

PHASE AND ELECTRON MICROSCOPE STUDIES OF THE
INTERRELATIONSHIP OF CYTOCHONDRIA AND
MYOFIBRILS IN PIGEON BREAST MUSCLE*

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The problem of mitochondrial structure has been the subject of extensive research during the past few years. Since Palade (1) published his "crista" concept, several other workers have proposed various interpretations of structure.

From sections of mouse pancreas and of guinea pig retinal rods, Sjöstrand (2) suggested that the mitochondria are surrounded by a double "membrane" and contain transverse double "membranes" rather than ridges described by Palade. This structure was also seen in the proximal convoluted tubule cells of the mouse kidney (3). Chapman (4), however, has been unable to substantiate presence of a double external "membrane" in insect muscle mitochondria, although the inner transverse double structures appear to have surface attachments which are interpreted as junctions with a limiting "membrane." Glimstedt and Lagerstedt (5) claim that their preparations of rat liver mitochondria show "... cable-like elements consisting of equal sized granules piled in a string one after the other ..." contained as a bundle within an external "membrane." A departure from the theories of transverse lamellae was presented by Beams and Tahmisian (6) who worked on *Helix aspersa* gonad and constructed a model demonstrating "longitudinal coaxial lamellae." There are now two schools of thought concerning the presence or absence (7, 8) of double walled "membranes."

Biochemical and phase microscopy studies in this laboratory (9-11) have adduced no conclusive proof for the presence of mitochondrial membranes. On the contrary, they give evidence for the probable existence of a gel structure. Therefore, this study was initiated for the purpose of correlating mitochondrial ultrastructure with previous findings. Since the completion of this work Powers, Ehret, and Roth (8) have reported the lack of external mitochondrial "membranes" in *Paramecium aurelia* or *P. bursaria*.

Some confusion still exists concerning the use of the terms *mitochondria*

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and *sarcosomes* in reference to sarcoplasmic inclusions, and many workers have used these terms interchangeably. In this paper the term *mitochondria* applies to those sarcoplasmic inclusions which appear cytologically similar and act biochemically like the mitochondria of other tissues. The term *sarcosomes* is restricted to smaller spherical bodies which are found only in muscle and are distinct from mitochondria. Because of their specific occurrence in muscle these small particles are more correctly designated sarcosomes (muscle bodies). In accord with previously published work (12, 13) all sarcoplasmic inclusions, excluding nuclei and myofibrils, are grouped together and considered under the inclusive term *cytochondria*.

Materials and Methods

The tissue used in this study was the pectoralis major muscle of the domestic pigeon, *Columba livia*. All preparations were obtained from decapitated animals within 5 minutes of death. Tissue used for electron microscopy was fixed in Palade's buffered osmic acid (14). Immediately after exposing the pectoralis major, an area of superficial fascia and a thin layer of the underlying muscle were stripped off and discarded. Strips of muscle less than 2 mm. thick, 2 mm. wide, and 10 mm. long were rapidly separated and dropped into osmic acid buffered at pH 7.4. After allowing the tissue to harden for a few minutes, the strips were cut into 2 mm. lengths, giving pieces approximately 2 mm.³

The cubes of muscle remained in the fixative for 4 hours and were then transferred to distilled water for 1 hour (3 changes). At this point two alternative methods of dehydration were employed. In one method, as recommended by Palade (14), the tissue was transferred from water directly to 70 per cent ethanol, whereas in the other method it was more slowly dehydrated by placing it successively in 35 and 50 per cent ethanol. In both methods the tissue remained overnight in 70 per cent ethanol, and on the following day dehydration was continued through 95 per cent and absolute ethanol for 1 hour each. The specimens were next transferred for 1 hour to a 1:1 mixture of absolute ethanol and methacrylate monomer mixture (inhibitor removed: 1 part ethyl methacrylate, 3 parts *N*-butyl methacrylate).¹ They were then infiltrated with the methacrylate monomer mixture using three 1 hour periods. Embedding was accomplished in No. 4 gelatin capsules which contained a small pellet of polymerized methacrylate mixture. Polymerization was induced, without the addition of a chemical catalyst, by overnight exposure to a Westinghouse FS 20T12 fluorescent sun lamp at a distance of 2.5 cm. (15).

