ELECTRON MICROSCOPIC STUDIES ON THE INDIRECT FLIGHT MUSCLES OF *DROSOPHILA MELANOGASTER*

II. Differentiation of Myofibrils

S. AHMAD SHAFIQ, D.Phil.

From the Department of Anatomy, University of Washington, Seattle. Dr. Shafiq's present address is Institute for Muscle Disease, Inc., New York

ABSTRACT

The differentiation of the indirect flight muscles was studied in the various pupal stages of *Drosophila*. Fibrillar material originates in the young basophilic myoblasts in the form of short myofilaments distributed irregularly near the cell membranes. The filaments later become grouped into bundles (fibrils). Certain "Z bodies" appear to be important during this process. The "Z bodies" may possibly be centriolar derivatives and are the precursors of the Z bands. The first formed fibrils (having about 30 thick myofilaments) are already divided into sarcomeres by Z bands. These sarcomeres, however, seem to be shorter than those of the adult fibrils. The H band differentiates in fibrils having about 40 thick myofilaments; the fibrils constrict in the middle of each sarcomere during this process. The individual myofibrils increase from about 0.3 μ to 1.5 μ in diameter during development, apparently by addition of new filaments on the periphery of the fibrils. The ribosomes seem to be the only cytoplasmic inclusions which are closely associated with these growing myofibrils. Disintegration of the plasma membranes limiting individual myoblasts was commonly seen during development of flight muscles, supporting the view that the multinuclear condition of the fibers of these muscles is due to fusion of myoblasts.

INTRODUCTION

The elaborate structure of the striated muscles makes them very suitable material for studies in cytodifferentiation. Already there is considerable literature in the field. Of the various aspects of muscle differentiation, the origin of myofibrils and the origin of striations in them have attracted special attention (26, 28, 15, 10, 21, 14). However, many aspects of these problems still remain largely controversial. It was, therefore, decided to study the origin and differentiation of myofibrils during the development of the flight muscles of *Drosophila melanogaster*. The fine structure of the flight muscles in the adults of *Drosophila* has already been described (23); the present paper deals with an electron microscopic study of their differentiation.

MATERIALS AND TECHNIQUES

Pupae from a wild type laboratory stock of *Drosophila melanogaster* were used. The developing flight muscles from different pupal stages were dissected out in a 2.5 per cent aqueous solution of osmium tetroxide buffered with *s*-collidine (pH 7.4) (1). The muscles were then fixed in the above mixture for about an

hour and embedded in an Epon resin (17). The resin blocks were sectioned on a Porter-Blum microtome and the sections stained with uranyl acetate or lead hydroxide. They were then examined in a RCA EMU 2A or 2C microscope operating on specially designed power supplies.

Tissues embedded in Epon or methacrylate resins and sectioned (at about 1 to 2 μ in thickness) on the Porter-Blum microtome were also used for light microscopy. They were stained with toluidine blue (pH 7–8) or by Brachet's technique (3) for ribonucleic acids.

OBSERVATIONS

The indirect flight muscles develop during the pupal period in *Drosophila*. It is known from several earlier studies (*e.g.*, 24, 26) that the process begins with the accumulation of myoblasts in those regions of the newly formed pupa which correspond to the positions of the different flight muscles of the adult fly. The myoblasts then elongate and become spindle-shaped, produce fibrillar material, and finally fuse to form the muscle fibers. It is also known that differentiation in the myoblasts does not proceed synchronously, so that myoblasts at different stages of development may be found alongside one another. Further, the young fibers have been described as growing by progressive addition of free myoblasts which usually surround such fibers (26). In the present study these last mentioned features of muscle development have been used to identify the young mybolasts (in which the fibrils have not yet developed) from other unspecialized cells of the early pupa. Thus, only the elongated cells either investing the young fibers (as shown, *e.g.*, in Fig. 1) or closely associated with those myoblasts which have clearly acquired fibrillar material (as shown, *e.g.*, in Figs. 2 and 3) were identified as young myoblasts.

The young myoblasts are spindle-shaped cells with a centrally placed nucleus about 6 μ in diameter. The nucleus usually has a prominent nucleolus about 2 μ in diameter (Fig. 1). The cytoplasm of these cells appears strongly basophilic by Brachet's technique. Electron microscopy shows large numbers of granules in the basophilic regions of the cytoplasm. The granules are thus, presumably, ribosomes. They either are associated with the membranes of endoplasmic reticulum or lie free in the cytoplasm (Fig. 3). A few small rod-shaped mitochondria are also noted in the cytoplasm.

Fibrillar material in the form of myofilaments becomes discernible in some myoblasts which otherwise look very similar to the young myoblasts described above. Such first formed filaments can be seen in the micrographs of Figs. 3 and 4. The filaments are about 100 A in diameter and are distributed irregularly in groups near the cell membrane. They all appear to be less than 1 μ in length. Examination of some serial sections of myoblasts at this stage seemed to confirm that the first formed filaments originate close under the cell membrane of the myoblasts, are only about 1 μ in length, and are not arranged in well defined bundles.

