STUDIES ON THE STRUCTURE OF MUSCLE

III. Phase Contrast and Electron Microscopy of Dipteran Flight Muscle*

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A considerable literature relating to the electron microscopy of muscle has appeared in recent years.[1] This has been largely concerned with the examination of fragmented fixed muscle (13–15, 19, 22, 29, 45) and of isolated myofibrils of fresh muscle (2, 3, 40). More recently, however, a number of papers have appeared on the application of thin sectioning techniques to the problem of muscle structure (11, 24, 26, 27, 32, 41, 46, 61). Little work has been reported on insect muscle (19, 26, 27) during this period, but interest in it has been stimulated recently by work on the sarcosomes (20, 53, 58) which are especially abundant in the highly specialized flight muscles of the Diptera.

Some preliminary results on the fine structure of flight muscle have already been described (27), while a more detailed study using phase contrast and electron microscopy has been presented recently (26). Hanson (23) described briefly the changes in band pattern during contraction induced by adenosinetriphosphate (ATP) in isolated myofibrils of both vertebrate and insect (*Dytiscus*) muscle. More recently, Huxley and Hanson in a series of papers on vertebrate striated muscle (24, 32–34) have put forward a model of the structure involving two distinct arrays of filaments in the A band, and have indicated a possible distribution of actin and myosin in terms of their picture.

The myofibrils in the flight muscles of the blowfly are large and exceptionally uniform in diameter, and are easily dispersed to yield suspensions suitable for phase contrast or polarization optical studies, especially of the changes accompanying contraction induced by ATP. The results obtained in the present studies appear to differ from the conclusions of Huxley and Hanson in that only a single array of myofilaments has been observed. The myofilaments run

* References 14 and 19 are to be regarded as parts I and II respectively of this series.

[1] The literature on muscle is enormous, and for this reason, references in the present paper will be confined mainly to relatively recent work. References to biochemical and physiological aspects, and to earlier histological and polarization optical studies can be found in many publications (*e.g.*, references 6, 8, 9, 50, 55, 59, and 60).

continuously through all bands of the sarcomere in both fresh and glycerinated flight muscle, both before and after application of ATP. The recently published observations of Aloisi *et al.* (1) and Szent-Györgyi *et al.* (57) on vertebrate muscle appear to be more consistent with the type of structure described in the present paper than with the interdigitating model of Huxley and Hanson. However, it must be emphasized that the present results do not necessarily apply to the relatively non-specialized skeletal muscles of insects or vertebrates.

Materials and Methods

The main source of flight muscle was the blowfly, *Lucilia cuprina*.[2] However, similar results have been obtained from other flies including the housefly, *Musca*. Both fresh and glycerinated muscle (55) were used. Flies for examination were usually sacrificed several days after emergence. They were rendered inactive by placing in the refrigerator for a few minutes, then rapidly slit in the median sagittal plane with a safety razor blade. The two halves of the thorax thus obtained, containing the thoracic musculature in an essentially intact state and approximately at rest length, were then either placed in 50 per cent (*v/v*) glycerol in the cold or in fixative for appropriate periods of time.

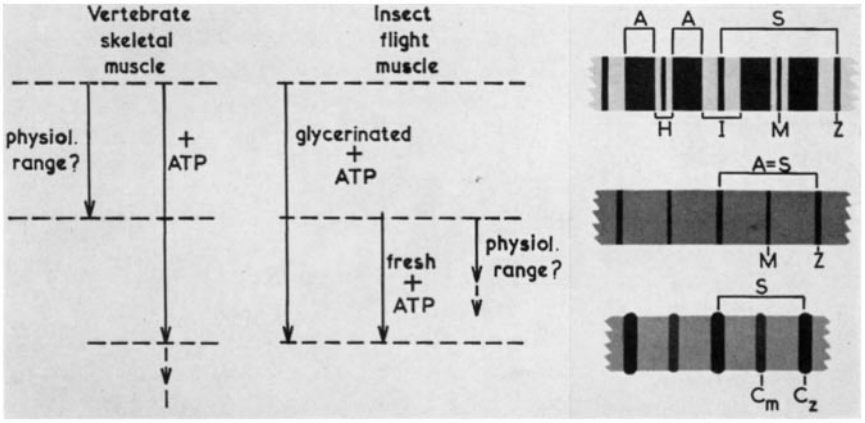
Initial fixation of the fresh or glycerinated muscle was usually in 1 per cent osmium tetroxide buffered to pH 7.4 with acetate-veronal buffer (38). In most cases, this was followed by staining in 1 per cent phosphotungstic acid (PTA), either in unbuffered aqueous or 95 per cent ethanol solution. Specimens were embedded in *n*-butyl methacrylate (37) after dehydration in an ethanol series, and ultrathin sections for electron microscopy were then obtained using the method described by Hodge, Huxley, and Spiro (28). Sections were examined in the electron microscope (RCA model EMU-1) without removal of the plastic and using a 25 μ externally centerable objective aperture (18). The instrumental magnification was calibrated by an interferometric method (17).

Suspensions of myofibrils for phase contrast examination were conveniently obtained from both fresh and glycerinated flight muscle by gentle trituration of small pieces of muscle in suitable media. The muscle, which appeared to lack strong connections at the Z band level, was easily dispersed to yield a suspension containing many single myofibrils as well as larger fibre fragments and large numbers of sarcosomes. In most cases, the medium was an aqueous solution containing 0.1 M KCl, 0.001 M $MgCl_2$, but some suspensions were made up of the glycerinated material in 15 or 50 per cent glycerol. Drops of suspension were examined at room temperature under coverslips, and the myofibrils irrigated with various media by drawing the fluid through with small pieces of filter paper (23). It was found advantageous to cement the coverslip to the slide at several points with paraffin wax in order to minimize drifting of the focus during irrigation. Media investigated included 0.1 M KCl, 0.001 M $MgCl_2$ (solution A), in which myofibrils could be maintained for considerable periods without apparent physical deterioration or loss of response to ATP, Guba-Straub solution (0.3 M KCl, 0.15 M phosphate buffer, pH 6.5), and Hasselbach-Schneider solution (0.47 M KCl, 0.01 M pyrophosphate, 0.1 M phosphate buffer, pH 6.4) containing 0.001 M $MgCl_2$ as used by Hanson and Huxley (24). The above solutions were also prepared with admixtures of Na ATP[3] (usually between 10^{-3} and 10^{-6} M).

[2] I am indebted to Dr. M. F. Day, Division of Entomology, Commonwealth Scientific and Industrial Research Organization, Canberra, for a generous supply of pupae.

[3] Kindly supplied by Dr. R. K. Morton, Biochemistry Department, University of Melbourne.

Suitable myofibrils were selected under phase contrast conditions using an oil immersion objective (N.A. 1.35) and photographed both before and after irrigation at room temperature with the various media. By selecting fields containing free ends of myofibrils, it was possible to obtain unambiguous data concerning the changes in the band pattern occurring on exposure to ATP. In many instances myofibrils were photographed between crossed polaroids both before and after irrigation in order to compare the distribution of birefringence with the corresponding phase contrast band patterns.



TEXT-FIG. 1. Diagram to illustrate the three main types of band pattern observed in both blowfly flight muscle and vertebrate skeletal muscle under phase contrast. Intermediate stages are also commonly observed. The changes observed on exposure of myofibrils to ATP are summarized at left, the diagram illustrating the range of action under various conditions.

RESULTS

1. Phase Contrast

Fresh Muscle.—Freshly isolated myofibrils suspended in solution A or 15 per cent glycerol are characterized by a very simple band pattern (Fig. 3 *a*) in which only the Z bands (and occasionally the M bands) can be identified with certainty. The A substance is distributed uniformly throughout the sarcomere. This type of band pattern is observed in vertebrate striated muscle only in myofibrils which are partly contracted (*e.g.* reference 14, Fig. 5). On irrigation with solution A or 15 per cent glycerol containing ATP, there is a marked change in the band pattern of most of the myofibrils. The A substance migrates rapidly (less than 1 second) to form dense contraction bands at the Z band level (Fig. 3 *c*) and somewhat less dense bands also appear at the M band level. It seems desirable to name these bands specifically, and it is therefore proposed that the dense regions forming on contraction at the Z and M bands be designated as the C_z and C_m bands respectively (see Text-fig. 1). Fig. 3 *b* shows a myofibril in a slightly less contracted form. It is clear from the marked clearing of the sarcomere which accompanies the formation

of the C_z and C_m bands (Figs. 3 *a*, *b*, *c*) that contraction (at least that induced by ATP *in vitro*) is definitely associated with a migration of refractile material, thus confirming in general the striation reversal observed by Jordan (35) and others (*e.g.* 22, 16). In contrast to the behaviour of suspensions of vertebrate myofibrils, which shorten greatly on ATP addition unless restrained (3, 48), free myofibrils of blowfly flight muscle shorten only to about 90 per cent of rest length, a peculiarity which is probably related to their specialized function *in vivo*.

Glycerinated Muscle.—The majority of myofibrils from glycerinated flight muscle display a band pattern (Figs. 1 *a*, 2 *a*, 3 *d*, 3 *e*) generally regarded as typical of the relaxed state in vertebrate striated muscle, the Z, A, I, and H bands being clearly recognizable (see Text-fig. 1). This suggests that the fine structure of the flight muscle is probably not fundamentally very different from that of vertebrate striated muscle. On irrigation with ATP-containing media at room temperature, glycerinated myofibrils undergo rapid changes in the band pattern and shorten by up to 25 per cent. Figs. 1 *a* and *b* show a typical field of such fibrils before and after exposure to ATP, the changes in band pattern observed in unrestrained myofibrils being clearly illustrated. There is a definite migration of the refractile A substance to the Z bands, where it forms dense accumulations (C_z bands). In some instances, the migration of the A substance seems to be incomplete (Figs. 3 *f* and *g*). However, in most cases, the myofibril contraction is accompanied by almost complete migration yielding a typical contracted band pattern (Fig. 3 *h*) identical with that found in ATP-treated fresh myofibrils (Fig. 3 *c*). On prolonged standing, some shrinkage takes place as the medium dries, and the band pattern becomes more prominent (Fig. 3 *i*). Myofibrils which are unable to shorten appreciably because of their adhesion to the coverslip behave in a significantly different manner when exposed to ATP. Figs. 2 *a* and *b* show such a fibril before and after irrigation with ATP-containing medium. There is clearly an accumulation of material at the Z bands together with formation of the C_m band, and the density of the A band has been reduced. Despite these changes, the I band has persisted, thus indicating that migration of the A substance is possible across the I band. This observation seems incompatible with the interdigitating filament model of Huxley and Hanson (24, 32, 34) (see Discussion).

