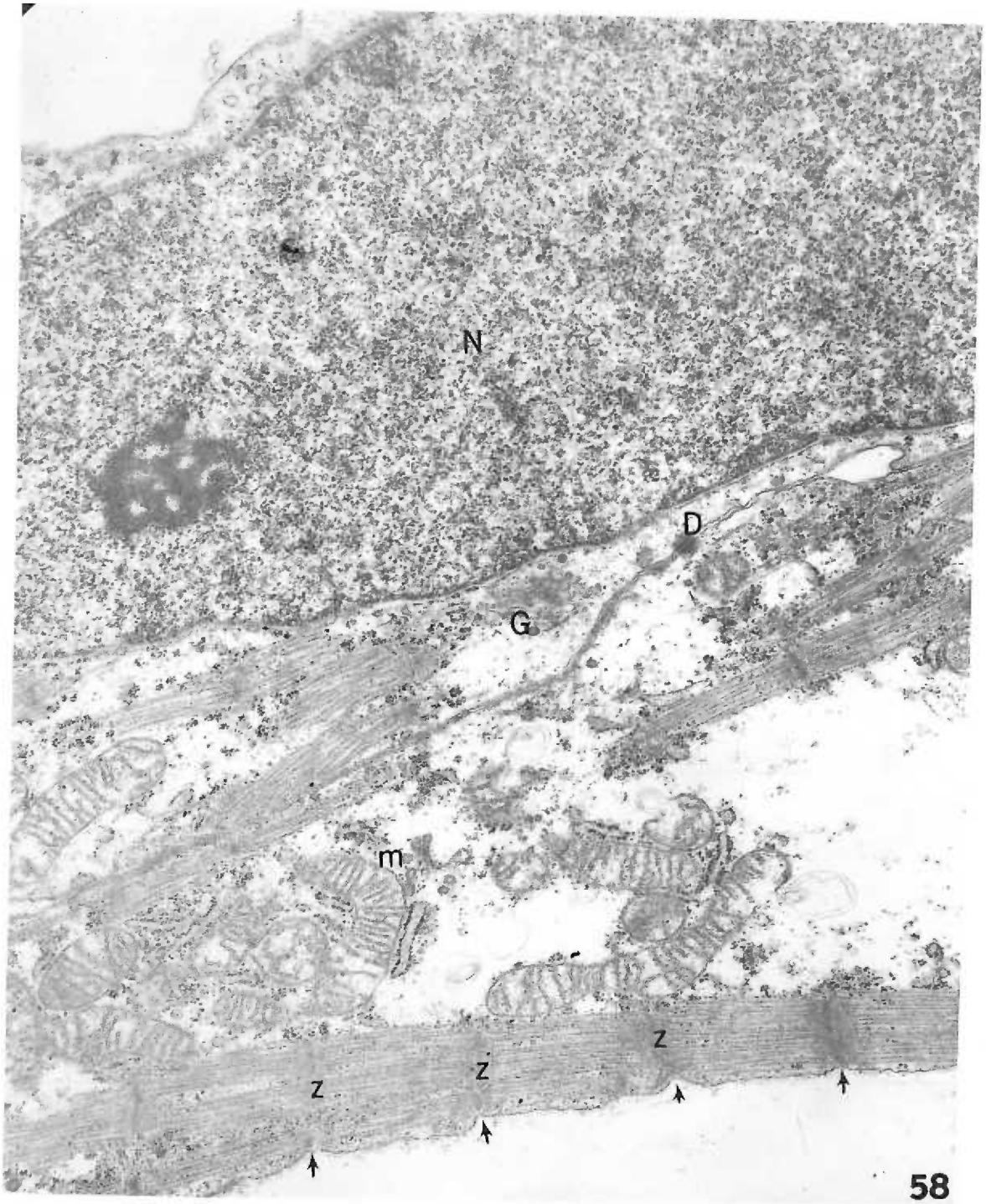

MF
I
D
z
m
Nfl
NP
RBC
57

Figure 58. 13-Day Myocardium

The sarcolemma scallops and makes contact with a myofibril at the Z disc regions (Z and arrows). The myofibril runs parallel to the sarcolemma and seems to be in contact with it at several places. Tubular cristae are recognizable in some of the mitochondria (m).

N-nucleus; D-desmosome; G-Golgi region; Z-Z disc; m-mitochondria.

X 17,880



58

Figure 59. 13-Day Myocardium

The sarcolemma contributes the two leaflets of the intercalated discs, which have undergone further differentiation since the previously noted stages. The plicated nature of the intercalated disc is more apparent than in earlier stages (ID, upper right). Myofibrils seem to fan out in different directions from the intercalated disc. Fuzzy vesicles (V) are seen next to the sarcolemma. Endoplasmic reticulum contains an amorphous material. ID-intercalated disc; ER-endoplasmic reticulum; m-mitochondria; Ri-ribosomes; V-fuzzy vesicles.

X 17,880

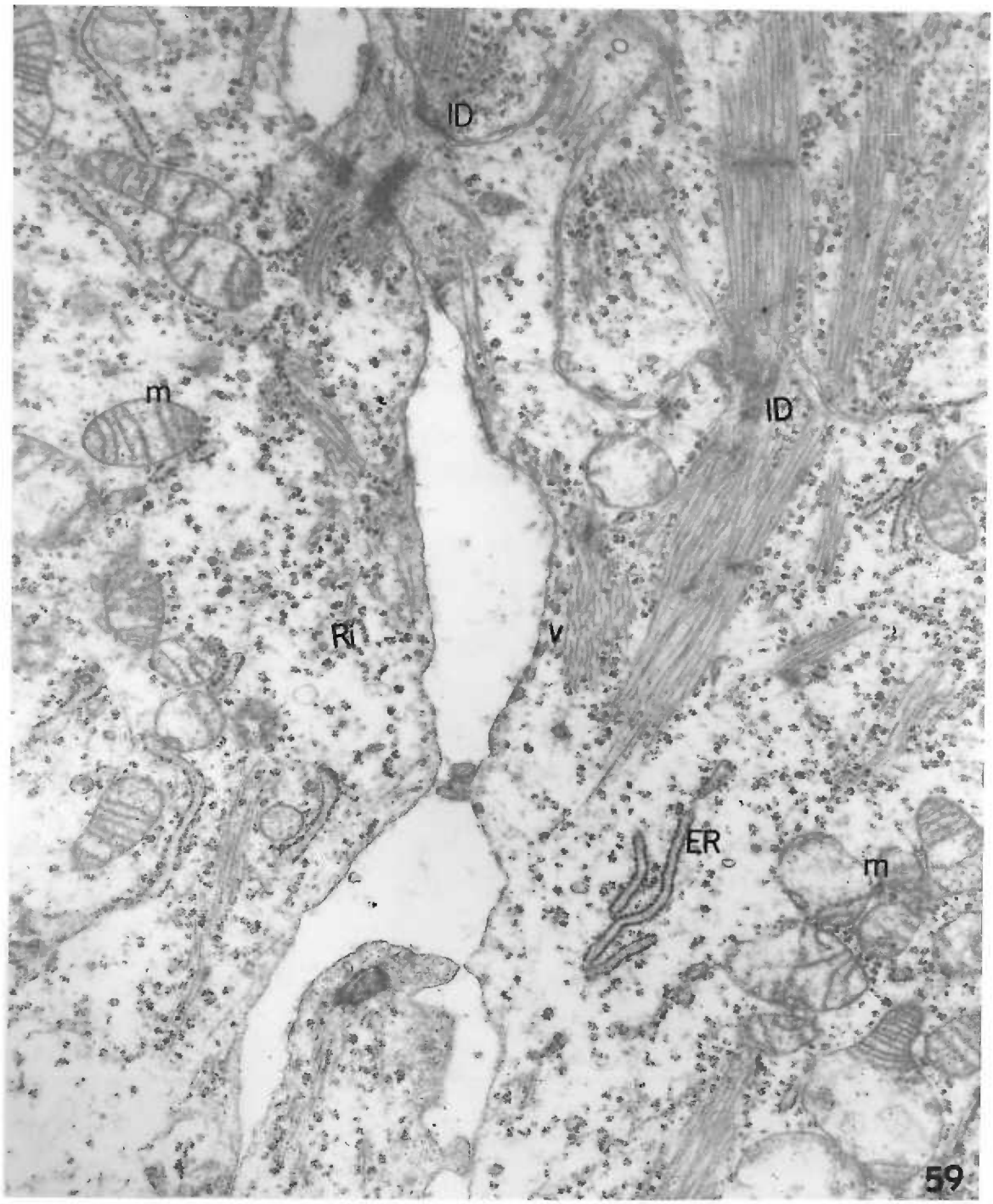

ID
m
ID
Ri
v
ER
m
59

Figure 60. 13-Day Myocardium

A mitotic cell showing isolated myofibrils and filaments (MF) is shown. Several microtubules (Mt) representing remnants of the spindle apparatus are recognizable. As previously noted, the phenomenon of simultaneous process of mitosis and myofilament synthesis in the same cell is frequently observed in rat embryonic myocardium.

Chr-chromatin material; MF-myofibrils and filaments; G-Golgi region; ER-endoplasmic reticulum; Mt-microtubules; m-mitochondria.

X 23,760

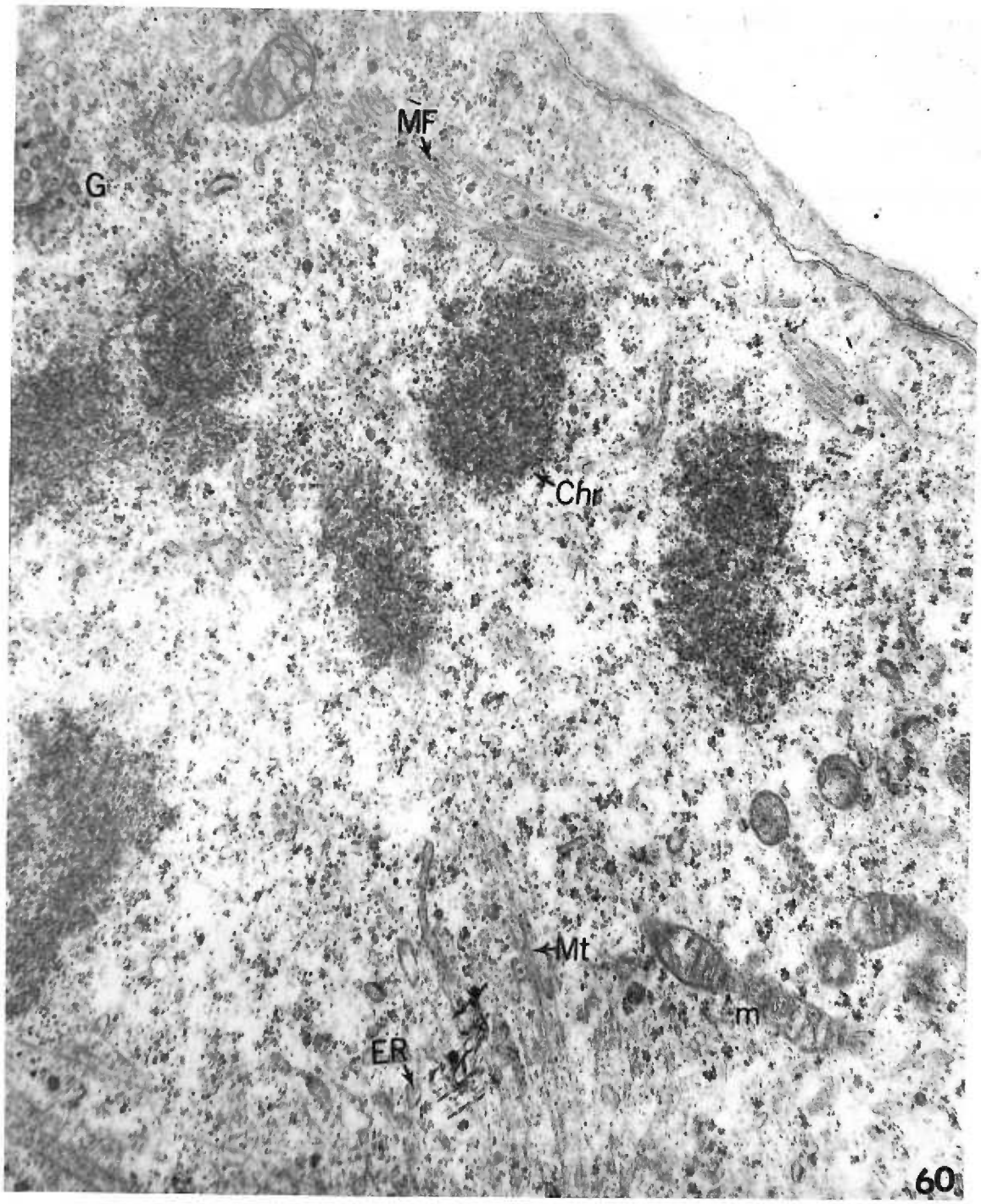

MF
G
Chr
Mt
m
ER
60

Figure 61. 13-Day Myocardium

Numerous sites of branching of the myofibrils are recognizable at the Z disc regions (Z) and at the regions of intercalated discs (ID). The myofibrils seem to fan out in different directions from the intercalated discs (ID, bottom right). The sarcolemma is thrown into folds.

ID-intercalated discs; ER-endoplasmic reticulum; Z-Z disc regions; Ri-ribosomes; m-mitochondria.

X 22,350

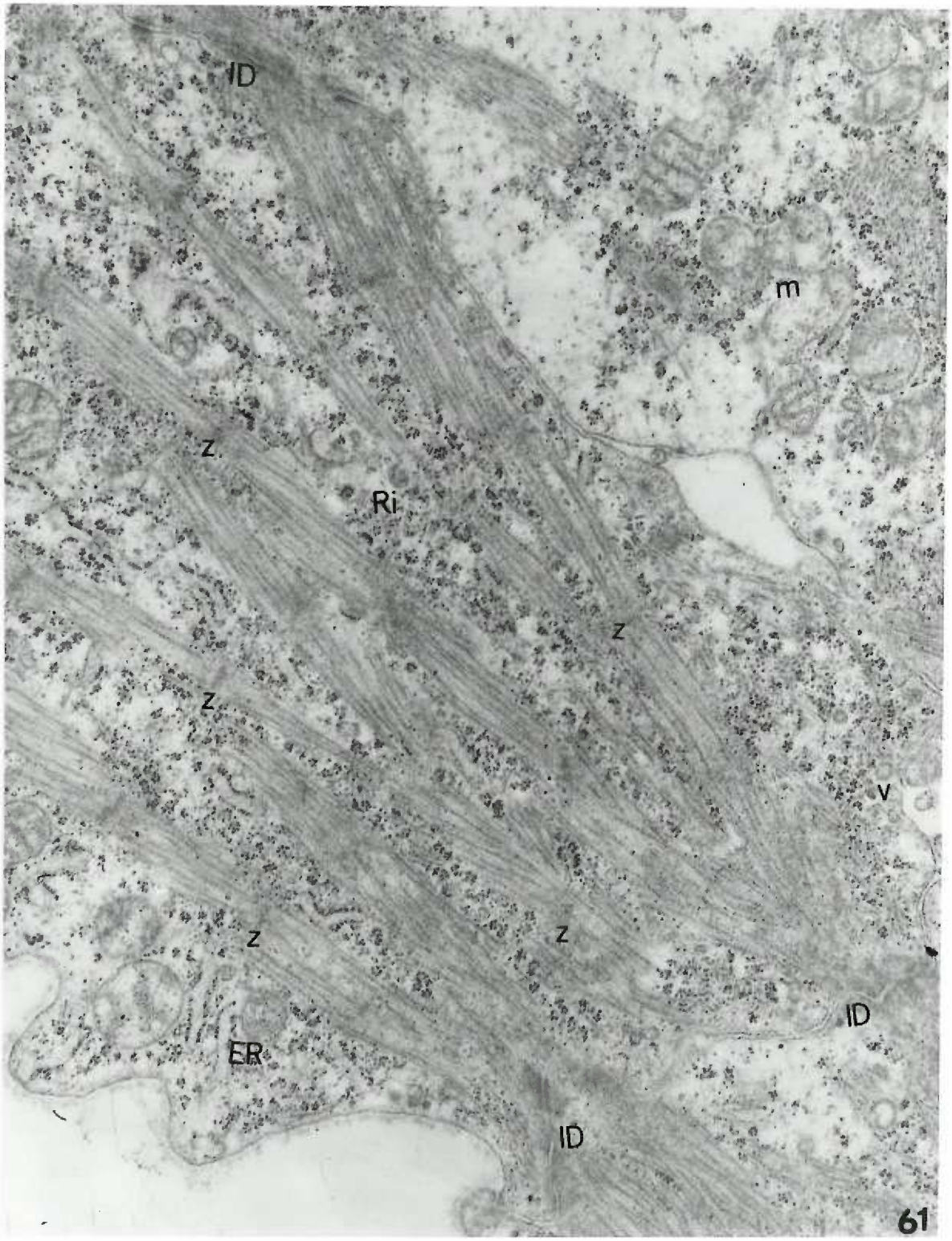

ID
m
z
Ri
z
z
z
v
z
z
ID
ER
ID
61

Figure 62. 13-Day Myocardium

The deep penetration of a nerve cell process (NP)*between myocardial cells (Myc) is noticeable. This phenomenon brings about contact between neural elements and the remote regions of the myocardium. Fine neurofilaments (Nfl) are visible in the axoplasm.

Myc-myocardial cells; NP-neural process; Nfl-neurofilaments; Z-Z band, where branching of the fibril is discernible; N-nucleus; Nu-nucleolus.

X 15,975

* See appendix

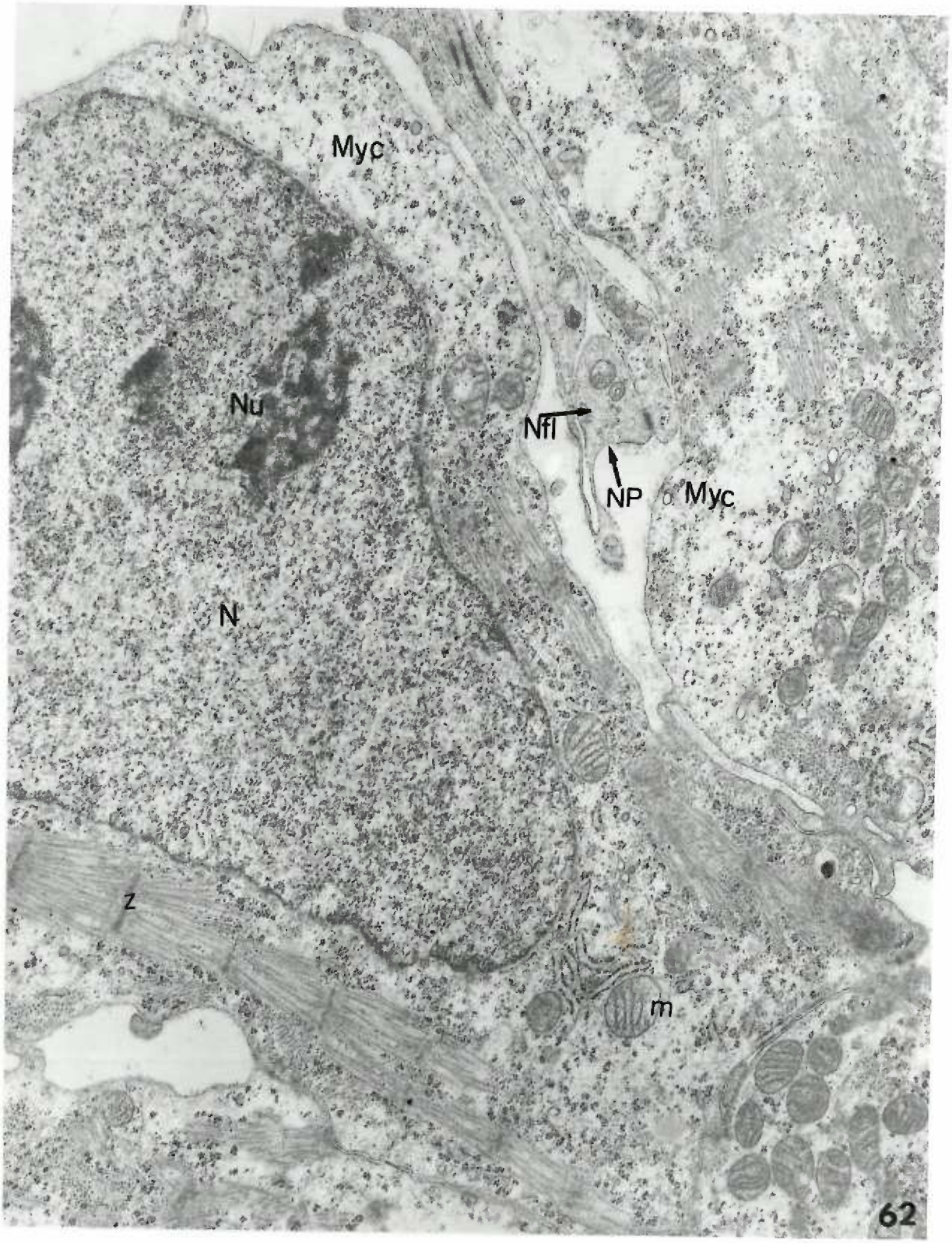

Myc
Nu
Nfl
NP
Myc
N
z
m
62

Figure 63. 13-Day Myocardium

Extensive distribution of Golgi elements (G) is noticeable. All the three elements of the Golgi bodies, namely, the cisternae, vacuoles and vesicles, are recognizable, with a preponderance of vesicles and vacuoles. Clusters and rosettes of ribosomes (Ri) are freely dispersed in the cytoplasm.
ID-developing intercalated disc; Ri-ribosomes; N-nucleus; G-Golgi region; m-mitochondria.

X 22,350

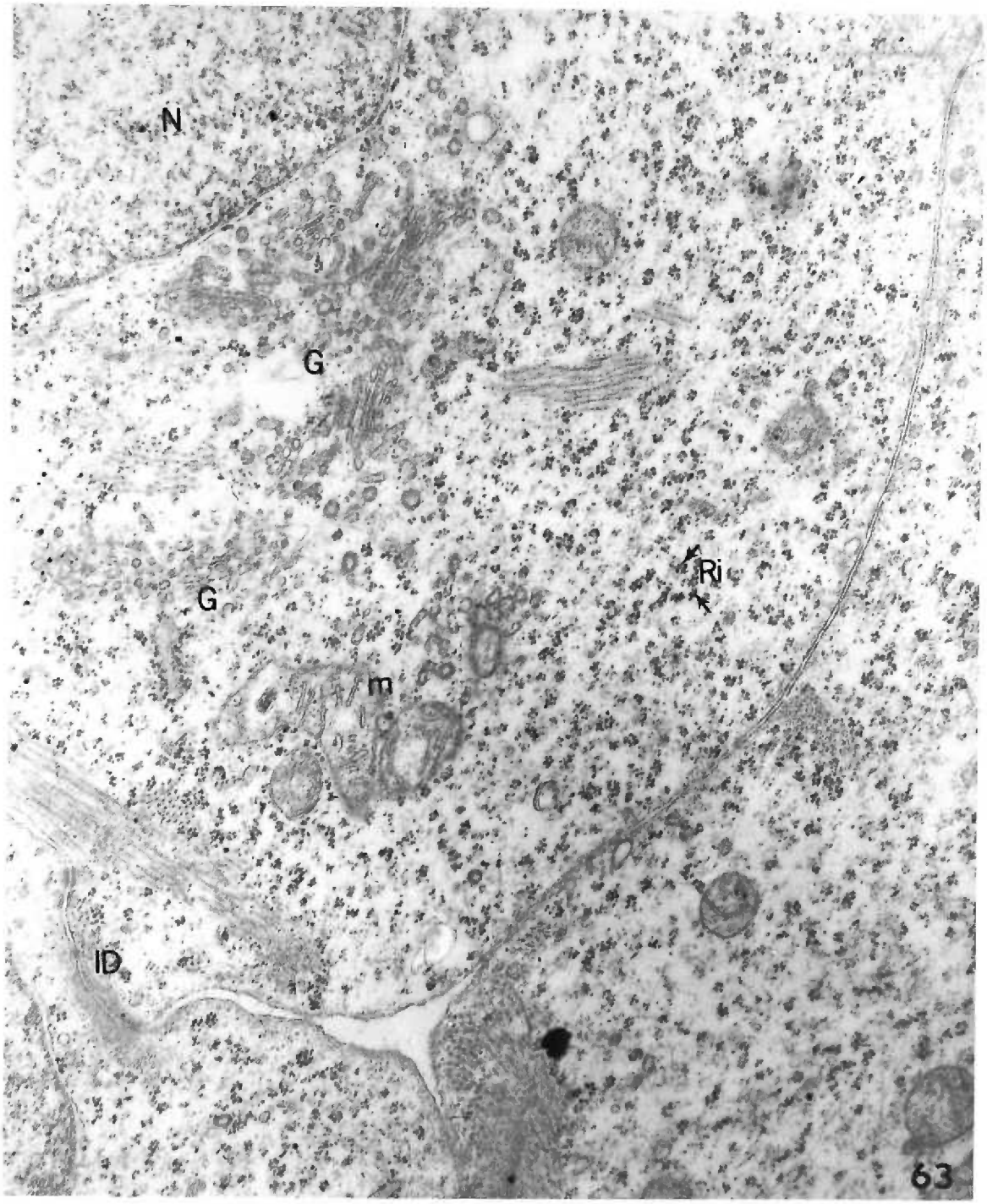

N
G
G
Ri
m
ID
63

Figure 64. 13-Day Myocardium

Increasing numbers of mitochondria are recognizable in this stage of development as compared to the previous stages. The cristae are well-formed and tightly packed. Several mitochondria are seen to be undergoing multiplication by a process of constriction and division (m, arrows). Large regions of Golgi elements are visible (G). The vacuoles and vesicles (v) predominate, many of which contain a flocculent material. Some of these vacuoles are in the intermediate stage of forming secretory granules. MF-myofibril; G-Golgi region; v-vacuoles and vesicles; m-mitochondria; ER-endoplasmic reticulum.

