EXPERIMENTAL NEEDLE BIOPSY OF THE MYOCARDIUM OF DOGS WITH PARTICULAR REFERENCE TO HISTOLOGIC STUDY BY ELECTRON MICROSCOPY*

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Experimental cardiac lesions in the laboratory animal are difficult to evaluate during life. In rodents and other small mammals, examination of cardiac tissue usually entails dispatch and autopsy. Surgical biopsy of the heart has been possible in larger animals. Recently Casten and Marsh (1) devised a simpler method for obtaining myocardial biopsies in dogs. Their technique included surgical fixation of the heart to the anterior thoracic wall. After healing, myocardial tissue was obtained by direct puncture with a Vim-Silverman needle (2).

In the course of studies of experimental collagen disease in dogs, it became necessary to secure serial myocardial biopsies for histologic examination. For this purpose it was desirable to avoid surgical manipulation of the heart and mediastinal tissues, and to ascertain whether tissues obtained from the actively beating heart would be suitable for light and electron microscopy. We utilized a procedure for myocardial biopsy, employing the Vim-Silverman biopsy needle, which was a simplification of the method of Casten and Marsh. This communication is concerned with a brief description of the normal histology of the myocardium and the technique of biopsy.

Method

Mongrel dogs of 10 to 30 pounds were used. Each animal was anesthetized with sodium pentobarbital and fixed in supine position. The area overlying the heart was shaved and washed with soap and water. Alcohol (70 per cent) or tincture of iodine was applied to the skin. The instruments were kept in 1:1000 zephiran solution. Further asepsis was not employed. A cutaneous stab wound was made at the point of maximum cardiac impulse. A Silverman cannula with obturator was inserted into the thoracic cavity until cardiac movements were felt against the instrument. The needle was directed toward the midline and slightly head-

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ward. The obturator was removed without changing the position of the cannula and the split stylus was inserted and thrust into the heart. While the stylus was held stationary, the cannula was advanced as far as its hub would permit, locking a small cylinder of tissue in the split stylus. The entire assembly was then rotated and withdrawn from the chest.

The tissue was prepared for light and electron microscopy by Palade's method (3). The specimens were fixed for 4 hours in 1 per cent osmic acid in half-strength Ringer's fluid with acetate-veronal buffer (pH 7.5). They were washed in distilled water, and then dehydrated by successive hourly changes through 70, 95, and 100 per cent ethanol with final transfer to a mixture of equal parts of ethanol and methacrylate for 1 hour. The tissues were then soaked for 3 hours in 3 changes of pure methacrylate. Last, the tissues were embedded in butyl methyl methacrylate with added benzoyl peroxide catalyst. Polymerization was carried out at 47°C. for 24 hours.

Sections were cut with a Minot microtome equipped with a glass knife as described by Dempsey and Lansing (4). Thin sections (about 250 A) were examined without removing the plastic in an RCA model EMU electron microscope, with 40 micra aperture in the objective lens and a centerable condenser aperture. Electron micrographs were taken at an original magnification of 1000 to 5000, and enlarged photographically as desired. In addition, thick sections (2 micra) for light microscopy were cut and stained with hematoxylin and eosin, periodic acid-Schiff stain, and Wilder's ammoniacal silver stain (5).

The animals were observed closely in the postoperative period. They were sacrificed at intervals varying from ½ hour to 2 months following biopsy in order to observe the immediate and chronic effects of the procedure.