A later stage in the development of the myoblasts is seen in Fig. 5. There is now a larger

FIGURE 1

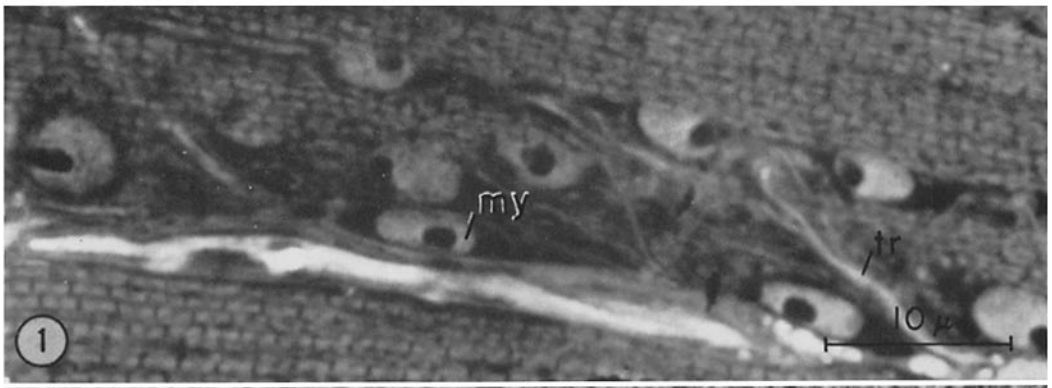
Photomicrograph of a longitudinal section of young fibers of flight muscle from a pupa of *Drosophila*. The fibers have large numbers of striated fibrils in them and are still accompanied by unincorporated myoblasts (*my*). Tracheoles (*tr*) associated with the muscle fibers are also seen. × 2,400.

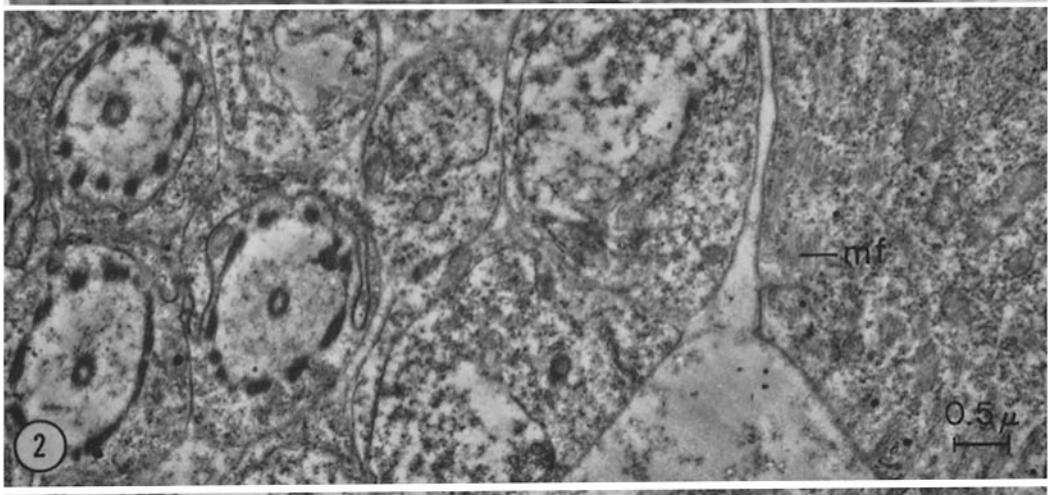
FIGURE 2

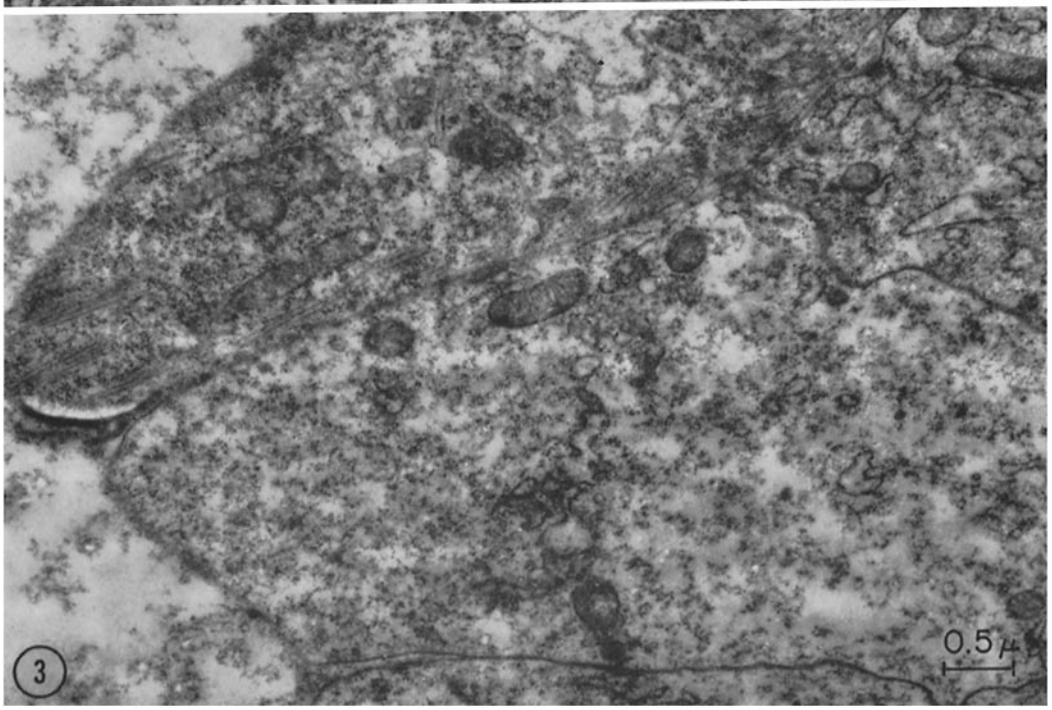
Electron micrograph of a group of young myoblasts. The myoblast on the right shows some of the earliest myofilaments (*mf*). The other myoblasts do not appear to have them. Small rod-like bodies associated with membranes are seen in three myoblasts on the left side of the picture. × 11,300.