X 22,350

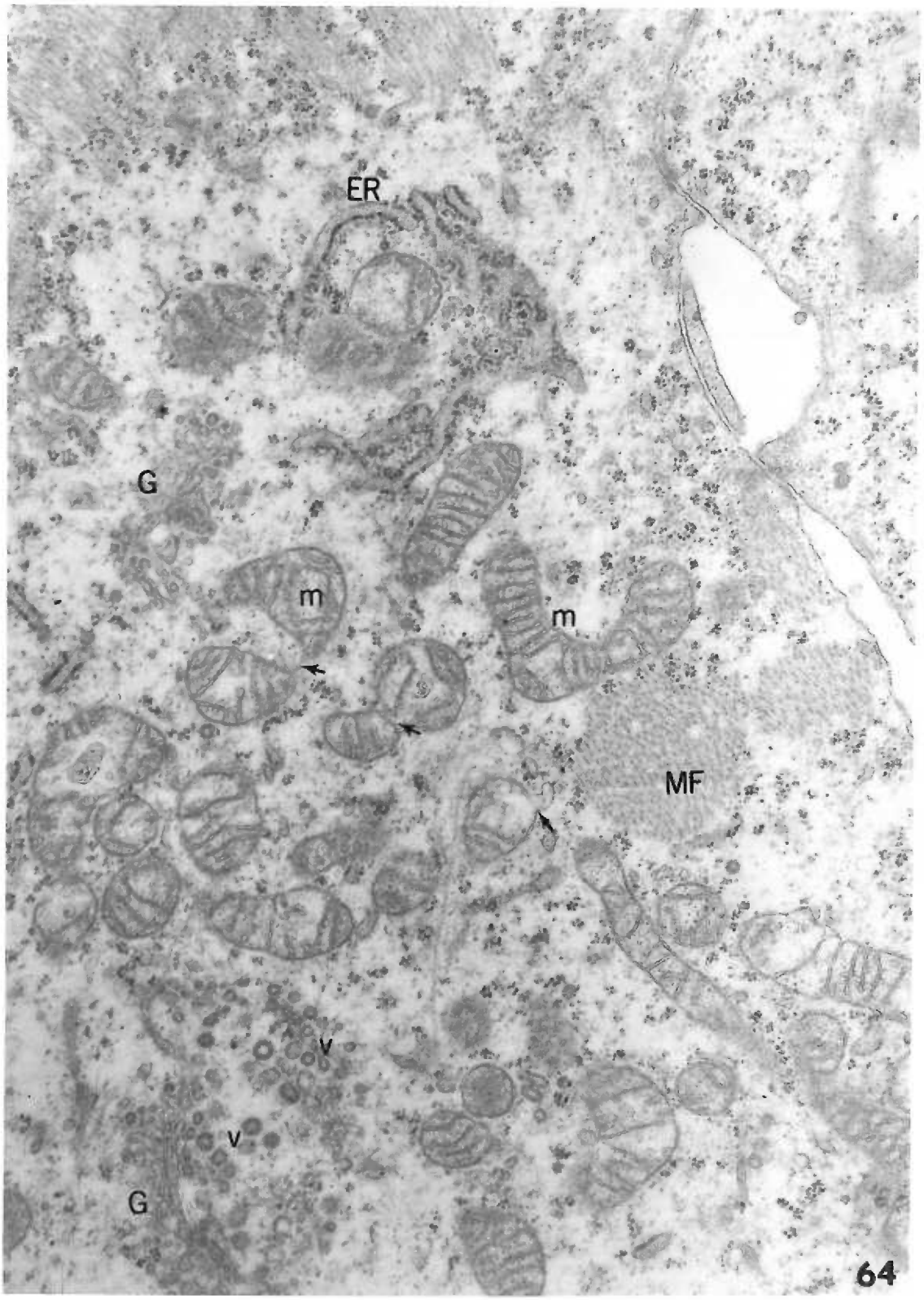

ER
G
m
m
MF
v
v
G
64

Figure 65. 13-Day Myocardium

The sarcolemma shows complementary projections and depressions so that the two cells fit together snuggly. The endoplasmic reticulum (ER) is seen to encircle the mitochondria (m). The developing T-system can be visualized (Ts). Several vesicles and vacuoles (v) are recognizable in the cytoplasm. N-nucleus; Ts-T-system; m-mitochondria; ER-endoplasmic reticulum; v-vacuoles.

X 22,350

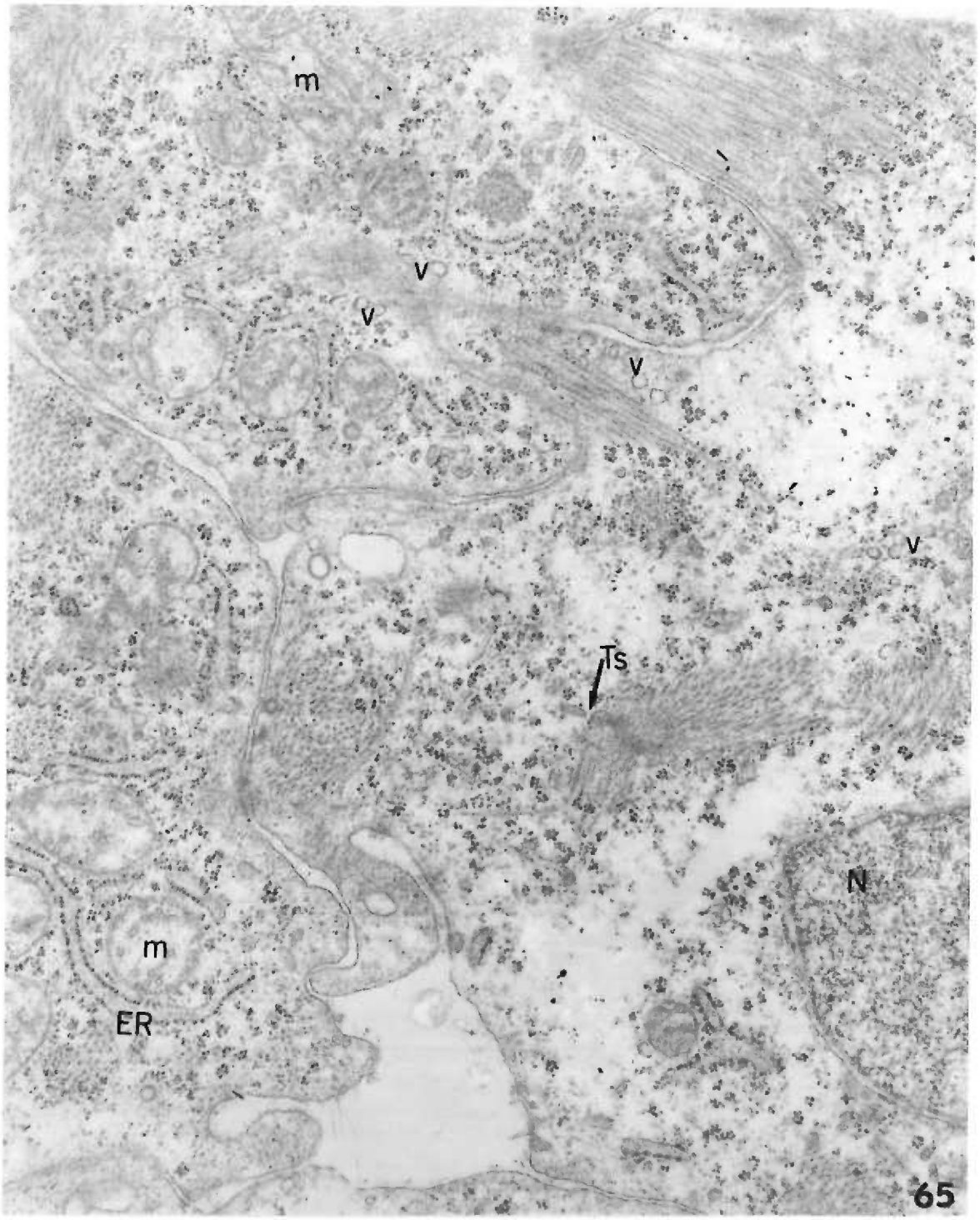

m
v
v
v
v
Ts
N
m
ER
65

Figure 66. 13-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. The final reaction product is deposited as very fine granules or particles (arrows). The intensity of reaction is considerably reduced compared to 10 and 11-day myocardium. Stacks of parallel cisternae of the endoplasmic reticulum (ER) are recognizable. The cytoplasm contains several vesicles and vacuoles (V). N-nucleus; ER-endoplasmic reticulum; V-vacuoles.

X 22,350

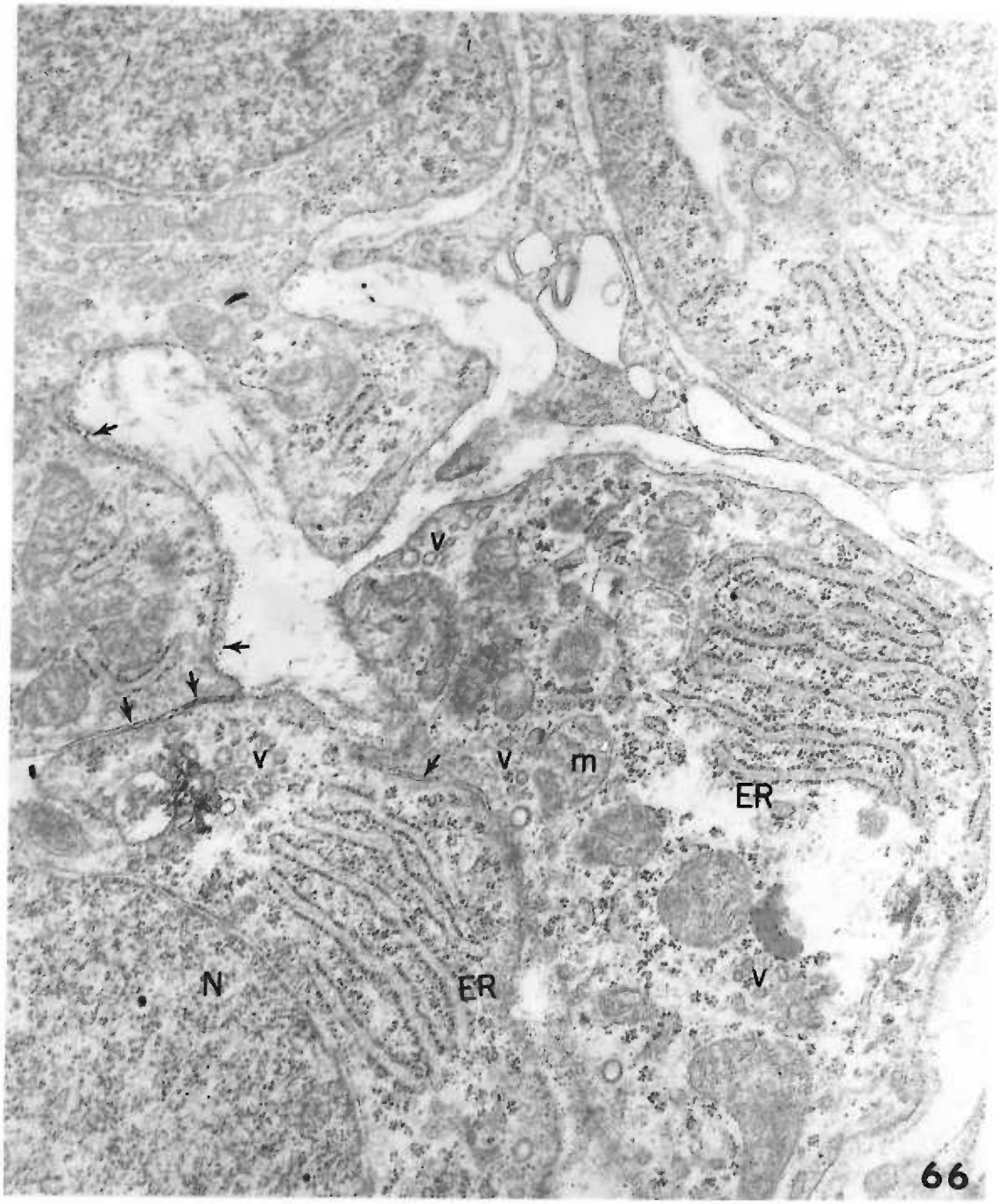

v
v
v
m
ER
N
ER
v
66

Figure 67. 13-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. The final reaction product is deposited as very fine granules (arrows) on the sarcolemma, unlike the thick granules of final reaction product discernible in the 10 or 11-day myocardium.

X 22,350

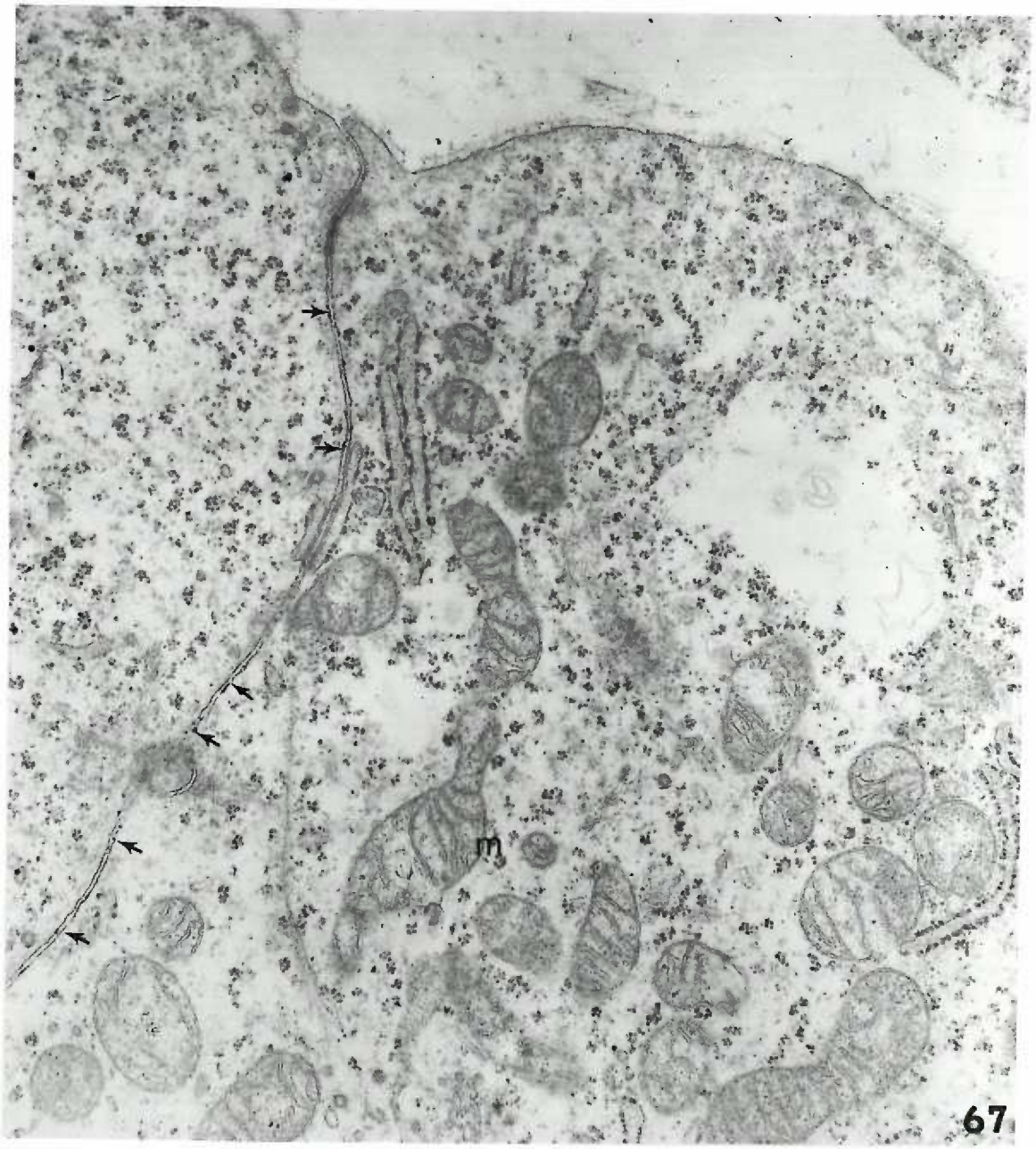

m
67

Figure 68. 13-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. The final reaction product is deposited as very fine granules (arrows) unlike the thick precipitate observed in 10 and 11-day myocardium.

MF-myofibrils; m-mitochondria; Ri-ribosomes.

X 15,975

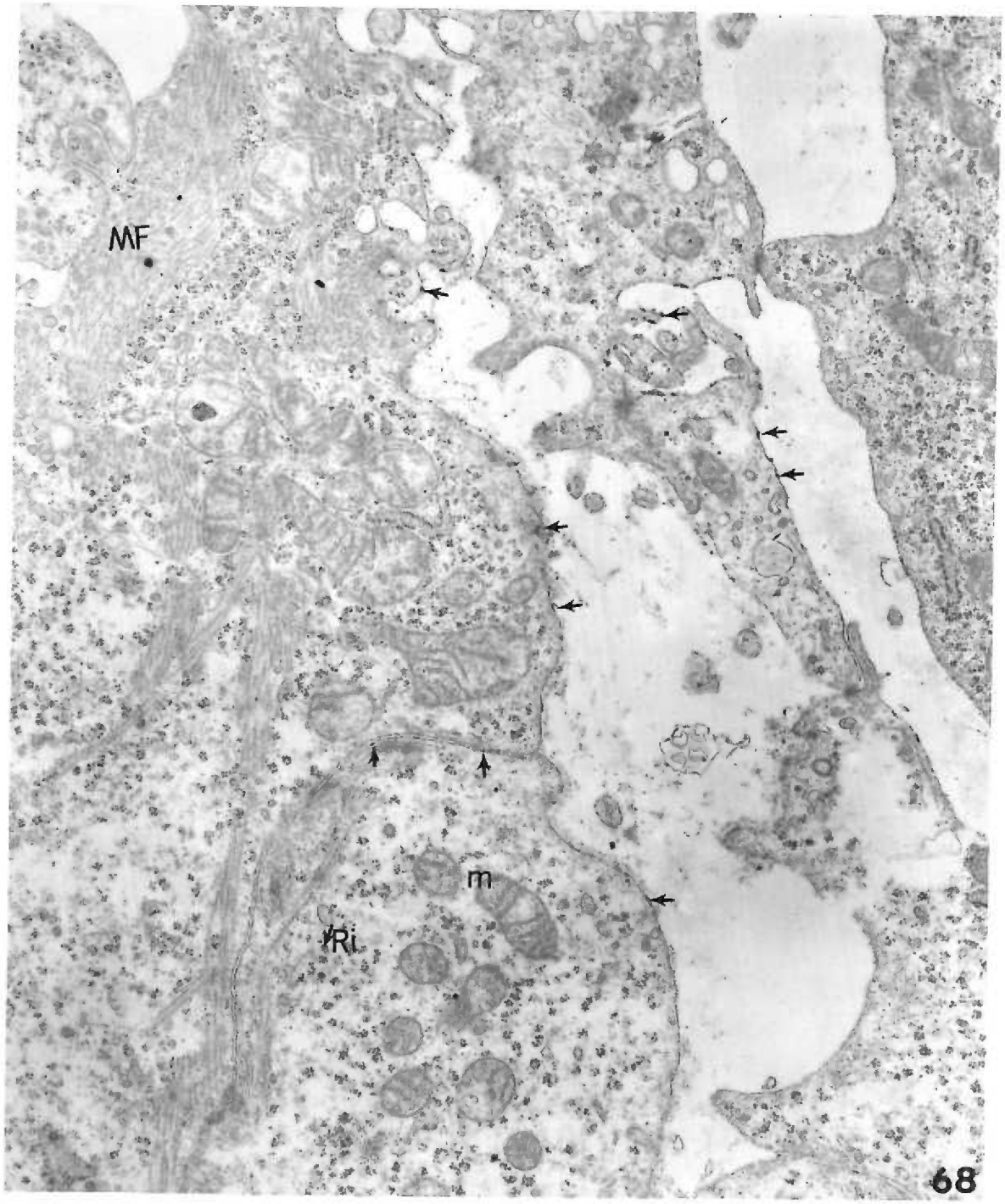

MF
m
Ri
68

Figure 69. 14-Day Myocardium

The presence of neural tissue*is frequently observed in this stage of development. Neural elements appear as long cell processes (NP) between the myocardial cells (Myc). Nerve-muscle "contact" appears to be established by the evaginations or protrusions of the sarcolemma and neurolemma (arrows) towards each other. Neurofilaments (Nfl), distended endoplasmic reticulum (ER) and ribosomes are recognizable in the axoplasm.

Myc-myocardial cell; MF-myofibrils; Nfl-neurofilaments; NP-nerve process; m-mitochondria; ER-endoplasmic reticulum.

X 22,350

* See appendix

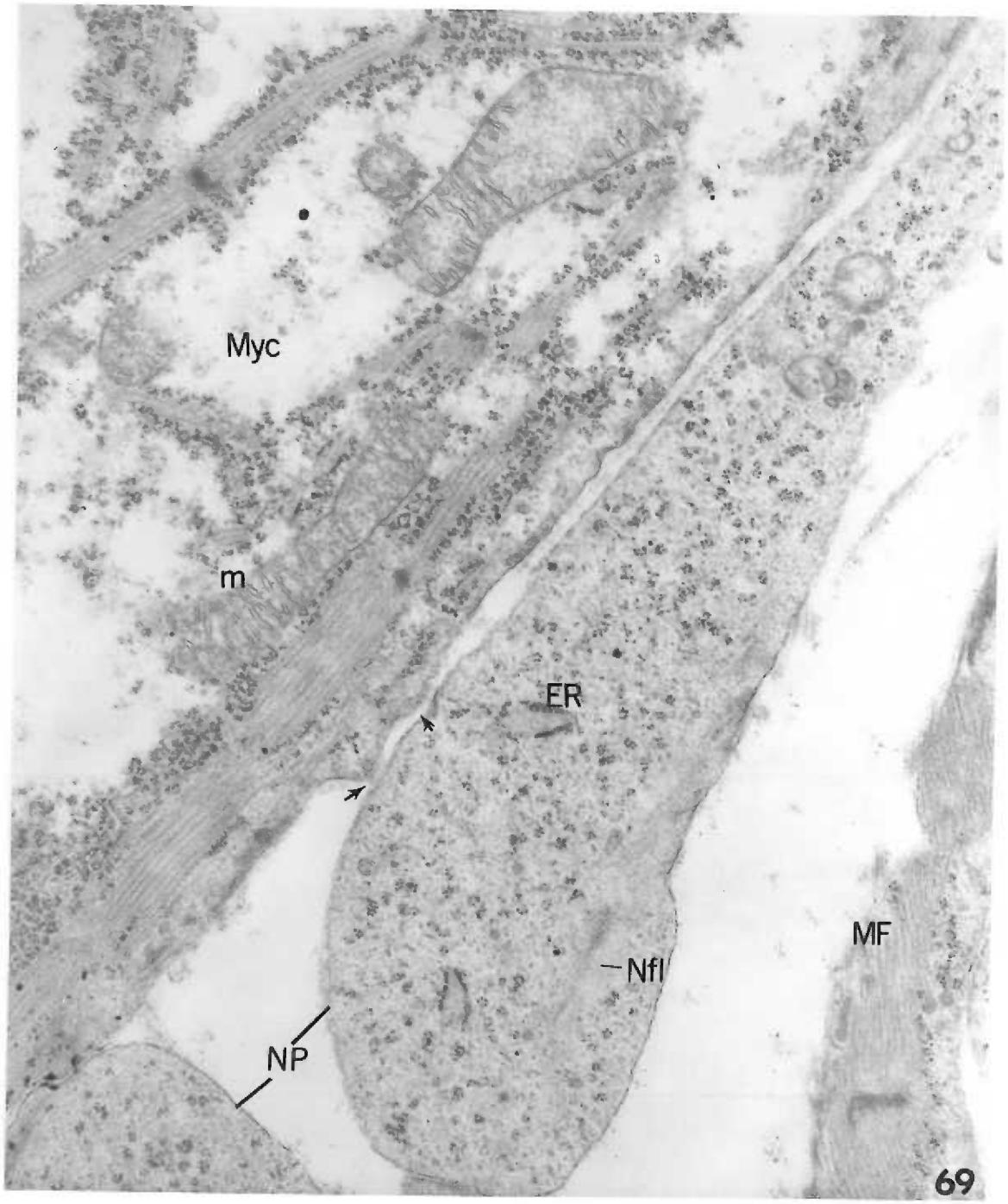
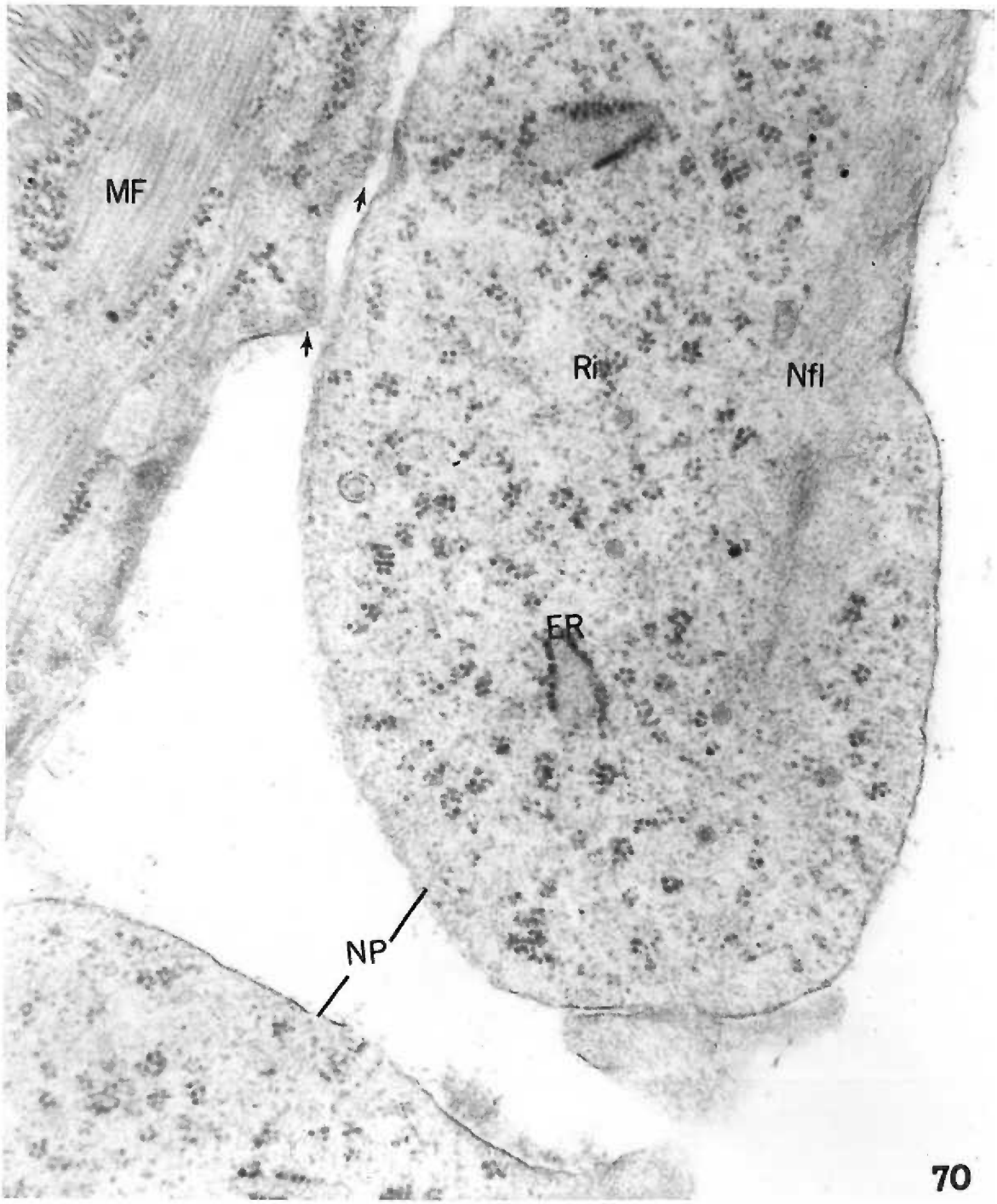

Myc
m
ER
Nfl
NP
MF
69

Figure 70. 14-Day Myocardium

A high magnification picture of figure 69. A long neural process (NP)* is noticeable between myocardial cells. The neuro-muscular "contact" is readily recognizable (arrows). The cell surfaces of both the myocardial and neural elements are covered with a filamentous material called the external lamina or glycocalyx. The axoplasm contains clusters of ribosomes (Ri), distended endoplasmic reticulum (ER) and fine neurofilaments (Nfl).
NP-nerve process; Nfl-neurofilaments; MF-myofibrils; Ri-ribosomes; ER-endoplasmic reticulum.

