THE DOUBLE ARRAY OF FILAMENTS IN CROSS-STRIATED MUSCLE

By H. E. HUXLEY, Ph.D.

(From the Medical Research Council, Department of Biophysics, University College, London)

PLATES 206 TO 218

(Received for publication, February 27, 1957)

INTRODUCTION

Until about five years ago, the view was generally held that the contractile structure in striated muscle consisted of a single set of longitudinal filaments which extended continuously through each sarcomere (*i.e.*, from one Z line to the next) and which were, perhaps, even continuous between successive sarcomeres. In the A bands, some extra material, the A substance, was believed to be present, lying either on or between the filaments. It was believed that some internal folding or coiling of the filaments was responsible for contraction.

Since then, studies on striated muscle by x-ray diffraction (1), electron microscopy (2), and phase contrast light microscopy (3), have suggested that a rather different type of structure is present. The essence of this structure is that it consists of two separate sets of longitudinal filaments in each sarcomere, which overlap in certain regions and thereby produce the characteristic band pattern of striated muscle. In this model, changes in the length of the muscle are brought about by a process in which the two sets of filaments slide past each other. The changes in the visible band pattern of muscle which occur as a result of stretch or contraction can be explained in a very straightforward way by the model, as can a number of other aspects of the behaviour of muscles (4–6).

Despite the apparent straightforwardness of the observations and arguments which led to this model, its validity has been questioned by some workers recently (7–9) primarily on the basis of their own electron microscope observations; and attempts have been made to revive the so called "classical" model. It is the author's view that the force of the original arguments, particularly those deriving from the light microscope observations, has not been fully appreciated in these attempts; and that in consequence, certain electron microscope observations have been accepted at their face value, and theories constructed from them (which conflict with other observations on muscle) perhaps a little too readily. However, electron microscope observations should

be able to stand on their own; and so it is important to establish what the facts of the situation really are, and to find out just at what point the error in argument or interpretation has crept in.

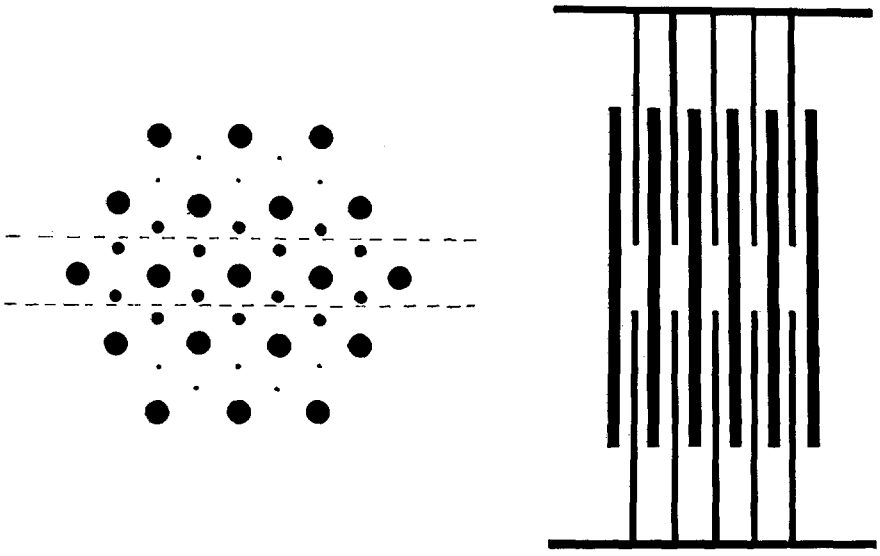
The issue revolves primarily around the appearance of longitudinal sections of muscle in the electron microscope. At first sight, one would expect the double array of filaments to be readily visible in such sections. However, it is usually found that the sections show what appears to be only a single set of filaments. It is this observation which has led some people to call into question the original interpretation of the cross-sectional views of muscle, which did appear to show a double set of filaments. The purpose of the present paper is to discuss the conditions under which one might expect to see the double array of filaments, if it exists, in longitudinal sections of striated muscle, and to describe the appearance of such sections when the requisite conditions are fulfilled.

Section Thickness and Visibility of Filaments

The separation between the filaments which form the primary array in the A bands of striated muscle is, in fixed and embedded material, about 300 to 350 A. The separation between adjacent layers of filaments in this lattice will depend on the plane in which the particular set of layers is drawn, relative to the hexagonal axes of the lattice. The maximum separation of layers will be found in the direction indicated in Text-fig. 1 (the $10\bar{1}0$ crystallographic direction), and will be approximately 250 A; the distance between primary filaments in these layers will be the fundamental lattice spacing, 300 to 350 A. The next largest layer separation will be found in the direction indicated in Fig. 2 (the $11\bar{2}0$ crystallographic direction); the separation of these layers will be about 150 A, and the distance between primary filaments in the layers will be 520 to 600 A. The lattice can of course be divided up into layers in an infinite number of other directions, but these will all give layer separations smaller than the ones already described, and the filaments in any given layer will be further apart.