Myosin Extraction.—Attempts to observe the changes in band pattern on selective removal of myosin by the methods used by Hanson and Huxley (24) on vertebrate myofibrils were uniformly unsuccessful, both for the fresh and glycerinated myofibrils of flight muscle. This is perhaps not surprising since the myosin[4] of insect flight muscle is known to be difficult to extract (21).

[4] It is here assumed that insect flight muscle contains components similar to those found in vertebrate skeletal muscle; *i.e.*, actin, myosin, tropomyosin, etc. Unfortunately, relatively

Irrigation of suspensions with Hasselbach-Schneider solution or with Guba-Straub solution in the absence of ATP produced no apparent changes in band pattern. However, in the latter solution, some swelling occurred and the myofibrils became fainter on prolonged exposure. While it is possible that some slow extraction of the myosin was taking place, it seems significant that such myofibrils would still contract with typical band changes on exposure to ATP. On irrigation with Guba-Straub solution + ATP as used by Hanson and Huxley (24), the changes in band pattern were identical with those resulting from exposure to either solution A plus ATP or 15 per cent glycerol containing ATP; *i.e.*, a rapid migration of A substance and the formation of C_s and C_m bands. It seems likely, therefore, that the changes observed may be ascribed almost solely to the presence of ATP in the media, with no significant extraction of myosin taking place. A clear need exists for the development of solutions suitable for selective extraction of flight muscle components, so that comparisons can be made with the results of such experiments on vertebrate muscle (25, 57, 1).

2. Polarization Optical

The changes in birefringence (BR) of the myofibrils on addition of ATP were correlated with those observed under phase contrast conditions. The results obtained were in many respects similar to those of Engelmann (16) and Schmidt (see 49). The distribution of birefringence in a typical relaxed fibril, having a band pattern of the type shown in Fig. 3 *d*, is illustrated in Fig. 3 *j*. As expected, the A band is bright and the I and H bands dark. After ATP-induced contraction (Fig. 3 *k*), despite the striking shift of refractile material to form the C_s and C_m bands evident under phase contrast, the birefringence pattern is still strikingly similar to that of the relaxed myofibril; *i.e.*, the sarcomere is bright except for the Z and M regions (C_s and C_m bands). The magnitude of the birefringence is not greatly reduced, a seemingly reasonable result in view of the fact that the shortening does not exceed *ca.* 20 per cent. It is of interest to note that the accumulation of A substance (which is thought to be mainly responsible for the BR of the A band) in the C_s and C_m bands does not result in strong BR in those regions, but rather the reverse. If in the relaxed myofibril the A substance exists as an oriented fibrous material, it would seem to follow that after contraction it must be present in the C bands in a disordered, folded, or coiled state.

3. Electron Microscopy

Fresh Muscle.—The field shown in Fig. 4 is fairly typical for transverse sections of fresh flight muscle fixed in buffered osmium tetroxide without fur-

little is known concerning the properties of these components except in the case of rabbit and frog muscle (and some others), on which the bulk of reported work has been carried out.

ther staining. A high proportion of the fibre cross-section is occupied by the myofibrils and sarcosomes which are immersed in a granular matrix (the cytoplasm or sarcoplasm), the whole being enclosed by a thin limiting membrane, the sarcolemma, which is devoid of connective tissue fibrils such as those investing the sarcolemmata of vertebrate skeletal muscle fibres.

The tracheal system of flight muscle is of some interest because of the very high energy turnover characteristic of this type of muscle, and has been investigated by both thin sectioning and isolation procedures. In *Lucilia*, large tracheae or tracheoles (the distinction seems rather arbitrary) run the length of the fibre, often just beneath the sarcolemma, and give off major branches, which turning inwards, branch repeatedly. The finest tracheolar branches (0.1 to 0.2 μ diameter) ramify throughout the cytoplasm and end blindly. They have not been observed to penetrate either the myofibrils or sarcosomes. The tracheolar system can readily be isolated by mild maceration or proteolytic procedures such as those employed by Richards and Anderson (43, 44), and is then amenable to study by the conventional method of drying on to a grid. The appearance of such a preparation in the electron microscope is illustrated in Fig. 7. The tracheal system is characterized throughout its ramifications by the presence of regularly spaced thickenings or folds of the epicuticle, the taenidia, which, in *Lucilia* at least, are annular and not helical. The finest tracheolar branches, a number of which are visible in the upper part of Fig. 7, exhibit taenidia, right up to the point where they end blindly. The lumina of a number of tracheoles are visible in Fig. 4. The smaller ones are distinguishable only with difficulty from elements of the endoplasmic (sarcoplasmic) reticulum (11) which also appear in the sarcoplasm.

Further information concerning the fine structure of the tracheoles can be derived from thin sections. In the finer branches, the taenidia appear to arise by simple folding of the thin epicuticle. However, in the larger tracheoles and in the tracheae, there is present within these folds a dense material, presumably exocuticular (62), so that the taenidia here are in the form of exocuticular annuli girdling the epicuticular tube. Fig. 8 gives some indication of this, and in addition shows that the epicuticle, at least in the larger tracheoles and tracheae, has a system of fine longitudinal thickenings or folds. These, together with taenidia, are also present in the walls of the large air sacs of *Lucilia*.

The sarcosomes appear as rather dense bodies of irregular rounded shape. In transverse section (Fig. 4), they often display a quite regular array of circular regions of lower density. These may represent longitudinal pores or channels, which possibly serve to increase the effective surface area of the sarcosomes, or to facilitate passage of the reactants and products of numerous enzymic processes known to be localized in these bodies (20, 47, 58). It is possible that diffusion is aided by active mechanical pumping through these

channels, since the sarcosomes are probably compressed laterally during contraction. Each sarcosome is limited by a thin apparently single membrane (Fig. 5), the internal space being densely packed with double membrane structures similar to those described by Sjöstrand (51), Palade (39), and Spiro (53). However, the packing density is much higher than for other types of mitochondria so far described in the literature, a factor, which together with the relatively very high volume fraction occupied by the sarcosomes, probably reflects the very high activity of the flight muscle.

As has already been shown elsewhere (26, 27), the myofibrils of insect flight muscle comprise a regular hexagonal array of myofilaments, about 100 A in diameter and spaced about 300 A apart in the fixed and sectioned material. A comparison of the diameters of fresh myofibrils under phase contrast and in electron micrographs of transverse sections indicates that the spacing in the living state may be as high as 600 A. In the present work, further details of myofibrillar fine structure have been obtained by examining sections of muscle treated with PTA to improve the contrast conditions. Fig. 6 shows a typical longitudinal section of fresh flight muscle treated with PTA after fixation in buffered osmium tetroxide. The myofilaments are evenly spaced and run continuously throughout the sarcomere. The continuity of the myofilaments through the Z band has already been demonstrated (26), and may be just discerned in Fig. 6. The wavy pattern of alternating light and dark regions has been described as pseudostriation (27). In Fig. 6, the section is of such a thickness that the dark regions represent areas in which two consecutive layers of the myofilament lattice are included simultaneously within the thickness of the section. The lighter regions contain only a single layer. In still thinner sections, regions devoid of myofilaments alternate with areas containing a single layer. From simple geometrical considerations, it is clear that the disposition of the pseudostriations is a representation of the orientation of the myofilament lattice with respect to the plane of the section. The Z and M bands are clearly visible, the former appearing as dense transverse continua penetrated by the myofilaments, the latter as discrete accumulations of dense material on the myofilaments, having little if any transverse continuity, and situated in the centre of the rather light and narrow H band. The band pattern is essentially one of uniform density throughout the sarcomere, except for the Z, M, and H bands, and corresponds with that visible in freshly isolated myofibrils under phase contrast conditions (Fig. 3 *a*). The I band is usually absent or very narrow in fresh flight muscle, the A substance being uniformly distributed through the sarcomere, but absent from the H band. In Fig. 6, the extremely narrow I bands are just detectable as regions of lower density immediately adjacent to the Z bands.

Figs. 9 to 14 illustrate the appearance in transverse section of the same preparation of fresh flight muscle; *i.e.*, fixed in buffered osmium tetroxide fol-

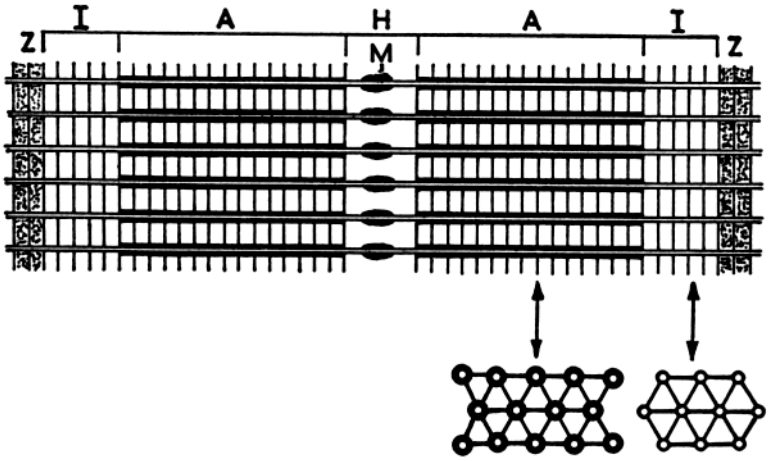
lowed by PTA treatment. This treatment in general has markedly deleterious effects on the structure of the cytoplasm and sarcosomes, particularly if carried out in unbuffered aqueous solution at low pH, but usually results in a very satisfactory improvement in contrast together with a high degree of preservation of structural order within the myofibrils. Staining is much less intense if carried out at pH 5 or above.