X 40,175

* See appendix



70

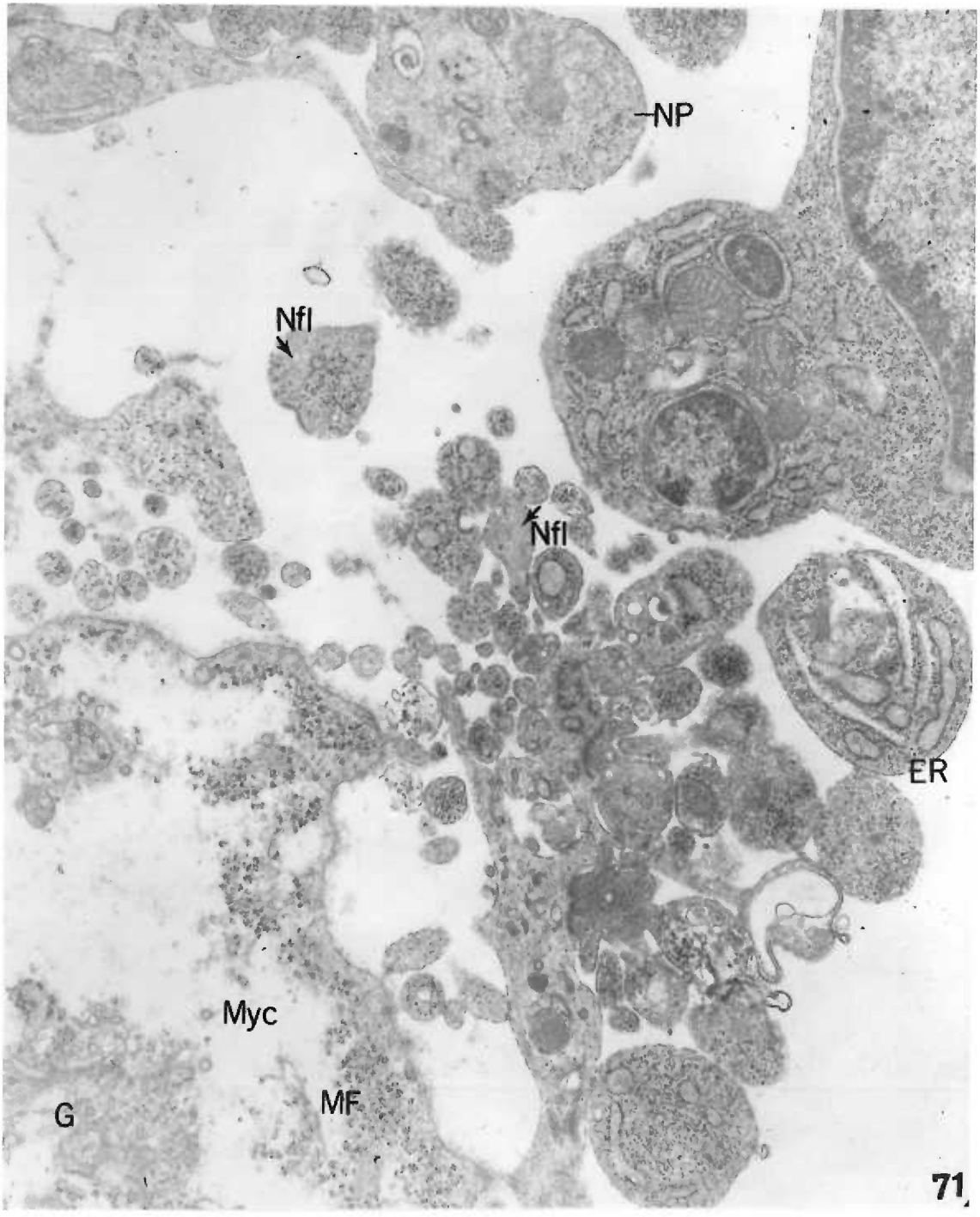
Figure 71. 14-Day Myocardium

A cross sectional view of several nerve processes*(right of the picture) in close contact with the myocardial (Myc) cells (left of the picture) is shown. Fine neurofilaments are recognizable in some processes (Nfl). Cross sectional profiles of greatly distended cisternae of endoplasmic reticulum (ER) are very conspicuous in these processes.

Myc-myocardial cell; MF-myofibrils; Nfl-neurofilament; NP-nerve process; ER-endoplasmic reticulum; G-Golgi region.

X 17,880

* See appendix



71

Figure 72. 14-Day Myocardium

Neural tissue* is intimately associated with the embryonic myocardium. Structures resembling synaptic vesicles (NT) are recognizable in the myocardial cell (Myc).

NP-neural process (cross section); NT-neural tissue, synaptic vesicles; MF-myofibrils; Myc-myocardial cell; N-nucleus.

X 22,350

* See appendix

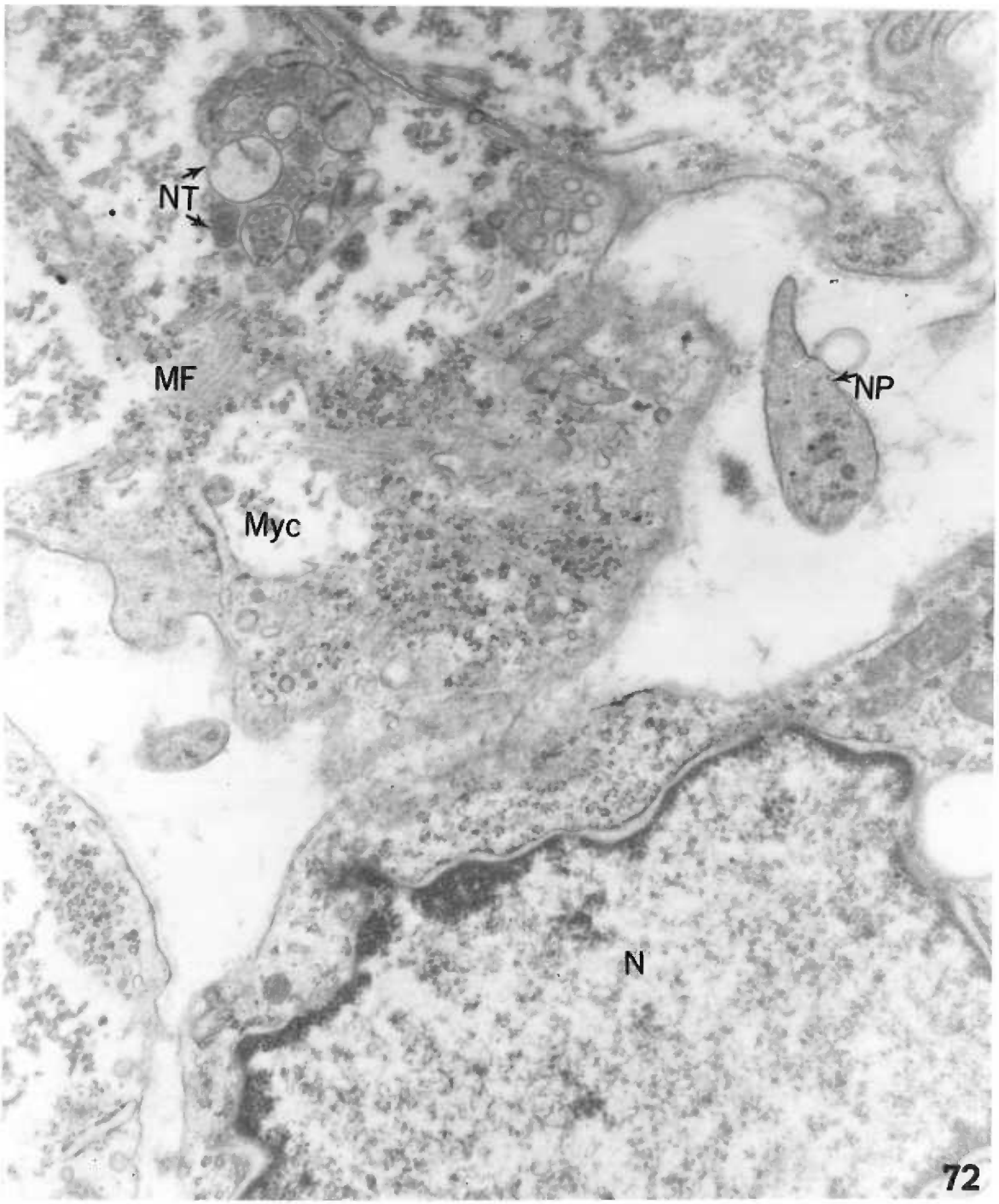

NT
MF
NP
Myc
N
72

Figure 73. 14-Day Myocardium

Myofibrils (MF) appear to be more tightly packed or grouped together than in 11-day myocardium. At several Z disc regions, the developing elements of T-system can be recognized (Z, Ts and arrows).

D-desmosome; ID-developing intercalated disc; MF-myofibrils; SR-developing sarcoplasmic reticulum; Ts-T-system; Z-Z disc; m-mitochondria; N-nucleus.

X 15,975

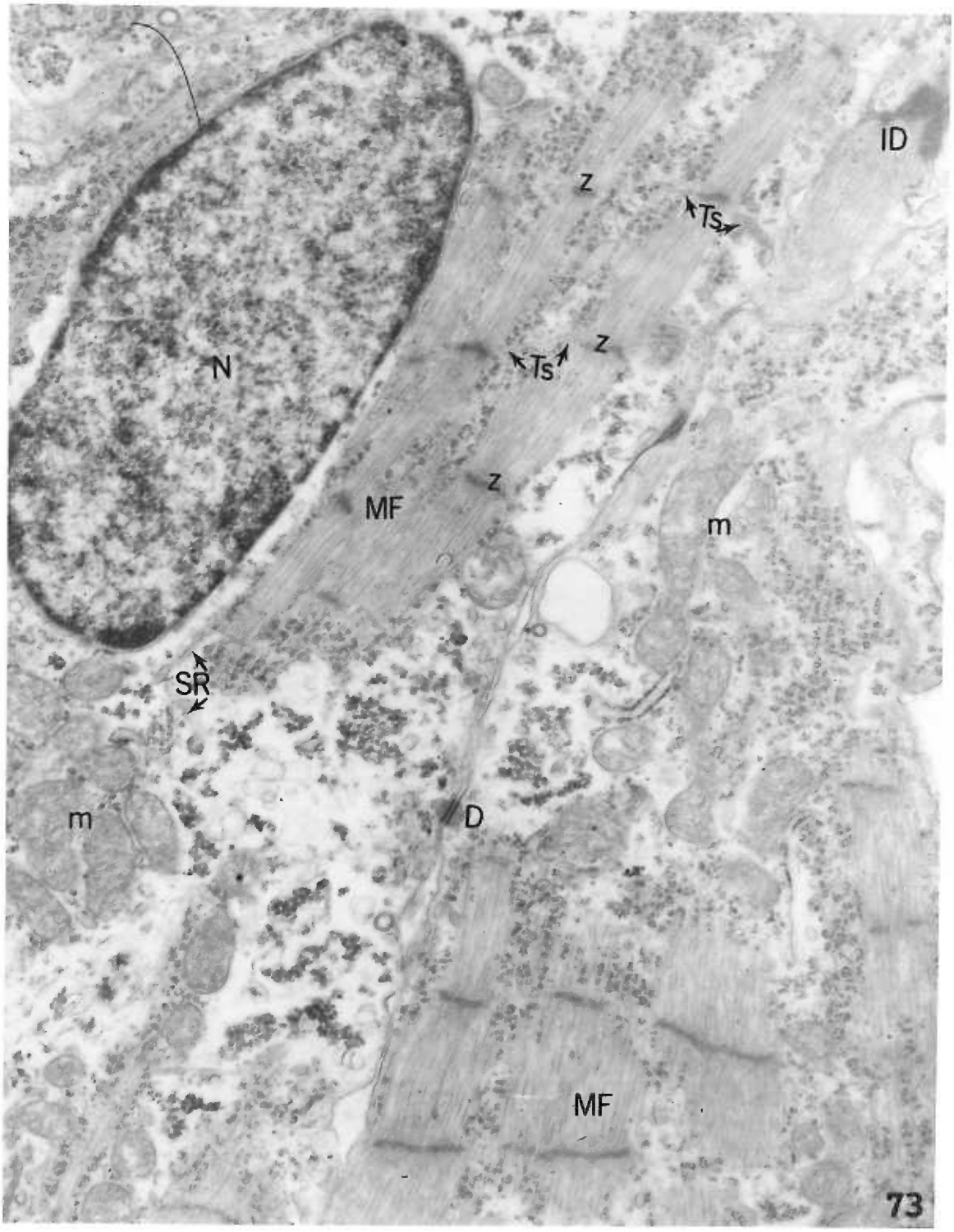

ID
z
Ts
Ts
z
N
z
MF
m
SR
m
D
MF
73

Figure 74. 14-Day Myocardium

The development of T-system can be recognized near the Z disc regions (Z, arrows). The sarcolemma scallops and makes contact with the myofibrils at the region of the Z discs (x). Two developing intercalated discs are shown (ID); notice that these discs appear one sarcomere length away from the preceeding Z discs.

ID-intercalated disc; Z-Z disc; m-mitochondria; Mvb-multivesicular body; x-regions of Z disc-sarcolemma contacts; Ts, arrows-T-system.

X 12,780

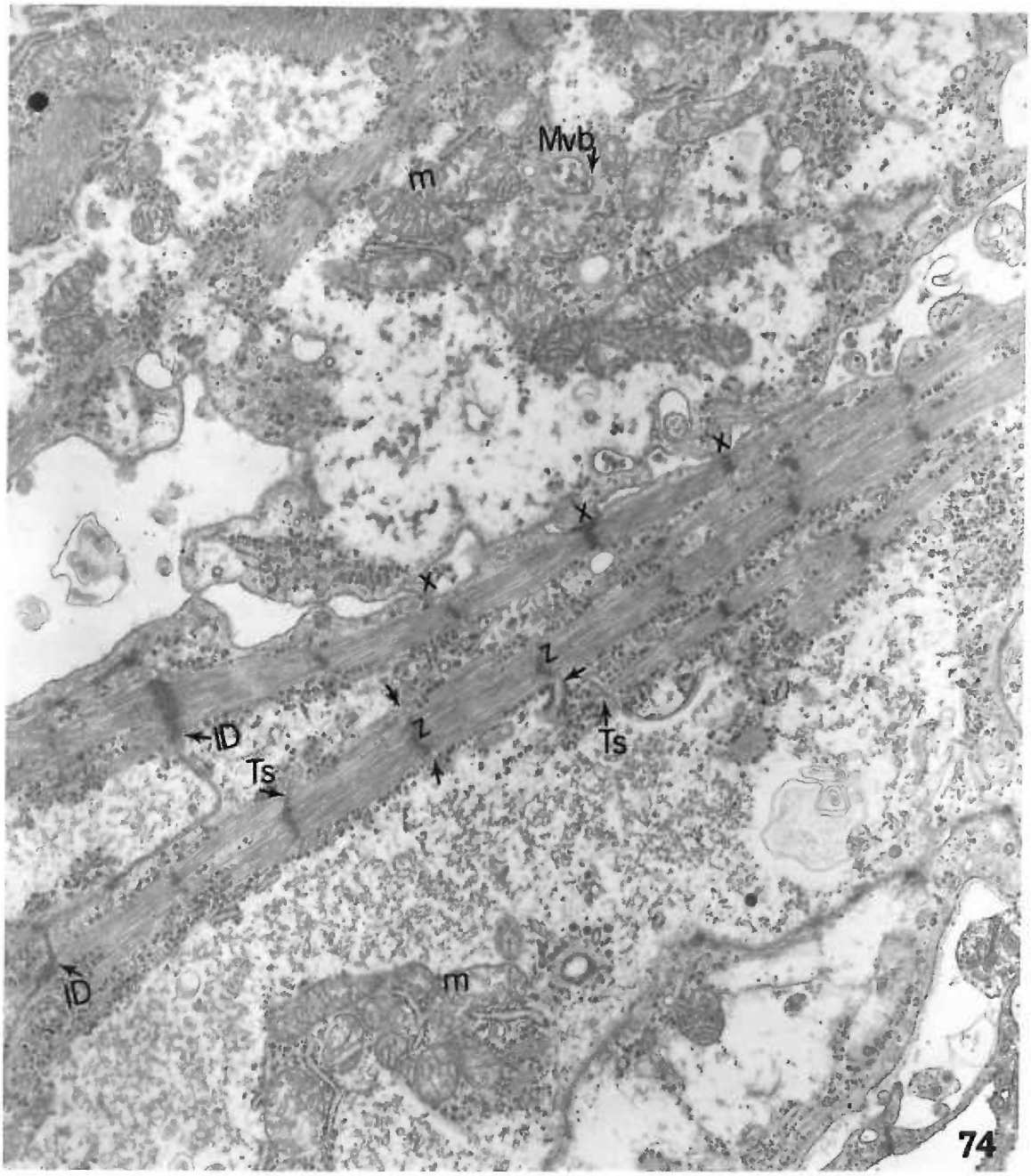

Mvb
m
x
x
x
z
z
z
ID
Ts
Ts
ID
m
74

Figure 75. 14-Day Myocardium

The myofibrils are fully formed and appear much wider and thicker than previously noted under the same magnification. Z, I, A and H bands are well-delineated. In the same cells, aggregates of myofilaments, not yet organized into fibrils, can also be recognized (Mfl). The sarcolemma scallops and makes contact with the fibrils at the Z disc regions (x). At several Z disc regions, the developing T-system can be visualized (Ts and arrows). The close association of mitochondria (m) with myofibrils (MF) is very apparent.

MF-myofibrils; Mfl-myofilaments; Ts and arrows-developing T-system; A, H, I and Z-their respective bands; m-mitochondria; x-regions of Z disc-sarcolemma contacts.

X 22,350

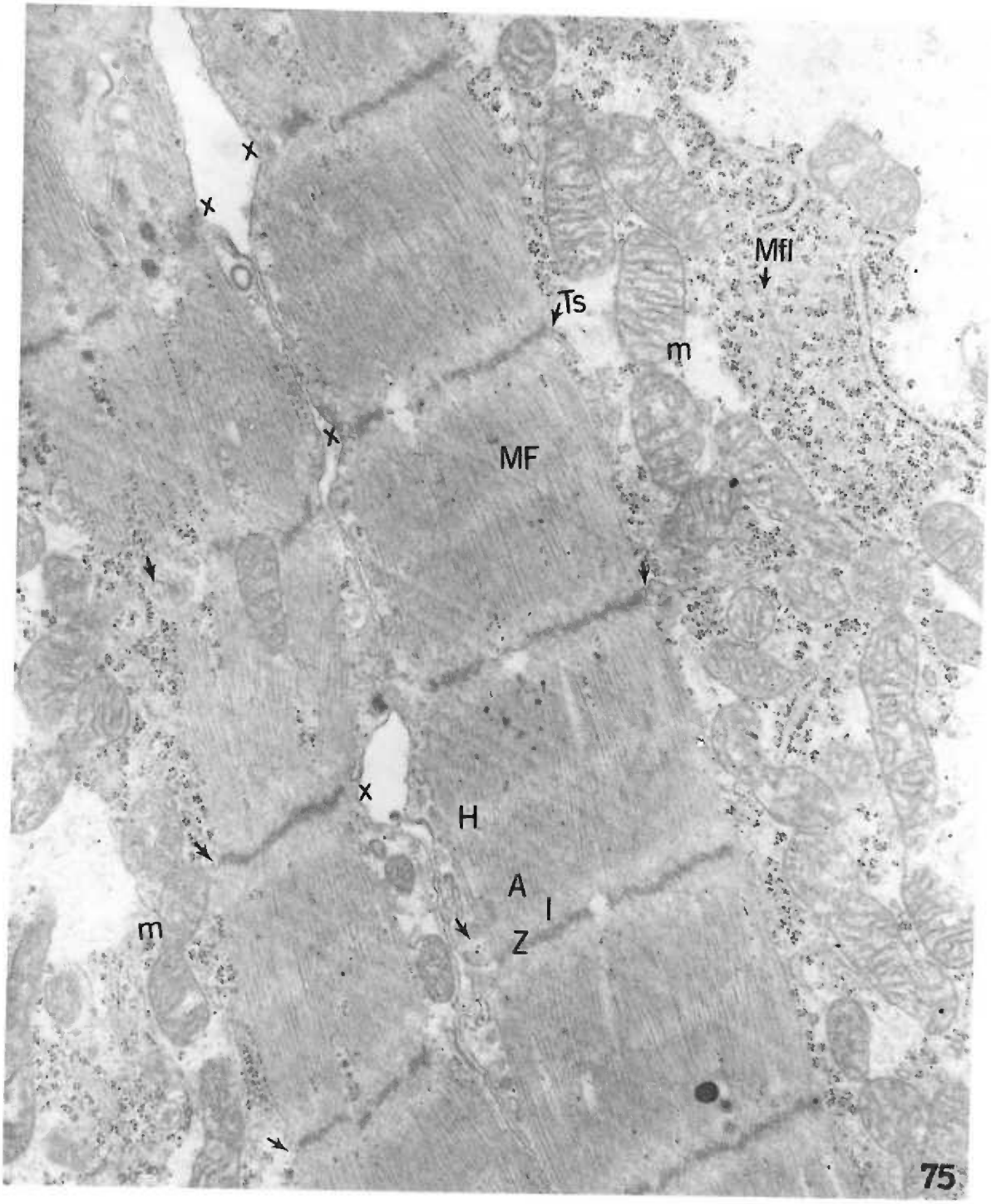

x
x
x
x
Mfl
Ts
m
MF
H
A
I
Z
m
75

Figure 76. 14-Day Myocardium

A deep indentation of the sarcolemma at the Z disc region is shown at the top right (x). The development of the T-system at Z disc regions can be visualized (Ts and arrows). The branching of the fibrils can also be recognized at the Z disc regions (Z_1, Z_2 and Z_3). Chains of polyribosomes (PRi) are discernible in close association with myofibrils; clusters of ribosomes (Ri) are freely dispersed in the sarcoplasm. The distribution of mitochondria is very extensive (m).