Thus the appearance of longitudinal sections through this structure will depend rather critically both on the plane of sectioning and on the section thickness. If the section thickness exceeds the layer separation in that particular direction, then obviously the section will contain two or more superimposed layers of filaments. The appearance of the section in the electron microscope (whose depth of focus is of course much greater than the layer separation), will then be governed by the way in which the filaments in successive layers are stacked above each other. When two or more filaments lie vertically above each other in the sections, they appear as a single dense filament. In general, however, this will not happen, and a wide variety of different patterns will result. This is indeed what one observes in the electron microscope with the usual type of longitudinal sections of muscle, but of course one tends to select

those areas in which the "filaments" show up most clearly. The possibility of seeing secondary filaments in sections which contain more than one layer of primary filaments will be governed by the extent to which, in projection, they overlap with each other and with primary filaments. Examination of Text-fig. 1 will show that even in the most favourable case (layers parallel to the $11\bar{2}0$ direction), and even with idealized filament profiles, the secondary filaments in the projected view of sections of thickness 300 A and upwards



1 *a* 1 *b*

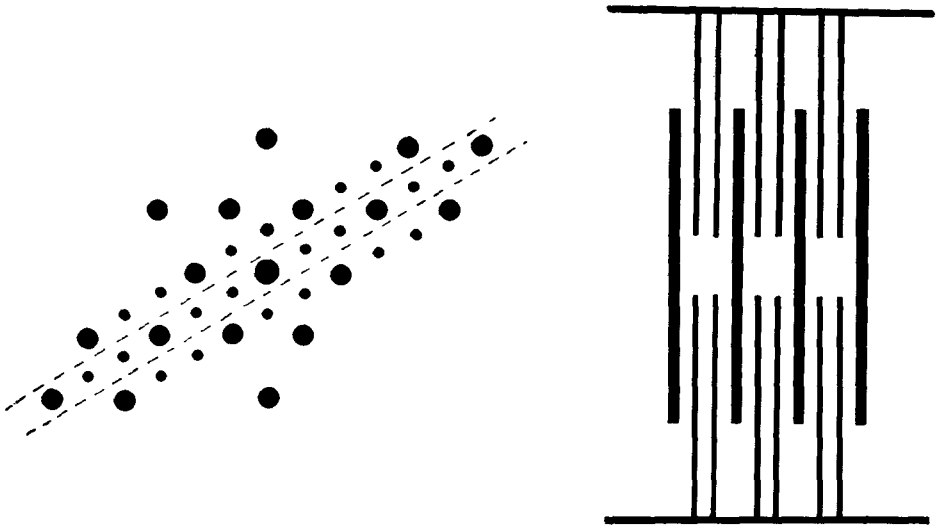
TEXT-FIG. 1 *a*. Diagram showing end-on view of a double hexagonal array of filaments; the two dotted lines indicate the outline of a longitudinal section, about 250 A in thickness, parallel to the $10\bar{1}0$ lattice planes, at the appropriate level to include one layer of primary filaments, and two layers of secondary filaments.

TEXT-FIG. 1 *b*. Diagram showing expected appearance of longitudinal section, cut as indicated in Text-fig. 1 *a*. Note simple alteration of primary and secondary filaments. The latter will represent two filaments lying vertically above each other in the section.

would almost touch each other and the primary filaments alongside them. And imperfections in the sharpness of the outline of the filaments, and in their alignment, will aggravate this effect. The result would be that the space between the primary filaments would appear to be filled with material of more or less uniform density. In other directions, the overlapping will be worse, and in the case of layers parallel to the $10\bar{1}0$ planes, primary and secondary filaments would lie in the same vertical register. This argument will be illustrated later by the superposition of photographs of single layers of the structure.

We see, then, that although these sections may in some instances give some

sort of a picture of the main array of filaments, they will be of little use for showing details of the structure between these primary filaments. In order to allow any reasonable possibility of seeing secondary filaments (if they exist), the section must contain only a single layer of primary filaments. This can be achieved if sections of the type shown by the dotted lines in Text-figs. 1 and 2 are employed. These represent section thicknesses of 250 A and 150 A respectively. And although it is frequently stated or implied that sections



2 *a* 2 *b*

TEXT-FIG. 2 *a*. As in Text-fig. 1, but with dotted lines showing a longitudinal section about 150 A in thickness, cut parallel to the $11\bar{2}0$ planes of the lattice; primary and secondary filaments in this case all lie in the same layer, and two secondary filaments occur between each pair of primary ones.

TEXT-FIG. 2 *b*. Diagram showing expected appearance of longitudinal section, cut as in 2 *a*. Note characteristic appearance of two secondary filaments between each pair of primary filaments.

of this thickness have been used, we will see in a moment that there is reason to believe that the real thickness has often been substantially greater.

For any given filament to remain within a 200 A thick section for a distance of, say, 1 to 2 microns, its orientation must be correct to better than one degree, and it must not diverge from a straight course by more than 100 A. Now these are rather exacting requirements, and it is surprising, to say the least, that what appear to be perfectly oriented layers of filaments can so often be seen all over the field when longitudinal sections of muscle, cut without taking any particular precautions to obtain perfect orientation, are examined in the electron microscope.