RESULTS

Biopsy Procedure

Thirty-five myocardial biopsies were performed on 19 dogs. All but one animal survived the biopsy and recovered from the anesthesia without apparent harm. Three weeks after the last of 2 or 3 biopsies most of the dogs were given an overdose of anesthesia and autopsied. Neither blood nor adhesions were found in the pericardial sacs, and the epicardial and pericardial membranes were normal in appearance. The biopsy site could be identified by a small gray spot of scar tissue on the anterior surface of the heart at or near the interventricular septal area. In one instance the biopsy puncture had included a primary branch of the left descending coronary artery. In other cases smaller branches of the coronary arteries or veins had been punctured. These points of damage caused no apparent alteration in the postoperative course. Four dogs were biopsied simultaneously and then sacrificed individually at intervals of ½ hour, 1 hour, 2 hours, and 24 hours. The 3 animals examined within the first 2 hours after the procedure were found to have 30 to 60 cc. of blood in the pericardial cavity. There was no evidence of active bleeding or tamponade. Sponging of the epicardial surface to locate the puncture site occasionally provoked seepage of blood from the wound. The pericardium of the dog killed at 24 hours contained about 40 cc. of clotted blood and serum.

The myocardium of one dog was punctured three times at 15 minute intervals. For a few minutes after the third puncture the heart sounds became

distant and the heart rate was increased; however, the dog recovered from the anesthesia in the usual time with no evident cardiovascular embarrassment, and during the next 6 months had 4 more biopsies. The heart of another dog was biopsied under direct vision through an operative incision with the pericardium opened. Immediately after the needle was withdrawn from the left ventricle, a thin stream of blood issued 5 or 6 feet with each contraction. The stream subsided to slow oozing within a few minutes.

The one dog that expired following cardiac puncture had also received an injection of an antigenic substance which occasionally caused death. Autopsy showed only a left pneumothorax. It was not possible to assign the exact cause of death.

Microscopic Studies

(*a*) *Light Microscopy.*—Photographs of the tissue sections taken for light microscopy are shown in Fig. 1. Examination of these sections indicates that there was no recognizable distortion of cellular elements in the small core of tissue comprising the biopsy specimen. In hematoxylin-eosin stained preparations, the coloration was the same as in ordinary tissue fixation and embedding. Reticular fibers and sarcoplasm were emphasized by silver stains (Fig. 2). The periodic acid–Schiff reagents stained the sarcoplasm intensely (*cf.* Fig. 1).

(*b*) *Electron Microscopy.*—There have been few published reports of myocardial histology studied by electron microscopy. The majority of these studies were carried out with fragmented muscle tissues which were inadequately fixed. Technical progress now permits fixation in buffered osmic acid so that no morphological change in cultured cells is evident by darkfield microscopy (limit of detection 0.006 micron) (6). It seems probable that with proper osmic acid fixation and sectioning, electron micrographs display nearly perfect examples of cellular anatomy.

General Description.—The myocardium comprises an anastomotic network of muscle fibers which are 16 to 20 micra in diameter. Capillaries, extravascular red cells, and small amounts of supportive tissue are present in the interstitial spaces. The myocardial fiber is a unit defined by its enveloping sheath, the sarcolemma. The internal structure of the fiber consists of myofibrils, mitochondria (sarcosomes), nuclei, and amorphous granular material embedded in the sarcoplasm (*cf.* Figs. 4 to 7).

Capillaries.—The capillaries appear as tubes formed by juxtaposed endothelial cells invested by a delicate condensation of connective tissue, the basement membrane (Figs. 5 and 6). The endothelial cell wall is approximately 150 A thick and is known as the plasma membrane. The portion of the plasma membrane which is adjacent to the basement membrane is called the basal plasma membrane; the remainder of the cell wall is in contact with the capillary luminal contents and is called the inner plasma membrane. These plasma

membranes may send invaginations into the cytoplasm, and occasionally give rise to villous-like structures which project into the capillary lumen. The endothelial cell cytoplasm is clear and electron-lucent, containing small membrane-bounded invaginations from the plasma membranes, and an occasional mitochondrion. An endothelial cell nucleus is occasionally included in the plane of section (*cf.* Fig. 6).

Sarcolemma.—The muscle fiber is surrounded by the sarcolemma which at low magnification appears as a single electron-dense line (Figs. 4 to 6). At higher power, two distinct membranes separated by a clear space (200 A wide) can be identified constituting the sarcolemma (Fig. 8). The inner membrane (150 A thick) is more dense than the outer. It is the plasma membrane of the muscle fiber. The outer membrane is 200 A thick. Fibers fray from its surface. These are probably collagen fibers as described by Pease and Baker (7), although the typical collagen periodicity of 640 A was not observed in our sections.