MF-myofibril; m-mitochondria; Ts and arrows-developing T-system; Ri-ribosomes; PRi-polyribosomes; Z_1, Z_2, and Z_3- Z disc regions where branching of the fibrils can be visualized.

X 22,350

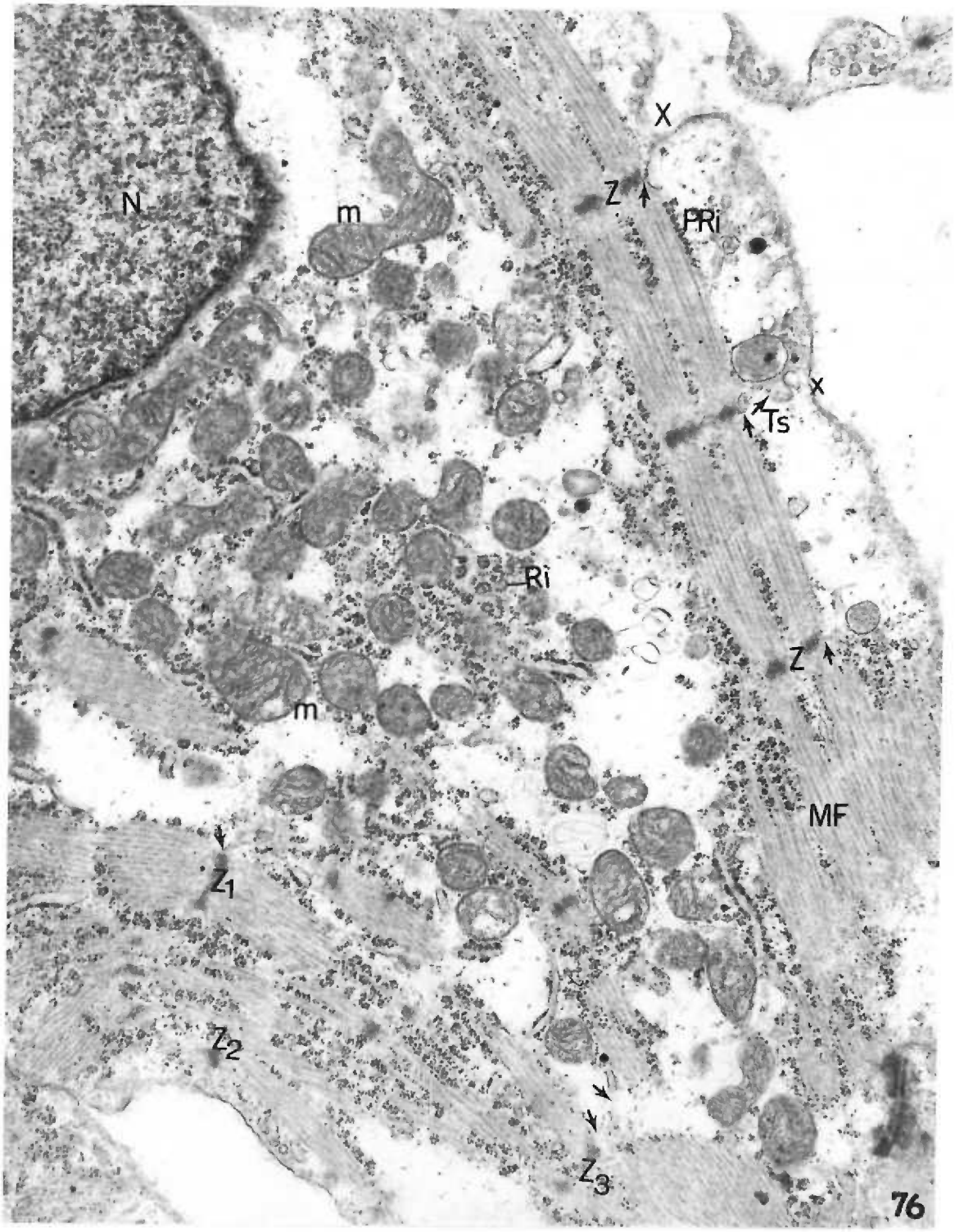

N
m
X
Z
PRi
x
Ts
Ri
Z
m
MF
Z1
Z2
Z3
76

Figure 77. 14-Day Myocardium

Golgi bodies (G) appear very active, and numerous rows of membrane-bound secretory granules (SGr) are recognizable in the region of this organelle. The Golgi cisternae are filled with a flocculent material, and the granules seem to pinch off from these cisternae (long arrows). Large amounts of vacuoles and vesicles are visible in the Golgi region, and some of the vacuoles are in the intermediate stage of formation of secretory granules. Stackes of parallel cisternae of endoplasmic reticulum (ER) are recognizable at top left. Developing T-system is associated with Z disc regions (Ts, Z). Several myofibrils (MF) appear to be "fused" together to form "sheets" of fibrils. A single centriole is discernible (C).

C-centriole; ER-endoplasmic reticulum; G-Golgi region; Ts-T-system; SGr-secretory granules; MF-myofibril; m-mitochondria.

X 23,760

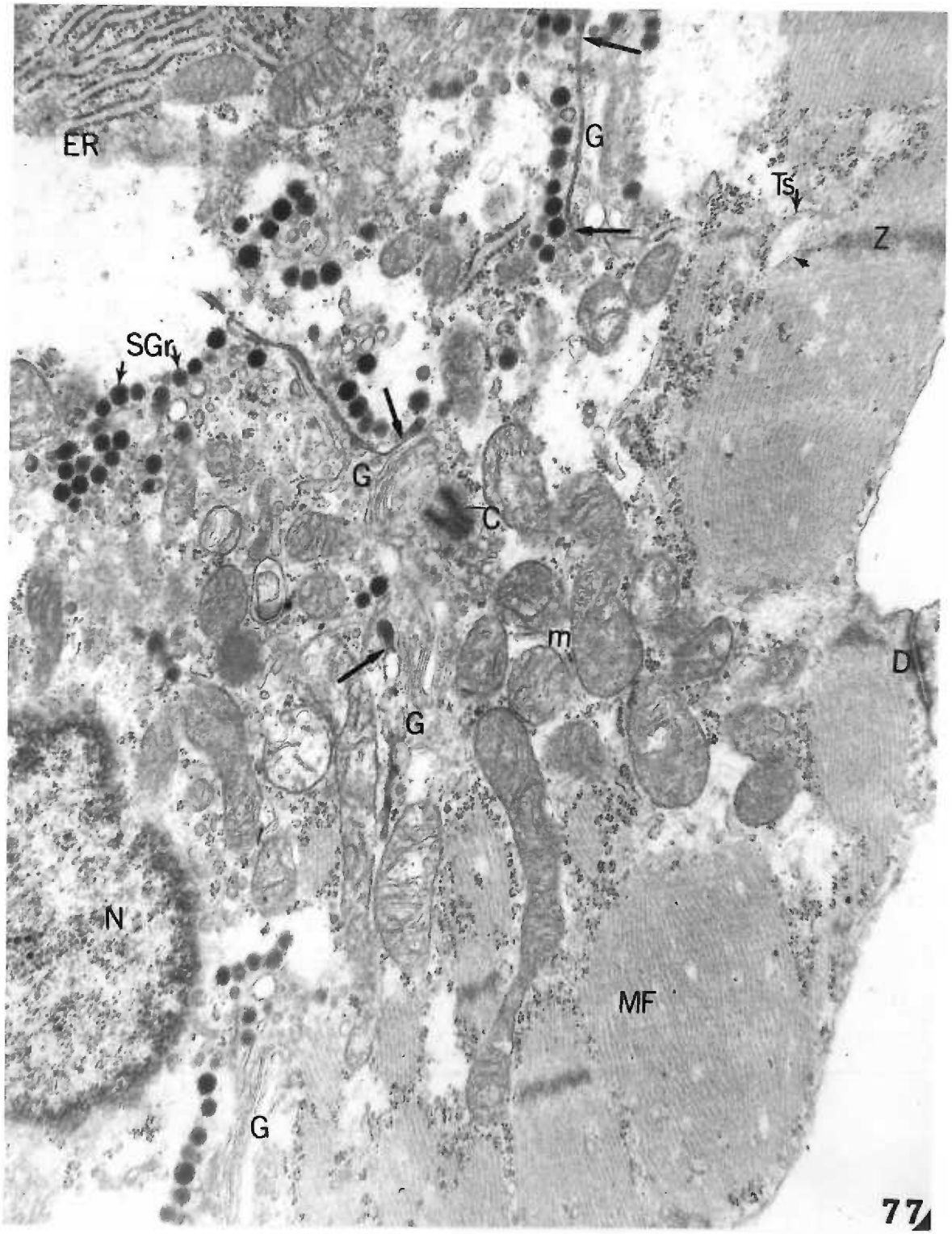

ER
G
Ts
Z
SGr
G
C
m
D
G
N
MF
G
77

Figure 78. 14-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. The final reaction product (FRP and arrows) is deposited as fine granules on the sarcolemma, unlike the clusters of granules noted in 10 and 11-day myocardium. In some regions where the final reaction product is deposited, the myofibrils are in contact with the sarcolemma (MF). A neural process (NP) is shown.

FRP-final reaction product; NP-neural process; MF-myofibril; ID-developing intercalated disc; G-Golgi region.

X 6,850

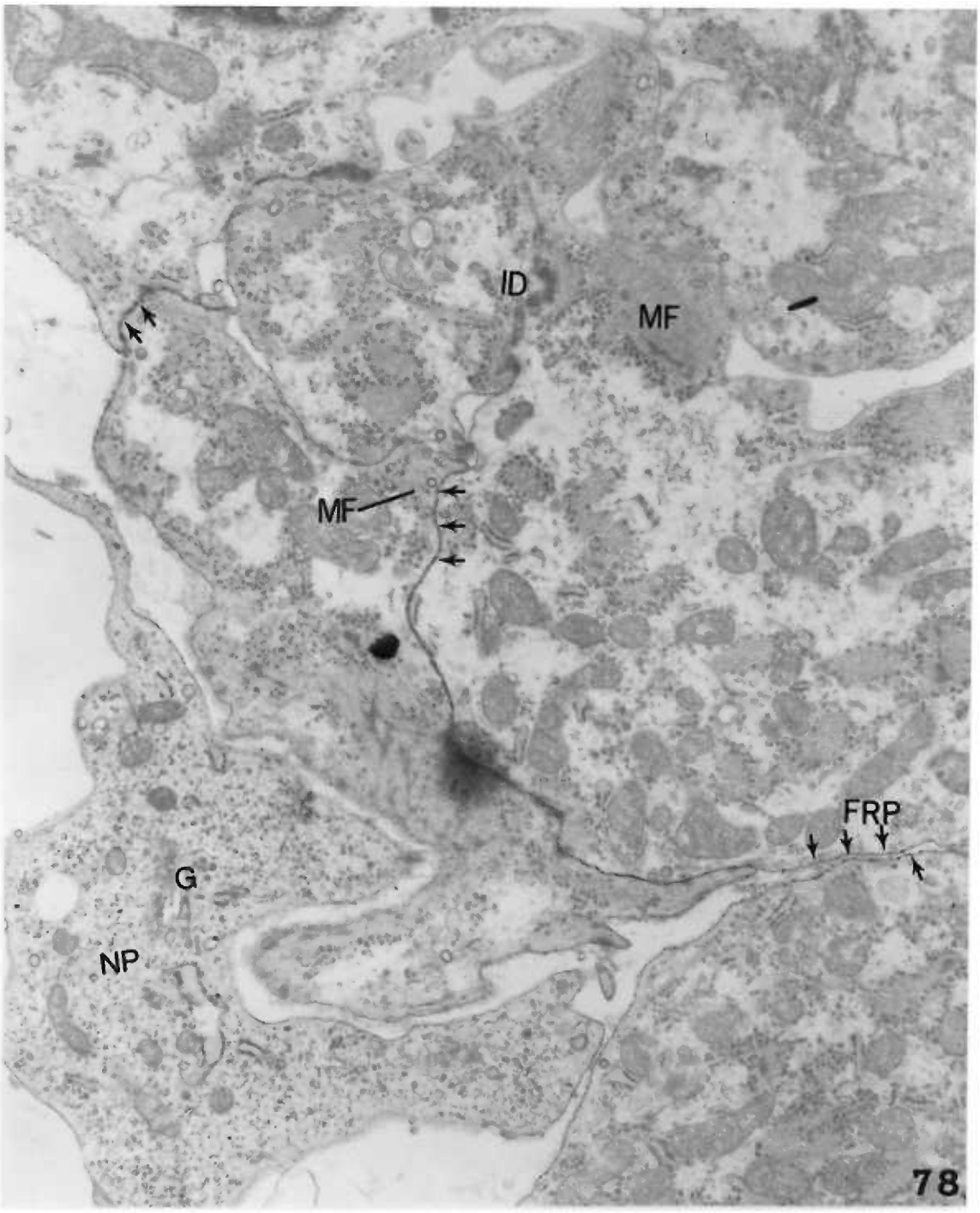

ID
MF
MF
FRP
G
NP
78

Figure 79. 14-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. The final reaction product (FRP and arrows) is deposited as fine granules on the sarcolemma, unlike in the 10 and 11-day myocardium where the reaction product appears as heavy clusters of granules.

Mfl-myofilament; FRP and arrows-final reaction product; G-Golgi elements; ID-developing intercalated disc.

X 15,975

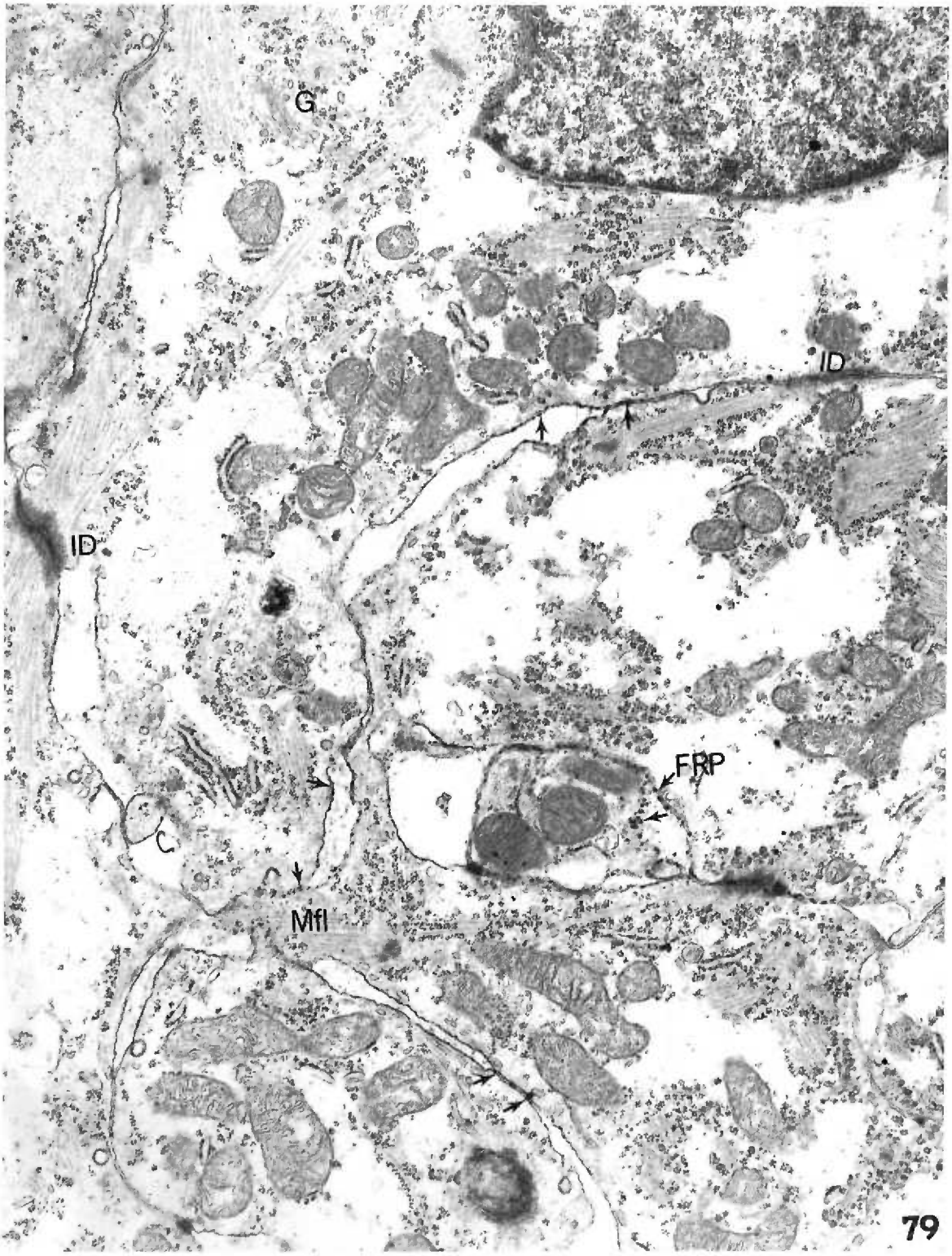

G
ID
ID
FRP
C
Mfl
79

Figure 80. 14-Day Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. The final reaction product (FRP and arrows) is deposited as fine granules on the cell surfaces, unlike the clusters of granules observed in 10 and 11-day myocardium.

MF-myofibrils; N-nucleus; m-mitochondria; FRP and arrows-final reaction product.

X 15,975

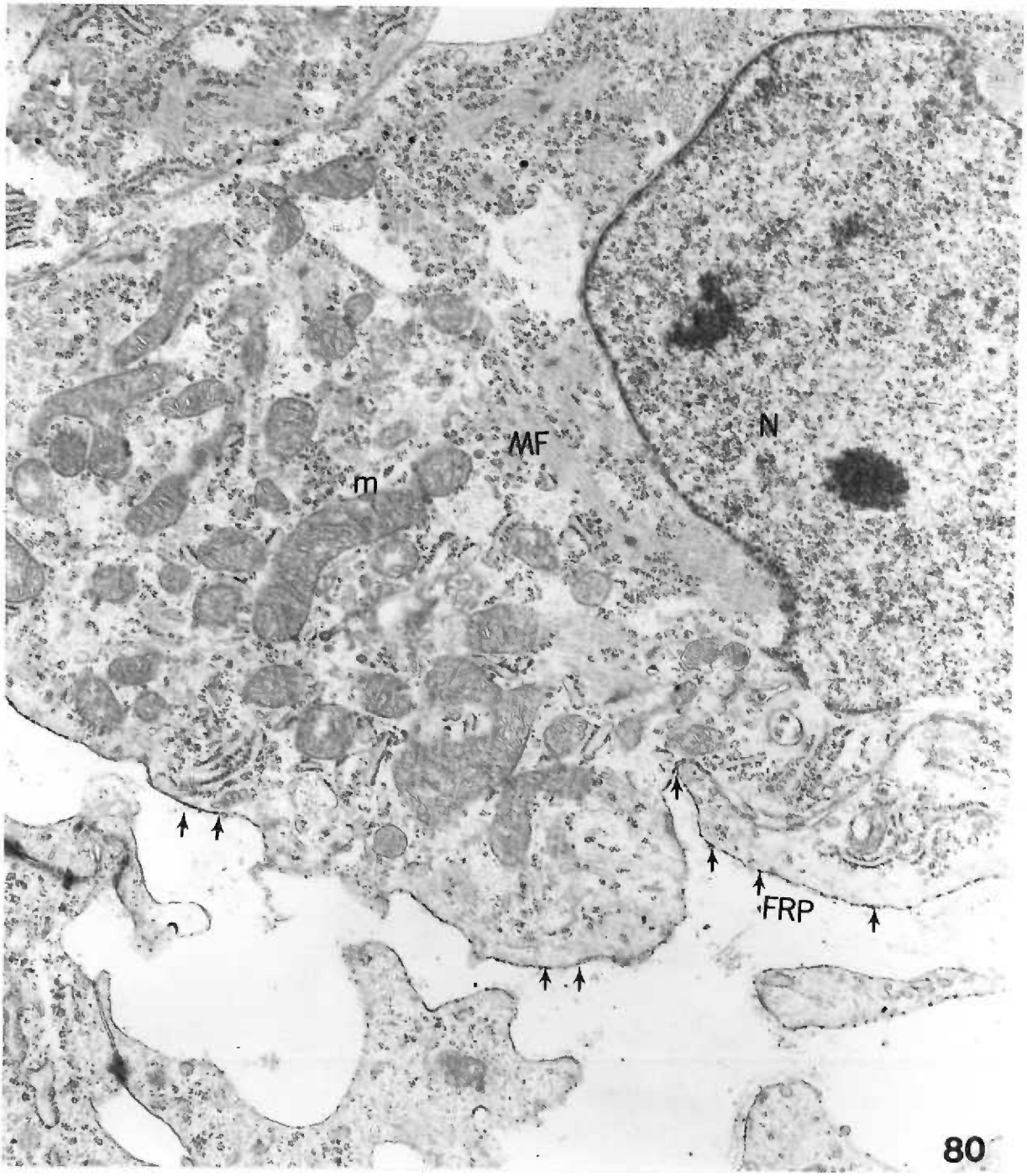

N
MF
m
FRP
80

Figure 81. New Born Myocardium

Several features of new born myocardium are observable in this picture. Compared with earlier stages of development, the sarcoplasmic reticulum (SR) and T-system (Ts) are in advanced stages of differentiation. The sarcoplasmic reticulum establishes a system of interfibrillar network of anastomosing channels (SR and arrows). The sarcolemma (SL) makes exaggerated indentations so as to make contact with the Z disc regions. Pinocytotic activity is intense (Pv). Both fuzzy or spiny vesicles (Fv) and smooth vesicles (Pv) are recognizable. Elaborate arrangement of granular endoplasmic reticulum is discernible (ER). Clusters and chains of ribosomes (Ri) are associated with ER.

SR-sarcoplasmic reticulum; Ts-T-system; m-mitochondria; ER-endoplasmic reticulum; Ri-ribosomes, clusters and chains; Fv-fuzzy or spiny vesicles; Pv-smooth contoured pinocytotic vesicles; SL-sarcolemma.

X 26,000

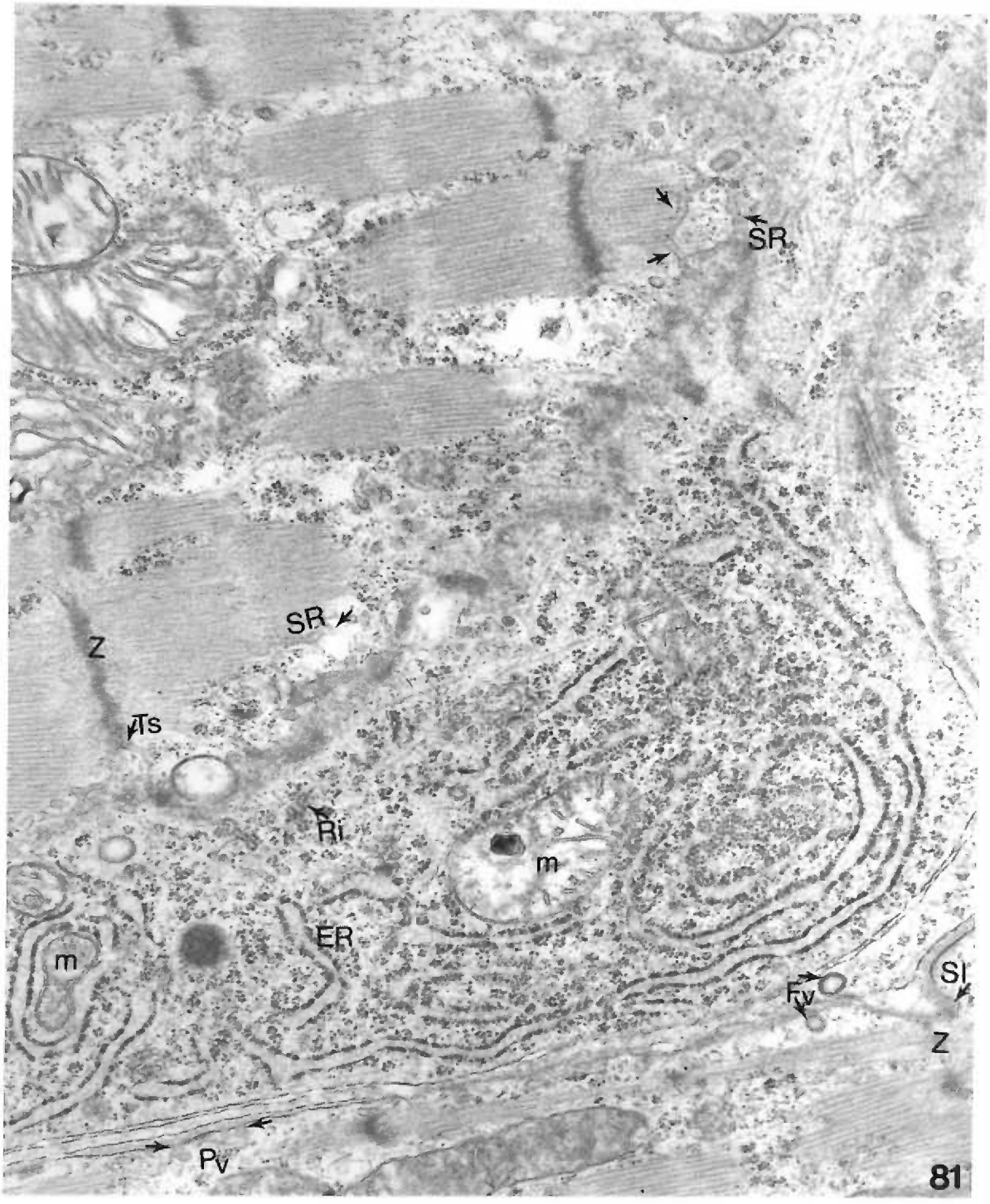

SR
SR
Z
Ts
Ri
m
ER
m
Fv
Sl
Z
Pv
81

Figure 82. New Born Myocardium

The new born myocardium is well-vascularized. A portion of capillary located between myocardial cells is shown. The vesicular cytoplasm of endothelial cells (Enc) is apparent. The capillary lumen encloses an erythrocyte (RBC) from which the nucleus has already been extruded, and thus the structure shown consists mainly of hemoglobin. A few particles of ribosomes (dark dots) are recognizable in the red blood cell(RBC).