The explanation lies in the fact that the "bright silver" sections often used in electron microscope studies represent layers of the structure which, before sectioning, were considerably more than 300 A in thickness. The actual thickness of such sections, as measured in the interference microscope, is in the range 600 to 1000 A. However, these sections may be reduced in length by as much as a factor of 30 per cent in the cutting direction, as compared with the block face, without any increase in their width; it follows that an increase in thickness must have taken place, and that the thickness of the sectioned layer was 400 to 700 A. Thus the usual silver sections will contain more than one layer of filaments, and in those areas where the filaments can be seen at all clearly, *i.e.* where they are in vertical register, the fact that they may be tilted about a horizontal axis with respect to the plane of the section will not be detectable for there will always be *some* filaments in the section, to give the appearance of a continuous "filament," and the density variation as one filament leaves the section and another one enters will not be very great. When the sections contain only one layer of filaments, however, they exhibit quite a different appearance, and even with the most carefully oriented material, it can immediately be seen that most of the filaments are not accurately parallel to the plane of the section and remain in it for only a relatively short distance. This provides quite a good test of section thickness. And it will be clear from what has gone before that sections which do not pass this test will be too thick for secondary filaments to be separately resolved.

The best conditions for seeing the structure in between the primary filaments are obtained with sections of the kind illustrated in Text-fig. 2. These contain only one primary and one secondary filament in the thickness of the section, which needs to be about 150 A. The primary filaments in the layers will be about 500 A apart, and the appearance of an array with this rather large spacing, therefore, provides another indication of section thickness. If secondary filaments exist, we should expect to find two of them between each pair of primary filaments, as shown in Text-fig. 2 *b*. The sections appear dark grey in colour when floating on a water surface and viewed by reflected light. The muscle shows the same kind of "pseudo striations" (as layers of filaments enter and leave the section), as have previously been observed (10) in insect flight muscle, where the spacing of the array is greater and the pseudo striations easier to obtain. With a little care, it is usually possible to find examples of layers of filaments which stay in the section for the whole length of an A band.

Sections of the kind shown in Text-fig. 1 also contain only a single layer of primary filaments, but would contain two secondary filaments in vertical register; these should, however, still be seen separately from the primary filaments, as indicated in Text-fig. 1 *b*; the sections in this case are about 250 A in thickness, and they also appear dark grey in colour.

The reduction of the dimension of the section in the cutting direction has already been remarked upon. This, of course, will produce changes in the embedded tissue, and if the filaments are parallel to the knife edge, they will be moved towards each other and the whole appearance obscured. In order to see the arrangement of the filaments most clearly, they should therefore be sectioned perpendicular to the knife edge, a fact already remarked upon by Spiro (7).

Thus the longitudinal sections of striated muscle which are likely to show the secondary filaments (if they exist), are ones which have been cut with the fibre axis perpendicular to the knife edge, and which are so thin that they appear dark grey when examined in reflected light, and show muscle with pseudo striations when examined in the electron microscope. It is this type of section which has been used in the present studies.

Materials and Methods

The main source of material was rabbit psoas muscle, both fresh and glycerinated (11); fresh muscle from frog sartorius and frog musculus extensor longus digiti IV was found to give results almost indistinguishable from those obtained with fresh psoas muscle. Specimens were fixed for varying lengths of time in buffered osmium tetroxide (12). Optimum results were obtained using a fixation time of ½ hour at room temperature. The material was dehydrated in an ethyl alcohol series and embedded in methacrylate in the usual way in No. 4 gelatin capsules. A polymerization temperature of 60°C. was employed; no improvement in the appearance of the tissue was noted when prepolymerized plastic was employed. Additional staining was usually provided by dissolving 1 per cent phosphotungstic acid (PTA) in the absolute alcohol used in the dehydration. This rendered the tissue very hard and as a result it was more difficult to cut good sections. Thick, damaged sections of tissue stained with PTA can often give the impression that the PTA has itself caused structural damage. But when thin, smooth sections are obtained from the same block, they show that the tissue is preserved perfectly satisfactorily. It was often found advantageous to use a harder plastic to compensate for the hardness of the tissue; mixtures containing up to 50 per cent methyl methacrylate were quite practicable. Sections were cut on an extensively modified Hodge-Huxley-Spiro microtome (13, 14), using a glass knife. They were collected on Smethurst-Highlight copper grids coated with carbon films (15).

The sections were examined in a Siemens Elmiskop I. A beam current of 13 μa., an accelerating voltage of 80 kv., and a condenser aperture of 200 microns were used. Condenser 1 was set at the fifth click position from zero on the coarse control; this gave a reasonable compromise between specimen contamination and overheating. A molybdenum objective aperture 35 μ in diameter was employed; the astigmatism was kept below about $\Delta f = 0.2\ \mu$ (3 clicks on the fine focus control). An instrumental magnification of 40,000 was the one most commonly employed. Photographs were taken on Ilford special contrasty lantern plates, and were developed in Ilford PQ universal developer.