The Myofibril.—Myofibrils, the contractile elements of muscle cells, are seen as anastomosing bundles, 0.2 to 10.0 micra in width, running the length of the fiber. The myofibril has no limiting membrane, and its extent is ill defined since its weaving course frequently takes it out of the plane of section. High magnification shows that the myofibril is composed of many minute parallel threads, the myofilaments. Huxley (8) has described two types of myofilaments in skeletal muscle. He noted thin filaments 40 A in diameter in the isotropic and anisotropic bands. Thick filaments, 110 A in diameter, were found only in the anisotropic bands. In cross-sections the thick filaments were distributed evenly in hexagonal array. Hanson and Huxley (9) suggested that the thick filaments were myosin and that the thin filaments were actin. The localization of myosin in the anisotropic bands has been indicated by other studies (10–12). In our micrographs of cardiac muscle the thin and thick filaments are evident (Fig. 8), but cannot be delimited to the specific bands.

The Z membrane (or band) divides the myofibrils into units (sarcomeres) (Figs. 4 to 8). The Z membrane (most prominent of the transverse striations) appears to be a continuous structure across the breadth of the fibril, forming a connecting bridge between individual filaments as well as between fibrils. It blends indistinguishably into the plasma membrane at the cell margin. The Z membrane may be perforated by rows of mitochondria, by nuclei, or by subsarcolemmal sarcoplasm. The myofilaments proceed from one sarcomere to another through the Z band without interruption (Fig. 8). Contracted muscle fibers present a picket fence appearance due to segmental outward bulging of the sarcolemma. When the myofibrils shorten, the non-contractile sarcolemma is thrown into periodic folds because of its points of attachment to the Z membrane (Figs. 4 to 8). These observations are consistent with those of Beams *et al.* (13) and others (14, 15). Barer (16) and Weinstein (17), however, present a different interpretation of Z membrane structure.

The other cross-bands of striped muscle are less distinct than the Z membranes, and do not bridge the sarcoplasmic interval between filaments or fibrils. The Z band together with the adjacent electron-lucent zone constitutes the I (isotropic) band (Figs. 4 and 5). The I band is inconstant as it tends to disappear with contraction. The remainder of the sarcomere is designated the A (anisotropic) band. It is bisected by the electron-lucent H band which in turn is bisected by a dark line, the M band (Figs. 4 and 5).

Intercalated Discs.—At infrequent intervals along a muscle fiber double membraned structures are present which appear to transect the fiber in an uninterrupted, tortuous course (Fig. 7). These are the intercalated discs and may represent modified cell boundaries interrupting the continuity of the myocardial syncytium, as suggested by Sjöstrand and Anderson (18). At the fiber edge the membranes of the intercalated disc are the same thickness and density as the plasma membrane, with which they are continuous (Fig. 7). The intercalated disc generally follows along the course of one or two Z bands. For the distance in which the intercalated disc runs in a Z band, the membranes of the disc appear dense and thick, apparently due to the presence of Z band material. When the disc jumps a gap between two Z bands, the disc membranes again assume the characteristics of the plasma membrane. The distance between membranes of the disc varies from 100 to 200 A. Greater separations do occur for short intervals, however, giving the appearance of vacuoles interrupting the course of the disc. The gap between intercalated disc membranes is filled with a material which varies from moderately electron-dense in Z band regions, to electron-lucent in the intervals between Z bands.