Embedded tissues were sectioned at 0.05 μ and 0.025 μ using an International ultrathin sectioning microtome equipped with a glass knife (16), liquid trough (17), and rotary displacement specimen holder (18). The microtome was equipped with an 80:1 reduction worm and worm gear assembly in place of the standard 40:1 assembly. The sections were collected in a liquid trough containing 10 per cent acetone in water and later transferred to distilled water in a 37°C. incubator until flattened. For ease of handling, the flattened sections were allowed to return to room temperature before being mounted on *formvar*²-coated 200 mesh nickel *lectromesh* screens.³

Electron micrographs were prepared with the use of an RCA EMU-2 electron microscope equipped with an intermediate projection lens, compensated objective pole piece, and an

¹ Obtained through the courtesy of Rohm and Haas, Philadelphia.

² Polyvinyl formal, Shawinigan Resins Corp., Springfield, Massachusetts.

³ C. O. Jelliff Mfg. Corp., Southport, Connecticut.

adjustable 0.002 inch objective aperture. Ilford N.60 photomechanical plates were selected as the sensitized material of choice and were developed to high contrast in Ilford ID-2 developer.

Two types of preparation were used for phase microscopy, both prepared by the centrifugal fractionation procedure described by Kitiyakara and Harman (12). The method was modified by elimination of sodium bicarbonate from the sucrose solution. A coarse preparation obtained from the strained product of the initial homogenization was used for morphological relationship studies between the cytochondria and myofibrils. This preparation contained many small fragments which were easily studied as fresh mounts with the phase microscope. Observations on the mitochondria and sarcosomes were made directly on the cytochondrial fraction obtained from the complete fractionation procedure.

During the course of phase microscope observations, studies were made of the effect of a wetting agent on mitochondrial morphology. A wet mount of cytochondria suspended in 0.25 M sucrose was examined with the phase microscope. After initial examination a drop or two of 1:200 *photo-flo*⁴ in distilled water was introduced under the coverglass, and the morphological changes of the cytochondria observed.

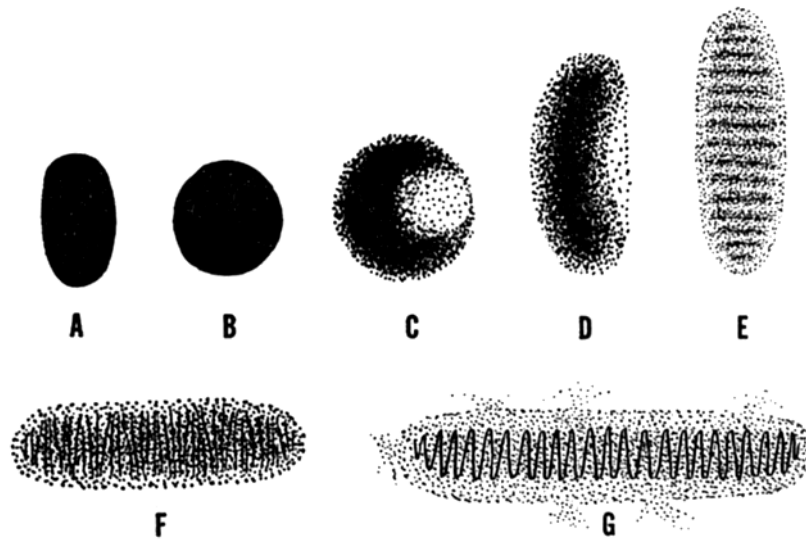
RESULTS

Mitochondria.—Phase microscope observations reveal that the mitochondria exist as short rods and occasional spheres lying within the sarcoplasmic reticulum between the myofibrils. The rods are normally oriented with their longitudinal axes parallel to the longitudinal axes of the myofibrils. There is a tendency for the poles of the mitochondria to be located adjacent to the loci of the "Z" lines, although this is sometimes difficult to demonstrate in muscle having a very high concentration of mitochondria. Harman and Kitiyakara (10) have already described the morphological changes resulting from thermal effects, swelling with hyposmolar solutions and shrinkage with hyperosmolar solutions. In these experiments the presence of an external limiting mitochondrial "membrane" was not demonstrated. Therefore, mitochondrial suspensions were subjected to the action of a wetting agent. As was expected from the previous observations, the mitochondria swell almost immediately. Although they swell to many times their original volume, they do not burst, but undergo gradual disintegration. It is also noted that the swollen mitochondria tend to agglutinate and coalesce.