As is evident from the figures, the myofibrils comprise a highly regular single array of myofilaments, the pattern being one of hexagonal symmetry except when distorted by compressional or other effects arising during the sectioning procedures. The interstitial spaces in the myofilament lattice appear to contain a material of density higher than that of the background, at least in those regions in which the section has passed through the A band (see especially Figs. 9 to 11). This material, possibly identical with the A substance, possesses no structure within the limits of resolution of the present investigation. The myofibrils usually have a roughly hexagonal cross-section with rounded corners (Figs. 9 and 13). Occasionally defects occur in the myofilament lattice, the most common, though rather rare, being the absence of a single myofilament.

Further information concerning the finer details of myofibrillar structure can be obtained from very thin sections of PTA-treated material. The myofilaments, which are spaced about 300 A apart, display a "tubular" or compound cross-section (Figs. 13 and 14) which is most clearly seen when the section passes through the myofibril in the A band. This is observed only in those regions of the section in which the myofilaments are oriented accurately normal to the plane of the section, the orientation being more critical for thicker sections. A lightly stained medullary region about 40 A in diameter is surrounded by a thin dense cortex, the over-all diameter of the myofilament being about 100 to 120 A. This compound structure is not visible or barely so in osmium-fixed muscle when the PTA treatment is omitted. While it is possible that the "tubular" appearance may be due to incomplete penetration of the myofilaments by the PTA, nevertheless, the fact that prolonged exposure to PTA does not alter the appearance suggests that there is here a true structural differentiation. As will be shown later, the lightly staining medulla or core of the myofilament appears to be structurally continuous with the filaments of the H, I, and Z bands (Text-fig. 2).

In addition to the compound nature of the myofilament, another feature of interest is illustrated in Figs. 13 and 14. Each myofilament is linked to its six nearest neighbours by thin, apparently filamentous, bridges of relatively dense material, which are between 40 and 60 A wide as seen in transverse sections. These bridges are observed in fresh muscle at all levels of the sarcomere with the exception of the H and M bands (see Text-fig. 2). In longitudinal sections (Figs. 17 to 19), it can be seen that the bridges are not struc-

turally continuous in the plane of the fibre axis, but are discrete elements which repeat at intervals along this axis. Their absence in the H band is also clearly demonstrated. Similar cross-bridges were observed by Bennett and Porter (11) in longitudinal sections of chicken breast muscle. Hoffmann-Berling and Kausche (29) noted regularly spaced disc-like cross-members in fragmented frog muscle, and similar structures are visible in some of the micrographs of Draper and Hodge (14), particularly in Fig. 7. The latter authors at that time interpreted the fine cross-striation as being an intrinsic



TEXT-FIG. 2. Schematic representation (not to scale) of the main structural findings in blowfly flight muscle fixed with buffered OsO_4 and stained with PTA. For the sake of clarity, interstitial materials which may be present in both the A and I bands have been omitted. At the bottom right are schematic representations of transverse sections through the A and I bands (*cf.* Figs. 11, 13, and 14). The myofilaments in the I, Z, and H bands are here represented as possessing a thin dense cortex. This compound structure, however, is not as clearly defined as is the thick dense cortex of the A band.

property of the myofilaments. However, some of their later unpublished work showed that the filamentous elements released by digestion with pepsin were not striated. Similar results were obtained by Farrant and Mercer (19) using arthropod muscle. The distribution of residual mineral content in fine cross-bands after electron-induced microincineration (13, 15) also lends support to the presence of cross-bridges linking the myofilaments.

The differences in structure of the various bands in relation to the myofilaments and lateral bridges can be conveniently followed in slightly oblique transverse sections which pass in succession through these bands within the confines of a single myofibril. Thus Figs. 10 and 12 illustrate the appearance in sections which pass from the A band into the H band into the M band. It is clear from longitudinal sections (Fig. 6), that the myofilaments in the A

band are continuous with those in the H and M bands, and as is to be expected, there is seen in the slightly oblique transverse sections (Figs. 10 and 12) an unbroken regular array of myofilaments as these bands are successively traversed. The myofilaments, which appear "tubular" in the A band, become less dense as they enter the H zone, then much denser and larger in diameter in the M band. They are not obviously "tubular" in the H and M bands. The lateral filamentous bridges which link the myofilaments in the A band are clearly absent from the H and M bands. The above features of the A, H, and M bands are also visible in both transverse (not illustrated) and longitudinal (Figs. 17 to 19) sections of glycerinated muscle.

The structural relations of the A, I, and Z bands are also made clear by an analysis of the myofilament array in a thin section passing through these bands (Fig. 11). The dense "tubular" myofilaments in the A band become thinner and less dense as they pass into the I band, where they appear to lack the densely staining cortex which characterizes them in the A band (see Text-fig. 2). However, under favourable conditions (Fig. 11), the I band filaments also appear compound, but with a thinner cortex. The transverse bridges linking the myofilaments are clearly present in both the A and I bands. The hexagonal pattern formed by the myofilaments and lateral bridges proceeds without any break in continuity from the A through the I and into the Z band. In the latter band, the pattern is obscured by the dense Z band material, but is easily observed when prints are made under high contrast conditions. It is clear that there is only a single array of myofilaments in insect flight muscle, and that the myofilaments, despite differences in structure in the various bands, are structurally continuous elements extending throughout the sarcomere. The continuity of the myofilaments through the Z bands has already been demonstrated in longitudinal sections (26) and this may also be seen in Fig. 15. The present results show that the myofilaments of fresh flight muscle form a single continuous array irrespective of the presence or absence of the I band, a result apparently incompatible with the model of Huxley (32).

Glycerinated Muscle.—The band pattern seen in thin sections of glycerinated muscle is in agreement with that observed under phase contrast (compare Figs. 3 *d* and *e* with Fig. 16). The H band is wider than in fresh muscle, in which it is barely distinguishable under phase contrast, and I bands are present (Fig. 16). The myofilaments again run continuously through all bands of the sarcomere. This appearance is almost certainly not due to a fortuitous superposition of two interdigitating sets of filaments such as those postulated by Huxley (32) for vertebrate muscle, since it is consistently observed in myofibrils, the hexagonal axes of which must be randomly oriented with respect to the plane of the section. The appearance of transverse sections through the A band is similar to that observed in fresh muscle, but with less

preservation of order. The filaments are less clearly defined in the I band and much more subject to disordering influences during fixation and embedding than those in the A band. Their density is considerably lower than in the A band; this seems to be due to lack of the densely staining cortex characteristic of the A band. The paleness of the I band also seems to be due in part to a relatively low stainability of the material in the interstitial spaces between the myofilaments.

Longitudinal sections of glycerinated muscle treated by immersion in solution A + ATP prior to fixation show myofibrils with band patterns characteristic of the contracted state, the sarcomere lengths generally being less than in fresh muscle or relaxed glycerinated myofibrils. However, the penetration of ATP is often insufficient to produce strong contraction, so that contraction bands (C_z and C_m) are not visible (Figs. 17 to 19). In contracted myofibrils, the myofilaments are again clearly continuous throughout the sarcomere.

Axial Periodicity.—In sufficiently thin longitudinal sections of both fresh and glycerinated flight muscle, the bridge network linking adjacent myofilaments is seen as an axially periodic system of thin filaments oriented normal to the muscle fibre axis (Figs. 17 to 19). This appearance is not due to specimen movement or lens asymmetry. Unfortunately, the bridges have not been observed as clearly in longitudinal sections as in the transverse sections. Alignment and superposition effects are possibly more critical when the section is longitudinal, or the bridges themselves may be more diffuse along the fibre axis than in the direction normal to it. Nevertheless, enough evidence has accumulated to suggest that the bridges are aligned across the whole width of the myofibril, each set being regularly spaced along the fibre axis (Text-fig. 2). In ATP-contracted glycerinated muscle (Figs. 18 and 19), the axial spacing is about 250 A. Work is in progress to correlate possible variations in this spacing with the state of contraction of the myofibril. The periodicity may be the equivalent in flight muscle of the very regular 400 A spacing observed in vertebrate skeletal muscle by both small angle x-ray diffraction (10, 32, 33) and electron microscopy (14, 22, 27) and the value of which appears to vary with the state of contraction as judged by sarcomere length and band pattern (14).

It seems appropriate at this point to summarize briefly the main structural findings in flight muscle (Text-fig. 2). The myofilaments comprise a lightly staining core which runs continuously through all bands and from sarcomere to sarcomere. They are joined laterally by regularly spaced cross-bridges, which are present in all bands, except the H band. In the A band of fresh muscle, the cores of the myofilaments are covered by a densely staining cortex. This is absent or much thinner in the I band. Interstitial material appears to be present to some extent in all bands. The Z bands consist of ex-

tremely dense interstitial material and there is definite continuity of the myofilaments through them.

With the above picture in mind, we shall proceed to a comparison of the structure and functional behaviour of flight muscle with those of vertebrate skeletal muscle. In order to do this profitably, it is necessary to assume that both types of muscle are basically similar both with respect to their general structural organization and their protein and other components. Although this is perhaps a dangerous assumption in our present state of knowledge, it seems not unreasonable in view of the similarities in band pattern and behaviour with respect to ATP. In any event, the reader is forewarned that such comparisons are possibly not valid.