Mitochondria.—In longitudinal sections the mitochondria (known as sarcosomes in muscle) are aligned in parallel rows between columns of myofibrils (Figs. 4 to 8). Frequently there is one mitochondrion for each sarcomere as was observed by Hodge *et al.* (14) in rat myocardium. Such profuse distribution of mitochondria is seen only in rapidly metabolizing muscles such as the heart and diaphragm. Pappenheimer and Williams (19) have shown that muscle mitochondria are highly active enzymatically. They probably regulate the intracellular metabolic processes essential to life. A mitochondrion may be identified structurally by its double membraned external wall from which arise many internal folds arranged in laminar fashion (Fig. 8).

The Nuclei.—Nuclei are centrally located in myocardial muscle fibers. They are ovoid in shape and lie with the long axes parallel to the fiber axis. When the muscle fiber is contracted, corrugations appear in the lateral walls of the nuclei (Fig. 4). Amorphous material, and occasionally nucleoli, may be seen within the nuclei.

The Sarcoplasm.—The sarcoplasm (muscle cytoplasm) surrounds the intracellular organelles. It is electron-lucent and contains numerous granules. The finer granules may represent precipitated glycogen, since the muscle is known to be rich in glycogen and the sarcoplasm stains with both the Schiff and

the ammoniacal silver reagents. The coarse dense granules may represent ergastoplasmic granules (Figs. 7 and 8). None of our sections reveals ergastoplasmic membranes, or Golgi membranes or vacuoles. Accumulations of sarcoplasm occur at the poles of the nuclei, around the columns of mitochondria, and in zones subjacent to the sarcolemma (Figs. 4 to 8).

COMMENT AND SUMMARY

Our experience with needle biopsy of the heart in dogs indicates that myocardial tissue can be sampled one or more times in each animal with comparative safety. Tamponade, pericarditis, serious arrhythmias, or myocardial infarction due to the interruption of coronary vessels was not observed. Excellent specimens were obtained for critical study by light and electron microscopy. Casten and Marsh (1) have used biochemical techniques to study myocardial tissue obtained in similar fashion. Histochemical methods would also be applicable.

Although limited to animal studies at present, the technique may conceivably be adapted to the study of human disease. Myocardial puncture has been carried out (20–22) in patients for the recording of intracardiac pressures and for other diagnostic purposes without apparent harm.

Our study of the myocardium of dogs by electron microscopy generally confirms the observations of other workers, except that presence of significant numbers of red blood cells in the extravascular spaces of the heart had not been previously described (and is possibly an artifact). Nevertheless, it is notable that the tissue cells, cellular membranes, and intracellular structures appeared to be intact and undistorted in the tissue specimens which were obtained, fixed, and examined by these methods.