The sequence of morphological alterations following addition of the wetting agent (Kodak *photo-flo*) was carefully observed by phase microscopy. Spherical mitochondria (Text-fig. 1 B) develop a vesicle at one side with the denser portion of the mitochondrion assuming a crescent-like configuration (Text-fig. 1 C). These mitochondria remain spherical for a short time while their volume increases. This is followed by rapid straightening of the crescent portion with a simultaneous repositioning of the vesicular material along the new rod-like configuration (Text-fig. 1 D). Simultaneously the short rod mitochondria (Text-fig. 1 A) swell and lengthen slightly, and swollen rods derived from either

⁴ A proprietary wetting agent manufactured by the Eastman Kodak Company, Rochester, New York, the formula for which is not available.

spheres or short rods are indistinguishable. With further swelling the rods show dark medullary and light cortical regions. Transverse striations appear in the medullary region and become more pronounced as swelling progresses (Text-fig. 1 E). Subsequent swelling is mainly longitudinal. The transverse striations gradually separate, when it is noted that they are not parallel (Text-fig. 1 F). Progressive swelling results in a gradual unfolding, revealing a folded "thread" structure (Text-fig. 1 G), followed by a slow disintegration of the cortical region.



TEXT-FIG. 1. Drawings from phase microscope observations showing the swelling effect of a wetting agent on mitochondrial morphology.

The experience obtained from the previous studies (10) on mitochondrial swelling serves as a control for the alterations observed under the influence of *photo-flo*. It is apparent from such observations that the essential nature of the swelling is similar to that seen in the previous study, but that certain structural features are accentuated and alterations accelerated by the wetting agent.

Marvin (19) has reported that in electron micrographs of the gastric gland cells of mice exposed to x-radiation, and rapidly fixed with osmic acid, mitochondria disintegrate, leaving a residual folded ribbon-like structure.

Electron microscopic examination of longitudinal sections through mitochondria reveals two forms: (a) coarse, irregular transverse lamination (Fig. 5); (b) fine, regular transverse lamination (Figs. 2 to 4). When tissue is dehydrated by direct transfer from water to 70 per cent ethanol, there is a higher percentage of coarsely laminated mitochondria. This is usually accompanied

by distention of the mitochondria and by separation of the myofibrils, resulting in over-all distortion. Osmium also tends to be deposited along portions of the mitochondrial surface, leading to the appearance of a "membrane."

In many mitochondria structures that appear like portions of an external limiting "membrane" upon closer examination are revealed to be fragments of lamellae. Often there is a marginal connection, or short loop, between adjacent lamellae (Fig. 3). The continuity between adjacent lamellae is clearly seen in those mitochondria with a coarse, irregular internal structure (Fig. 5). Some sections, however, show neither external limiting membrane nor interlamellar connections (Fig. 4). Cross-sections of mitochondria do not demonstrate an external membrane (Fig. 6). In both cross- and longitudinal sections numerous connections between adjacent mitochondria and other intracellular structures are demonstrated (Figs. 2 and 6).

Sarcosomes.—Phase microscopy reveals small, highly refractile spherical bodies adjacent to the mitochondria. There is a tendency for them to be located at the poles of a mitochondrion, although some lie between the mitochondria and adjacent myofibrils (Fig. 1).

Electron microscopy demonstrates spherical structures, closely related to myofibril "Z" lines, in the same positions as the sarcosomes visualized by phase microscopy. These bodies are usually the most electron-opaque, *i.e.* the most osmiophilic, structures in the section. The opaque body is usually suspended in a clear area, within a mitochondrial depression, by fine strands which often contain small, dense granules (Fig. 2). These strands interconnect with adjacent mitochondria and myofibrils.

Kitiyakara and Harman (12) report that sarcosomes are more osmiophilic and refringent than mitochondria, do not undergo osmotic swelling, and are highly soluble in organic solvents. Harman and Osborne (13) have demonstrated negligible oxidative enzyme activity for sarcosomes as compared to mitochondria. In addition, it has been reported (20) that sarcosomes have a higher RNA content than mitochondria.