DISCUSSION

It is of interest to compare the present phase contrast observations with those carried out on isolated myofibrils of vertebrate skeletal muscle. Schick and Hass (48) showed that such myofibrils contract on the addition of ATP to the medium. Hanson (23) using phase contrast, and Ashley *et al.* (3) by use of the electron microscope, have shown that the A substance migrates into the Z region during ATP-induced contraction. The myofibrils, if unrestrained, usually shorten greatly, ending up as rounded structureless masses. More recent work (31) indicates that in physiological contraction, the sequence of changes in the band pattern probably does not proceed beyond that state in which the I bands have disappeared but contraction bands (C_z and C_m) have not yet formed. There does not appear to be any detectable change in band pattern during isometric contraction of frog muscle fibres (31). The behaviour of isolated blowfly flight muscle fibrils is in strong contrast with the above observations.

On isolation, the band pattern of fresh flight muscle is similar to that observed in semicontracted vertebrate muscle. The I bands are absent or very narrow (Text-fig. 1) and the A substance is distributed essentially uniformly throughout the sarcomere, but is absent from the H band. Shortening on addition of ATP seldom proceeds beyond about 85 per cent of rest length in unrestrained myofibrils, although the formation of well defined C_z and C_m bands is similar to that in vertebrate muscle. Most glycerinated myofibrils (Figs. 3 *d* and 3 *e*) display a band pattern apparently identical with that of relaxed vertebrate muscle. However, shortening on the addition of ATP proceeds no further than with the fresh myofibrils, the final band patterns being identical (compare Figs. 3 *c* and 3 *h*).

The above observations, which are summarized schematically in Text-fig. 1, suggest that the structure of insect flight muscle is fundamentally very similar to that of vertebrate muscle, and that such differences in function and behaviour as exist are most probably related to the highly specialized

function of the former. The need here is apparently for a muscle capable of exerting large deformation forces on the insect integument several hundred times a second, the degree of shortening required presumably being relatively small. There appears to be little direct evidence concerning the level of organization at which the physiological peculiarities of flight muscle are manifested in terms of structure, but the observation of Gilmour and Calaby (21), that the myosin of the flight muscle of *Locusta migratoria* is extracted with difficulty while that of the femoral muscles is easily brought into solution, suggests that, in part at least, such differences are to be found at the macromolecular level of the contractile proteins. In this respect, it is relevant to note that there is good evidence (12) for the existence within the class Insecta of at least two physiologically distinct types of flight muscle, *viz.*, (*a*) the relatively slow muscles of the Orthoptera, Lepidoptera, etc., and (*b*) the very rapid "fibrillar" muscles of the Hymenoptera and Diptera. The latter type, with which we are concerned in this paper, might reasonably be expected to exhibit the more extreme modifications of a structural pattern presumably basic to all striated muscles. It seems likely that the intractability of flight muscle with respect to selective extraction of myosin is related in some manner to its structural specialization.

It seems clear from phase contrast and other data on both vertebrate skeletal and insect flight muscle that the A substance is a refractile material capable of migration under the influence of ATP into the Z region to form the C_s band. The formation of the C_m band also appears to be due to the migration of material within the sarcomere, but whether this material is identical with that forming the C_s band remains to be determined. Possibly the A substance consists of two distinct components which migrate selectively to the Z and M bands respectively. Certainly, the material accumulating in these two bands does not confer any additional birefringence on them, but rather the reverse (Figs. 3 *j* and *k*). If it is assumed that in the relaxed myofibril the A substance is in the form of elongated molecules oriented parallel to the fibre axis, then it must exist in the C_s and C_m bands (*i.e.*, in contracted muscle) in a disordered or highly coiled configuration. The apparent ability of the A substance to migrate to the Z band without prior disappearance of the I band in restrained myofibrils (Figs. 2 *a* and *b*) lends further support to the concept that the A substance is a mobile component which is, at least under some conditions, not structurally continuous with the basic framework of the myofibril.

The view that myosin is confined to the A bands has been advocated by a number of authors (*e.g.*, 24, 25, 34, 45). According to Hasselbach (25), a framework of actin filaments runs continuously through the sarcomere in vertebrate muscle. Superimposed on this in the A band is the myosin, which may be selectively extracted leaving the actin framework held together by

the Z band material. There seems little doubt that at least one component of the myofibril is structurally continuous through all bands of the sarcomere. Farrant and Mercer (19) have shown that the myofilaments of arthropod muscle appear "tubular" and are apparently continuous through the Z bands. The present results and those presented earlier (26) show clearly that the myofilaments extend continuously through all bands, at least in dipteran flight muscle. Thus, in all probability, the basic longitudinal framework consists of F actin. However, the distribution of myosin is not so clear, and in this respect, the recent results of Szent-Györgyi *et al.* (57) are of great interest. They found that myosin accounts for only 65 to 75 per cent of the protein extracted when the procedures for selective extraction of myosin used by Hasselbach (25) and Huxley and Hanson (24, 34) are carried out. The remainder of the extract consists of protein, as yet not fully characterized and of low viscosity, the amount of which is sufficient to account for the refractive index difference between the A and I bands. This protein appears to be held in the structure by myosin. Actin is extracted only in insignificant amounts under these conditions. Thus, the results of Hasselbach and of Huxley and Hanson seem compatible with a model in which myosin and actin are present in both the A and I bands. Furthermore, the properties of the unknown protein, *i.e.* low viscosity and molecular size (57), appear consistent with the migratory habit of the A substance, discussed in the preceding paragraph.

The finding that an axially periodic system of filamentous bridges links the myofilaments together laterally is clearly of importance in relation to the birefringence properties of muscle. In this respect, the recent polarization optical studies of Aloisi *et al.* (1) seem to be highly significant. Under conditions similar to those prevailing in the experiments of Hasselbach (25) and Huxley and Hanson (24, 34), in which both myosin and the unknown protein (A substance?) of Szent-Györgyi *et al.* (57) are selectively extracted, these authors noted a marked diminution in birefringence and disappearance of the cross-striation. On further extraction with alkaline media (conditions under which some actin is extracted), the muscle fibres became negatively birefringent throughout, again with no cross-striation remaining. These observations appear to be entirely compatible with a model in which F actin filaments form the skeleton of the myofibril and are joined laterally by filamentous bridges of transversely oriented protein. It does not seem necessary to postulate the presence of a negatively birefringent protein such as the N protein of Matoltsy and Gerendas (36).

In view of the findings discussed above, the following is presented as a possible picture of muscle structure. Filaments of F actin run continuously throughout the sarcomere in hexagonal array, thus providing structural continuity in the axial direction. They are linked together laterally at regular

intervals along the fibre axis by thin filamentous bridges. It is suggested that the latter may be composed of tropomyosin (5, 7), which is known to be associated with the myofibrils, comprises some 6 per cent of the total muscle protein (59), and appears to form complexes with actin, at least in uterine muscle (52). It should be noted that according to Bailey (5), tropomyosin is fixed to the "structural proteins" *in situ* and is still present in the acetone-dried muscle residue after extraction of actin by the method of Straub (54), indicating a strong association with the framework. The observations of Aloisi *et al.* (1) mentioned above are clearly compatible with such an acto-tropomyosin framework. L-Myosin molecules are present in both the A and I bands and are aligned parallel to the fibre axis (4). The birefringence properties of muscle under various conditions are due in the main to these three components. In the A band, another substance (the A substance, possibly identical with the unknown protein component of Szent-Györgyi *et al.* (57)) is present in the interstitial spaces, and migrates on contraction at least *in vitro* to form the C_s and C_m bands. As pointed out by Weber and Portzehl (59), the rate of migration is compatible with an electrophoretic process, but not with diffusion, and in this connection, the Z and M bands have obvious possibilities as "electrodes." In such a picture, it must be assumed that contraction primarily involves interaction between myosin and actin, the interaction perhaps being modified by the A substance, and certainly by the presence or absence of ATP. The ratio of A band width to I band width on stretching and contraction should offer clues on this problem. Unfortunately, the situation appears rather confused. Thus, it has been found that the width of the A band is relatively constant during stretching (31, 34). On the other hand, the results of Horvath (30) indicate a constant value of the A:I ratio during both stretch and contraction.

In view of the uncertainties discussed above, it seems premature to attempt a description of the structures observed in flight muscle in terms of actin, myosin, etc. However, this much can be said. The results are certainly consistent with a model in which F actin filaments form the core of the continuous myofilaments (see Text-fig. 2) and are joined laterally by regularly spaced filamentous bridges. As already mentioned, these may represent the tropomyosin of the myofibril. The micrographs reproduced here do not in themselves allow a decision between the two possible pictures of myosin distribution. They appear to be compatible with either a localization of myosin in the A band or a uniform distribution of it throughout the sarcomere. In the latter case, the increased thickness of the myofilament cortex in the A band could be ascribed to the presence of the unknown protein of Szent-Györgi *et al.* (57).

The recent finding that crystals of light meromyosin (LMM) exhibit a regular 420 A spacing in the electron microscope (42) is of great interest in

relation to the well established axial period of about 400 A in vertebrate skeletal muscle and the bridge system described here and by others. In unstained preparations of LMM crystals, the principal striation consists of a very thin dense line which may represent a concentration of salts (42). The distribution of density is similar to that of the residual mineral content in unstained myofibrils after microincineration in the electron beam (15), the resemblance extending to the spacing of the lines (*ca.* 400 A). As already discussed, there is good evidence from electron microscopic and polarization optical studies for the presence of transversely oriented filamentous bridges within the myofibril. Furthermore, the length of the LMM molecule (42) appears to be closely similar to the axial spacing of these bridges (*i.e.*, about 400 A). Hence it seems possible to consider the process of contraction in terms of an interaction between longitudinally oriented myosin and an actotropomyosin framework.