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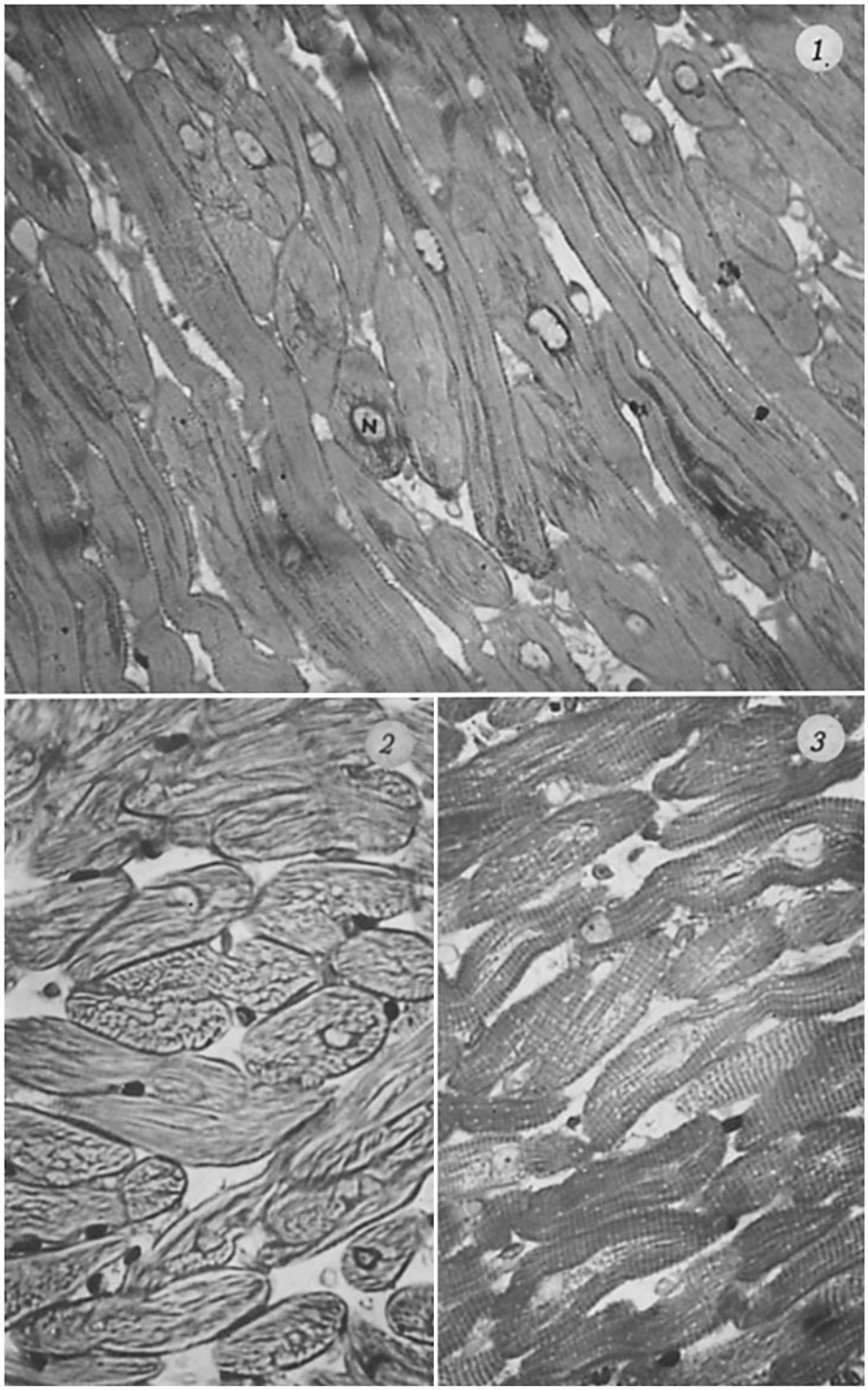
EXPLANATION OF PLATES

PLATE 49

FIG. 1. Light micrograph of a 2 micron thick section of plastic-embedded, osmic-fixed dog heart stained by the periodic acid–Schiff method. The nuclei (*N*) are centrally placed in the anastomosing muscle cells. The perinuclear and subsarcolemmal regions, rich in sarcoplasm, stain intensely with this method. The material which reacts positively is probably glycogen. × 600.

FIG. 2. Light micrograph of a 2 micron thick section of plastic-embedded, osmic-fixed dog heart stained with Wilder's ammoniacal silver method. In the muscle cells the sarcoplasm stains intensely, outlining the nuclei and accentuating the subsarcolemmal region. As with the periodic acid–Schiff method, the reacting material probably represents glycogen. Outside the muscle cells, the nuclei of capillary endothelial cells stain intensely. × 600.

FIG. 3. Light micrograph of a 2 micron thick section of plastic-embedded, osmic-fixed dog heart stained with hematoxylin and eosin. The A bands stain more intensely with hematoxylin and appear dark. × 600.