RESULTS

1. The Arrangement of the Filaments

The appearance of striated muscle in longitudinal sections of the required thinness and orientation is shown in Fig. 1. The pattern seen varies from one

fibril to the next, and from one sarcomere to the next in any given fibril, depending on the orientation of the axes of the hexagonal lattice of myofilaments with respect to the plane of sectioning. Sections through the lattice in the $11\bar{2}0$ direction are shown in Figs. 2 and 3. (In the latter figure, the orientation is inaccurate by about one degree and the section passes from one layer of the lattice to the next.) These electron micrographs should be compared with the theoretical diagrams shown in Text-fig. 2. Two thin filaments, lying in between each pair of thick ones, are invariably seen in such sections. The thick filaments are continuous from end to end of the A bands, and their distance apart is about 500 to 550 A, which agrees with the theoretical value. In fibrils at about rest-length, the thin filaments terminate before they reach the centre of the A band, leaving a gap in the middle, the H zone. This gap may be seen in the centre of each sarcomere over a wide field (*e.g.* Fig 1 and also Fig. 9) and therefore cannot be due to a chance displacement of the filaments. In slightly stretched muscle (Fig. 4), the ends of the secondary filaments are farther apart and a wider H zone appears; in slightly contracted muscle (Fig. 7), the ends of the thin filaments come together in the centre of the A bands and the H zone disappears. Intermediate stages are shown in Figs. 5 and 6.

The appearance of sections cut in the $10\bar{1}0$ direction is illustrated in Fig. 8. This should be compared with the theoretical diagram shown in Text-fig. 1. In this type of section too, a second set of filaments, terminating at the edges of the H zone, can be seen lying between the primary filaments which extend continuously from end to end of the A band. This time, however, a simple alternation of primary and secondary filaments is observed; the secondary "filaments" will in general really consist of two superposed secondary filaments, as reference to Text-fig. 1 will make clear. The different extent to which the secondary filaments penetrate into the A band at different muscle lengths is illustrated by Figs. 11 and 24 which show a stretched muscle with its wide H zone. Cross-bridges between primary and secondary filaments are prominent features of all these pictures.

If a slightly oblique section is cut through the structure, the appearance shown in Fig. 9 is obtained. This pattern may at first look very complicated, but longer inspection of it will show that it results simply from the section passing through successive layers of the double hexagonal lattice of filaments. The absence of the secondary filaments from the H zones is well demonstrated in this picture. Many of the sarcomeres have the appearance shown at higher magnification in Fig. 10. Reference to Text-fig. 2 will show how this has arisen. As the section passes out of one layer of filaments and enters the next, the thick filaments in this layer appear in a position midway between the thick filaments of the preceding layer; and between the thick filaments of the new set, pairs of thin filaments again appear. The ends of the thick and the thin fila-

ments, *i.e.*, the points where they leave the section, lie on the same straight line, and the two types of filaments in a given layer must therefore lie in the same plane. It will be apparent from all this that the secondary filaments cannot be "shaved-off edges" of the primary filaments in the layers immediately above and below.

If the muscle is fixed "unrestrained" (as opposed to being held at a fixed length), the characteristic appearance of primary and secondary filaments is still seen perfectly normally in longitudinal sections. Fig. 12 shows an example of a fibril fixed in this way. It will be seen that the only difference between this fibril and the one shown in Fig. 2 (which was held at a fixed length), is that the general orientation of the filaments is poorer, particularly in the I bands, where thin filaments enter and leave the section in many places. In the lower part of Fig. 12, the section, which must have been extremely thin, has passed through four secondary filaments between a very widely spaced pair of primary ones. This would correspond to a section parallel to the $21\bar{3}0$ crystallographic direction.

The filament arrangement can be illustrated in a slightly different way, by Fig. 15. This represents a transverse section through a fibril in the region of the H zone. The section is not quite accurately transverse, and it intersects the boundaries between the H zone and the rest of the A band. Thus in the centre of the field there is a simple array of filaments and on either side, a compound array of filaments; and the continuity of the rows of thick, primary filaments between the two regions, and the way in which the set of six secondary filaments appear around each of the primary filaments at the boundary can be readily seen.

The continuity of the secondary filaments in the A band with the I band filaments is difficult to demonstrate with diagrammatic clarity over a whole fibril, although it can be seen in restricted areas (Figs. 2, 13, and 14). The difficulty arises because the degree of order of the filaments in the I band is substantially lower than in the A band, and in so far as we are concerned with the separation of overlapping structures, we are already right on the limit of the present technique. The alignment of the I band filaments is considerably improved by holding the muscle at a fixed length, or under tension, during fixation, dehydration, and infiltration, but unfortunately, not to the extent that departures from linearity by about 100 A no longer occur; and this is sufficient to take a filament out of the section, or to bring it into the section if it lay just above or below. Thus the I band filaments rarely lie completely in the section over their whole course. And it will not help matters to use thicker sections, for then thick and thin filaments will overlap in the A bands. Furthermore, the Z lines seem to shrink sideways slightly during fixation and embedding, with the result that the array of I band filaments is constricted there, leading to further non-parallelism at the A-I boundary. The addition of these

complications to the initial requirements of section thickness and orientation has, so far, not allowed electron micrographs of the A-I boundary to provide a conclusive demonstration that the primary filaments *always* terminate there completely, and that the secondary filaments *always* continue on into the I band. The real evidence for this lies in the observations of the changes in the degree of overlap of primary and secondary filaments at different muscle lengths; it is very difficult indeed to see how such changes can be brought about unless the secondary filaments are connected to the Z lines. The electron micrographs referred to do, however, show examples of the termination of the primary filaments and the continuity of the secondary ones.