RBC-erythrocyte, mainly hemogolbin; Enc-endothelial cell; V-vesicles or vacuoles; ID-intercalated disc; m-mitochondria; EN-endothelial cell nucleus; Z-Z band.

X 18,000

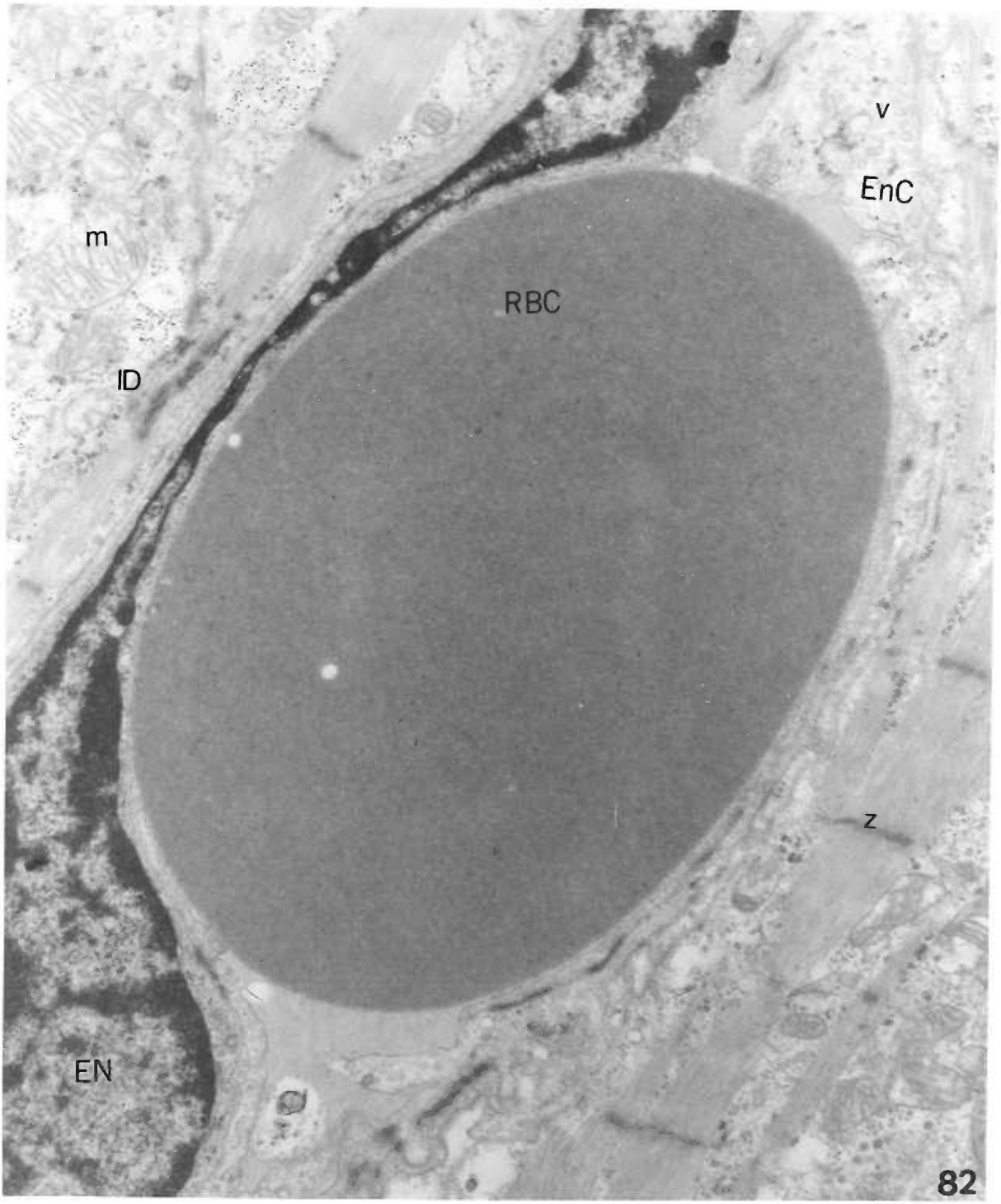

v
EnC
m
RBC
ID
z
EN
82

Figure 83. New Born Myocardium

A capillary located between myocardial cells is shown. The intense pinocytotic activity (Pv and arrows) of the endothelial cells and the vesicular cytoplasm (Enc) is observable. Desmosomal (D) attachments between endothelial cells are apparent.

EN-endothelial nucleus; N-myocardial nucleus; Enc-endothelial cell; Pv-pinocytotic vesicles; Cap L-capillary lumen; Myc-myocardium; m-mitochondria; D-desmosome.

X 18,000

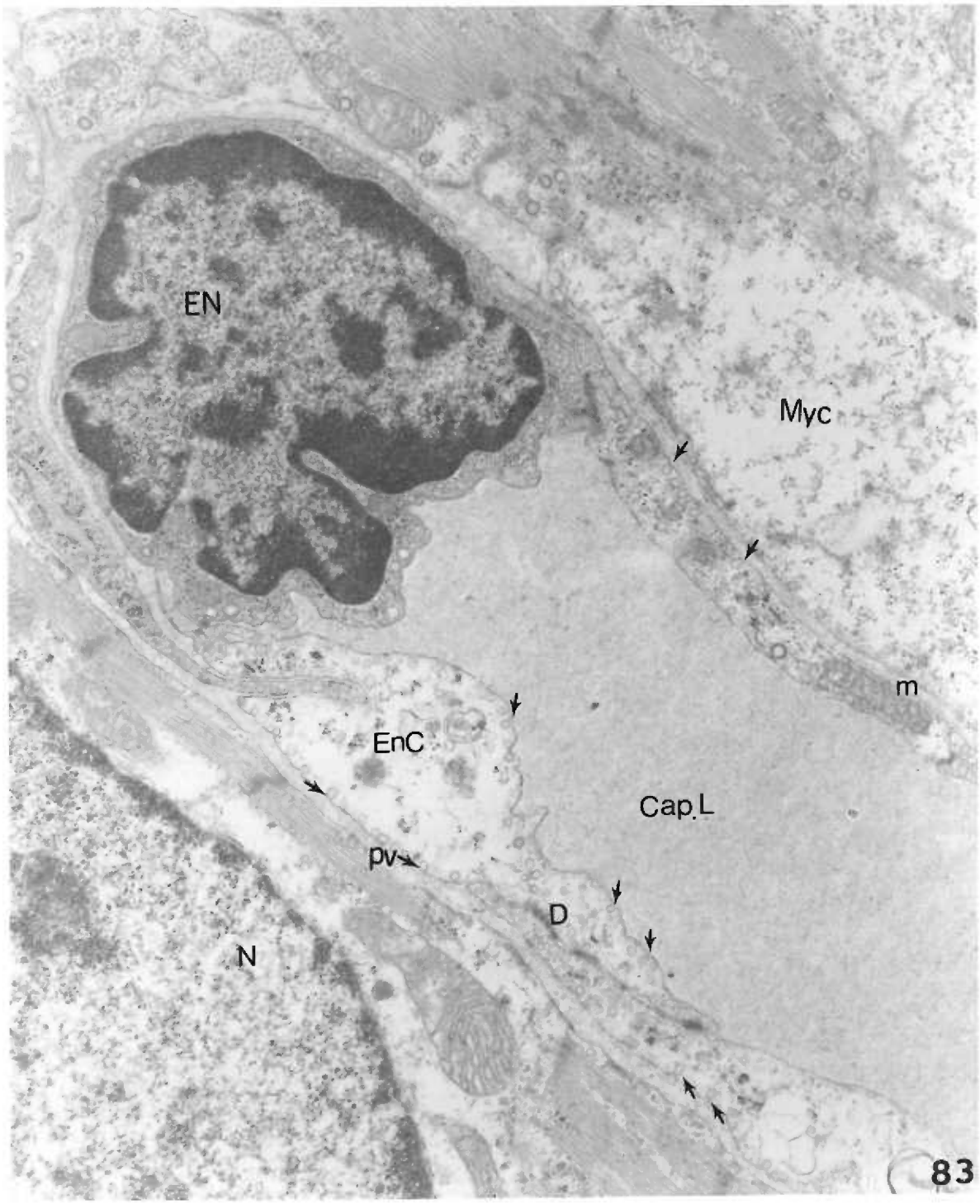

EN
Myc
m
EnC
Cap.L
pv
D
N
83

Figure 84. New Born Myocardium

Sarcolemma scallops and makes contacts with the Z disc regions at frequent intervals (X-Z). Intense pinocytotic activity (arrows) is associated with the sarcolemma. Smooth (Pv) and fuzzy or spiny vesicles are discernible in the cytoplasm. Mitochondria (m) assume a parallel arrangement to the fibrils in the long axis. It appears that there is a tubular connection between the mitochondria and the Z disc region (two long arrows).

G-Golgi region; Fv-fuzzy or spiny vesicles and vacuoles; Pv-pinocytotic vesicles (mainly smooth-contoured); N-nucleus; m-mitochondria; Z-Z disc; X-Z-sarcolemma-Z disc connections.

X 26,000

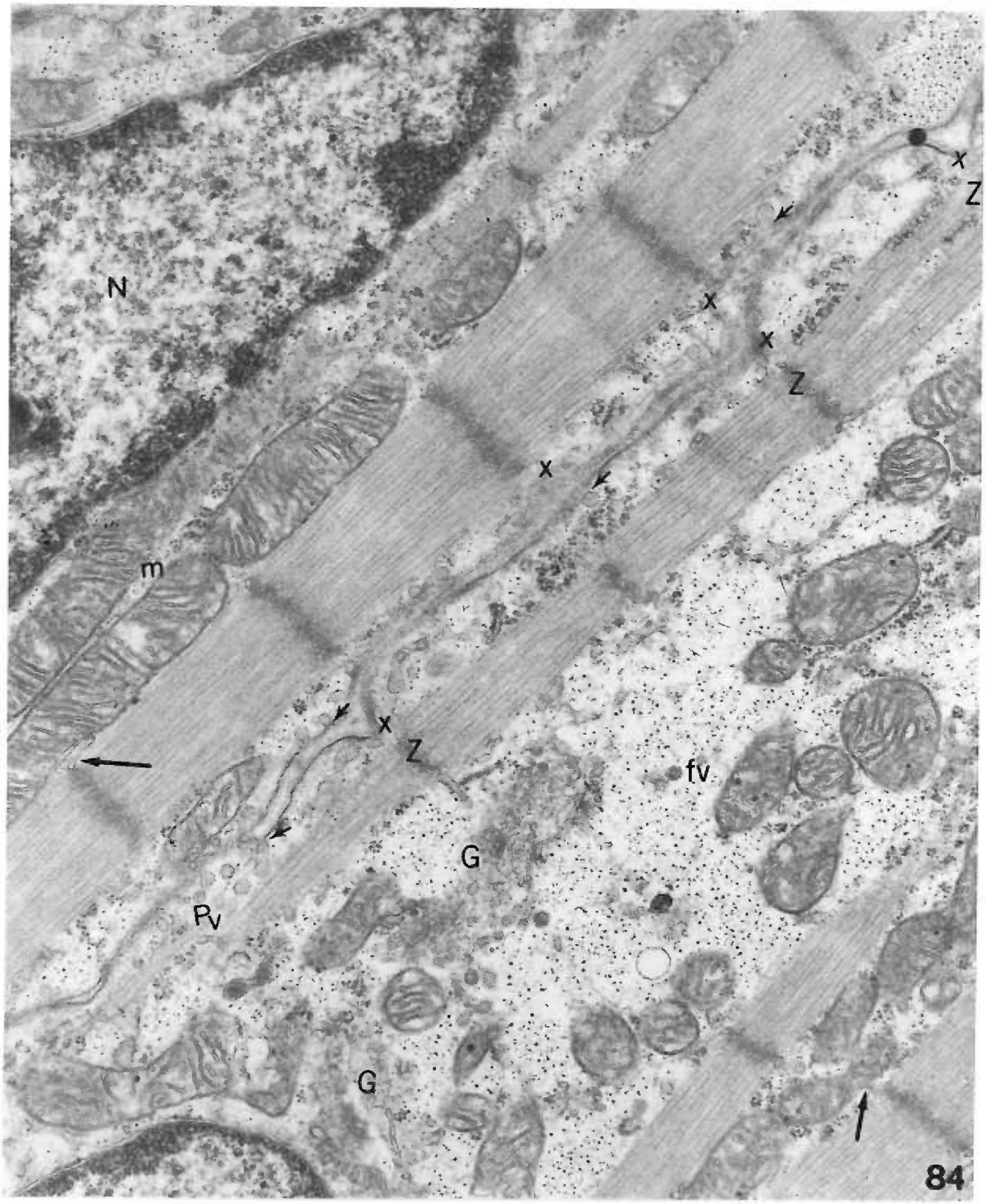

N
m
x
Z
x
x
Z
x
x
Z
x
Z
fv
G
Pv
G
84

Figure 85. New Born Myocardium

Nucleus is long and centrally placed in the cell (N). Associated with the nuclear envelope and the surrounding cytoplasm are numerous small vesicles and vacuoles (Fv, V, arrows). At the Z disc region (Z) developing T-system (Ts) and indentations of sarcolemma (Sl, long arrow, Z) are observable.

N-nucleus; Ts-T-system; Z-Z band; fv-fuzzy or spiny vesicles; Sl-sarcolemma.

X 18,000

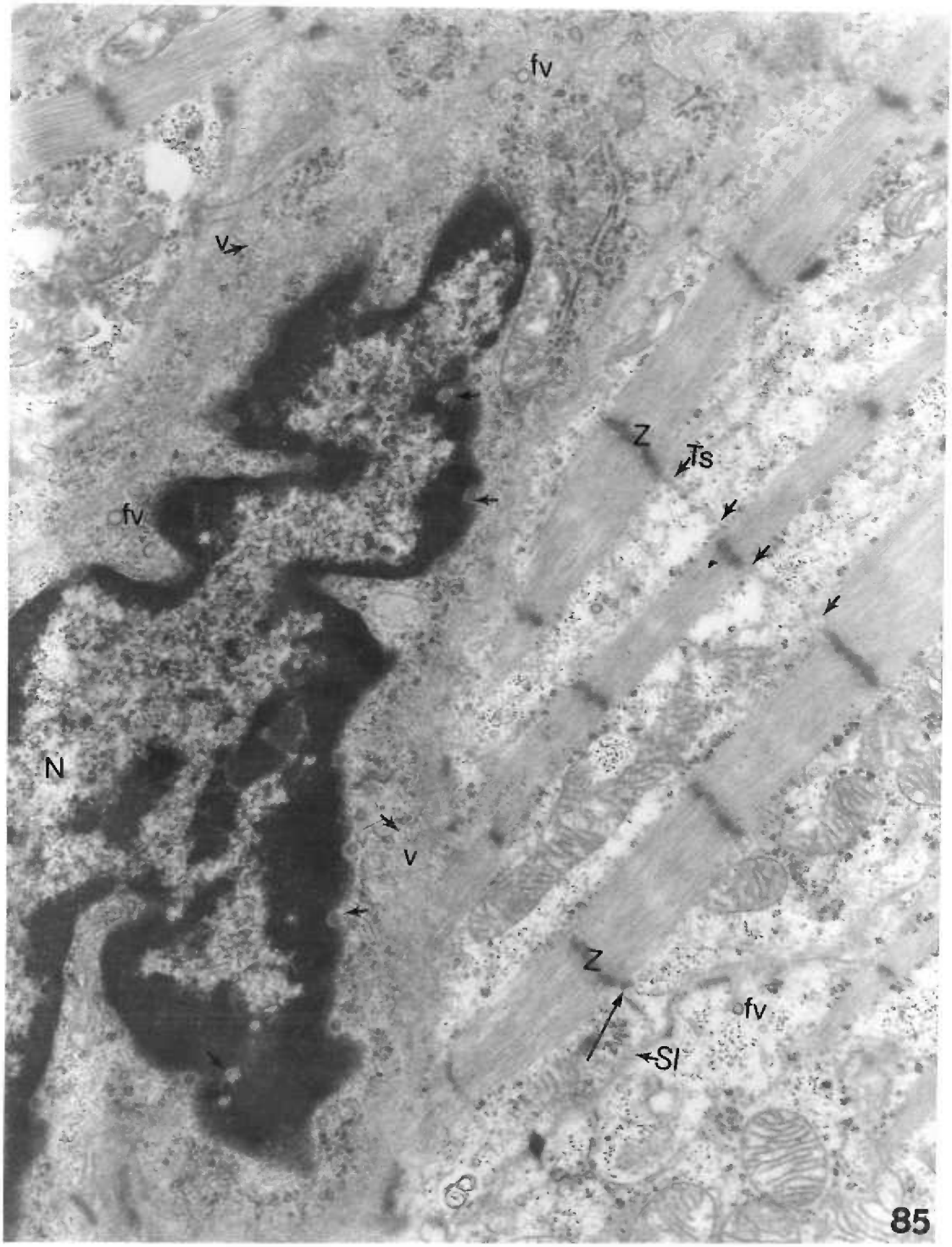

fv
v
Z
Ts
fv
N
v
Z
fv
Sl
85

Figure 86. New Born Myocardium

The sarcoplasmic reticulum forms extensive anastomosing channels in the cytoplasm (SR, arrows) between myofibrils. A suggestion of a structural continuity or open channels of communication between this organelle and mitochondria is indicated (long arrows, m, SR). In some mitochondria, longitudinal cristae are discernible. The intercalated disc has further differentiated in the new born myocardium (ID). Numerous pinocytotic vesicles (Pv) are associated with it.

ID-intercalated disc; SR-sarcoplasmic reticulum; m-mitochondria;Pv-pinocytotic vesicles.

X 18,000

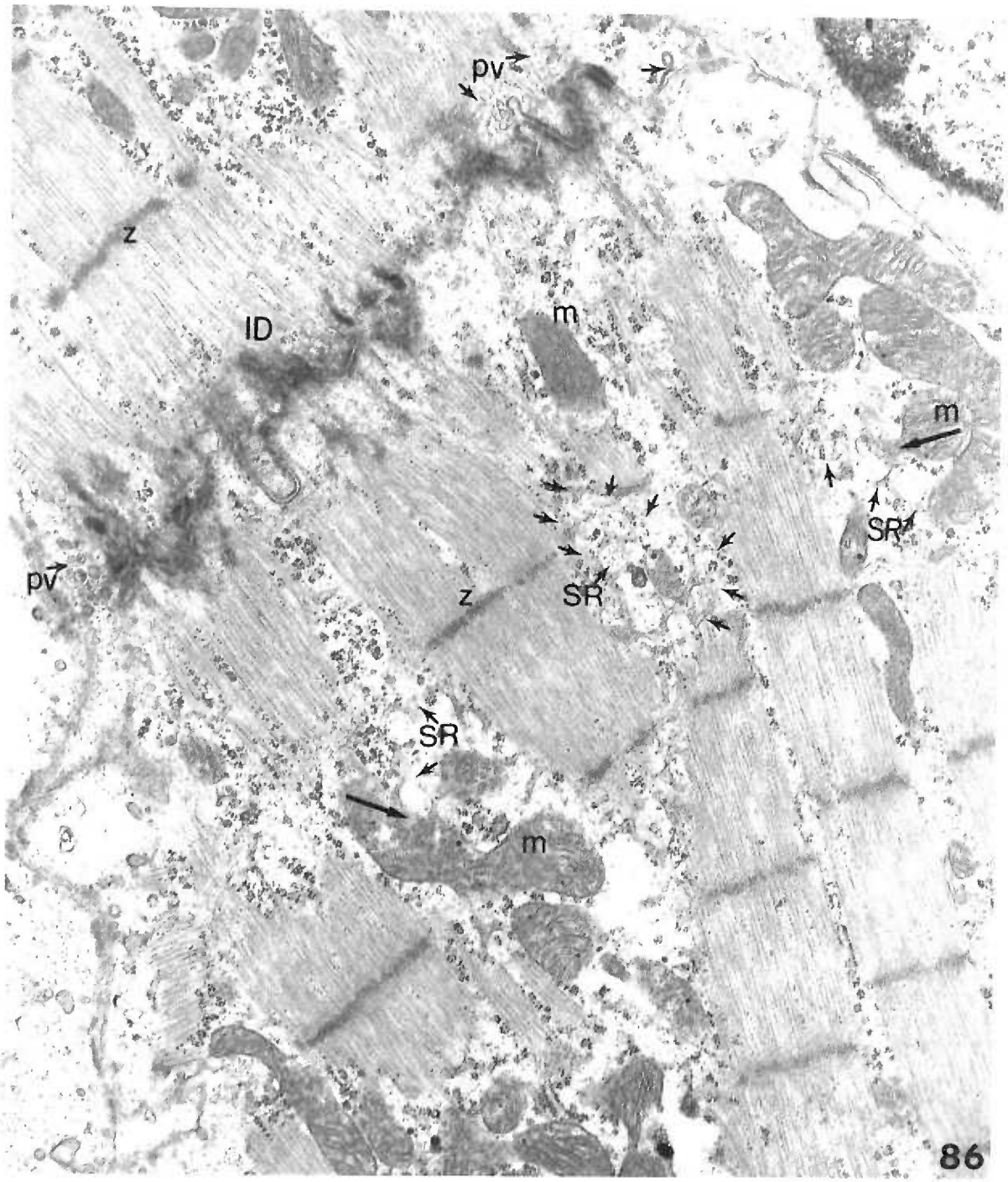

pv
z
ID
m
m
SR
pv
z
SR
SR
m
86

Figure 87. New Born Myocardium

Numerous small vesicles and vacuoles (Pv) are frequently observed to be associated with the regions of intercalated discs. Some of these vacuoles contain amorphous and granular material. In the picture shown, it seems as though there is a protoplasmic continuity between the two cells through a "gap" in the intercalated disc; a structure resembling a microtubule is seen stretched across the two cells through this gap (Mt, long and small arrows). An intermediate line can be observed in the macula occludens of intercalated disc; this line is formed by the fusion of the two outer leaflets of the apposing membranes, thus forming a trilaminar structure.

Z-Z disc; MO-macula occludens; Pv-vesicles and vacuoles; Mt-microtubule.

X 50,000

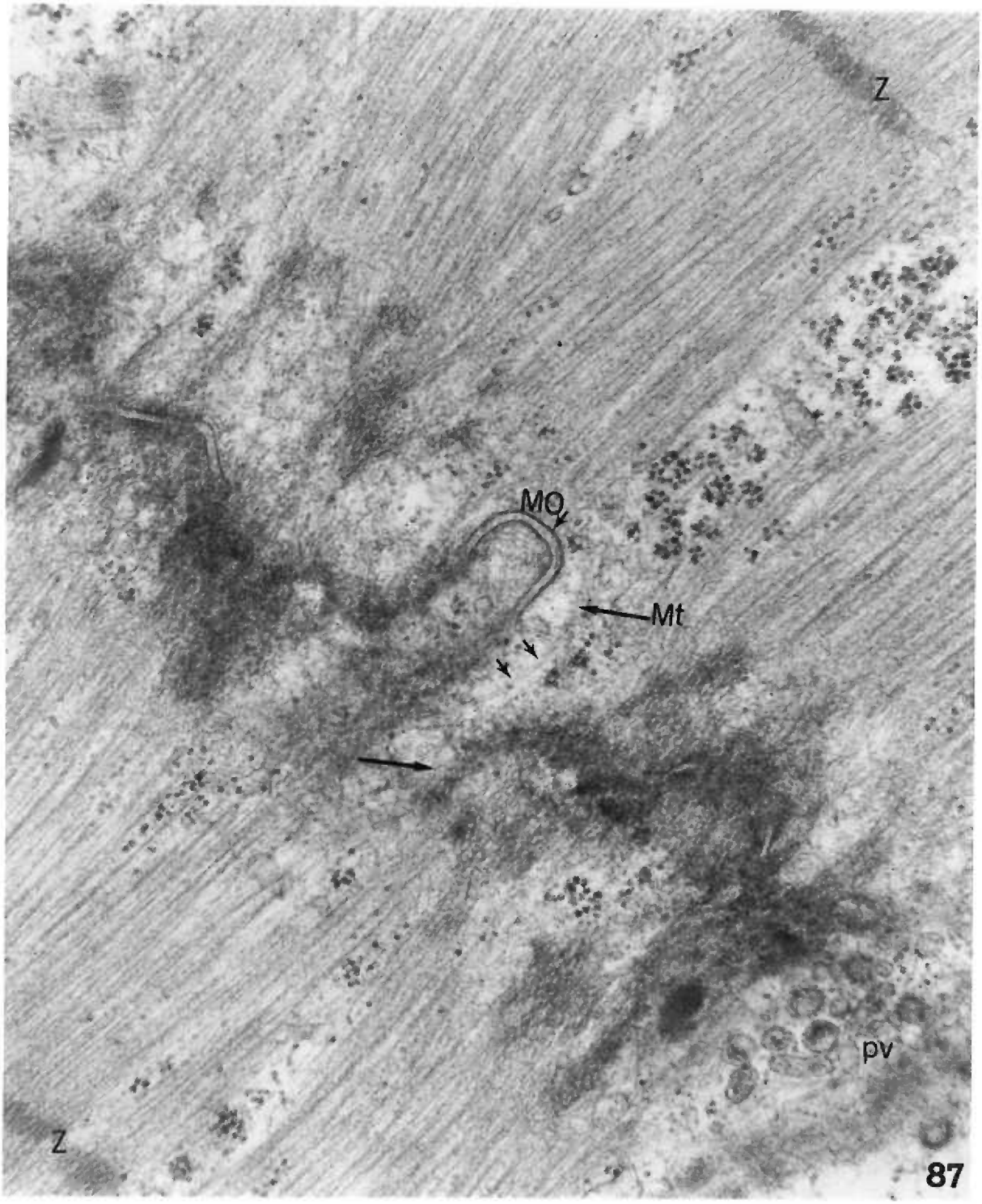

Z
MO
Mt
pv
Z
87

Figure 88. New Born Myocardium

The phenomenon of branching (B) is recognizable at the intercalated disc; two fibrils are attached to the intercalated disc by an angle (B). Several features of the disc are discernible. A desmosome is frequently associated with these discs.