Myofibrils.—The myofibrils manifest no evidence of a limiting membrane in electron microscope observations. The myofilaments, when seen in cross-section, appear to be oriented into well defined sheets (Fig. 6). Many fine "bridges" are observed between adjacent myofibrils and cytochondria, especially at the region of the "Z" lines. These "bridges" are especially apparent in sections which show extreme shrinkage and are strong enough to rotate the mitochondria into transverse orientation.

DISCUSSION

This study has revealed a symplasmic relationship between the cytochondria and myofibrils of pigeon pectoralis major muscle. It is most evident in the region of the myofibril "Z" lines and is in accord with the findings of Bennett

and Porter (21) in the breast muscle of the domestic fowl (*Gallus* sp.). In addition, no definite evidence was found for the existence of limiting "membranes" around the intracellular structures studied, and it was often difficult to determine a demarcation between these structures and the sarcoplasm.

It is consistent with the previous observations that the matrices of the cytochondria and myofibrils are of a gel nature and capable of maintaining both form and integrity without the presence of a limiting "membrane." This contention is supported by the electron micrographs showing ultrastructure, resistance to mechanical disruption, and the symplasmic relationship between the various intracellular structures. It has been found that cytochondria and myofibrils cannot be ruptured by relatively high mechanical pressure. Disruption of cytochondria requires prolonged exposure to high shearing forces (10). This does not support the presence of extremely delicate limiting "membranes." Further, the symplasmic relationship of the intracellular structures is completely destroyed in the preparation of suspensions by centrifugal fractionation, and artifacts would be expected at the sites of the broken bridges if limiting "membranes" existed. No such artifacts have been observed.

It is the opinion of the authors that the mitochondria are composed of a folded ribbon-like gel structure enclosed in a gel matrix (Text-fig. 2). The two gels seem to have different physical and chemical properties, and the matrix gel appears to be capable of swelling to a greater extent than the "ribbon" gel. Kitiyakara, Harman, and Weinreb (22) report that upon extraction of the mitochondrial proteins two distinct fractions have been detected. One is soluble in dilute neutral salt solution, suggesting that it has a non-fibrous structure. The other is soluble in concentrated salt solution and presumably has a fibrous structure. It is probable that the fibrous fraction forms the "ribbon" gel while the non-fibrous fraction forms the matrix gel.

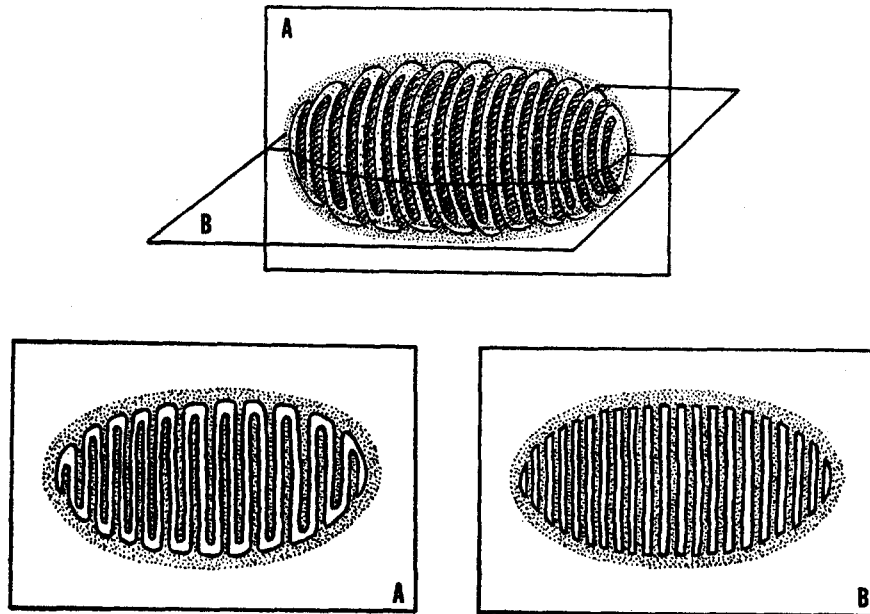
During swelling of the mitochondrion, the ribbon also swells and tends to unfold because of internal pressures. Simultaneously the swelling of the matrix between the folds forces them apart. This theory is supported by the phase microscope observations. In addition, in poorly fixed sections and sections that are subjected to rapid dehydration, the mitochondria swell and the folded internal structure is more clearly visible (Fig. 5). The lamellae are thicker in swollen mitochondria (approximately 320 Å) than in normal mitochondria (approximately 180 Å).