FIGURE 3

Three myoblasts at higher magnification. The individual myoblasts are separated from one another by their plasma membranes. The myoblast at the top of the figure shows some of the youngest myofilaments. The myoblasts show a large number of ribosomes either associated with the membranes of endoplasmic reticulum or lying free in the cytoplasm. × 17,000.


my
tr
10 μ
1


mf
0.5 μ
2


0.5 μ
3

number of filaments, but they still appear to be limited to the peripheral parts of the cell. Mitochondria and a large number of ribosomes (about 100 A in diameter) are seen in the cytoplasm. In addition, many dense structures with indistinct boundaries (marked by arrows in Fig. 5) can be seen. These will be called here "Z bodies" since they are precursors of the Z bands. They are seen more clearly in Fig. 6, which is a picture at higher magnification. Groups of myofilaments are seen to be associated with these bodies. It appears as if the filaments were becoming organized into myofibrillar units. The filaments, however, do not show good alignment with one another at this stage.

The earliest stage at which the filaments are distinctly organized in myofibrillar units is seen in Fig. 9. The filaments are at this stage much more symmetrically aligned with one another than at earlier stages; indeed, they appear to have acquired the hexagonal arrangement seen in transverse sections of such fibrils (Fig. 10). It should be noted that these first formed fibrils are not unstriated, but are already periodically divided into sarcomeres by Z bands (Fig. 9). The sarcomeres at this stage are only about 1.5 to 2 μ in length and show only one other broad band between each two Z's. This broad band may represent the future A. Comparison of these fibrils with those of earlier stages seen in Figs. 5 and 6 indicates that the Z bands are derived from "Z bodies." This interpretation is further supported by pictures of sarcomeres developing in late pupae (Fig. 7). The "Z bodies," here, are clearly present in the Z region.

The origin of the "Z bodies" is not clear. There is no evidence of their derivation from the myofilaments themselves. Preliminary observations indicate that they may be derived from certain small rod-like inclusions seen in the young myoblasts (Fig. 2). These rodlets are associated with membranes, and each of them appears to have a fine structure reminiscent of centrioles (Fig. 8).

The first formed myofibrils were mentioned above. They are about 0.3 μ in diameter and appear to consist of about 30 myofilaments of the thick type. These filaments present a hollow appearance in transverse sections (Fig. 10) similar to that of the thick filaments of the adult muscles (23). Some micrographs indicate that the thin filaments are also present in the first formed fibrils, but their development could not be studied satisfactorily.

It may be mentioned here that while the fibrils are forming in the myoblasts, changes in their plasma membranes also take place. They no longer appear as continuous membranes limiting individual cells (as shown, *e.g.*, in Fig. 3 or 4), but are now seen as broken irregular elements (Fig. 14). Ultimately, even these remnants disappear, indicating a complete fusion of myoblasts to form syncytial muscle fibers.

Observations on the later history of the development of fibrils relate to the origin of H and M bands and to the growth of the fibrils.

The H band in the muscles of adults of *Drosophila* was seen earlier (23) to be the region of the sarcomere where the thick myofilaments showed a homogeneous density in electron micrographs rather than the "hollow" appearance seen along the remainder of their length in the A band. This differentiation of the thick filaments apparently occurs when the fibrils have grown to about 0.5 μ in diameter and show about 40 thick myofilaments in them. The fibrils now become slightly constricted in the middle (Fig. 11) and the thick