2. Appearance of Thicker Sections

It is of some interest now to consider what the appearance of these structures would be in thicker sections. It has already been pointed out that the best chance of seeing secondary filaments in sections containing more than one layer of primary filaments would be given by sections cut in the $11\bar{2}0$ direction. And it was argued that even in this case, the secondary filaments would almost certainly be obscured by overlap. This effect can now be illustrated. Fig. 16 shows a photograph of a well oriented single layer $11\bar{2}0$ section. Fig. 17 shows the same photograph printed twice, with the second printing shifted so as to place the second layer of filaments in the position they would occupy in the projected view of two layers of the lattice. It will be seen that the secondary filaments are concealed very effectively, although the bridges still appear. Fig. 18 shows a typical electron micrograph of a thicker section (bright silver); again, the secondary filaments are completely obscured.

3. Fine Details of Structure

Bridges are observed between primary and secondary filaments. These bridges form part of the primary filaments, and in stretched muscles can still be seen projecting into the space from which the secondary filaments have been withdrawn. The secondary filaments, on the other hand, can, in the I bands, be seen to have no projections on them.

Between any adjacent primary and secondary filament there seems to be a bridge about every 400 A. This distance is arrived at by counting the number of bridges (15 to 20) in one half of an A band, and dividing this number into the half-length of the band (0.75 μ) measured in the light microscope; because of a variable amount of dimensional change produced in the sections during cutting, it is not possible to measure the spacing directly—it usually appears much less than 400 A. The bridges between a secondary filament and the three primary filaments around it do not occur in register, but seem to be spaced out fairly evenly along the secondary filaments, as though they were attached about every 133 A. The bridges on any given primary filament do

not occur in register either; one often gets the impression that they are disposed in a helical fashion (*e.g.* Fig. 27), one turn of the helix occupying 400 A. The bridges do not seem to have any precisely fixed form; they are more or less at right angles to the filaments, and there does not appear to be any preferential direction of tilt, even in muscle fixed under continuous tension. But displacement of structures may occur during sectioning.

The ends of the primary filaments appear to be tapered, down to a point, for about the last 1,000 to 2,000 A of their course; this is seen too consistently for it to be an effect of section orientation.

The bridges, as has been remarked before (2) may also be seen in cross-sections through the double array of filaments (Figs. 19 and 20). The angular form of the cross-section of the filaments themselves is also apparent, a fact already commented on by Sjöstrand and Andersson (9). In the H zone, this effect is particularly noticeable, and all sorts of bizarre shapes appear when the section is sufficiently thin to give a projection of only a short length of filament (Figs. 21 to 23).

4. Appearance in Unextracted Muscle

The observations as they have been described were made on glycerinated psoas muscle. However, no essential difference was found when fresh muscle was fixed and examined, and the secondary filaments can be seen perfectly well in suitably thin longitudinal sections (Figs. 25 and 26). The contrast, though, is very much lower than in glycerinated material, and it is evident that a good deal of diffuse and granular background material is present. It seems likely that this represents some of the soluble muscle proteins (which make up 30 to 40 per cent of the total), which are partly removed during glycerol extraction and the subsequent washing—a procedure which leaves the contractile structure in almost full working order (6, 11).

DISCUSSION

1. Previous Work

The results which have been described above give full support to the "sliding filament model" of striated muscle, and they are not compatible with the other types of structure which have been proposed by Spiro (7), by Hodge (8), and by Sjöstrand and Andersson (9). The latter are all essentially single array, coiling filament models. As we have already observed, it was the misleading appearance of muscle in longitudinal section which gave rise to the doubts about the sliding filament model, and now that that difficulty has been resolved, the primary motive for considering other types of structure has been removed. However, the existence of these alternative models has created a certain amount of confusion; particularly as reasons other than the invisibility of the secondary filaments in longitudinal sections have been put forward in

their support. It appears from the present electron microscope evidence that these models are wrong, but in order to clear up a little of this confusion, it is desirable to discuss some of the other evidence as well.

In the first place, these three hypotheses are incompatible with a large part of the original evidence on which the idea of a sliding filament model was based, and in the main, this evidence was simply set aside during their elaboration. Certain parts of it were challenged on *ad hoc* grounds[1] which it may not be essential to discuss now. The evidence has been described in detail in previous papers (1–6) and it is unnecessary to repeat it here. The observations, and the conclusions drawn from them, still all hold good. And the identification of myosin as the A substance has been confirmed recently by comparative interference microscope and biochemical measurements (16, 17). However, it does seem desirable to draw particular attention to one of the original arguments in favour of the sliding filament model, one based on simple observations in the light microscope, which has either been overlooked or whose force has been greatly underestimated. It concerns the changes in the length of the H zone which accompany changes in sarcomere length.