Huxley and Hanson (24, 32, 34) have recently advanced a model for vertebrate skeletal muscle involving two distinct interdigitating arrays of filaments. The primary array, about 100 A in diameter and identified with myosin, extends only through the A and H bands. The secondary array, identified with actin, extends from the Z through the I into the A bands. In the A band, the secondary filaments occupy the trigonal positions between the primary array of myosin filaments. The hypothesis is based on the finding that there appears to be a thin secondary array of filaments in transverse sections of glycerinated muscle passing through the A band (32) and on the variation in the intensities of the first and second orders of x-ray diffraction from the 360 A equatorial spacings in fresh and glycerinated muscle (or muscle in rigor) (33). However, there is as yet no direct evidence for the continuity of the secondary A band filaments with those in the I band. More recently, Huxley and Hanson (34) have carried out a phase contrast and electron microscopic study by thin sectioning on the effects of selective extraction of myosin from the myofibril, using essentially the methods of Hasselbach (25). They have chosen to interpret their evidence, namely, that after myosin extraction only a single array of thin filaments is left in the A band, on the assumption that two arrays existed initially in the A band. However, their results are in no way incompatible with the view expressed here; *viz.*, that in the fresh flight muscle only a single array of compound myofilaments exists. On selective extraction of myosin, the cortical regions of the myofilaments in the A band would be dissolved leaving the actin cores intact. The residual filaments in the A band would then, as expected, be continuous with those in the I band. However, too close a comparison seems unwarranted since the possibility exists that the structure of dipteran flight muscle may differ fundamentally from that of vertebrate skeletal muscle.

SUMMARY

1. The flight muscles of blowflies are easily dispersed in appropriate media to form suspensions of myofibrils which are highly suitable for phase contrast observation of the band changes associated with ATP-induced contraction.

2. Fresh myofibrils show a simple band pattern in which the A substance is uniformly distributed throughout the sarcomere, while the pattern characteristic of glycerinated material is identical with that generally regarded as typical of relaxed vertebrate myofibrils (A, I, H, Z, and M bands present).

3. Unrestrained myofibrils of both fresh and glycerinated muscle shorten by not more than about 20 per cent on exposure to ATP. In both cases the A substance migrates during contraction and accumulates in dense bands in the Z region, while material also accumulates in the M region. It is proposed that these dense contraction bands be designated the C_z and C_m bands respectively. In restrained myofibrils, the I band does not disappear, but the C_z and C_m bands still appear in the presence of ATP.

4. The birefringence of the myofibrils decreases somewhat during contraction, but the shift of A substance does not result in an increase of birefringence in the C_z and C_m bands. It seems therefore that the A substance, if it is oriented parallel with the fibre axis in the relaxed myofibril, must exist in a coiled or folded configuration in the C bands of contracted myofibrils.

5. The fine structure of the flight muscle has been determined from electron microscopic examination of ultrathin sections. The myofibrils are of roughly hexagonal cross-section and consist of a regular single hexagonal array of compound myofilaments the cores of which extend continuously throughout all bands of the sarcomere in all states of contraction or relaxation so far investigated.

6. Each myofilament is joined laterally with its six nearest neighbours by thin filamentous bridges which repeat at regular intervals along the fibre axis and are present in the A, I, and Z, but not in the H or M bands. When stained with PTA, the myofilaments display a compound structure. In the A band, a lightly staining medullary region about 40 A in diameter is surrounded by a densely staining cortex, the over-all diameter of the myofilament being about 120 A. This thick cortex is absent in the I and H bands, but a thinner cortex is often visible.

7. It is suggested that the basic structure is a longitudinally continuous framework of F actin filaments, which are linked periodically by the lateral bridges (possibly tropomyosin). The A substance is free under certain conditions to migrate to the Z bands to form the C_z bands. The material forming the C_m bands possibly represents another component of the A substance. The results do not clearly indicate whether myosin is confined to the A bands or distributed throughout the sarcomere.

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Note Added in Proof.—The following recent publications on electron microscopy of insect muscle have appeared in the literature or been brought to my attention since this paper was submitted for publication. Edwards, G. A., Souza Santos, P., Souza Santos, H. L., and Sawaya, P., *Ann. Entomol. Soc. Am.*, 1954, **47**, 343, 459; *São Paulo, Univ., Fac. fil., ciên. e letras, Zool.*, 1954, Bol. No. 19, 391; *Ciência e Cultura*, 1953, **5**, 27; Edwards, G. A., Souza Santos, P., Souza Santos, H. L., Sawaya, P., and Ruska, H., *Rev. brasil. entomol.*, 1954, **2**, 97; Edwards, G. A., and Ruska, H., *Quart. J. Micr. Sc.*, 1955, **96**, 151. The present state of knowledge in relation to muscle structure has been briefly summarized in a recent article (Bennett, H. S., *Am. J. Physic. Med.*, 1955, **34**, 46).

A detailed and valuable account of the anatomy and histology of the flight musculature in Orthoptera, Homoptera, and Diptera has been published recently (Tiegs, O. W., *Phil. Tr. Roy. Soc. London, Series B*, 1955, **238**, 221). The structural evolution of the wing musculature is traced from the primitive type, exhibiting low frequency tetanic shortening, to the highly specialized fibrillar or Siebold type flight muscles of Diptera and Hymenoptera, which are adapted to high frequency unfused contraction under essentially isometric conditions.

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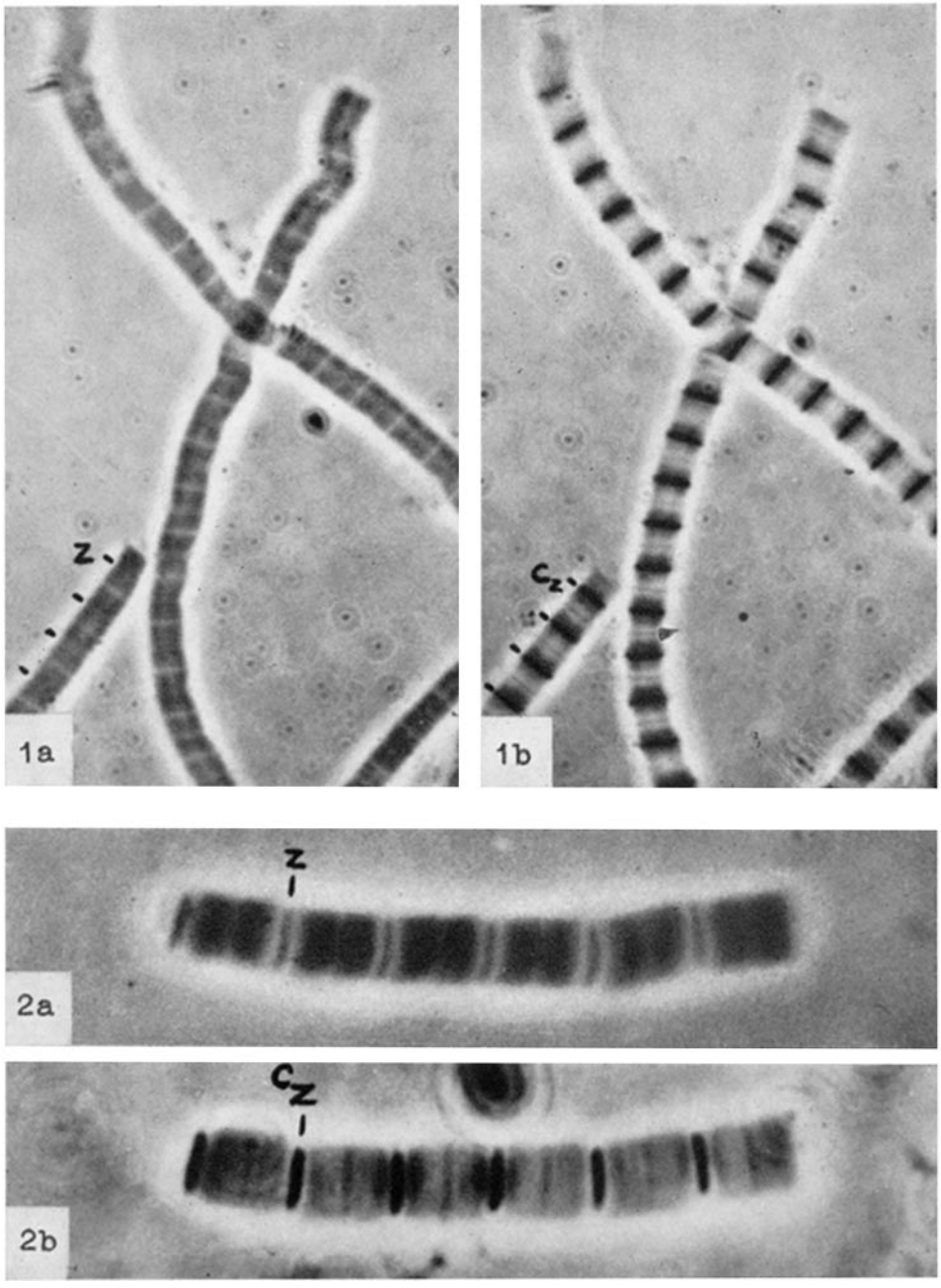
EXPLANATION OF PLATES

PLATE 92

(See Text-fig. 1 for the band notation.)

FIG. 1. Myofibrils of blowfly (*Lucilia*) flight muscle after glycerination showing the changes in band pattern on exposure to ATP. (*a*) In solution A (0.1 M KCl, 0.001 M $MgCl_2$). (*b*) Same field after irrigation with solution A + 10^{-4} M ATP. Note migration of A substance to *Z* region. Phase contrast, × 1600.

FIG. 2. Glycerinated myofibril of *Lucilia* restrained from shortening because of adhesion to coverslip. (*a*) In Guba-Straub solution showing A, I, Z, and H bands. (*b*) Same fibril after irrigation with Guba-Straub solution + ATP, showing migration of A substance and accumulation in *Z* and *M* regions despite persistence of I bands. Phase contrast, × 3750.