FA-fascia adherens; FO-fascia occludens; MA-macula adherens; D-desmosome; B-branching fibrils.

X 32,000

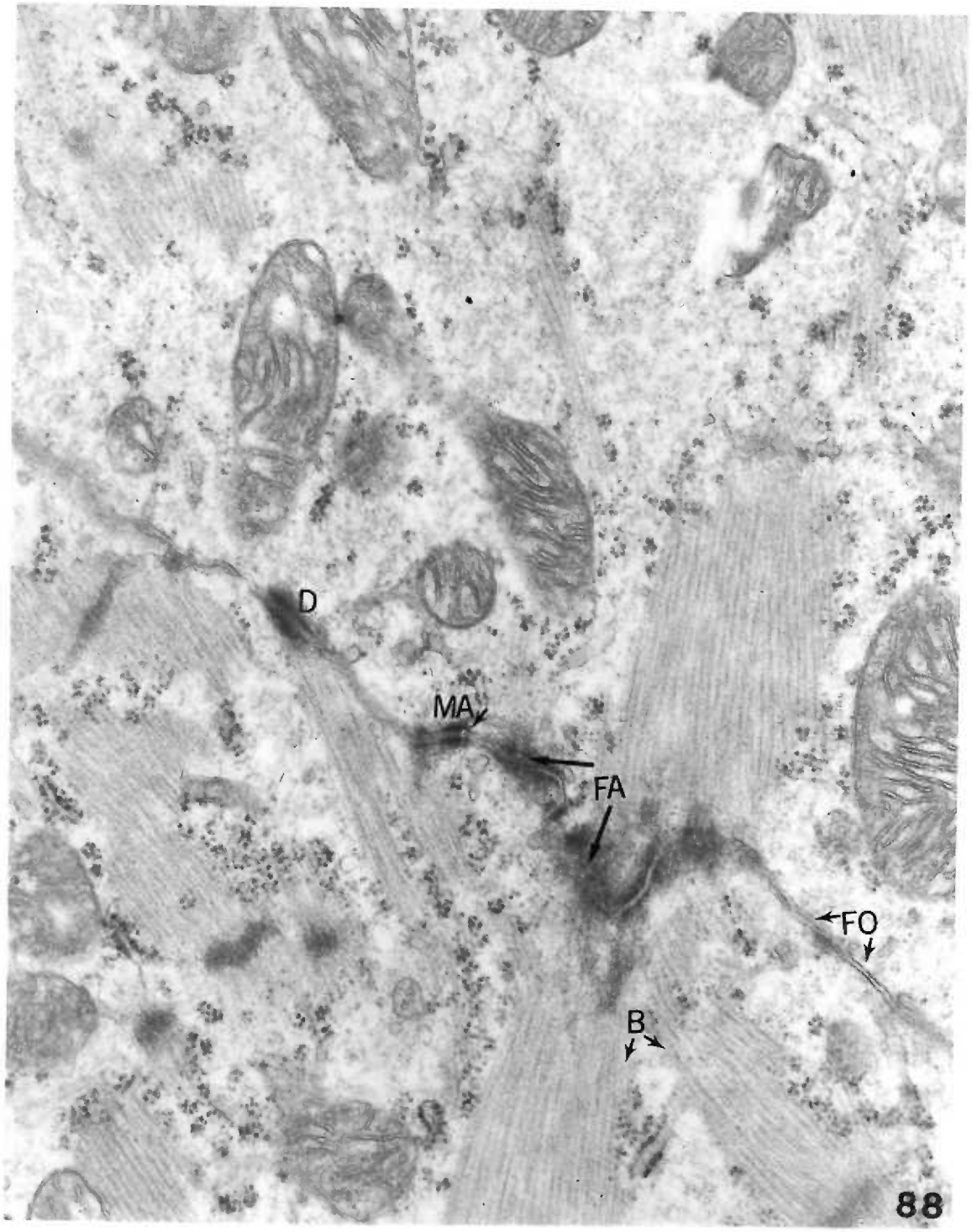

D
MA
FA
FO
B
88

Figure 89. New Born Myocardium

The phenomenon of branching of fibrils can be visualized at the Z disc regions and at intercalated disc regions (B). Many adult features of the intercalated disc are discernible. The discs have undergone further development relative to earlier stages, and an interdigitating configuration is observable. MA-macula adherens; MO-macula occludens; FA-fascia adherens; B-branching fibrils; Z-Z disc; m-mitochondria.

X 50,000

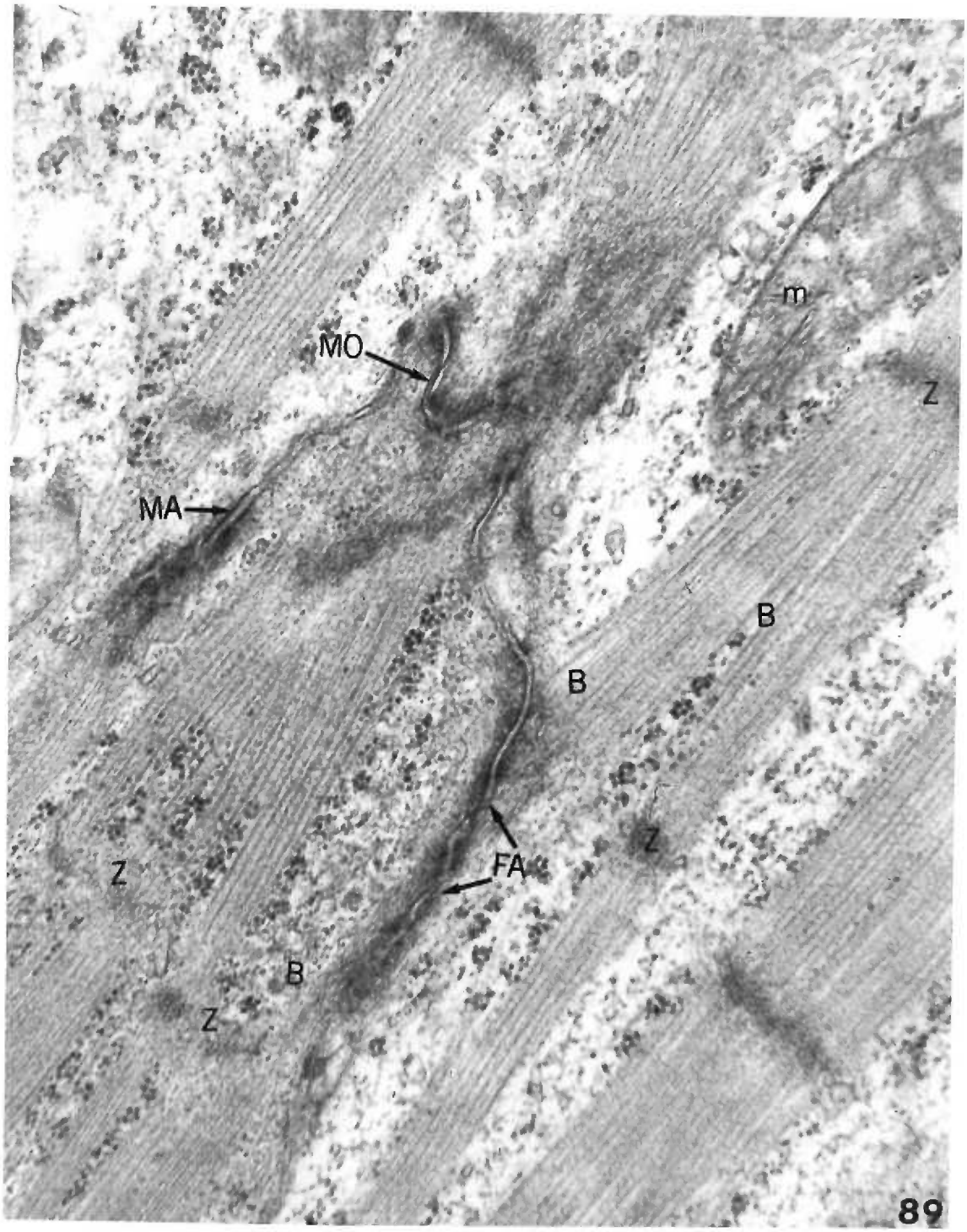

MO
MA
m
Z
B
B
Z
FA
Z
B
Z
89

Figure 90. New Born Myocardium

Frequently myofibrils are seen attached to two opposing intercalated discs with the intervention of a single Z disc (top left). I bands are observed on both sides of intercalated discs. Normally, intercalated discs occur at one sarcomere length away from the preceeding Z disc. Occasionally, when an intercalated disc is located more than one but less than two sarcomere lengths away from the closest Z disc, another Z disc intervenes (top right of the picture, Z-ID). In this case, the arrangement is Z band-I band-intercalated disc (top right). Numerous vesicles and vacuoles (V) are recognizable in the vicinity of intercalated discs. In some mitochondria longitudinal cristae are observable.

FA-fascia adherens; FO-fascia occludens; MO-macula occludens; Z-Z disc; m-mitochondria with longitudinal cristae; ID-intercalated disc; Li-lipid; D-desmosome; V-vacuoles and vesicles.

X 26,000

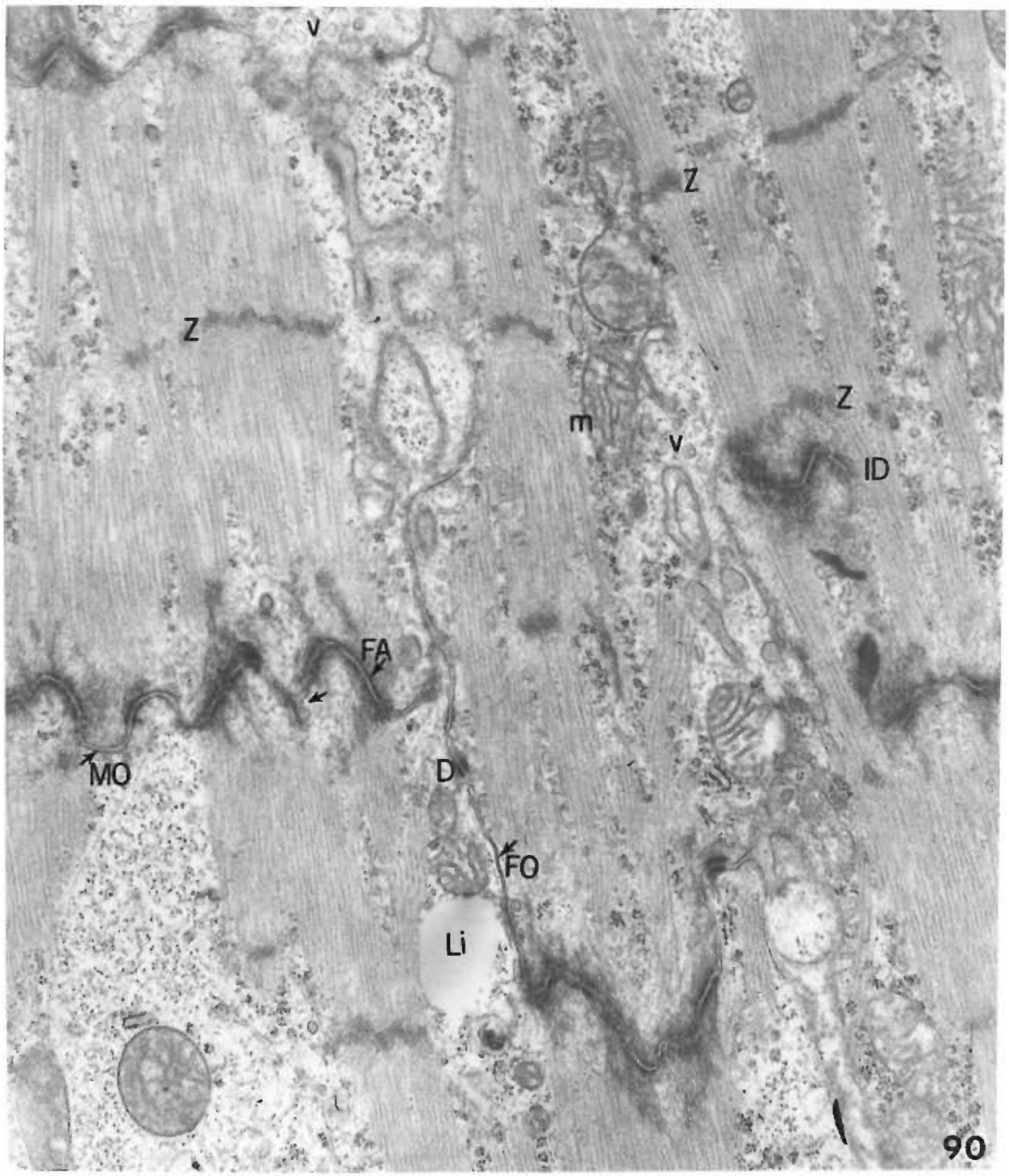

v
Z
Z
m
v
Z
ID
FA
MO
D
FO
Li
90

Figure 91. New Born Myocardium

Several myofibrils (MF) are attached to the intercalated disc (ID), shown on the left. Endoplasmic reticulum (ER) is arranged in ring-like configurations circling mitochondria. Mitochondria are closely associated with the myofibrils, and there seems to be a tubular connection between some mitochondria and myofibrils (m and arrows) (See also figure 84). Numerous vesicles and vacuoles are discernible (V).

ID-intercalated disc; MF-myofibril; G-Golgi bodies; m-mitochondria; V-vesicles and vacuoles; N-nucleus.

X 11,120

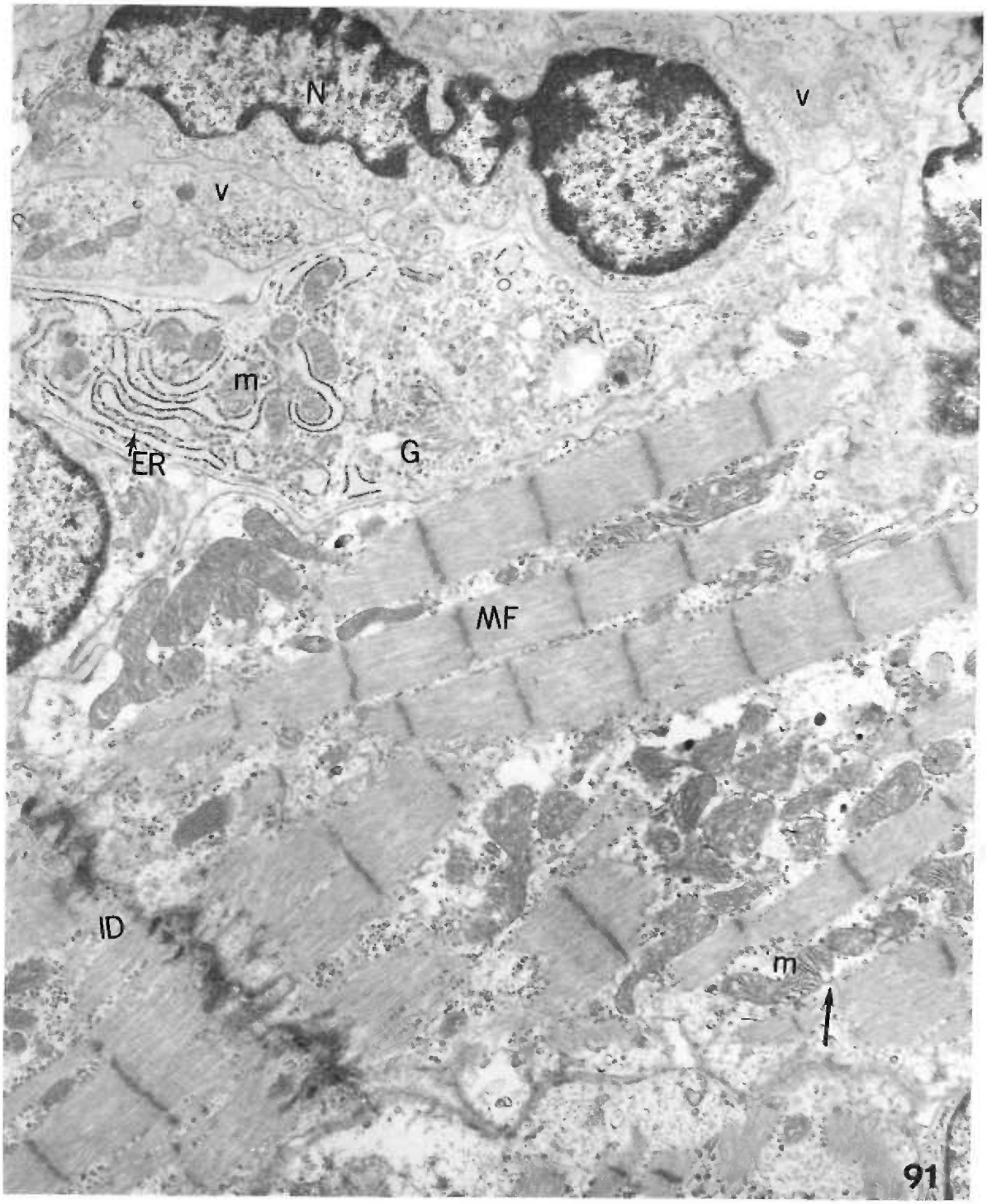

N
v
v
m
ER
G
MF
ID
m
91

Figure 92. New Born Myocardium

Several myofibrils are attached to the intercalated disc (ID). The phenomenon of branching of fibrils can be observed at several Z disc (Z) regions and at the intercalated disc. The number of mitochondria is considerably increased (m) in the new born myocardium.

m-mitochondria; ID-intercalated disc; Z-Z band; Co-collagen.

X 11,120

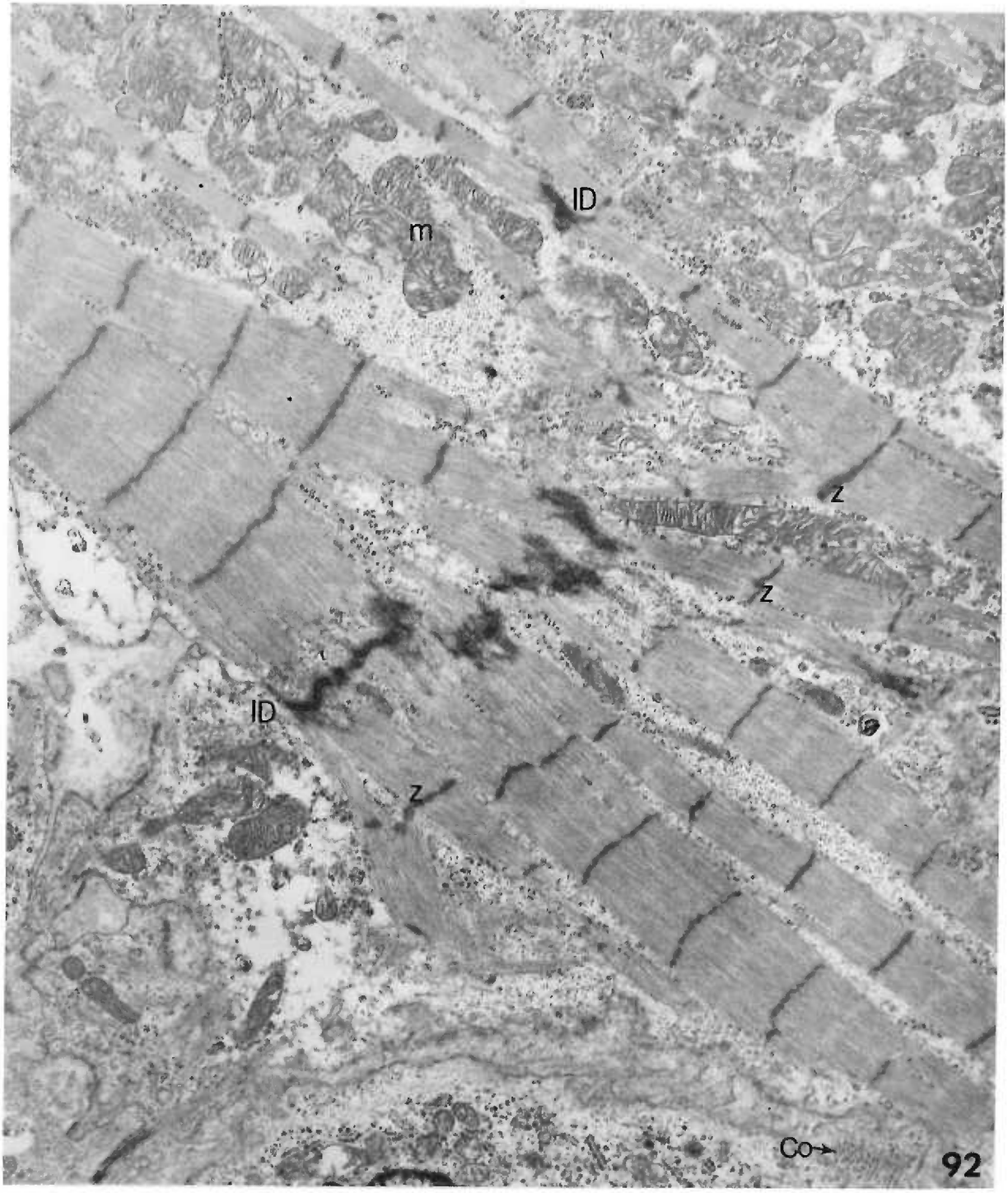

ID
m
z
z
ID
z
Co
92

Figure 93. New Born Myocardium

The nucleus (N) is longitudinally compressed and oriented parallel to the myofibrils in the long axis of the cell. Nucleolus (Nu) is not as prominent as in 10-day or 11-day myocardium. Chromatin is distributed peripherally. Several vesicles and vacuoles (arrows) associated with the nuclear envelope are found deep in the cytoplasm. At two Z disc (Z) regions, tubular indentations of the sarcolemma (Sl) can be visualized.

ID-intercalated disc; m-mitochondria; Z-Z disc; Sl-sarcolemma; N-nucleus; Nu-nucleolus; arrows-vacuoles and vesicles.

X 18,000

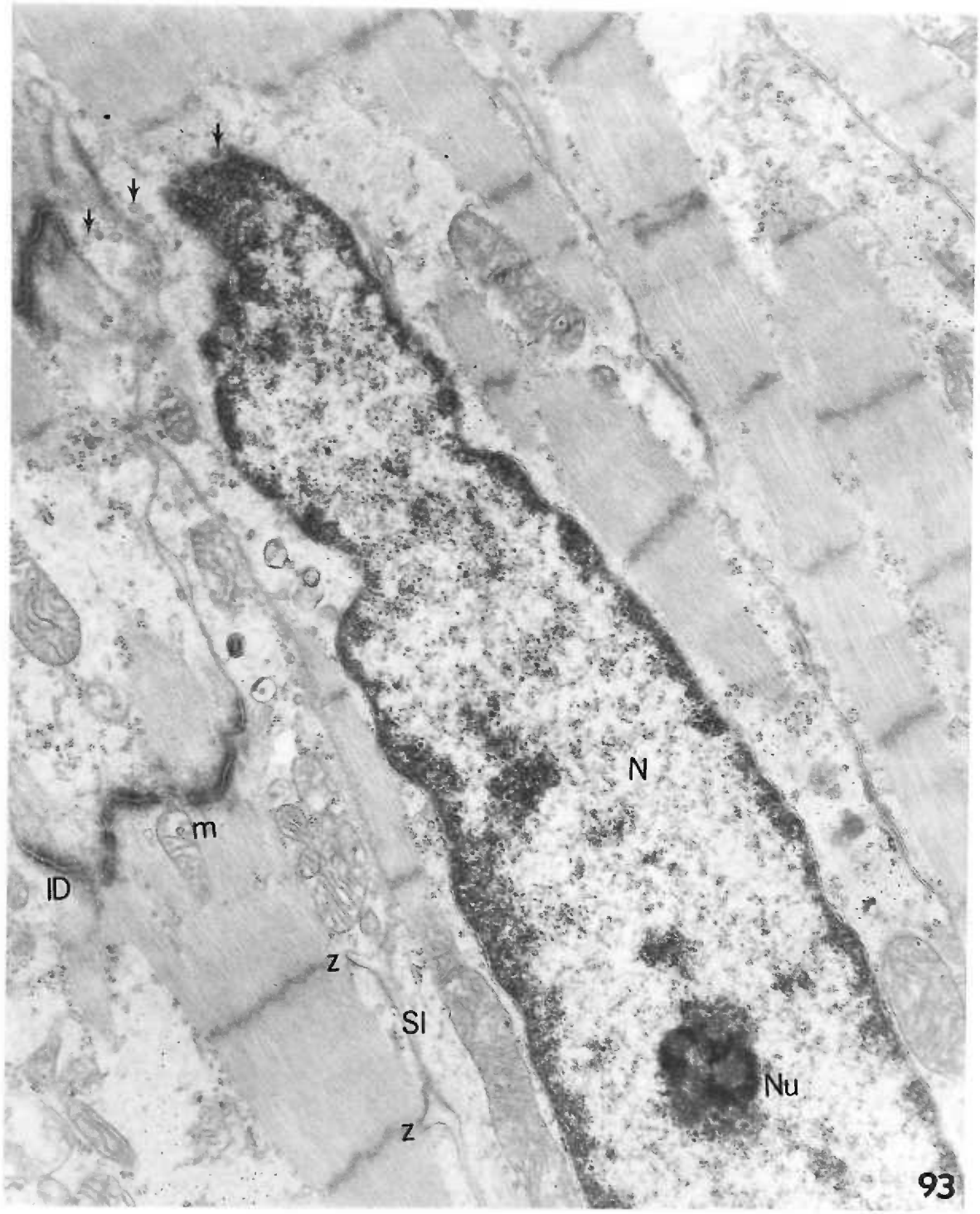

N
m
ID
z
SI
Nu
z
93

Figure 94. New Born Myocardium

An intercalated disc is seen to run diagonally across the cell in the middle of the picture. Pinocytotic vesicles, in the state of being formed from the membranes of the intercalated disc, are discernible (arrow), and numerous vacuoles are recognizable in the vicinity of the disc. Longitudinal cristae are discernible in the mitochondria (m).