FIGURE 4

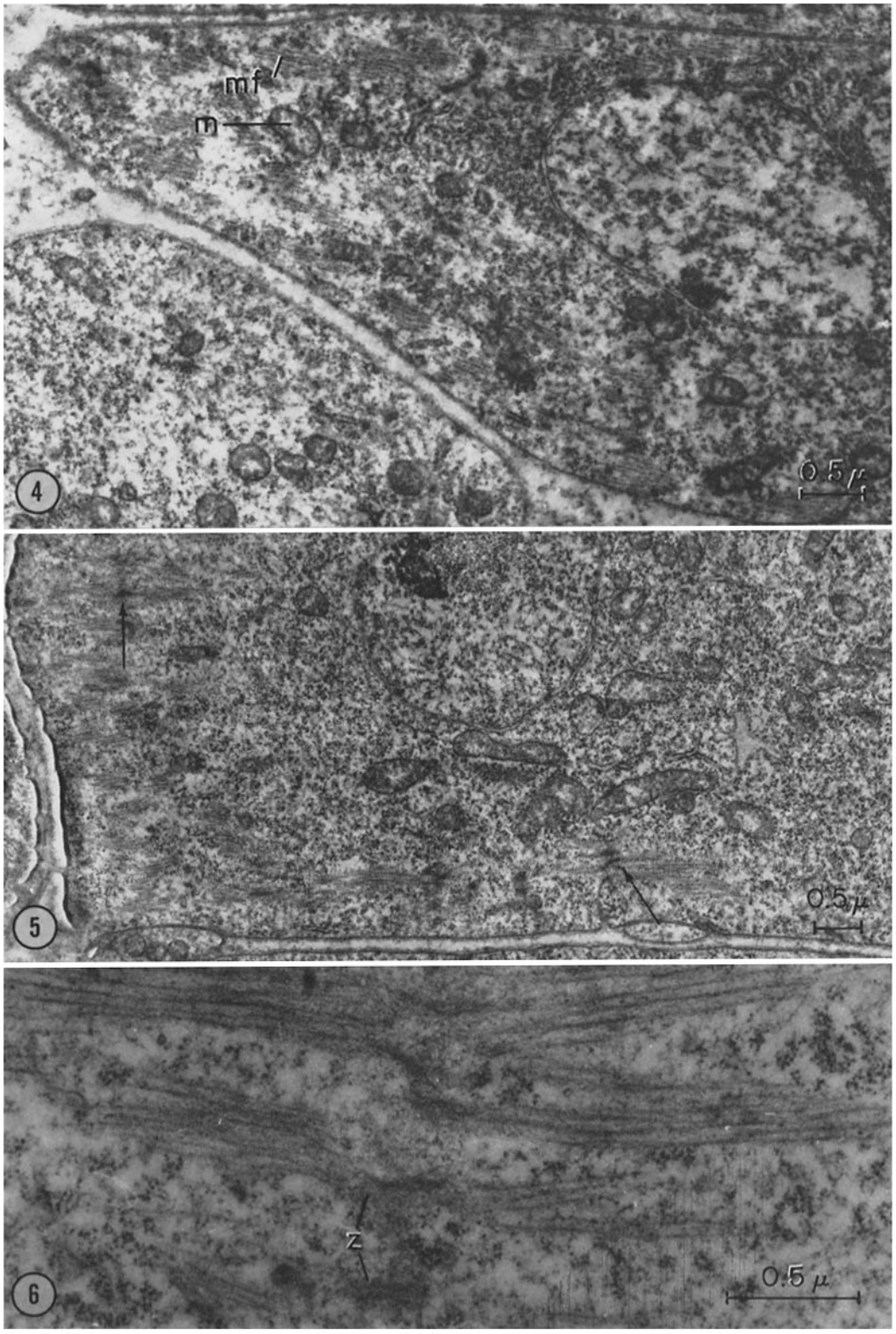
A myoblast showing that the youngest myofilaments (*mf*) appear as short threads distributed irregularly near the cell membrane. In the cytoplasm, mitochondria (*m*) and ribosomes are also seen. × 17,000.

FIGURE 5

A more advanced stage in the development of the myoblasts. The myofilaments have increased in number and appear to have become associated with the "Z bodies" (arrows) from which the Z bands will arise. × 11,000.

FIGURE 6

Higher magnification picture from a myoblast at about the same stage as in Fig. 5, showing association of myofilaments with the "Z bodies" (*z*). × 40,000.


mf
m
0.5 μ
4
0.5 μ
5
z
0.5 μ
6

filaments seem to acquire the characteristic uniform electron opacity in these regions (Fig. 12).

The M band of the adult muscle was described as the region in the middle of the sarcomere which showed a variable number of dense granules (23). In the developing fibrils such a distribution of granules could be recognized only after the fibrils developed beyond the stage at which the H band differentiates (as shown, *e.g.*, in Fig. 13).

The growth of fibrils appears to involve an increase in both the length and the diameter of the sarcomeres. Thus, the sarcomere length changes from 1.5–2 μ in first formed fibrils (Figs. 9 and 14) to about 3 μ in later stages (Fig. 13), and the diameter from 0.3 μ in the youngest fibrils to 1.5 μ in the adult stage (Fig. 16). The increase in diameter seems to be due to gradual additions of new myofilaments to the growing fibrils. The new filaments are probably added only on the periphery of the fibrils, as the central parts of the growing fibrils always show a hexagonal pattern (as shown, *e.g.*, in Figs. 10, 12, and 15). The distance between the filaments, however, seems to decrease during development (*cf.* Figs. 12, 15, and 16), so that the filaments of the adult fibrils seem to be more compactly arranged than those of the younger stages. It may also be mentioned that the ribosomes described earlier sometimes appeared in greater concentration immediately around the growing fibrils than elsewhere in the sarcoplasm (Fig. 15). Thus, it may be that the 100 A granules identified here as ribosomes are important in the formation of new myofilaments.

DISCUSSION

The problems of origin and development of fibrils and multinucleation of muscles may now be discussed with reference to the present findings on *Drosophila*.

The origin of myofibrils has been a subject of considerable speculation. Some of the opinions on the topic may be given here. Thus, Heidenhain (12) proposed that the fibrils develop from submicroscopic elements which are themselves derived from self-dividing "protomeres." Meves (19), Duesberg (7), and Levi and Chevremont (16) among others, however, thought that the fibrils originate from the mitochondria. Still others (*e.g.*, 9, 18, 11, 27, 26, 20) are of the opinion that certain "cytoplasmic granules" line up in a row and fuse to form the fibrils. Moscona (20) described these granules as being about 0.8 to 2.2 μ in diameter and containing polysaccharide and ribonucleoprotein. Finally, Holtzer *et al.* (15) deem the cell surface to have a special role in the formation of fibrils.

In the present study no association between the development of fibrils and mitochondria or the

FIGURE 7

Sarcomeres of an unidentified muscle developing in a late pupa. The "Z bodies" (arrows) are clearly distributed in the Z region of the fibrils. × 18,000.

FIGURE 8

Higher magnification picture of the young myoblasts seen in Fig. 2. The rod-like bodies have a fine structure resembling that of centrioles. × 28,000.