During stretch and contraction, the A bands remain at approximately constant length, and changes in the length of the muscle are accounted for by changes in the length of the I bands. Most significant of all, however, is the fact that the H zones (the lighter regions in the middle of the A bands) are longer in stretched muscle than in muscle at rest-length, and that they shorten and disappear as the muscle contracts. Over the range of lengths from the stretched condition down to the point at which the H zones close up completely, the distance between the Z lines and the edges of the H zones remains approximately constant. If myosin extraction is carried out at any particular sarcomere length in this range, then it is found that the "ghost" fibril, left after the removal of the A substance, consists of the Z lines and material extending from the Z lines up to the position of the edge of the H zones before extraction (4). These large, easily recognised differences in the length of the H zones, which are unaccompanied by any appreciable changes in the over-all length of the A bands, and which are very easy to observe either in isolated myofibrils or in sectioned whole muscle, provide, in the author's view, perhaps the most direct and compelling argument of all for the sliding filament model. It is very difficult to see how these observations, coupled with the present ones on the changes in the extent of overlap of primary and secondary filaments, can be explained in any other way. The alternative theories that have been put forward depend not on contesting this evidence, but on presuming that their incompatibility with it can in some unspecified way be

[1] *E.g.* that the "secondary filaments" seen in the end-on sections were really obliquely sectioned bridges; it was not clear how this effect could be produced with sections of conventional thickness.

removed; they take as their starting point the belief that there is no such thing as a system of two overlapping sets of filaments. Let us consider the positive evidence advanced in their support.

Spiro observed that the number of filaments present in the A bands, on either side of the H zone was, in rest-length muscle, three times as great as in the H zone itself. This is in agreement with the previous observations (2). In shortened muscle, however, only a single set of filaments was observed.

This latter observation has not been confirmed in the present studies, nor, contrary to the suggestion, has it been found that any difference, other than a straightening out of the I band filaments, results from holding a muscle at a fixed length rather than leaving it unrestrained during the preparative procedures. The evidence presented in support of a progressive transformation of thin into thick filaments is incomplete, for no intermediate stages were shown; the argument rests on the picture in which a complete "transformation" into thick filaments has occurred. It seems possible that the appearance of the structures has been affected by the freezing and thawing process used to produce the contraction; certainly considerable destruction of the thin filaments in the residual I bands has taken place.

Hodge presents a graph, apparently showing a linear relationship between sarcomere length and axial period, and suggests that it is this change in period which produces contraction. It is also suggested that the I band filaments may be incorporated into the A band filaments in some way. It is to be doubted, however, whether variations of $\pm$ 20 per cent (*i.e.* in the range 320 A to 480 A) are significant, for the measurements were made on mechanically fragmented material which had been allowed to dry down onto the electron microscope grid. During this process, variable amounts of shrinkage and distortion will affect both axial period and sarcomere length simultaneously. The two points on the graph at a sarcomere length of 1.5 μ and an axial period of 250 A seem to lie outside the range of scatter, but their significance is questionable until the effect is shown to be a reproducibile one correlated with changes in band pattern. One must point out that the most striking feature of very many of the published electron micrographs which show the axial period of muscle is that the number of periods in the A bands always seems to be constant (at about 38), while the number of periods in the I band varies with the degree of contraction. Specific attention is drawn to this fact by Edwards *et al.* (18), and their Fig. 6 shows the phenomenon with great clarity. It was also commented on earlier by A. F. Huxley (personal communication, 1953). Such a change in the number of periods in the I bands is incompatible with a linear relationship between sarcomere length and axial period. Moreover, the approximate constancy of the length of the A band has been well documented by observations in the light microscope (4–6), and if the number of periods there remains constant, then so must the length of each period, as x-ray measurements had indeed indicated (1).

The second argument used by Hodge derives from the apparent continuity, in many pictures, of the I band filaments with the main filaments in the A band. However, as we have already seen, it is almost impossible, even using the very thinnest sections, to prove which set of filaments in the A band is continuous with the I band filaments; in the pictures which have appeared previously in the literature, several layers of filaments are included in the sections, and no valid argument, either way, about filament continuity can be based on them. Indeed, in sections which show filaments in the A band most clearly, the secondary and primary filaments will lie in vertical register with each other.

The evidence from insect flight muscle will be considered in a separate paper, but a preliminary report (Huxley and Hanson (19)) has shown that in this type of muscle too, the secondary filaments can be seen clearly in thin, suitably oriented longitudinal sections as well as in cross-sections; in this case, however, the secondary filaments are located midway between *two* primary ones.