X 50,000

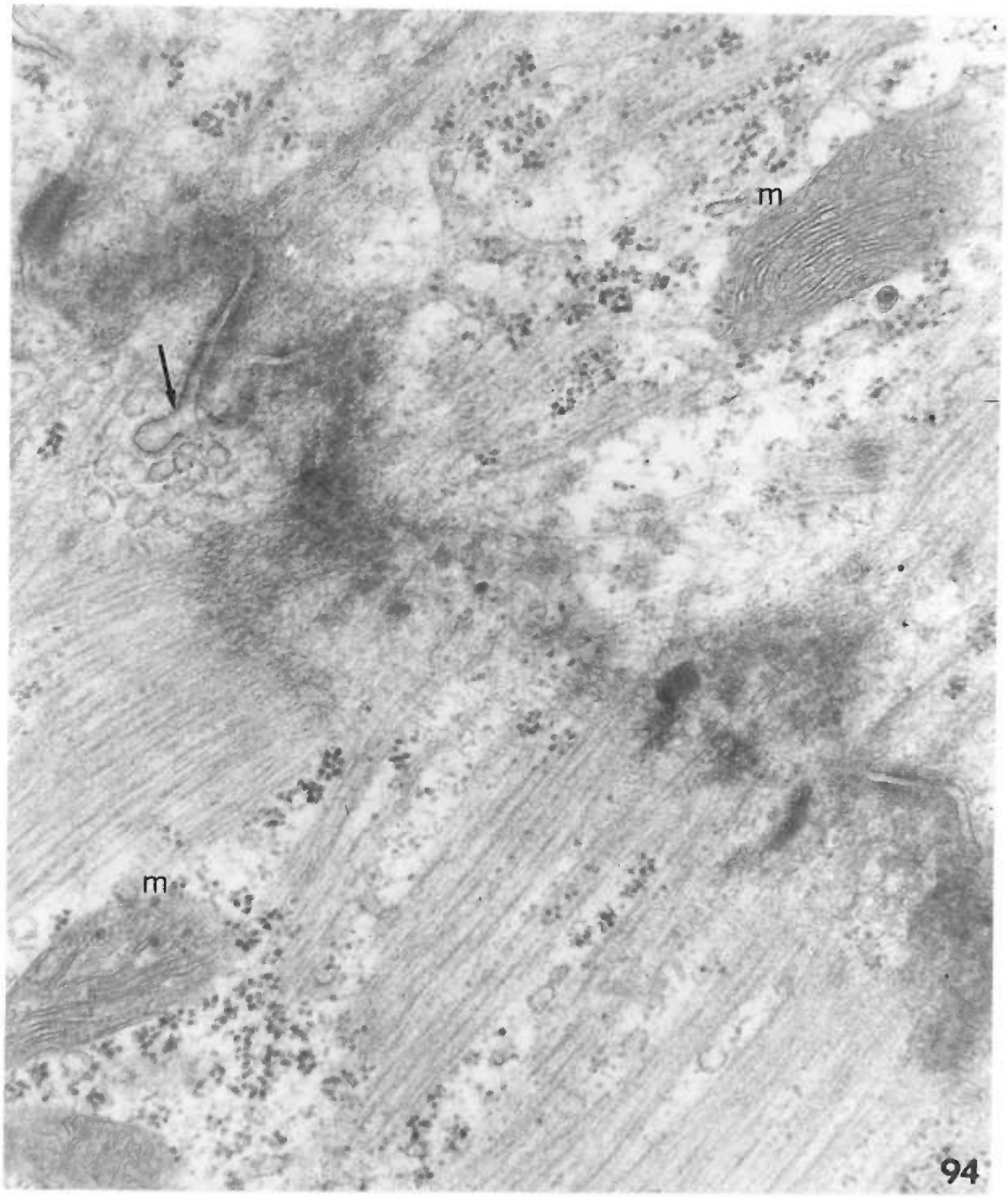

m
m
94

Figure 95. New Born Myocardium

Fibroblasts and connective tissue fibers are frequently found in the new born myocardium. A fibroblast cell (FB) containing bundles of collagen fibers (Co) is discernible between myocardial cells.

FB-fibroblast cell; Co-collagen fibers.

X 18,000

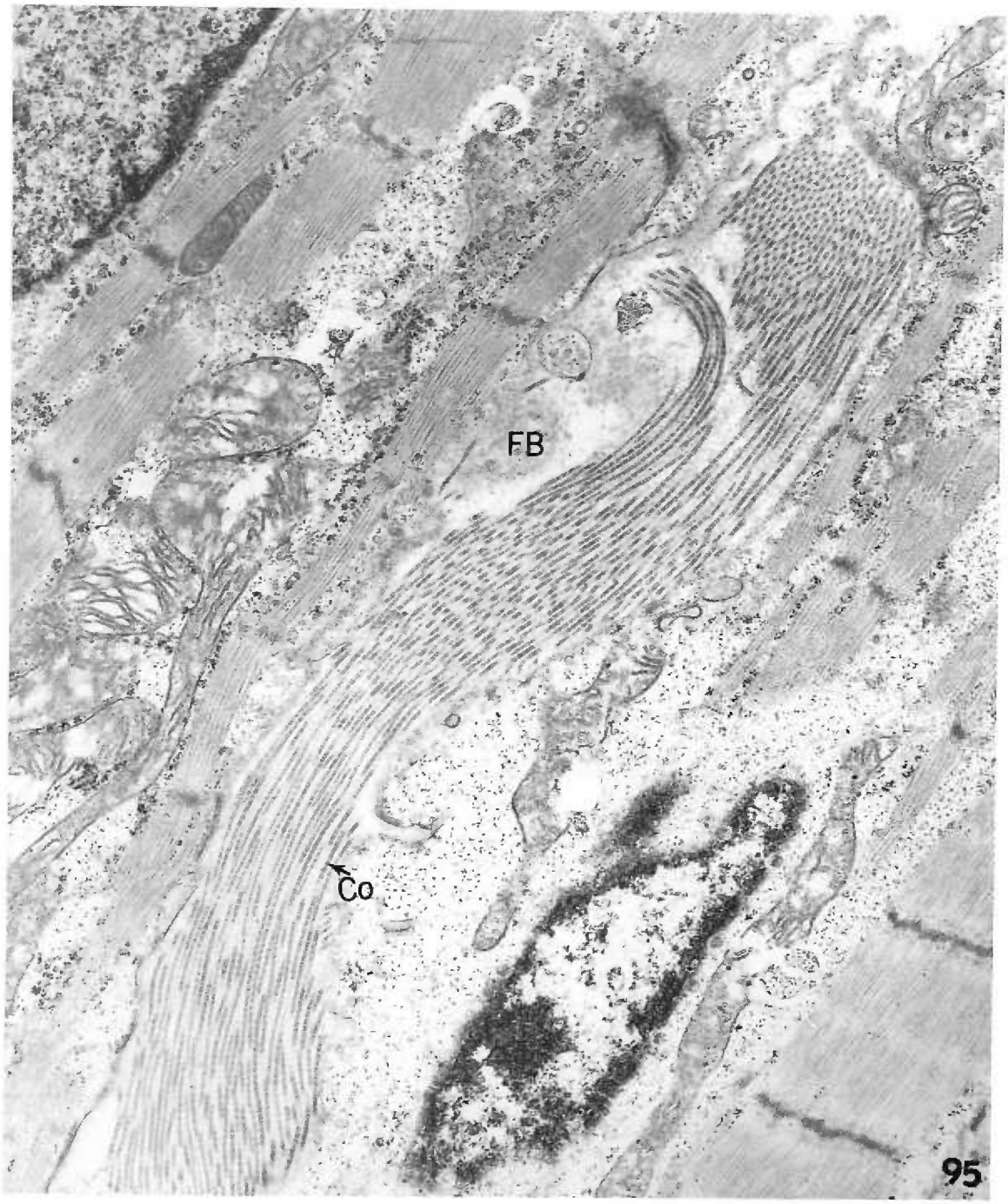

FB
Co
95

Figure 96. New Born Myocardium

Connective tissue elements are frequently observed in the new born myocardium. A fibroblast (FB) containing collagen (Co) fiber bundles is interposed between myocardial cells.

Co-collagen fibers; FB-fibroblast; Myc-myocardial cell.

X 26,000

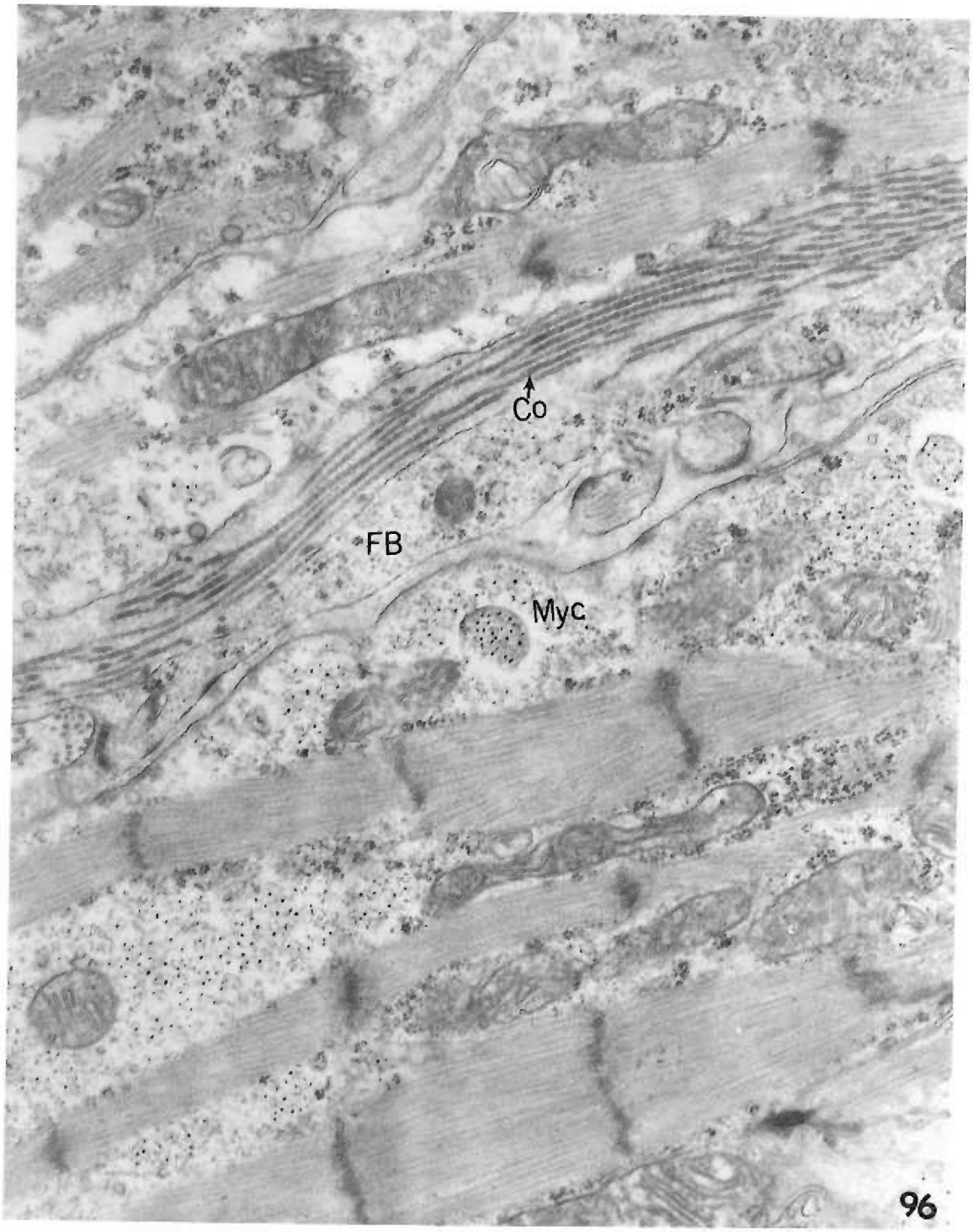

Co
FB
Myc
96

Figure 97. New Born Myocardium

In the contracting region of new born myocardium, two types of cells are recognizable (left and right). The cell on the left stains more darkly than the one on the right. The intercalated discs (ID) and sarcolemma serve as sharp and abrupt lines of demarcation between these cells. The myofibrils of the dark-staining cell (left) are in a state of intense contraction, whereas in the light-staining cell (right) the contraction is much less pronounced. The mitochondrial walls appear as dark, solid, thick lines and fuse with one another and with the myofibrils, thus suggesting a structural continuity between the individual mitochondria on one hand and between mitochondria and fibrils on the other. The cristae are disrupted.

m-mitochondria; Z-Z disc; ID-intercalated disc.

X 18,000

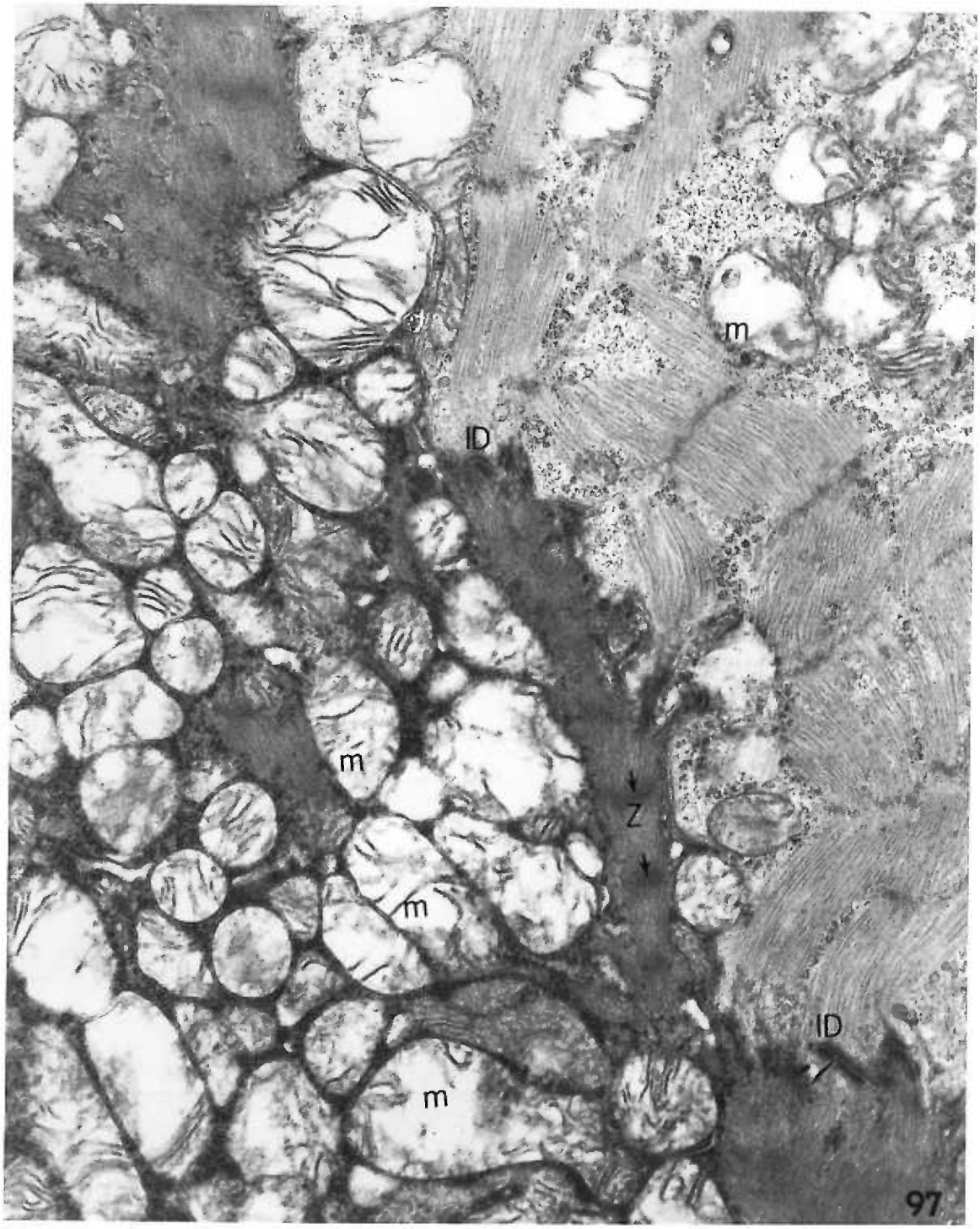

m
ID
m
Z
m
m
ID
97

Figure 98. New Born Myocardium

A region of new born myocardium in a state of contraction is shown. Dark-staining cell on the left is separated from the light-staining cell on the right by the intercalated disc (ID). The description given in figure 101 is applicable here.

Z-Z disc; m-mitochondria; ID-intercalated disc.

X 18,000

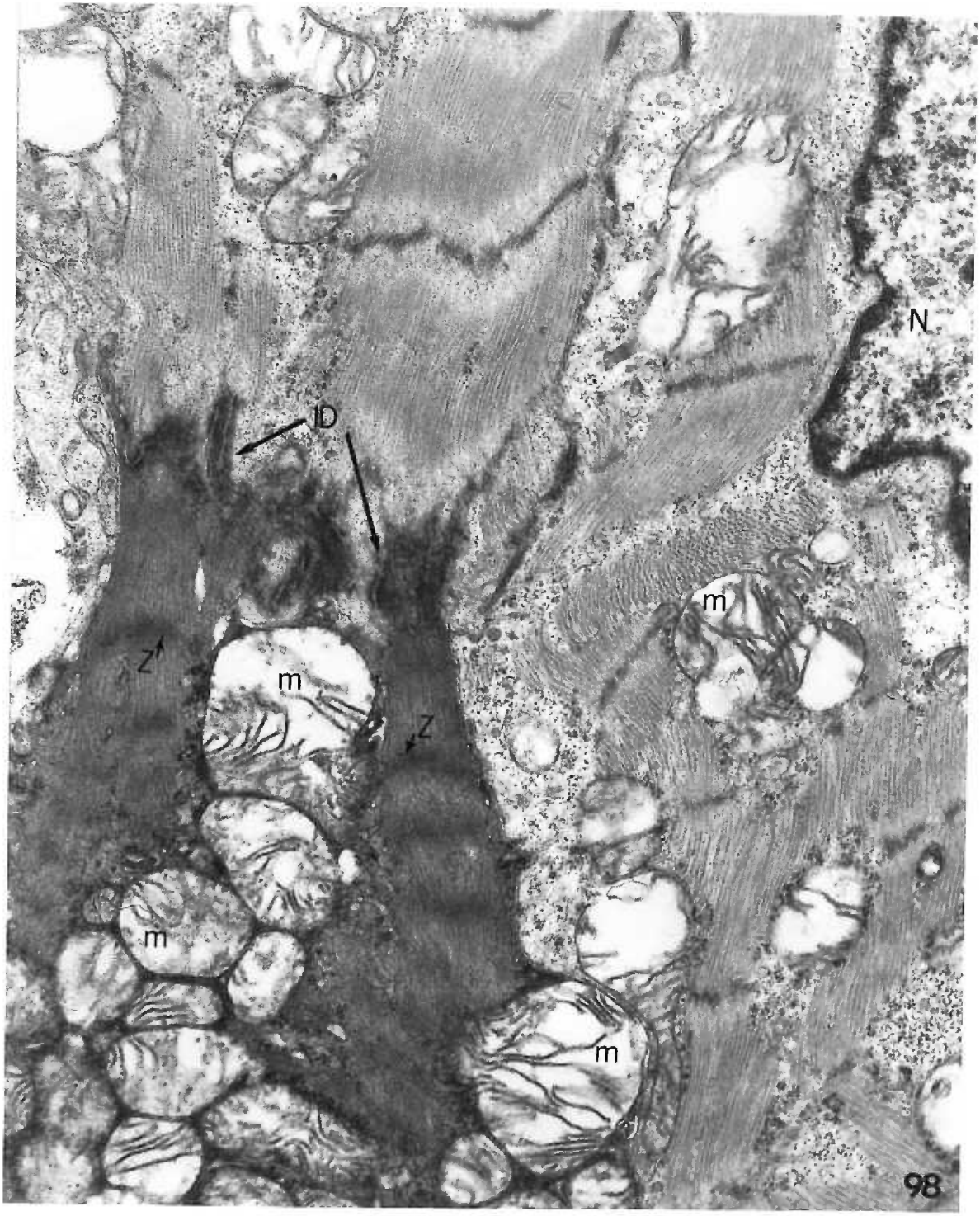

N
ID
m
Z
m
Z
m
m
98

Figure 99. New Born Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. The final reaction product is deposited as granules on membranes (FRP and arrows). The intensity of reaction in newborn is much reduced compared with the 10-day myocardium.

FRP-final reaction product.

X 18,000

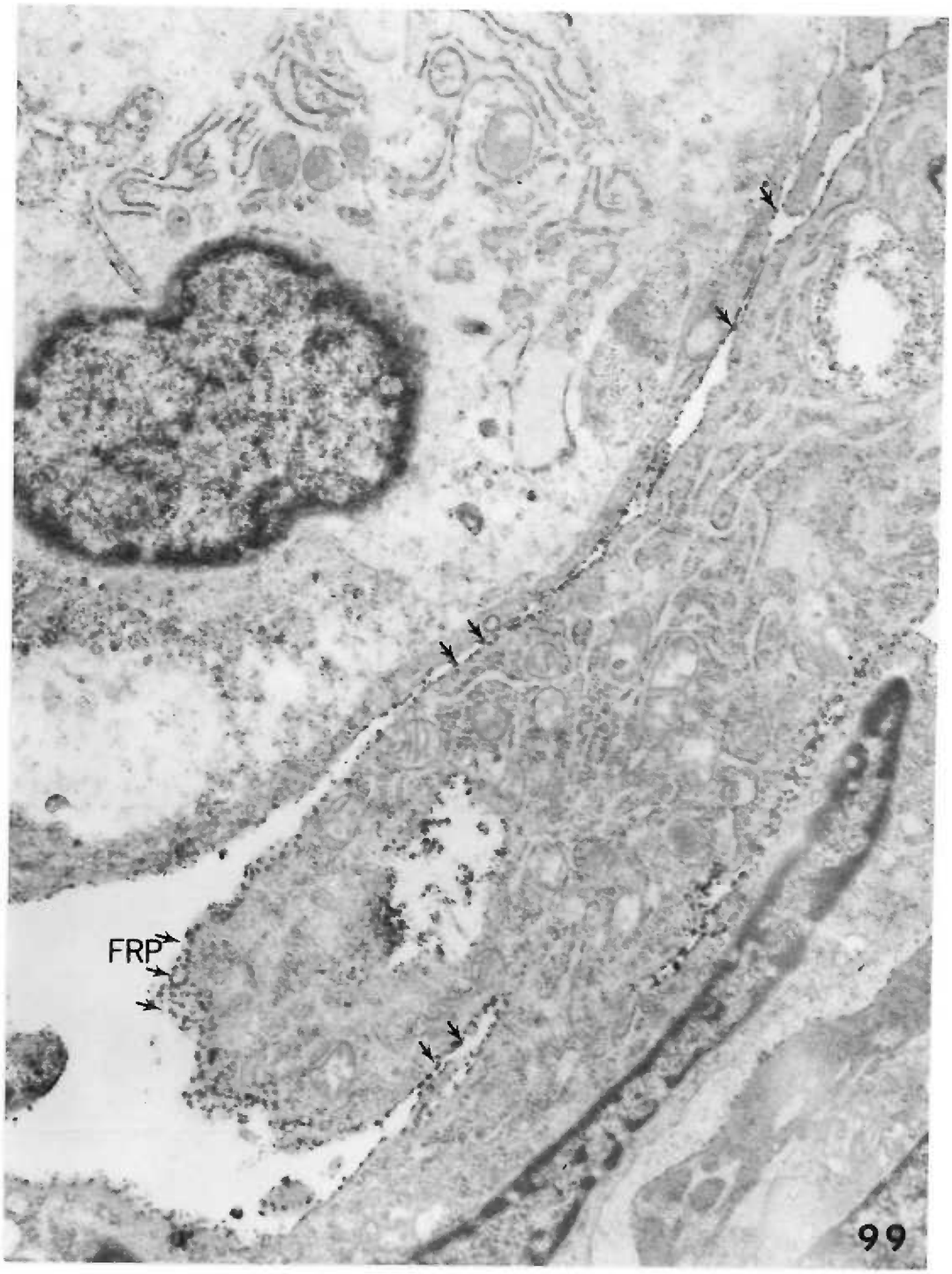

FRP
99

Figure 100. New Born Myocardium

Incubated in Wachstein-Meisel medium containing ATP as the substrate. The final reaction product (FRP and arrows) is deposited as granules primarily on the cell surfaces. The intensity of reaction is much reduced compared with 10-day myocardium.