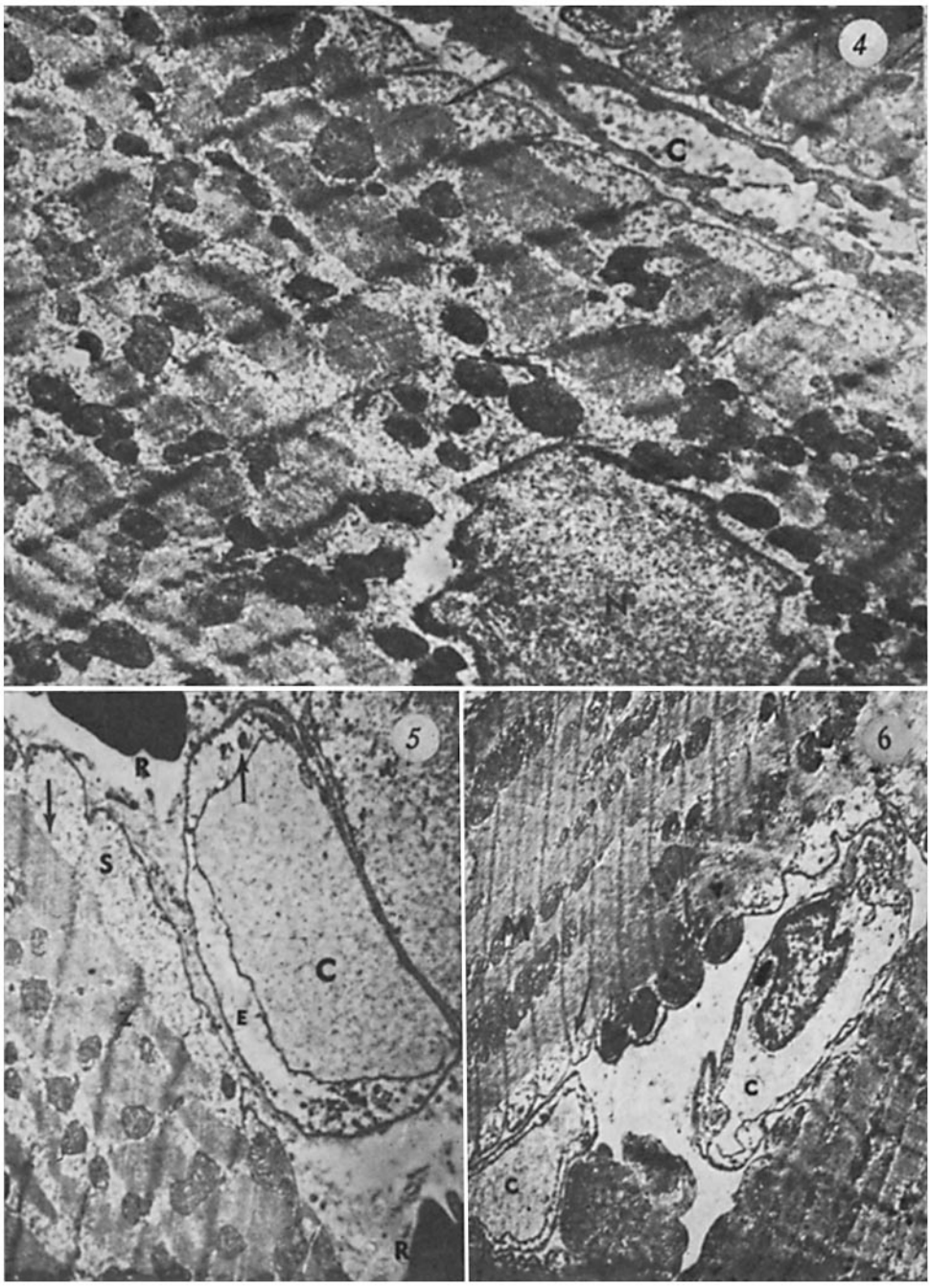
(Price *et al.*: Myocardium studied by electron microscope)

PLATE 50

FIG. 4. Electron micrograph of a portion of a heart muscle cell with a centrally placed nucleus (*N*). Accordion-like folds are present in the lateral nuclear membranes. The muscle cell is transected by Z membranes which appear to insert into the sarcolemma, pulling in at points of insertion to give a scalloped appearance to the sarcolemma. Midway between Z membranes are the dark M bands (arrow) which lie in the center of the lighter H regions. The granularity of the sarcoplasm around the nucleus, the mitochondria, and beneath the sarcolemma, is probably due to the presence of glycogen. In the extracellular space a capillary (*C*) is present. × 5,000