It is observed that a clear appearance of secondary filaments is difficult to obtain in cross-sections of muscle, and it has been suggested that this is due to a variability of the structure. But it is the present author's experience that the main difficulty with sections of conventional thickness is simply one of orientation. The secondary filaments are rather thin (about 50 A) and lie quite close to the primary filaments, and unless they are absolutely perpendicular to the plane of sectioning, the picture they give can be quite confused and misleading. Such a picture can be transformed into one which shows the secondary filaments, in transverse section, perfectly well all over the field, and, incidentally, all simultaneously in a given fibril (*e.g.* Fig. 19), simply by taking great care over the orientation of the block face.

Sjöstrand and Andersson, also, have expressed the view that only a single set of continuous filaments is present in striated muscle. But the picture of a longitudinal section which they have published (9) in support of this is not a convincing one, and, judging by the side spacing between filaments, contains more than one layer of them. It is also claimed that the axial distance between the cross-bridges which they have seen varies with the length of the sarcomere. However, the observations could equally well be accounted for by the reduction of the sarcomere length—and, of course, of any other axial periodicities—which takes place during sectioning unless the cutting edge is parallel to the fibres; this effect can reduce the dimensions by a factor of two or more in very thin sections. This criticism also applies to their claim that the A bands decrease in length when sarcomeres shorten.

It would seem, then, that the additional arguments advanced for the various alternative hypotheses are all rather insecurely founded. The hypotheses were neither designed to, nor are able to, account for much of the other evidence available concerning the structure of muscle. They were put forward

essentially because of the failure to observe a double array of filaments in longitudinal sections. This failure occurred because the sections used, though thin, were not thin enough for the purpose of studying a close-packed array of filaments.

2. The Present Results

There are many features of this type of structure which might profitably be discussed in relation to the mechanism of muscular contraction. However, most of them have already been considered at length in previous papers (1–6), and we will comment here on only a few special aspects.

One simple feature of the structures seen in these longitudinal sections is that, no matter whether the muscle is stretched, at rest-length, or shortened, the cross-bridges between primary and secondary filaments have the same form, *i.e.* they are, more or less, at right angles to the filaments. The only difference between the muscles at different lengths is that the two sets of filaments overlap to different extents. This must mean that as the filaments slide past each other during contraction, the bridges between them remain attached for a short distance only, and they must then detach from the secondary filaments and reattach at a point a little further along. We will refer to the separation between these points of attachment on the secondary filaments as the "step distance." Thus if contraction is brought about by some movement of, or interaction at, the bridges, then one complete contraction of the muscle will represent a number of cycles of operation of the contraction mechanism associated with the bridges. This idea is by no means a new one, but it is as well to draw attention to it again now that more direct evidence is available to support it. If the bridges represent sites of actin-myosin interaction and ATPase activity, if they represent the sites at which chemical energy is transformed into a mechanical deformation and hence into external work, then each site will be able to operate a number of times during a single contraction of the muscle. This possibility offers obvious advantages over a mechanism in which the active sites can function only once during each contraction.

In an earlier paper (4), the increase in the length of the I bands during stretch of muscle *in rigor* (*i.e.* when the contractile system was "locked"), was commented on. Probably the latter part of the rather large extensions used was non-physiological (although it was reversible); but the present observations of the difference in the degree of orientation of the I band filaments, as between muscle fixed when unrestrained, and when held at a fixed length, does direct attention again to the possibility that part of the series elastic component (21) might be accounted for by a tendency of the I band filaments, in normal circumstances, to depart slightly from perfect linearity. After all, the filaments are very thin, they are a long way apart, and they must be undergoing con-

tinuous Brownian movement. If this were the case, one would expect the effective length of the series elastic component to be different at different muscle lengths, *i.e.* to depend on the length of the thin filaments "exposed" in the I bands. This should be susceptible to experimental test.

The low birefringence of the I bands compared with that of the A bands is, of course, in part due to there being less oriented structural protein there (about ⅓ the amount). And the greater space available between the filaments might allow the concentration of the molecules of the soluble proteins to be higher there than in the A bands, thereby increasing the effective refractive index of the solution, and decreasing the form birefringence of the array. But the low birefringence may also be contributed to by an inherently poorer orientation of the array of I band filaments themselves.

The details of the arrangement of the cross-bridges between the filaments have several interesting features. Each secondary filament is connected to each of the three primary filaments around it once every 400 A. The three bridges involved are not in register but appear to be spaced out evenly along the secondary filaments. Thus one would expect these filaments to possess a threefold screw axis. A structure for actin has recently been proposed by Selby and Bear (20) on the basis of x-ray diffraction data. This structure is described in terms of a two-dimensional net, but the authors draw attention to, and indeed favour, an equally valid interpretation in terms of a two-chain helix with a 406 A axial period. It is interesting to note that when one builds this helical structure, it turns out to possess a threefold screw axis. The axial distance between nodes in the Selby and Bear structure is about 54 A. If these nodes represent actin monomers, to which bridges from the myosin may attach, then the minimum step distance would be 54 A; and as the nodes occur on a helix, the secondary filaments would have to spin about their long axes as the muscle contracted. A step distance of 54 A is compatible with a mechanism in which one molecule of ATP is split at each of the bridges for each step of the contraction when the muscle is exerting a maximum tension of about 4 kg./cm^2.