FIGURE 9

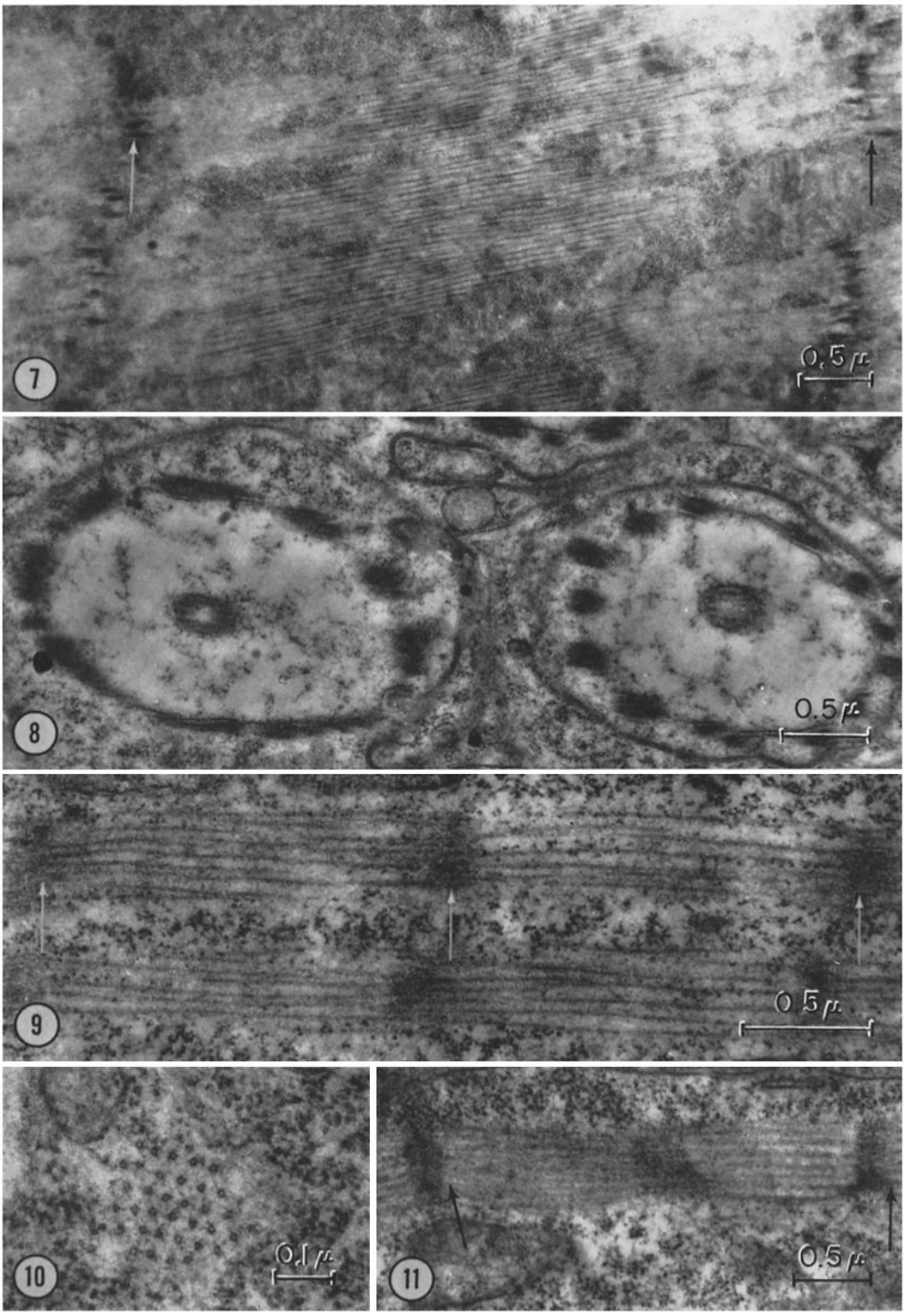
Longitudinal section showing the earliest stage at which the myofilaments become arranged into myofibrils. The fibrils are already divided into sarcomeres, two of which are seen in their entirety in each of the myofibrils in the figure. The Z bands are indicated by arrows. × 40,000.

FIGURE 10

Transverse section of a young myofibril showing about 35 thick filaments. The filaments have the characteristic "hollow" appearance and appear to be arranged in hexagonal pattern. × 94,000.

FIGURE 11

A more advanced stage in the development of myofibrils. The one shown appears to be slightly constricted in the middle of the sarcomere. The Z bands are indicated by arrows. × 25,000.


7
0.5 μ
8
0.5 μ
9
0.5 μ
10
0.1 μ
11
0.5 μ

granules described by Moscona (20) was found. The fibrils appear to originate by aggregation of the submicroscopic filaments as envisaged by Heidenhain and noted in the electron microscopic studies of muscle development by van Breemen (4) and Hibbs (13). As regards the involvement of cell surface during the development of fibrillar material (15), it may be mentioned that the first formed filaments do apparently originate close to the cell membrane, but this does not seem to be the case for the myofilaments which develop later. These appear to originate on the periphery of the growing myofibrils, and only the 100 A granules (which are probably ribosomes) seem to be associated with them.

The origin of striations has also been a controversial subject. One popular view appears to be that the first formed myofibrils are homogeneous and acquire striations only later in their development (26, 10, 8, 2, 21, 22). Hibbs (13), however, from electron microscopic study of the development of the cardiac muscle of the chick concluded that the Z bands appear concurrently with or shortly after the orientation of the myofilaments to form loose bundles (fibrils). In the current study of *Drosophila* an unstriated stage in the development of the fibrils could not be found. The first formed fibrils are already divided into sarcomeres by the Z bands; in fact, the latter are perhaps responsible for grouping the myofilaments into fibrillar units. There is an indication that the Z band may be a centriolar derivative, but a more detailed study of the problem is required. In this connection it is of interest that Wolbach (30) has already described divisions of centrioles to produce a large number of granules during myogenesis in tumors of human striated muscle.