(Hodge: Structure of muscle. III)

PLATE 93

(See Text-fig. 1 for the band notation.)

FIG. 3. (*a*) Typical freshly isolated myofibril of *Lucilia* in solution A (0.1 M KCl, 0.001 M $MgCl_2$) showing uniform distribution of A substance. Phase contrast, × 3500. (*b*) Same preparation as (*a*) showing partly contracted myofibril resulting from ATP addition. Phase contrast, × 3500.

(*c*) Same preparation as (*b*) showing a myofibril with well developed contraction bands (C_z and C_m). Photographic conditions in (*a*) and (*c*) identical. Phase contrast, × 3500.

(*d*) and (*e*) Typical myofibrils from glycerinated muscle in solution A, showing the well developed I bands. Phase contrast, × 2850.

(*f*) Same myofibril as in (*e*) after irrigation with solution A + ATP, showing incomplete migration of A substance. Phase contrast, × 2850.

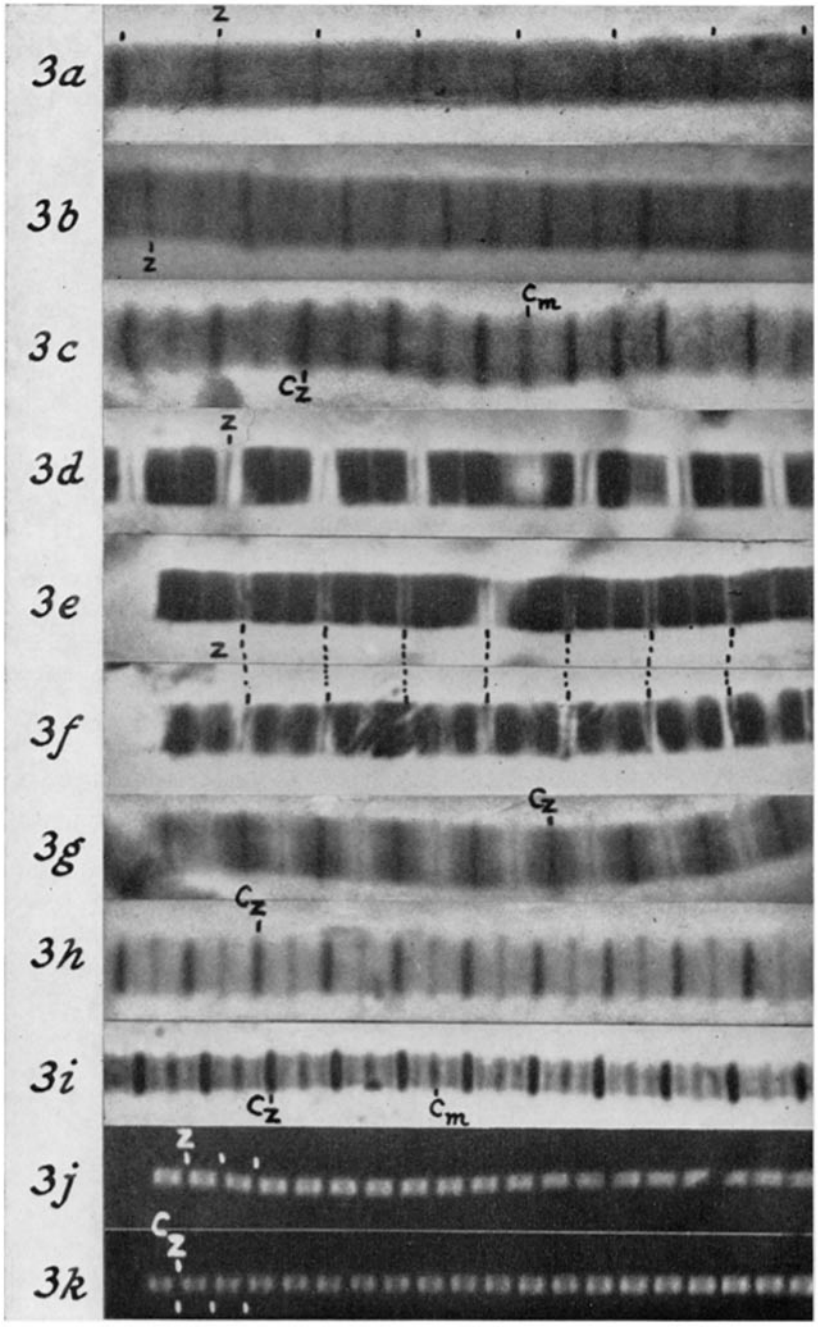
(*g*) Glycerinated myofibril contracted in Guba-Straub solution + 4 × 10^{-4} M ATP, showing incomplete migration of A substance similar to that in Fig. 1 *b*. Phase contrast, × 2850.

(*h*) Glycerinated myofibril contracted in solution A + ATP showing apparently complete migration with formation of well developed C_z and C_m bands. Phase contrast, ×2850.

(*i*) Fibril similar to that in (*h*) but left for several hours in Guba-Straub solution + ATP. Shrinkage has occurred due to evaporation of water from the preparation but there is no indication of extraction of myosin (compare with 3 *h*). Phase contrast, ×2850.

(*j*) Appearance between crossed polaroids of a glycerinated myofibril in solution A. The band pattern of this myofibril seen under phase contrast was similar to that in Fig. 2 *a*. The A bands are bright and I bands dark. × 1000.

(*k*) Appearance between crossed polaroids of a glycerinated myofibril after contraction in solution A + ATP. The band pattern under phase contrast was identical with that of Fig. 3 *h*. The over-all birefringence has decreased as compared with the relaxed fibril (Fig. 3 *j*), but its distribution is very similar. The sarcomere is bright except in the C_z and C_m regions. × 1000.

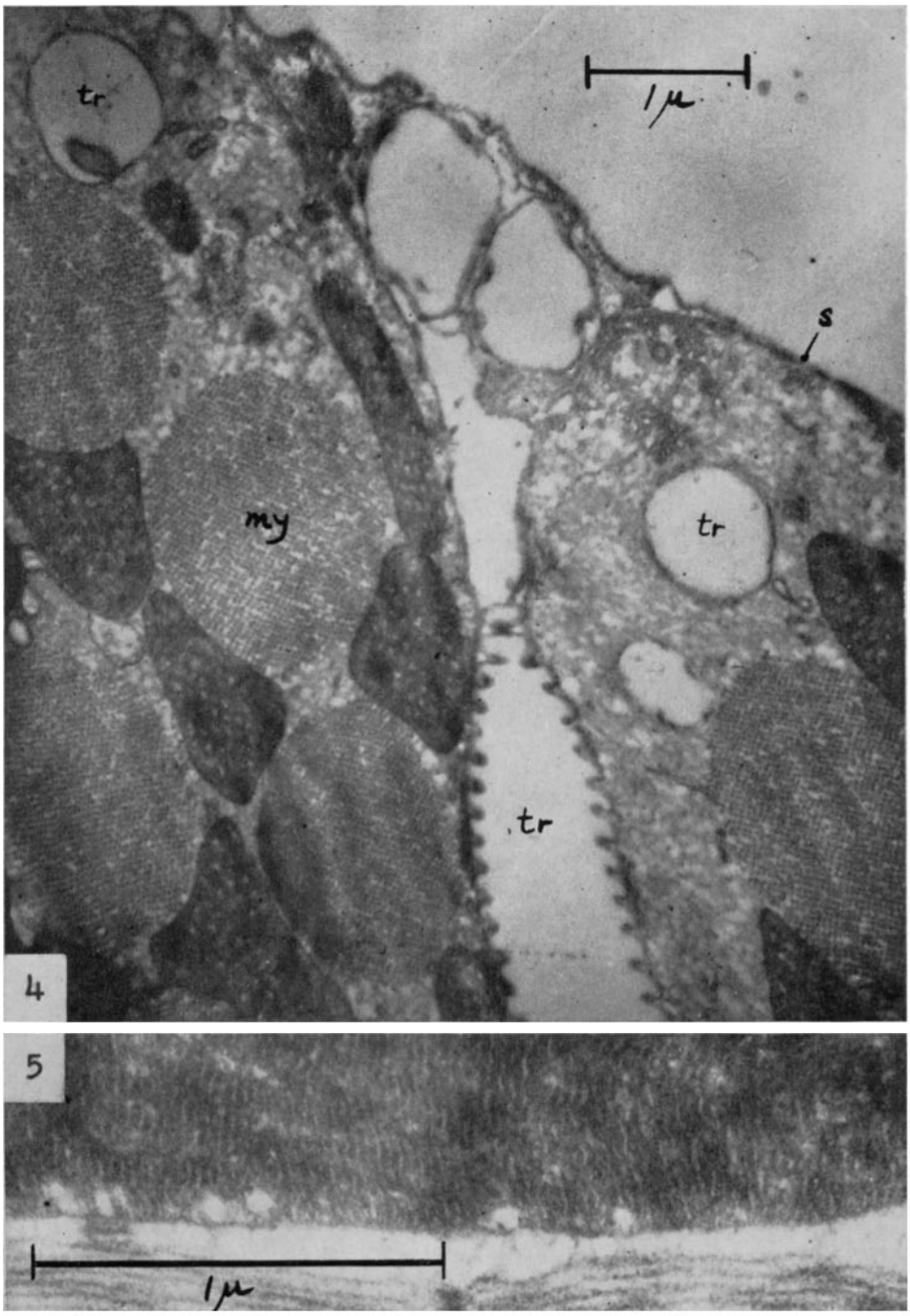


(Hodge: Structure of muscle. III)

PLATE 94

FIG. 4. Transverse section of blowfly flight muscle fibre (fixed in buffered osmium tetroxide) at the level of a large transverse tracheole (*tr*). Note the granular cytoplasm (sarcoplasm) containing elements of the endoplasmic (sarcoplasmic) reticulum, a number of small tracheoles, sarcosomes (mitochondria), and the myofibrils (*my*), all enclosed within the thin sarcolemma (*s*). The sarcosomes display a regular arrangement of pores. × 22,500.