MF-myofibril; FRP-final reaction product; arrows-granules of reaction product.

X 18,000

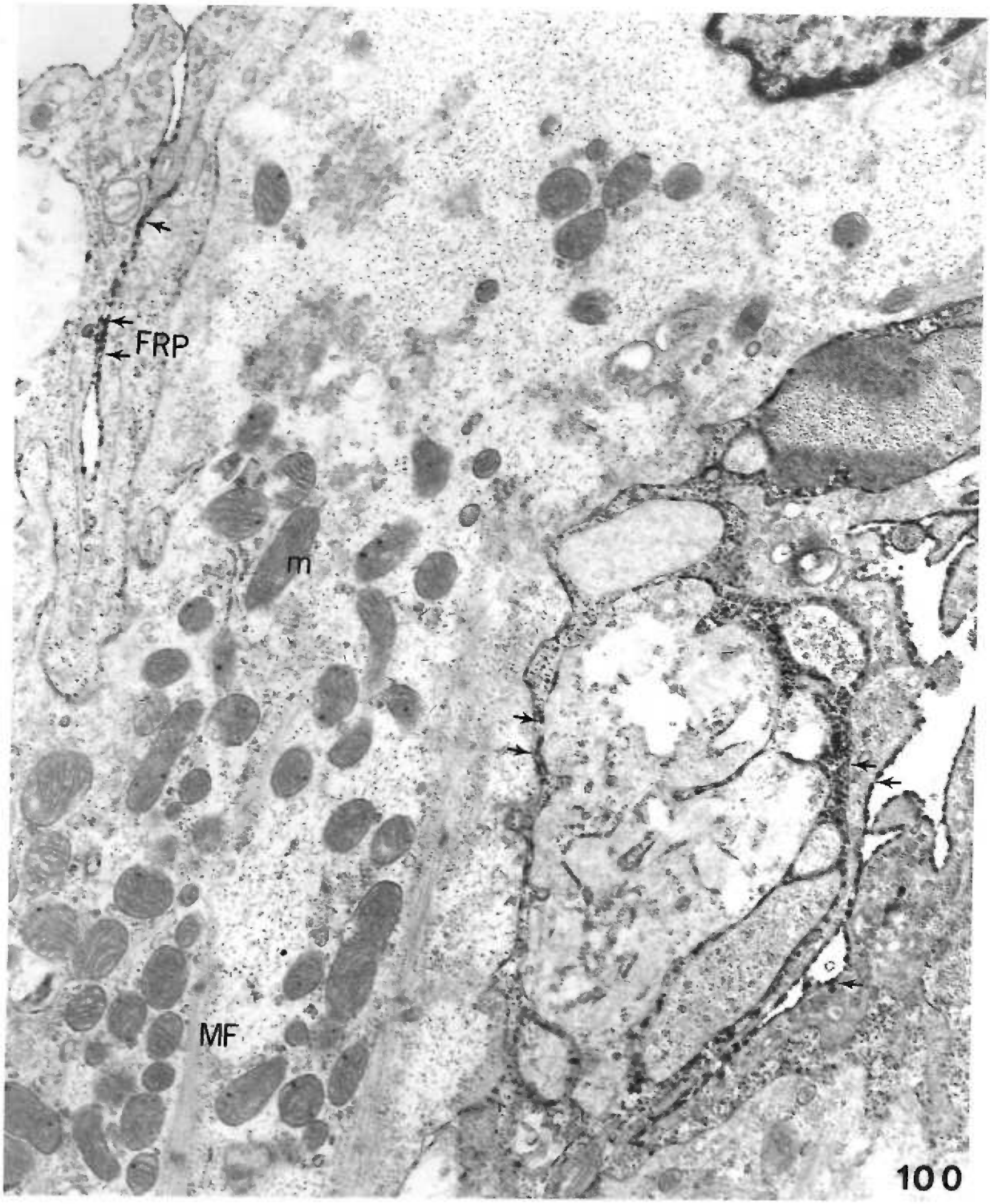

FRP
m
MF
100

Figure 101. New Born Myocardium

Incubated in a Wachstein-Meisel medium containing ATP as the substrate. The final reaction product (FRP and arrows) is recognizable on erythrocytes (RBC) and on fibroblasts (FB).

RBC-red blood cell; FRP-final product; arrows-final reaction product; FB-fibroblast; Co-collagen.

X 18,000

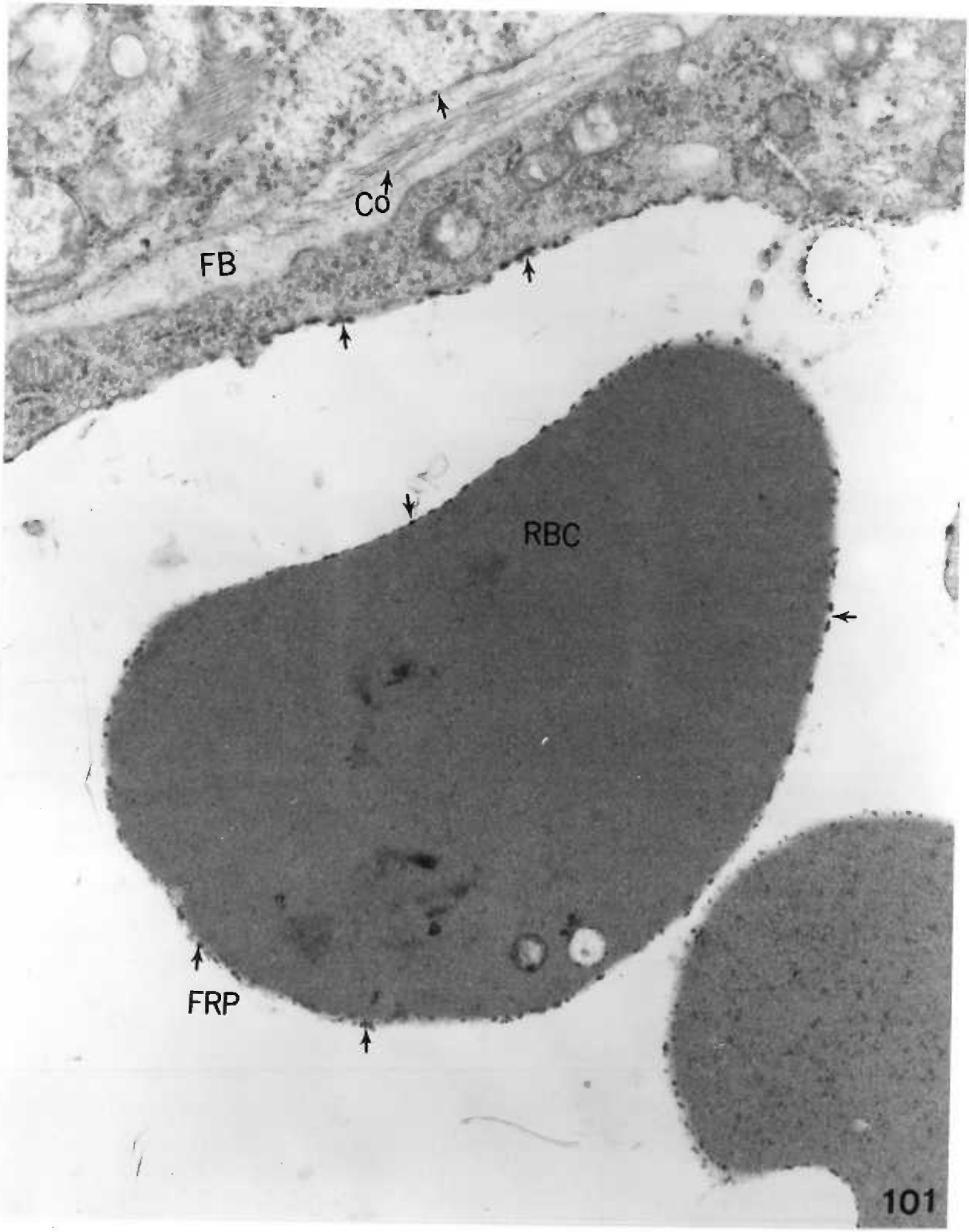

Co
FB
RBC
FRP
101

Figure 102. Control I.

Tissues were pretreated with osmium tetroxide before incubation in Wachstein-Meisel medium containing ATP as the substrate. Final reaction product is not recognizable on the cell membranes or any organelles (12-day myocardium).

MF-myofibril; m-mitochondria; N-nucleus; D-desmosomes.

X 18,000

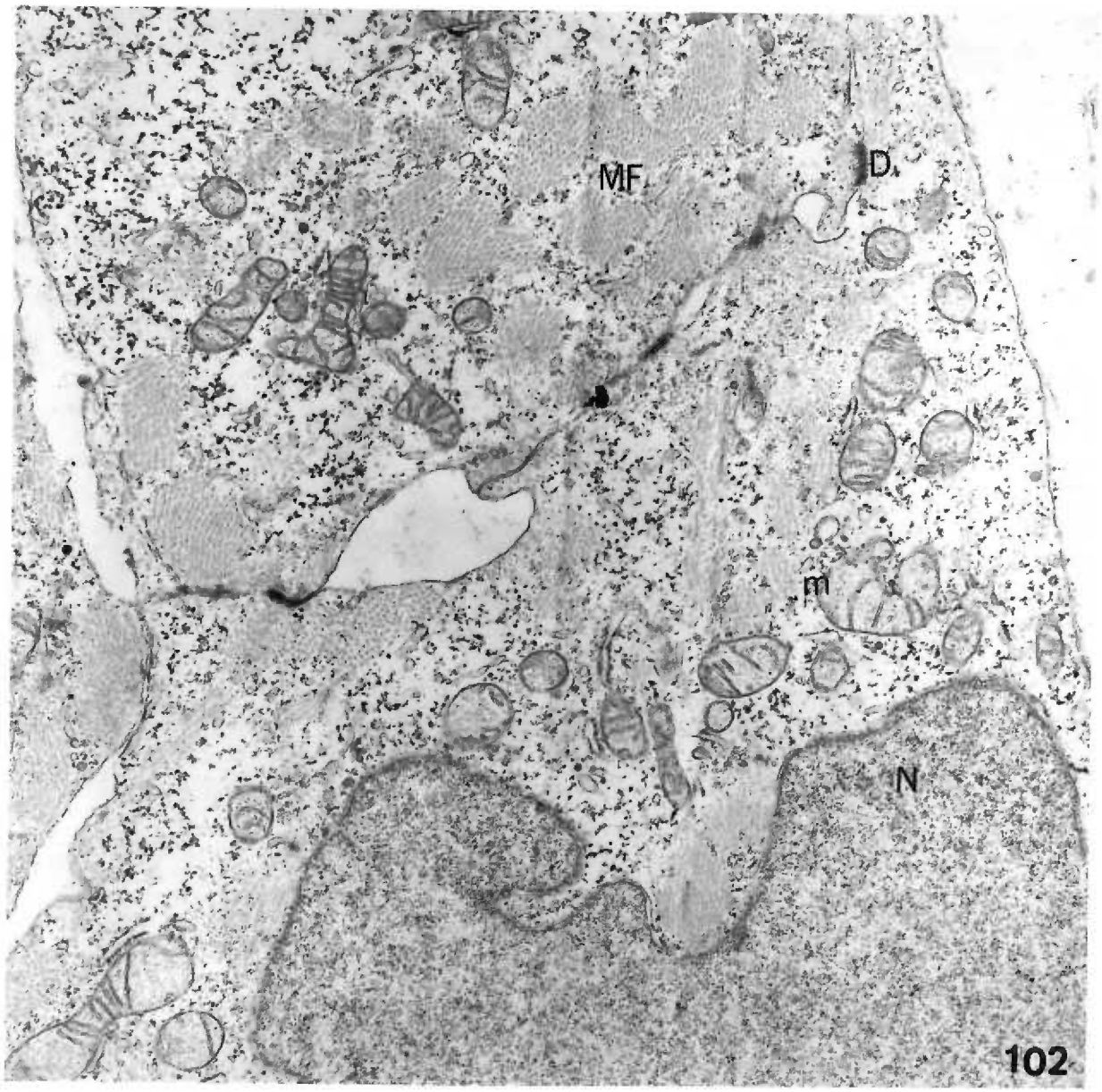

MF
D
m
N
102

Figure 103. Control II.

Incubated in Wachstein-Meisel medium containing ADP as the substrate instead of ATP. Final reaction product is not recognizable (10-day myocardium).

MF-myofibril; m-mitochondria; N-nucleus.

X 22,350

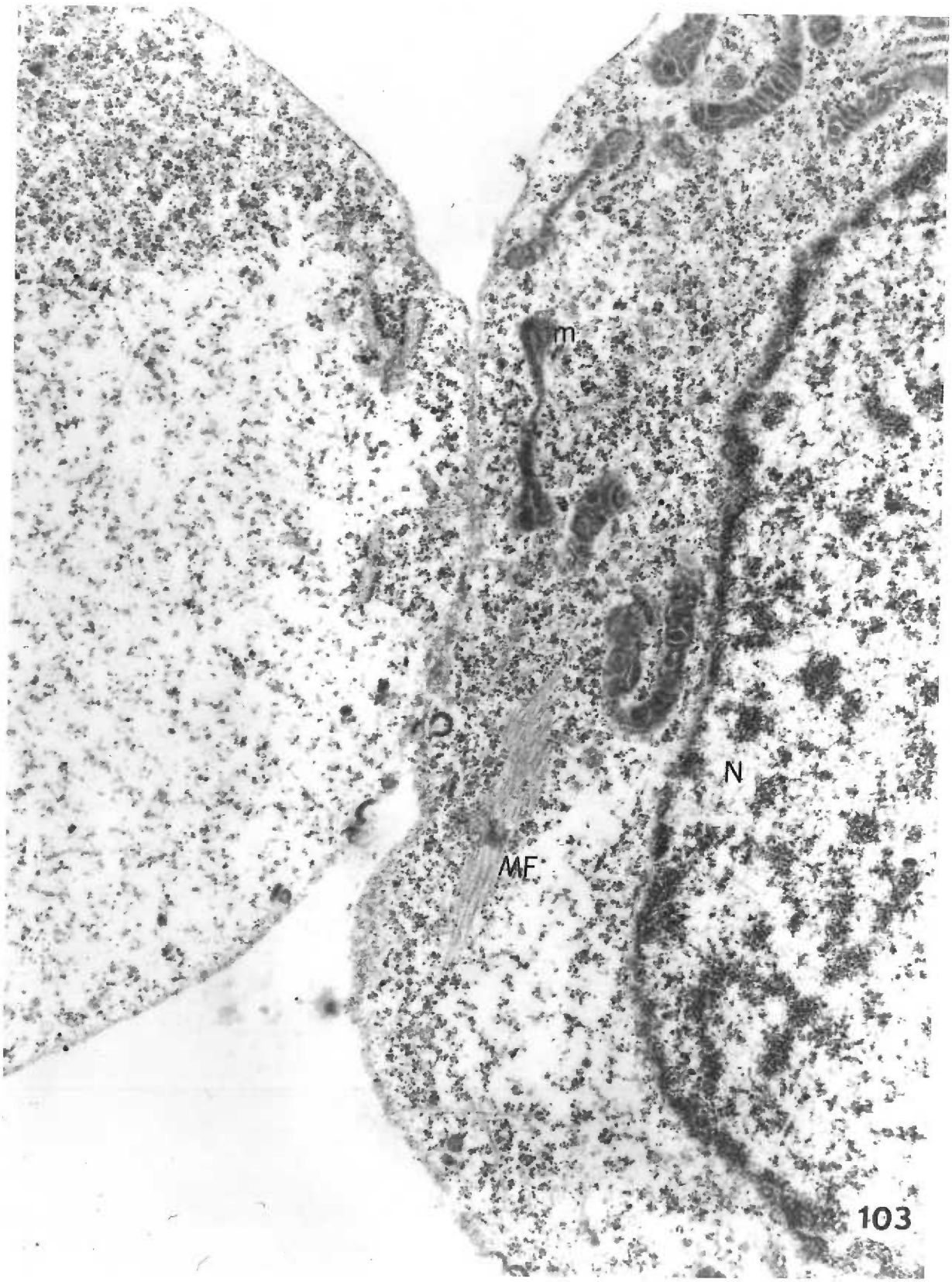

m
N
MF
103

Figure 104. Control III.

Incubated in Wachstein-Meisel medium containing sodium B-glycerophosphate as the substrate instead of ATP. Final reaction product is not visible (11-day myocardium).

ICS-intercellular space; MF-myofibril; m-mitochondria.

X 18,000

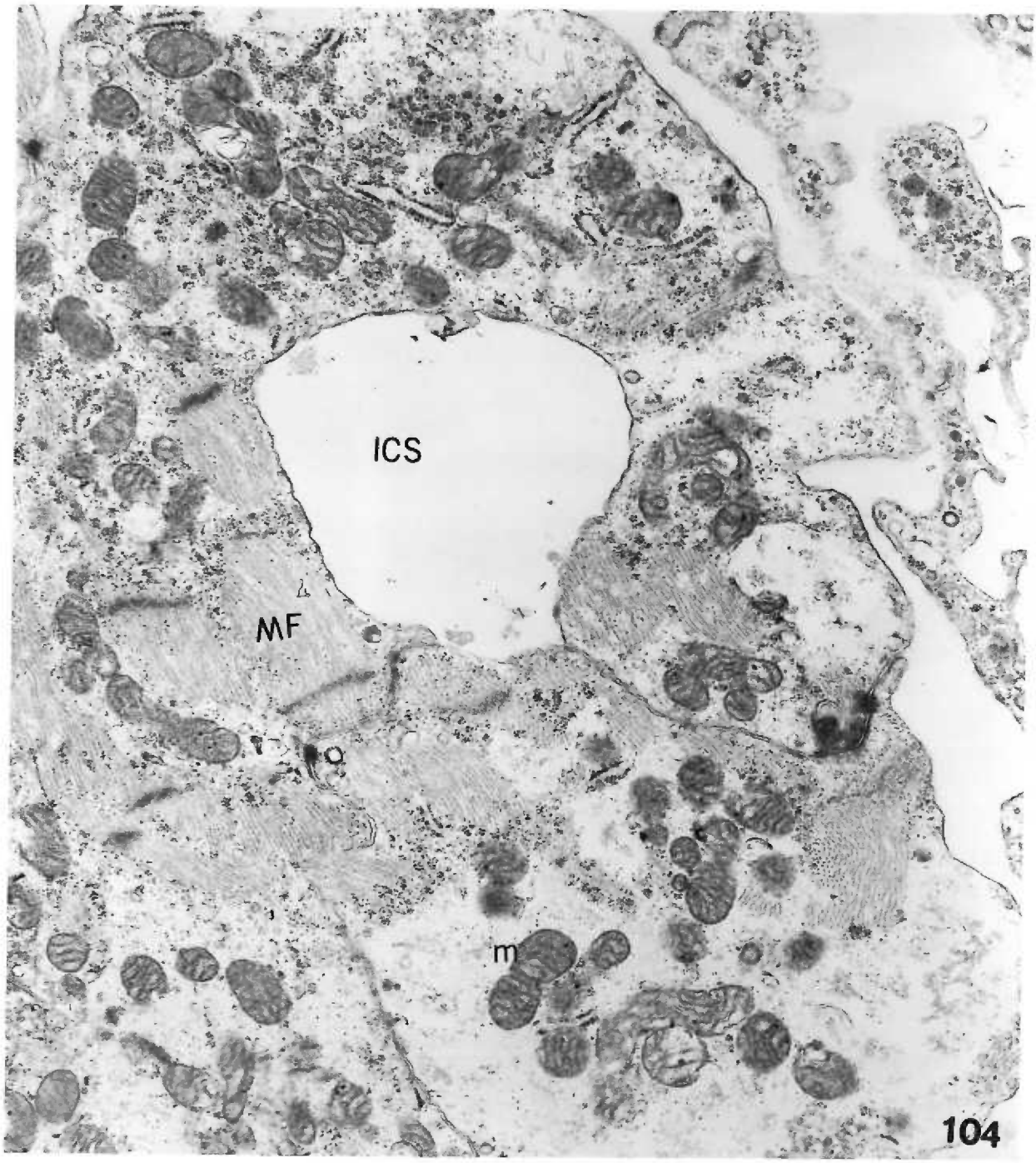
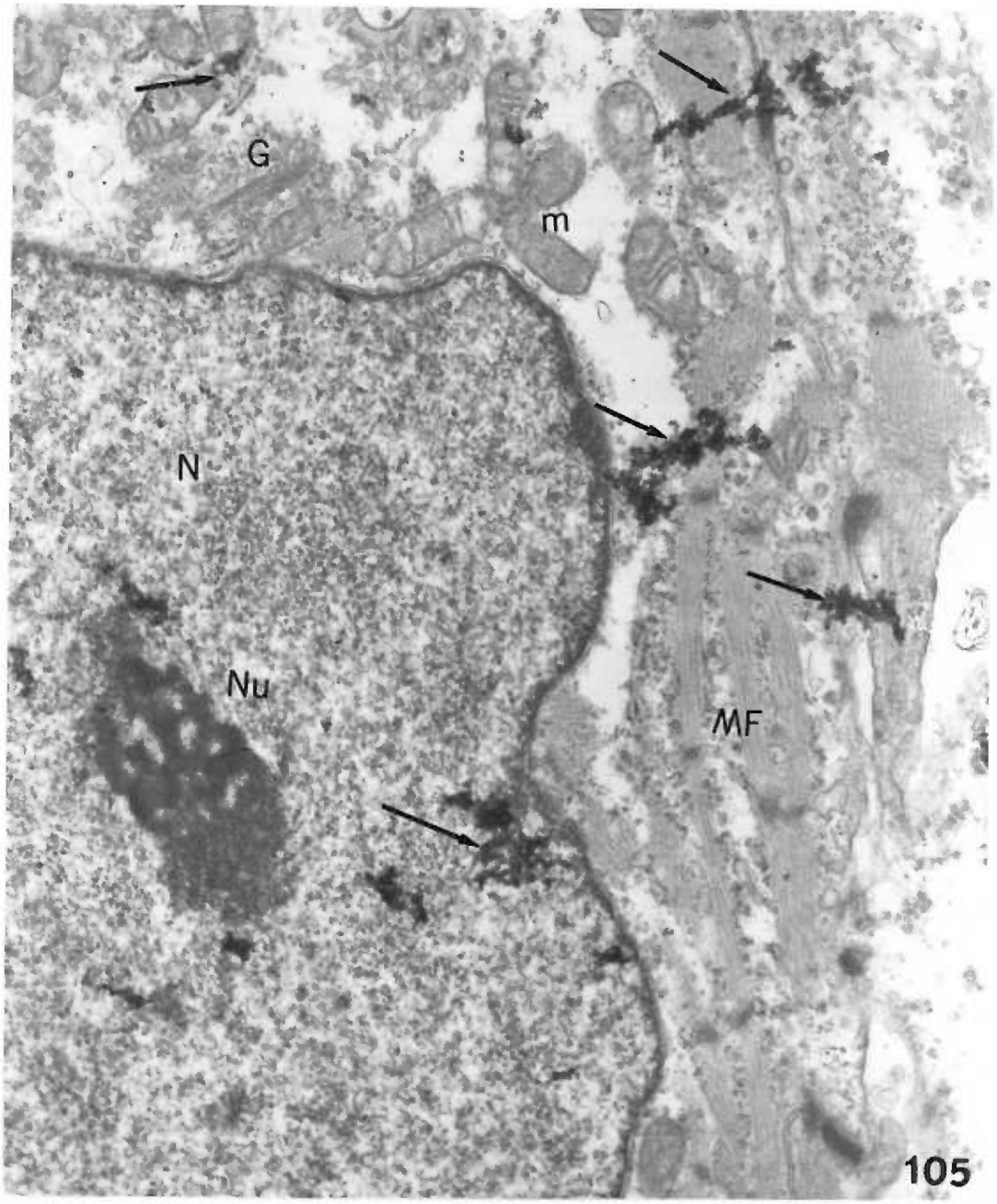

ICS
MF
m
104

Figure 105. Control IV.

Incubated in Wachstein-Meisel medium containing sodium phosphate instead of ATP. Large clumps of precipitate (arrows) are distributed at random without any specific localization (11-day myocardium).

N-nucleus; Nu nucleolus; MF-myofibril; G-Golgi bodies; m-mitochondria.

X 17,440


G
m
N
Nu
MF
105

APPENDIX

This appendix is presented here to stress the fact that the neural elements described in the text and labeled as such in the figures may be processes of mesenchymal origin. Although the author is of the opinion that these processes are derived from neural tissue, the data presented in this thesis does not permit this conclusion. The reader is cautioned that until a more definitive investigation has been accomplished it cannot be stated with certainty that these processes are not extensions of connective tissue or endothelial cells.

As indicated in the discussion section, it will be necessary to apply special histotechniques to make an accurate differential diagnosis of these cells. The similarity of the morphology of the cell processes described and demonstrated in this thesis to those reported elsewhere in the literature, however, does, in this author's opinion, permit the use of the term neural elements for purposes of identification of a cell type that differs from other components of the myocardium. Obviously, such use of terminology is only permissible when the reader is fully aware of the other possible interpretations of the data submitted.