Further evidence for the 'folded ribbon' theory is found in Fig. 4; in this mitochondrion the lamellae have no marginal connections. Examination of Text-fig. 2 shows that a longitudinal section through plane A gives the appearance of a folded structure, while rotating the cutting plane 90° to B results in separate lamellae. It should be noted that although the lamellae in Fig. 4 are well defined, there is no indication of an external limiting "membrane."

The question of the apparent "double walled" structure of the lamellae, or

ribbon, has been considered, and it is believed that this is a visualization of the interfaces between the different types of gels. As the tissue is washed and dehydrated, there are repeated movements of phase boundary waves through the tissue with the addition of each new solution. A certain amount of osmium may be carried along with each wave and deposited along interfaces.

This study indicates that there is variation of mitochondrial structure within a single tissue. There is, therefore, a probability of great differences between the structure of mitochondria of different tissue and different animals.



TEXT-FIG. 2. Diagrammatic conception of the structure of a mitochondrion. A and B represent two longitudinal sections through the mitochondrion with the cutting planes rotated 90° .

The ultrastructure of the sarcosomes is still uncertain. There is little doubt that the dense spheres shown with the electron microscope are really sarcosomes (12, 13), since their positions correlate so well with phase microscope observations. However, even when sectioned at 0.025μ , the dense portion still appears homogeneous, although one electron micrograph did give a vague impression of a finely laminated structure. If an internal structure exists, it is either too electron-opaque or too fine for resolution by the electron microscopy techniques employed in this study. In so far as the clear area surrounding the sarcosome is concerned, there has been either severe shrinkage or loss of a peripheral layer. This suggests the presence of large amounts of material

soluble in organic solvents (12), probably lipids, dispersed throughout the sarcosome or surrounding it as a cortical layer.

A final interesting observation concerns the dark granules which often appear in the suspending strands within the clear area surrounding the sarcosomes. They occur both dispersed and as dense aggregates. Their structure and possible function have not yet been determined.

SUMMARY

Mitochondria in pigeon breast muscle are composed of two protein gels: a fibrous gel, in the form of a folded ribbon, enclosed within a non-fibrous matrix. An external limiting "membrane" is not demonstrated, and there is poor demarcation between the mitochondria and adjacent structures or sarcoplasm.

No internal structure has been determined for sarcosomes. These structures, however, are symplasmic with mitochondria and usually are located within mitochondrial depressions. They apparently have a high lipid content.

The myofibrils also have no external limiting "membranes" and the mitochondria and sarcosomes are symplasmic with them. The mitochondria normally lie in the intermyofibrillar sarcoplasm oriented with their longitudinal axes parallel to the myofibrils. Cross-sections show that the myofilaments are oriented into well defined sheets.