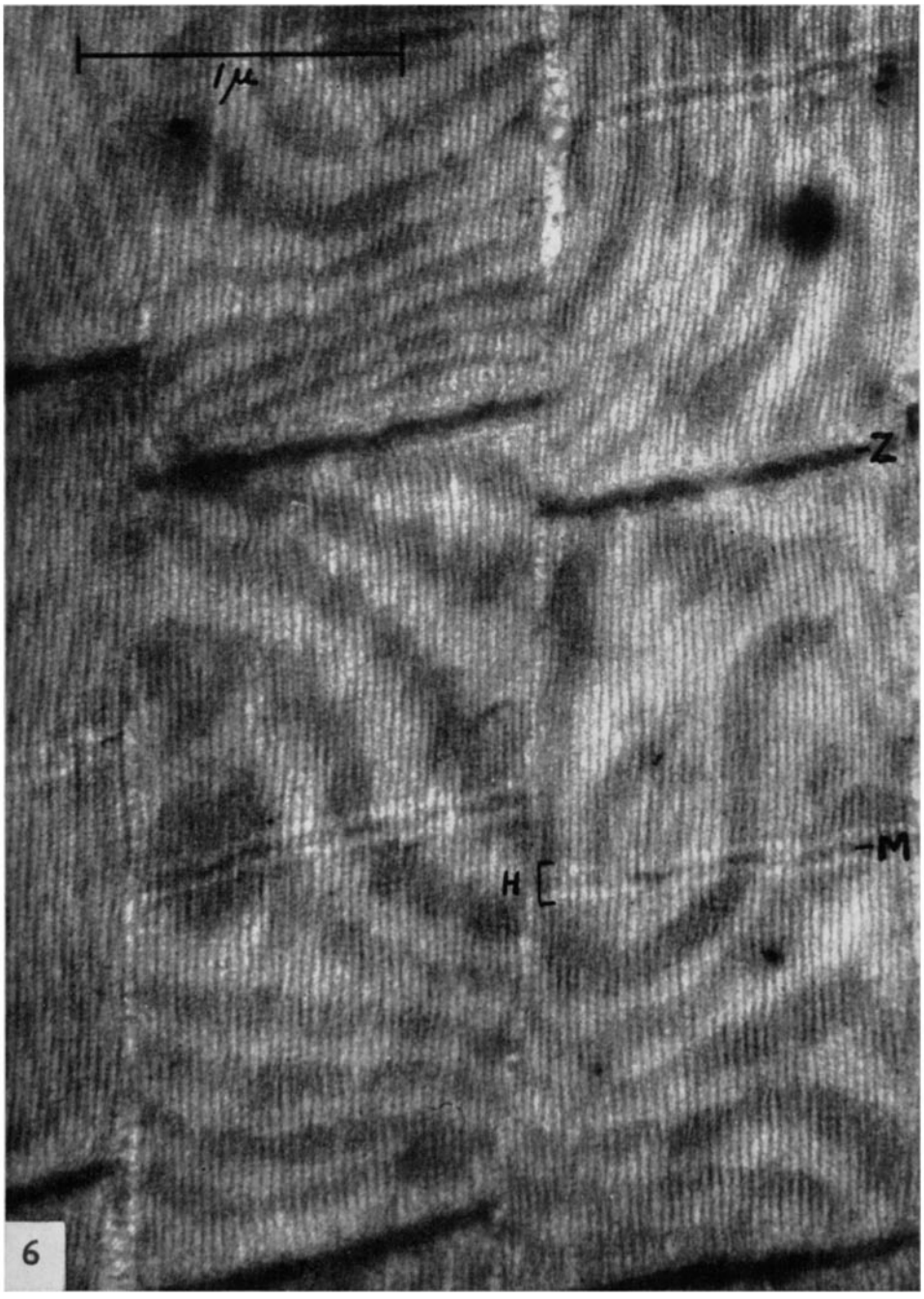
FIG. 5. Portion of a longitudinal section through a preparation similar to that in Fig. 4, showing the densely packed double membrane lipoprotein structures within a sarcosome, enclosed by a thin, apparently single, limiting membrane. × 57,000.



(Hodge: Structure of muscle. III)

PLATE 95

FIG. 6. Longitudinal section of fresh flight muscle fixed in buffered OsO_4 followed by PTA treatment, showing the regular array of myofilaments traversing all bands of the sarcomere and penetrating the Z band. Wavy pattern is due to pseudostriations (see text). Apart from the very narrow H bands, the band pattern corresponds to that in Fig. 3 *a*. Interstitial material is present between the myofilaments, and lateral bridges linking adjacent filaments are just visible. × 44,000.



(Hodge: Structure of muscle. III)

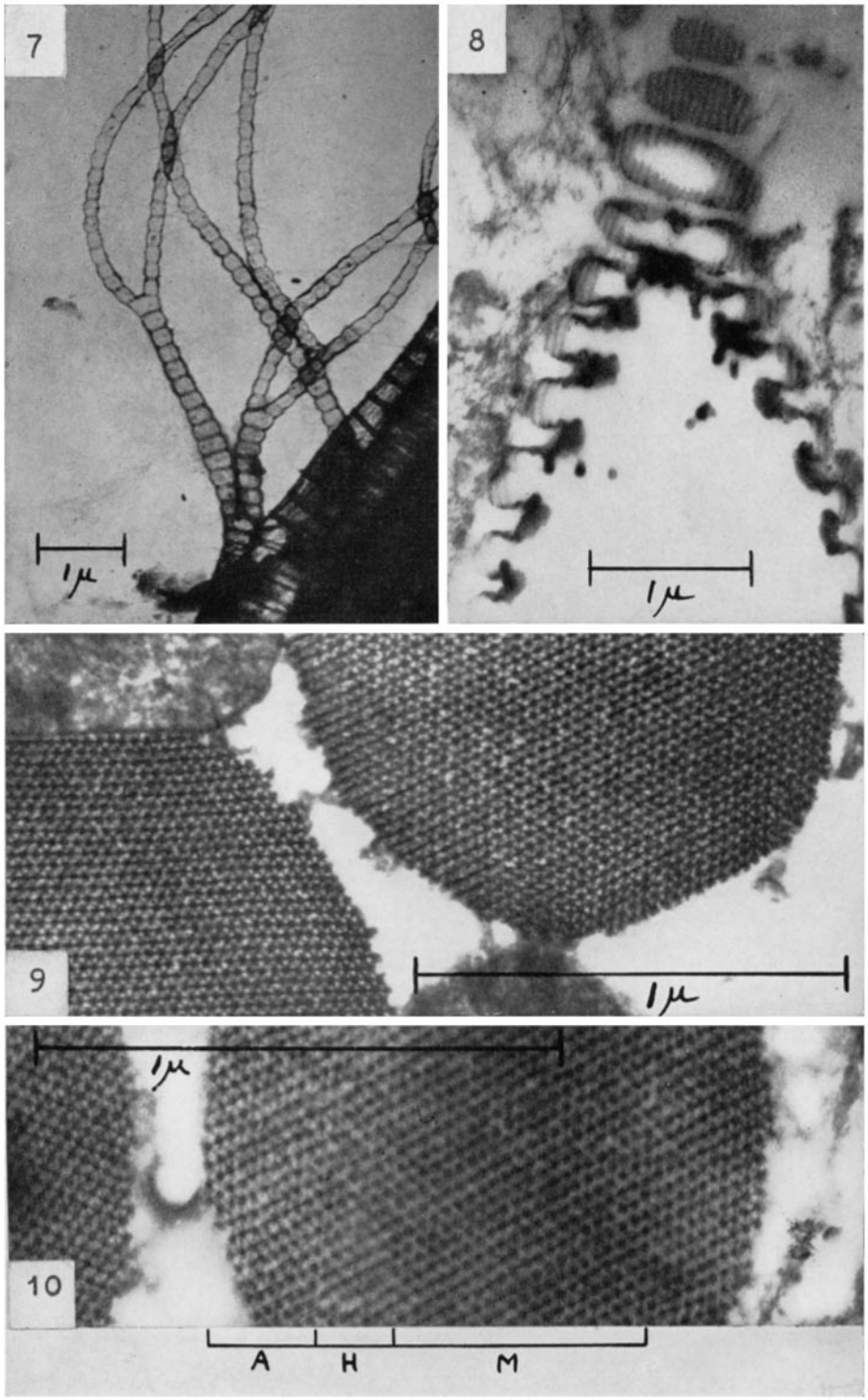
PLATE 96

FIG. 7. Isolated tracheoles of *Lucilia* flight muscle showing the regularly spaced annular taenidia. The tracheoles at the top are representative of the finest branches observed, and end blindly. × 12,000.

FIG. 8. Thin section passing obliquely through a relatively large trachea situated externally to a muscle fiber. Note the characteristically folded epicuticle, the presence of dense exocuticular material in the taenidial folds, and the longitudinal striation of the epicuticle. × 23,000.

FIG. 9. Transverse section through the A band of the same material as Fig. 6 (OsO_4, PTA) showing clearly the regular hexagonal myofilament array and the roughly hexagonal shape of the myofibrils. In some regions the lateral bridges linking adjacent myofilaments can be seen. × 62,000.

FIG. 10. Slightly oblique transverse section of the same material passing from the A band (at left) through the H and M bands back to the A band (at right). Note lateral bridges in the A band and their absence in the H and M bands, also the differences in myofilament structure in the various bands. × 75,000.