FIG. 5. Electron micrograph of a small region of cardiac muscle. In the lower left part of the field is a portion of a muscle cell. The Z membranes (*Z*) of the lower left cell do not reach the sarcolemma, but are interrupted by an expanse of sarcoplasm (*S*) which contains granular material probably representing glycogen (*cf.* Figs. 1 and 2). Scattered among the bundles of myofilaments, which penetrate the Z membranes, are numerous mitochondria. Banding of the myofilaments is evident in this micrograph. The electron-lucent H region, lying midway in the A region, is demidiated by a thin line, the M band (arrow down). The I region lies on both sides of the Z membrane (see Fig. 6 for finer detail). In the extracellular space is a capillary (*C*) lined by endothelial cell cytoplasm (*E*). The cytoplasm of the endothelial cell is markedly attenuated on the right where the inner and outer plasma membranes of the cell are apparently in contact. Besides a small mitochondrion (arrow up) and several vacuoles, the cytoplasm of the endothelial cells is remarkably clear. Outside the capillary are two red blood cells (*R*) lying free in the extracellular space. This is a constant finding in our biopsy material. × 4,000.

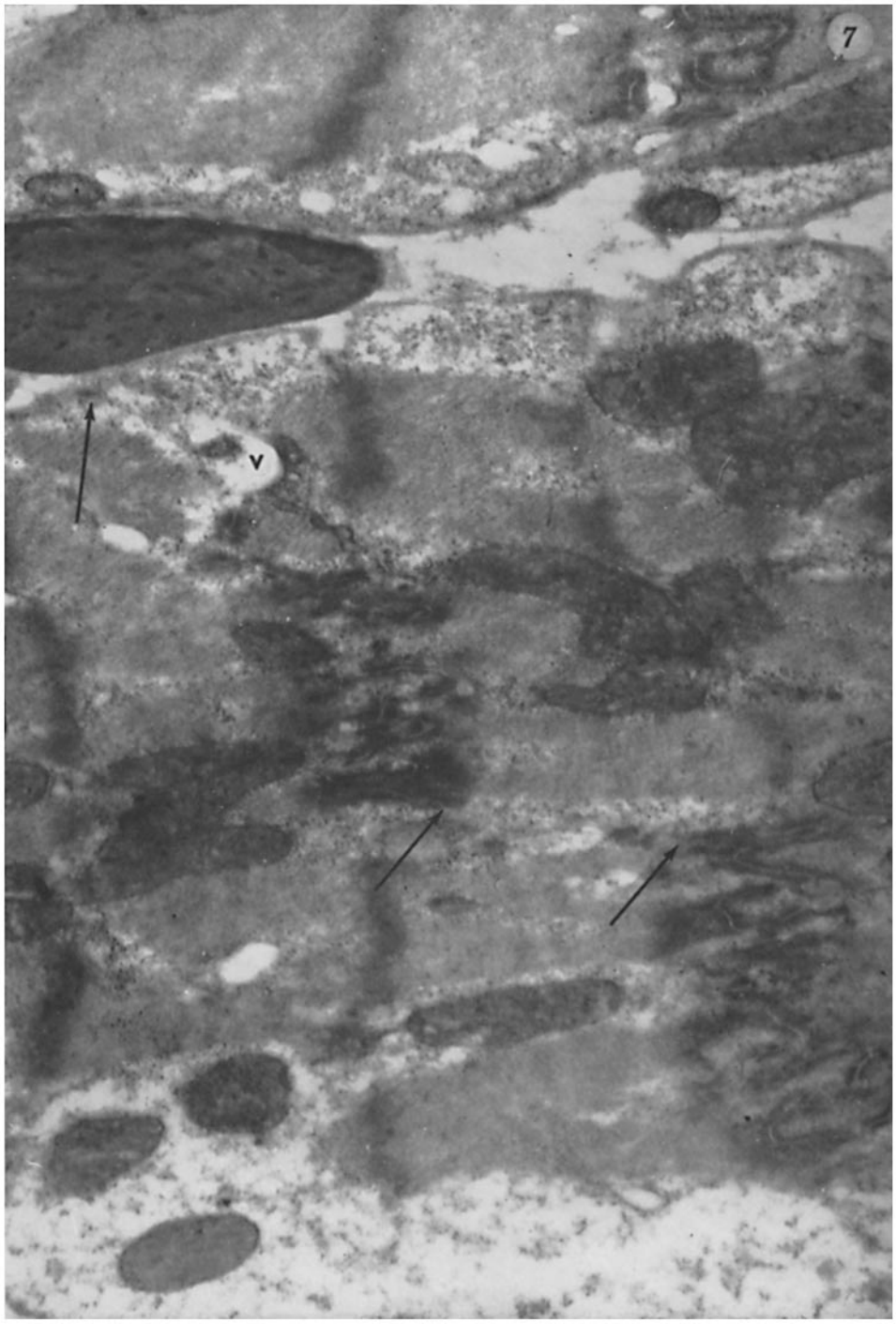
FIG. 6. Electron micrograph of a portion of heart muscle. The cells are transected by Z membranes which appear to insert into the sarcolemma (arrow), pulling in at the points of insertion and giving a scalloped appearance to the sarcolemma. Rows of mitochondria (*M*) alternating with bundles of myofilaments (myofibrils) are disposed parallel to the long axis of the cell. Large holes are formed in the Z membranes to accommodate the mitochondria. In the extra-cellular space are two capillaries (*C*) lined by endothelial cells, a nucleus of which can be seen in the upper capillary. The endothelial cell cytoplasm is clear except for membrane-bounded vacuoles which probably represent sections through invaginations of the cell membrane. × 2,000.