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EXPLANATION OF PLATES

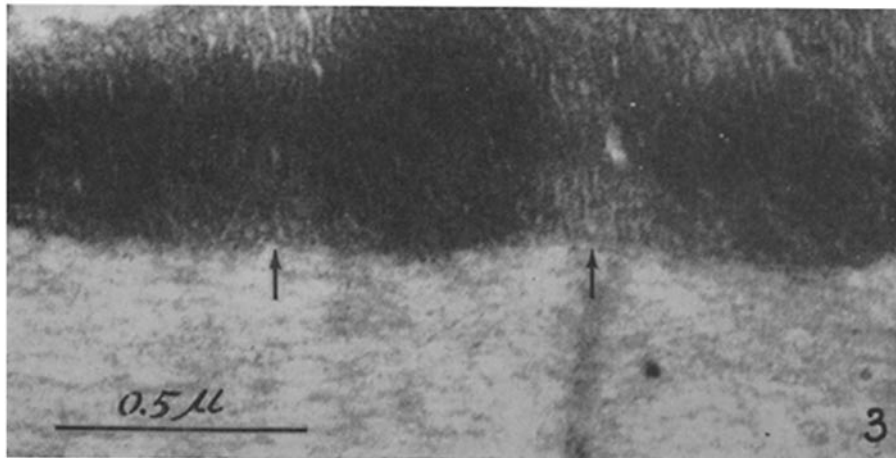
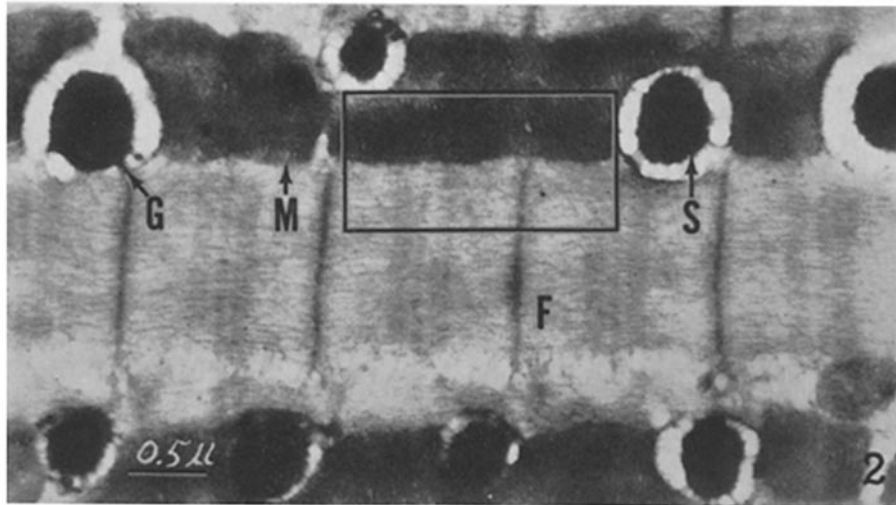
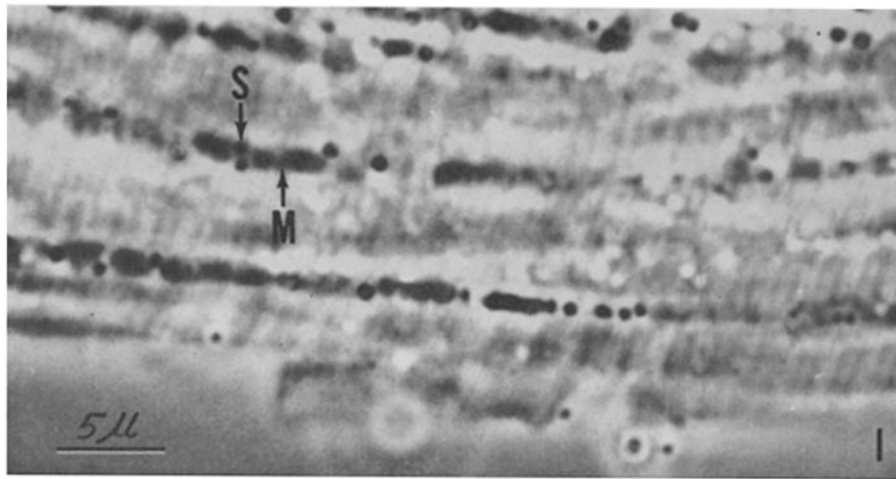
PLATE 28

F, myofibril; *G*, granules; *M*, mitochondrion; *S*, sarcosome

FIG. 1. Phase micrograph (dark contrast) of pigeon breast muscle fragments showing the interrelationship of cytochondria and myofibrils.

FIG. 2. Electron micrograph of pigeon breast muscle (longitudinal section) showing the interrelationship of cytochondria and myofibrils.

FIG. 3. Enlarged section of Fig. 2 showing connecting loops (arrows) between the mitochondrial lamellae.