The mode of development of the multinuclear condition of muscle fibers is also a disputed problem. Some workers (*e.g.*, 25, 27, 21, 10) believe that the nuclei of the myoblasts divide (probably by amitosis) without a separation of daughter cells, leading to a coenocytic condition. Others (*e.g.*, 26, 29, 5, 14, 6) believe that the multinuclear condition arises mainly by fusion of individual myoblasts. The extensive disintegration of the plasma membranes limiting the myoblasts seen in the present study of *Drosophila* development supports the latter view.

I wish to thank Professor H. S. Bennett for accommodating me in the Department of Anatomy and for his many suggestions and his guidance in the preparation of manuscript. I am also very grateful to Dr. R. L. Wood for his kind help and advice during the course of this work.

Received for publication, August 27, 1962.

Bibliography on page 372

FIGURE 12

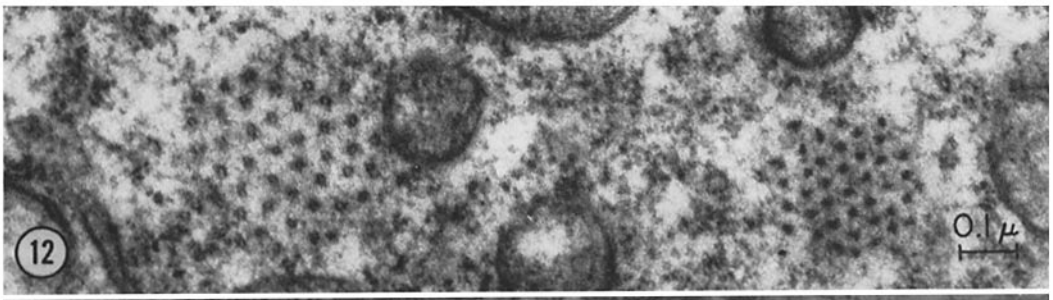
Transverse section of fibrils at about the stage of Fig. 11. The fibril on the left is cut at the level of an A band, showing the typical "hollow" appearance of the myofilaments, the fibril on the right, which has about the same number of filaments (approximately 40), is cut at the H level. The filaments here appear to be more uniformly dense. × 72,500.