(Price *et al.*: Myocardium studied by electron microscope)

PLATE 51

FIG. 7. Electron micrograph of portions of two heart muscle cells showing segments of two intercalated discs. The double membrane of the lower disc starts at an invagination of the plasma membrane (upper arrow). The membranes are difficult to see as they pursue a course downward and to the right. After a short interval the membranes separate to form a vacuole (*V*). The membranes are denser on the other side of the vacuole, as they start to run in the Z band region. In this region the membranes are quite dense, and the intercalated disc runs a tortuous course. Halfway across the fiber the disc abruptly skips across the interval between Z bands. In this interval (between the two lower arrows) the membranes are once again difficult to discern. As the intercalated disc again enters a Z band region (lower right arrow), its membranes become thick and electron-dense, and pursue a tortuous course. A second intercalated disc can be seen beginning in the cell at the top of the micrograph. A portion of a red blood cell can be seen lying free in the extracellular space between the two muscle cells. × 15,000.



(Price *et al.*: Myocardium studied by electron microscope)

PLATE 52

FIG. 8. Electron micrograph of a portion of a heart muscle cell. Myofilaments run across the field and are arranged in bundles, the myofibrils. The large filaments are 110 A in thickness. There are small filaments, about 40 A in thickness, also present. These may represent the small fibrils described by Huxley (8, 9) or tangential sections through the thicker fibrils, or both. Several rows of mitochondria (*M*) are present, lying parallel to the course of the myofilaments. Here again it appears that the mitochondria disrupt the continuity of the Z membranes (Z). Above, the Z membranes appear to be continuous with the plasma membrane, which forms the inner wall of the double membraned sarcolemma. The outer wall of the sarcolemma is less electron-dense and has a more frayed appearance than the plasma membrane. This outer line is probably analogous to the basement membrane of epithelial cells. Sarcoplasm (*S*) is present below the sarcolemma and around the mitochondria. The granules present in the sarcoplasm may represent ergastoplasm or glycogen, or both. × 36,000.

(Price *et al.*: Myocardium studied by electron microscope)