(Hodge: Structure of muscle. III)

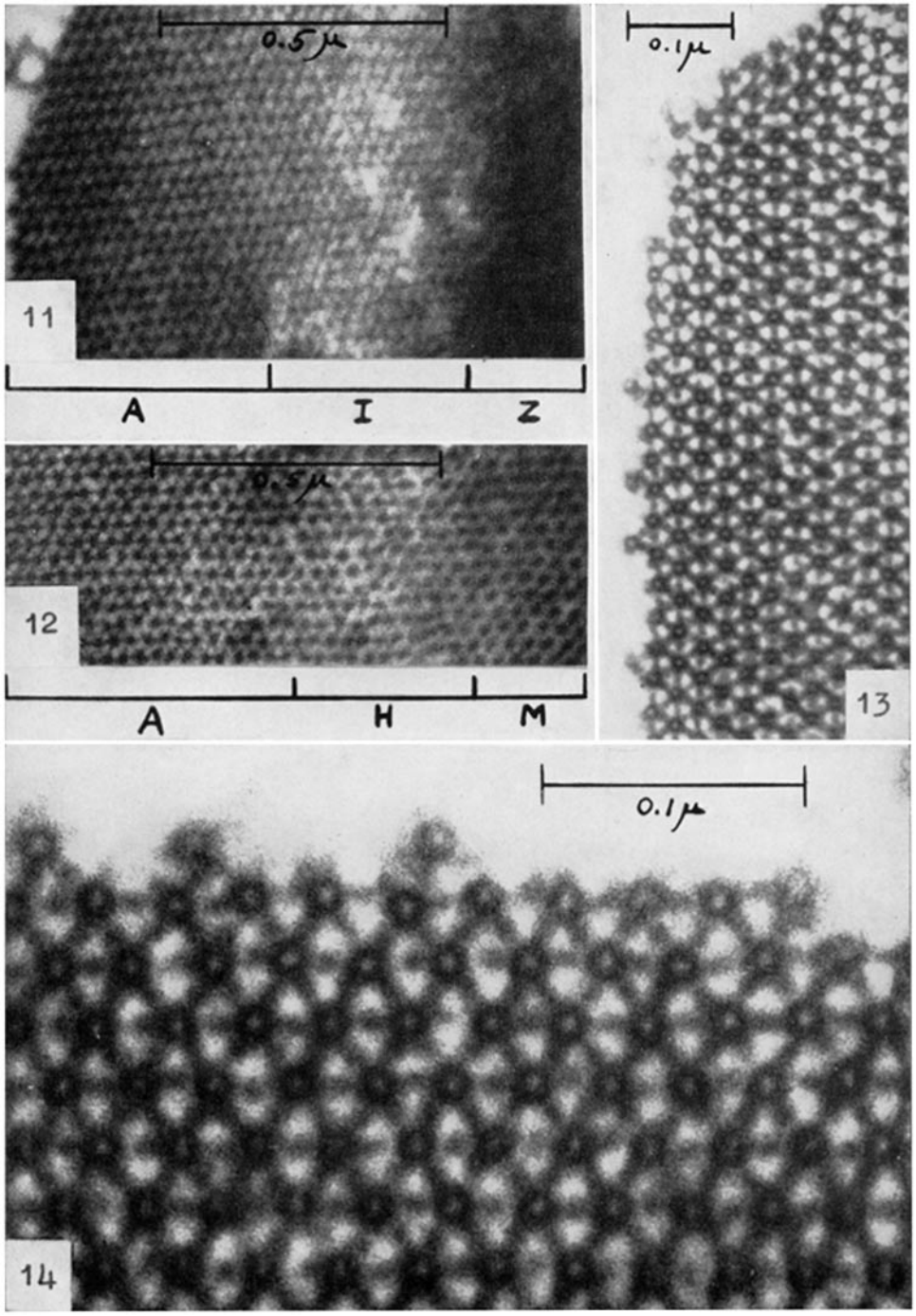
PLATE 97

FIG. 11. Slightly oblique transverse section of the same preparation passing successively through the A, I, and Z bands in a myofibril with narrow I bands. The myofilaments, which display a "tubular" structure in the A band become less dense and are devoid of the thick dense cortex as they pass through the I band into the Z band. However, a thin cortex is visible in some areas of the I band. The hexagonal array of myofilaments proceeds without interruption through all bands, as does the regular pattern of lateral bridges linking the myofilaments. × 80,000.

FIG. 12. Section similar to that of Fig. 9 passing successively through the A, H, and M bands respectively, but showing more clearly the differences in myofilament structure and the absence of the lateral bridges in the H and M bands. × 80,000.

FIG. 13. Ultrathin transverse section of the same material in the A band, showing clearly the compound nature of the myofilaments, the regular filamentous bridges linking nearest neighbours, and the absence of a secondary filament array. × 146,000.

FIG. 14. A part of the same field as Fig. 13, illustrating more clearly the regular hexagonal lattice of compound myofilaments and bridges. The finely dotted appearance is due to the photographic grain of the plate. × 370,000.



(Hodge: Structure of muscle. III)

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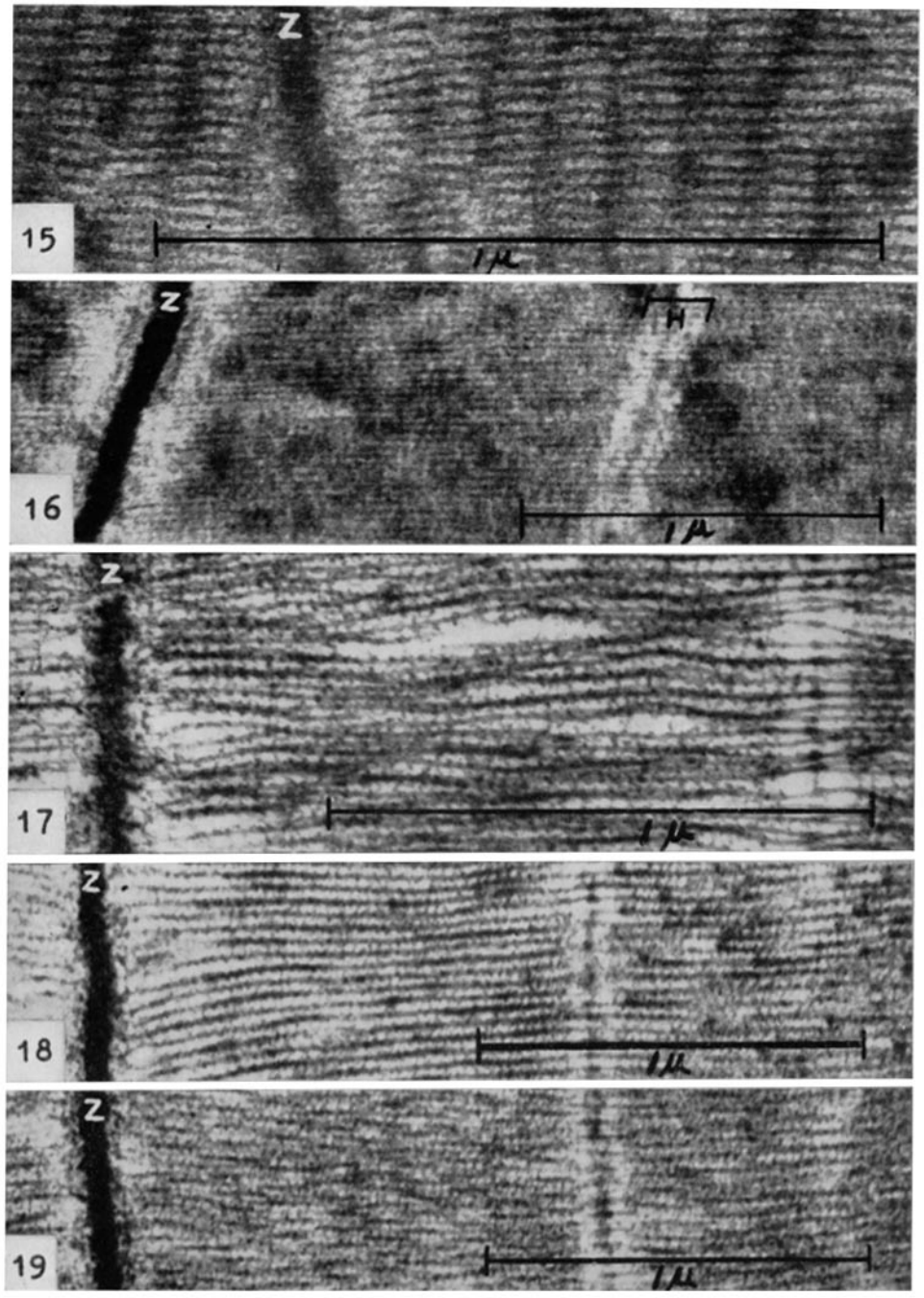
FIG. 15. Slightly oblique longitudinal section of fresh myofibril (same preparation as Figs. 7 to 14) showing the "tubular" appearance of the myofilaments and their penetration of the Z band. × 100,000.

FIG. 16. Longitudinal section of glycerinated flight muscle (OsO_4, PTA) with I bands present, illustrating the continuity of the myofilaments from the A into the I and H bands. × 50,000.

FIG. 17. Longitudinal section of glycerinated muscle, treated with solution A + ATP prior to fixation in buffered OsO_4, followed by staining in alcoholic PTA. The heavy staining has resulted in considerable disorganization, but the presence of the lateral bridges in the A band and their absence in the H band are clearly shown. × 75,000.

FIG. 18. Same preparation as the previous figure, but showing a myofibril with better preservation of order. The axially periodic arrangement of the lateral bridges is illustrated, the spacing in this instance being about 250 A. × 54,000.

FIG. 19. Another portion of the same micrograph as that shown in Fig. 18. Myofibril axis slightly oblique to the plane of the section, illustrating the different appearance of the array of bridges under these circumstances, presumably because of superposition effects. × 54,000.



(Hodge: Structure of muscle. III)