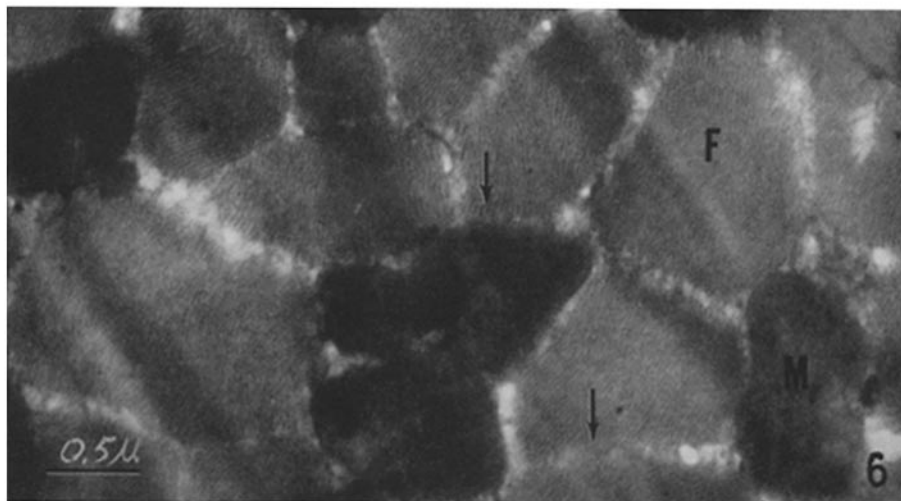
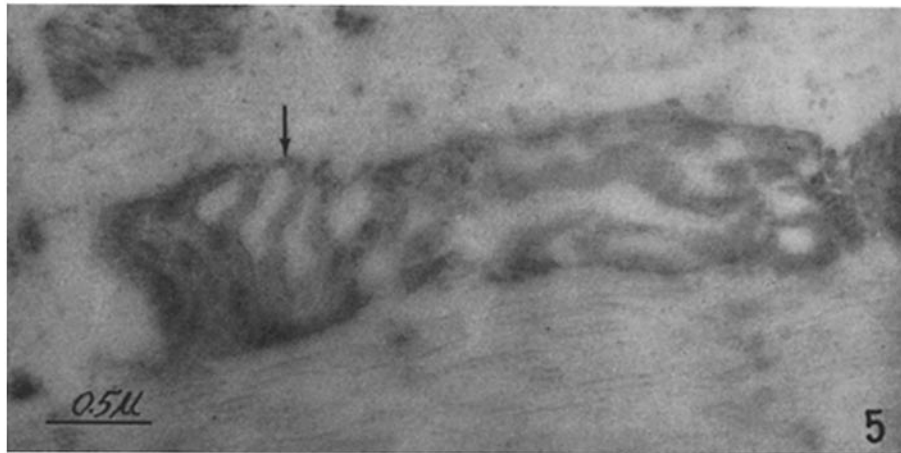
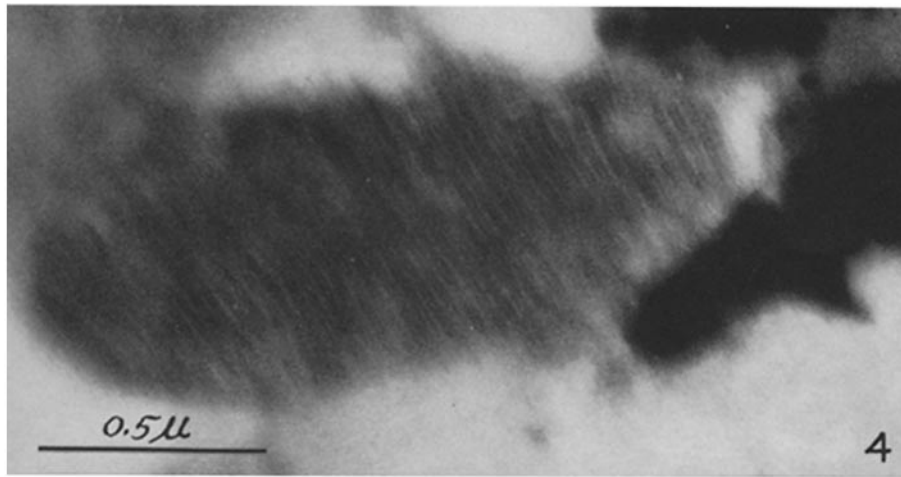
(Weinreb and Harman: Cytochondria and myofibrils)

PLATE 29

FIG. 4. Electron micrograph of a mitochondrion (longitudinal section) demonstrating the absence of both an external limiting "membrane" and interlamellar loops.

FIG. 5. Electron micrograph of a swollen mitochondrion (longitudinal section) showing interlamellar loops (arrow).

FIG. 6. Electron micrograph of pigeon breast muscle (cross-section) showing connecting bridges (arrows) between mitochondria and myofibrils.



(Weinreb and Harman: Cytochondria and myofibrils)