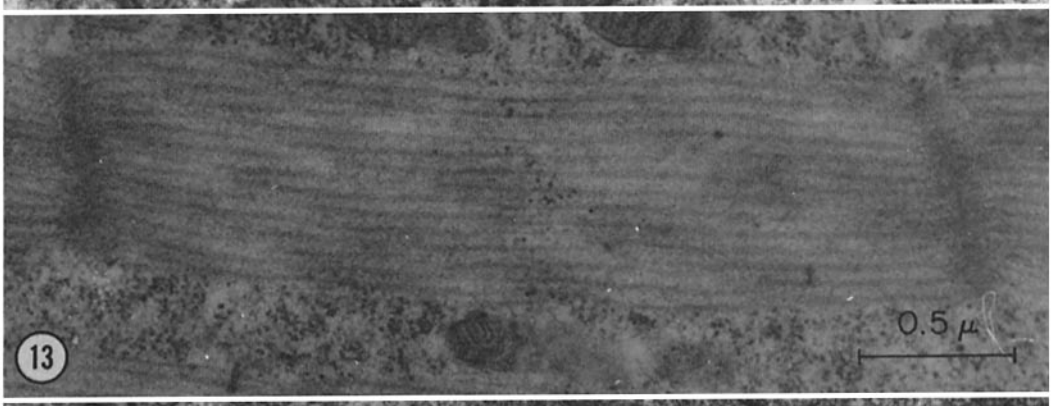
FIGURE 13

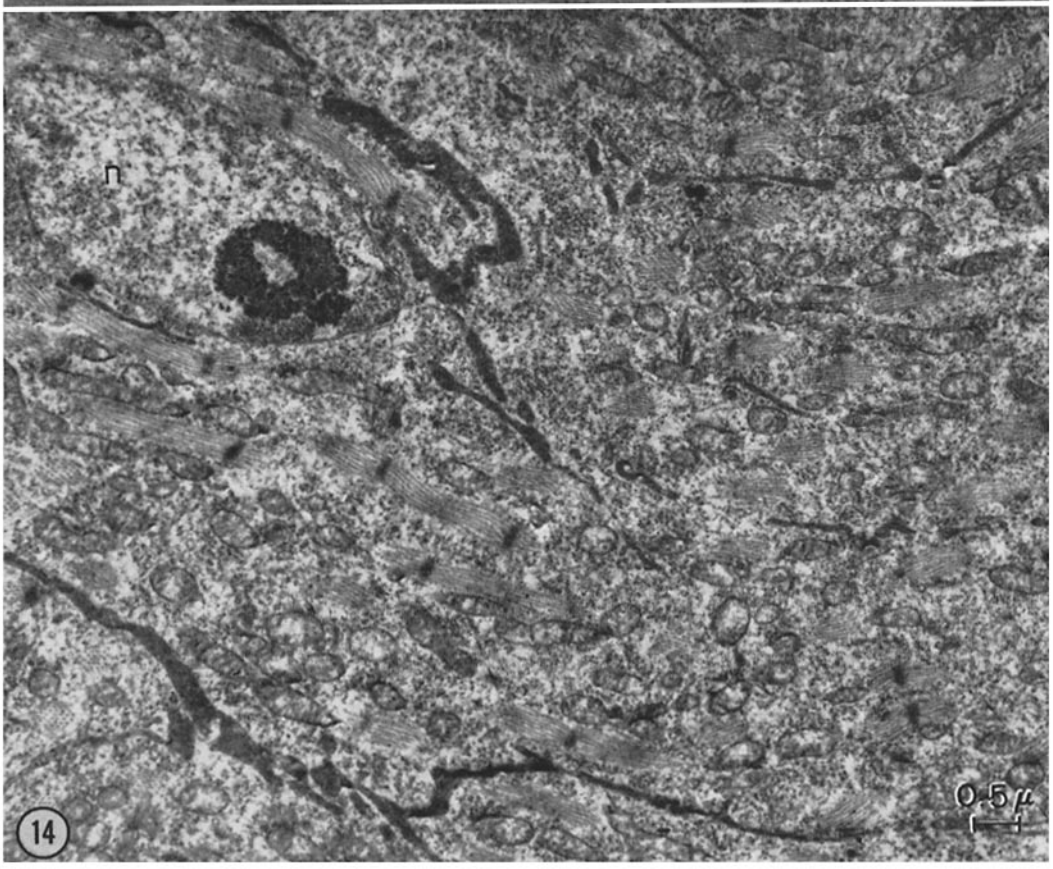
Longitudinal section of a fibril which has grown thicker than those in Figs. 9 and 11. The granules in the M band are now seen. × 40,000.

FIGURE 14

Section showing the disintegration of the plasma membranes as the myoblasts fuse. A nucleus (*n*) with a prominent nucleolus is also seen. The nucleolus appears to have a dense outer part and a lighter inner part. Such a structure of the nucleolus was seen in the various developmental stages only and not in the adult stage. × 11,000.


12
0.1 μ


13
0.5 μ


n
14
0.5 μ

BIBLIOGRAPHY

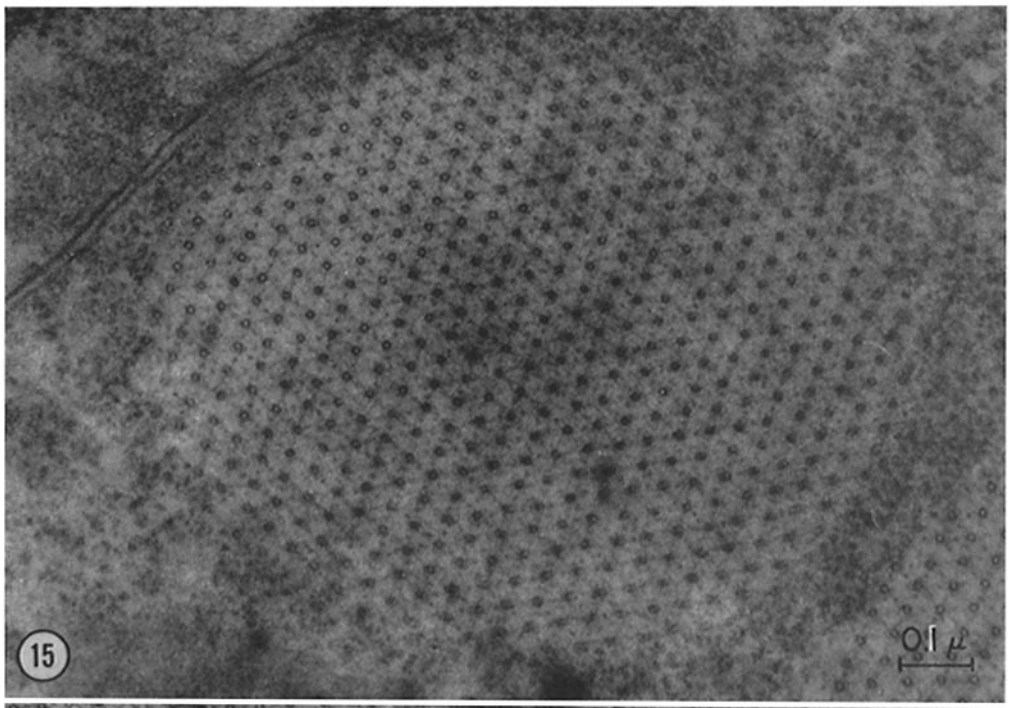
1. BENNETT, H. S., and LUFT, J. H., *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 113.
2. BOYD, J. D., *in* The Structure and Function of Muscle, (G. H. Bourne, editor), New York, Academic Press, Inc., 1960, **1**, 63.
3. BRACHET, J., *Quart. J. Micr. Sc.*, 1953, **94**, 1.
4. VAN BREEMEN, V. L., *Anat. Rec.*, 1952, **113**, 179.
5. CAPERS, C. R., *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 559.
6. COOPER, W. G., and KONIGSBERG, I. R., *Anat. Rec.*, 1961, **140**, 195.
7. DUESBERG, J., *Arch. Zellforsch.*, 1910, **4**, 602.
8. FERRIS, W., *Anat. Rec.*, 1959, **133**, 275.
9. GODLEWSKI, E., *Arch. mikr. Anat.*, 1902, **60**, 111.
10. GODMAN, G. C., *in* Frontiers in Cytology, (S. L. Palay, editor), New Haven, Yale University Press, 1958, 381.
11. HAGGQVIST, G., *Anat. Anz.*, 1920, **52**, 389.
12. HEIDENHAIN, M., *in* Handbuch der Anatomie des Menschen, (K. V. Bardeleben, editor), Jena, Gustav Fischer, 1911, 507.
13. HIBBS, R. G., *Amer. J. Anat*, 1956, **99**, 17.
14. HOLTZER, H., *in* Synthesis of Molecular and Cellular Structure, (D. Rudnick, editor), New York, Ronald Press Co., 1961, 35.
15. HOLTZER, H., MARSHALL, J. M., and FINK, H., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 705.
16. LEVI, G., and CHEVREMONT, M., *Arch. biol.*, 1941, **52**, 523.
17. LUFT, J. H., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
18. MCGILL, C., *Anat. Rec.*, 1910, **4**, 23.
19. MEVES, F., *Anat. Anz.*, 1907, **31**, 399.
20. MOSCONA, A., *Exp. Cell Research*, 1955, **9**, 377.
21. MURRAY, M. R., *in* The Structure and Function of Muscle, (G. H. Bourne, editor), New York, Academic Press, Inc., 1960, **1**, 111.
22. PICKEN, L., The Organisation of Cells and Other Organisms, Oxford, Clarendon Press, 1960, 245.
23. SHAFIQ, S. A., *J. Cell Biol.*, 1963, **17**, 351.
24. SHATOURY, H. H. EL, *J. Embryol. and Exp. Morphol.*, 1956, **4**, 228.
25. TELLO, J. F., *Z. Anat. u. Entwicklungsgesch.*, 1922, **64**, 348.
26. TIEGS, O. W., *Phil. Tr. Roy. Soc. London, series B*, 1955, **238**, 221.
27. WEED, I. G., *Z. Zellforsch. u. mikr. Anat.*, 1936, **25**, 516.
28. WIGGLESWORTH, V. B., *Quart. J. Micr. Sc.*, 1956. **97**, 465.
29. WILDE, C. E., *in* Cell, Organism and Milieu, (D. Rudnick, editor), New York, Ronald Press Co., 1959, 3.
30. WOLBACH, S. B., *Anat. Rec.*, 1928, **37**, 255.

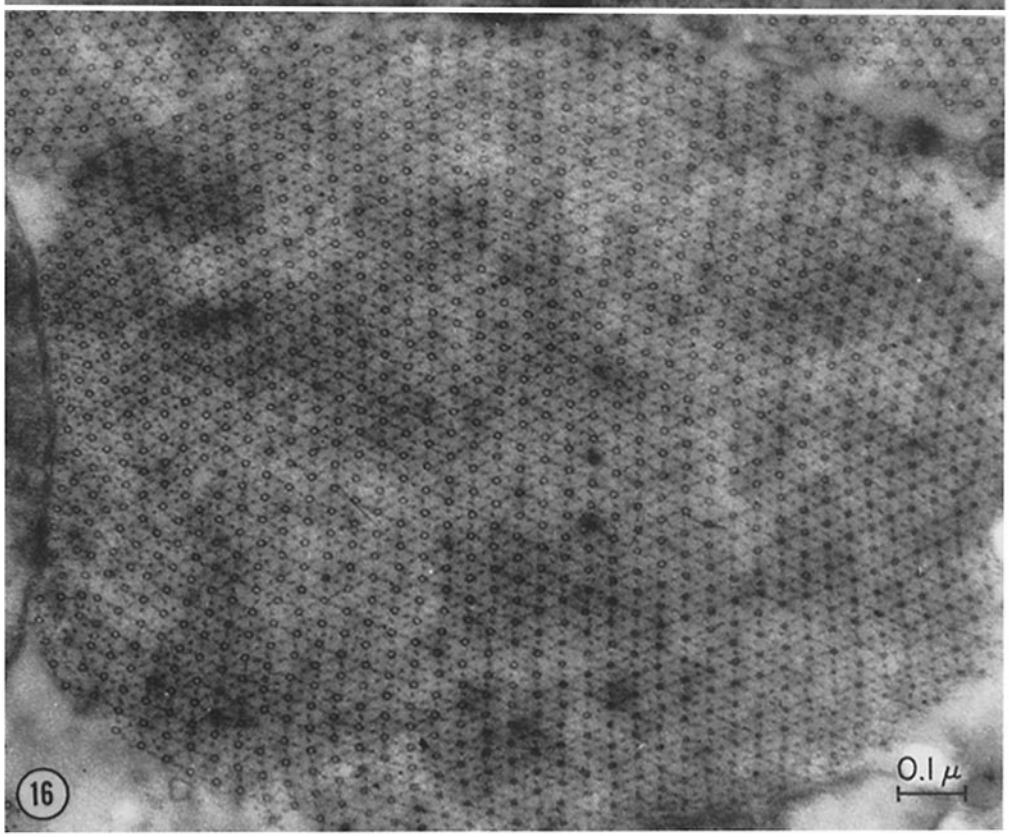
FIGURE 15

A more advanced stage in the growth of the myofibril. The fibril now has about 500 thick filaments and is surrounded by particles 100 A in diameter (identified here as ribosomes). × 94,000.

FIGURE 16

Transverse section of a fibril of the adult muscle. The fibril has about 1500 thick myofilaments arranged in the characteristic hexagonal pattern. × 94,000.


15
0.1 μ


16
0.1 μ