In the micrographs so far examined, the primary filaments also give the impression of having a helical structure, with the bridges protruding from them along a sixfold screw axis, with six bridges in 400 A. It is, incidentally, perfectly possible to build a structure from such filaments with the bridges in the right places for them to lie on a threefold screw axis on the secondary filaments. The tapered ends of the primary filaments would be a natural consequence of their being built from a helical array of long molecules, with their axes parallel to the helix, spaced out along it with a period less than the length of the molecules. The observation by Szent-Györgyi (22) that it is the H meromyosin subunit of myosin which combines with actin and which functions as an ATPase would suggest that the bridges or protuberances seen on the

primary filaments represent or contain this part of the myosin molecule. The total number of these bridges in one gram of muscle is easily computed, and is found to be approximately 5×10^{16}. The number of molecules of myosin present in one gram of muscle may be calculated from the molecular weight of myosin (23) and the known myosin content of muscle (16, 17, 24, 25), and is found to be in the range 4 to 8×10^{16}. Thus each of the bridges seen probably represents the active part of one molecule of enzyme.

Thus the details of the structure seem to be capable of interpretation in terms of the known properties of actin and myosin; but as yet, they do not reveal the nature of the basic mechanism involved in contraction.

SUMMARY AND CONCLUSIONS

The conditions under which one might expect to see the secondary filaments (if they exist) in longitudinal sections of striated muscle, are discussed. It is shown that these conditions were not satisfied in previously published works for the sections were too thick. When suitably thin sections are examined, the secondary filaments can be seen perfectly easily. It is also possible to see clearly other details of the structure, notably the cross-bridges between primary and secondary filaments, and the tapering of the primary filaments at their ends. The arrangement of the filaments and the changes associated with contraction and with stretch are identical to those already deduced from previous observations and described in terms of the interdigitating filament model in previous papers. There are therefore excellent grounds for believing that this model is correct. The alternative models which have been proposed appear to be incompatible both with the present observations and with much of the other available evidence.

I am indebted to Professor Bernard Katz for the encouragement that he has given to this work, to the Medical Research Council for their support, and to the Wellcome Trust for the provision of an electron microscope.

REFERENCES

1. Huxley, H. E., *Proc. Roy. Soc. London Series B*, 1953, **141,** 59.
2. Huxley, H. E., *Biochim. et Biophysica Acta*, 1953, **12,** 387.
3. Hanson, J., and Huxley, H. E., *Nature*, 1953, **172,** 530.
4. Huxley, H. E., and Hanson, J., *Nature*, 1954, **173,** 973.
5. Huxley, A. F., and Niedergerke, R., *Nature*, 1954, **173,** 971.
6. Hanson, J., and Huxley, H. E., *Symp. Soc. Exp. Biol.*, 1955, **9,** 228.
7. Spiro, D., *Exp. Cell Research*, 1956, **10,** 562.
8. Hodge, A. J., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** No. 4, suppl., 131.
9. Sjöstrand, F. S., and Andersson, E., *Exp. Cell Research*, 1956, **11,** 493.
10. Hodge, A. J., Huxley, H. E., and Spiro, D., *J. Exp. Med.*, 1954, **99,** 201.
11. Szent-Györgyi, A., Chemistry of Muscular Contraction, 1951, New York, Academic Press Inc., 144.

12. Palade, G. E., *J. Exp. Med.*, 1952, **95,** 285.
13. Hodge, A. J., Huxley, H. E., and Spiro, D., *J. Histochem. and Cytochem.*, 1954, **2,** 54.
14. Huxley, H. E., *Proc. Internat. Conf. Elect. Micr.*, London, 1954, in press.
15. Watson, M. L., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 183.
16. Huxley, H. E., and Hanson, J., *Biochim. et Biophysica Acta*, 1957, **23,** 229.
17. Hanson, J., and Huxley, H. E., *Biochim. et Biophysica Acta*, 1957, **23,** 250.
18. Edwards, G. A., Ruska, H., de Souza Santos, P., and Vallejo-Freire, A., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** No. 4, suppl., 143.
19. Huxley, H. E., and Hanson, J., *Proc. 1st European Regional Conf. Elect. Micr.*, Stockholm, Almqvist & Wiksell, 1956, 202.
20. Selby, C. C., and Bear, R. S., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 71.
21. Hill, A. V., *Proc. Roy. Soc. London Series B.*, 1953, **141,** 104.
22. Szent-Györgyi, A. G., *Arch. Biochem. and Biophysics*, 1953, **42,** 305.
23. Holtzer, A., *Arch. Biochem. and Biophysics*, 1956, **64,** 507.
24. Hasselbach, W., and Schneider, G., *Biochem. Z.*, 1951, **321,** 461.
25. Szent-Györgyi, A. G., Mazia, D., and Szent-Györgyi, A., *Biochim. et Biophysica Acta*, 1955, **16,** 339.

EXPLANATION OF PLATES

General Notes: (*1*) All photographs shown are of material fixed in buffered osmium tetroxide for ½ hour.

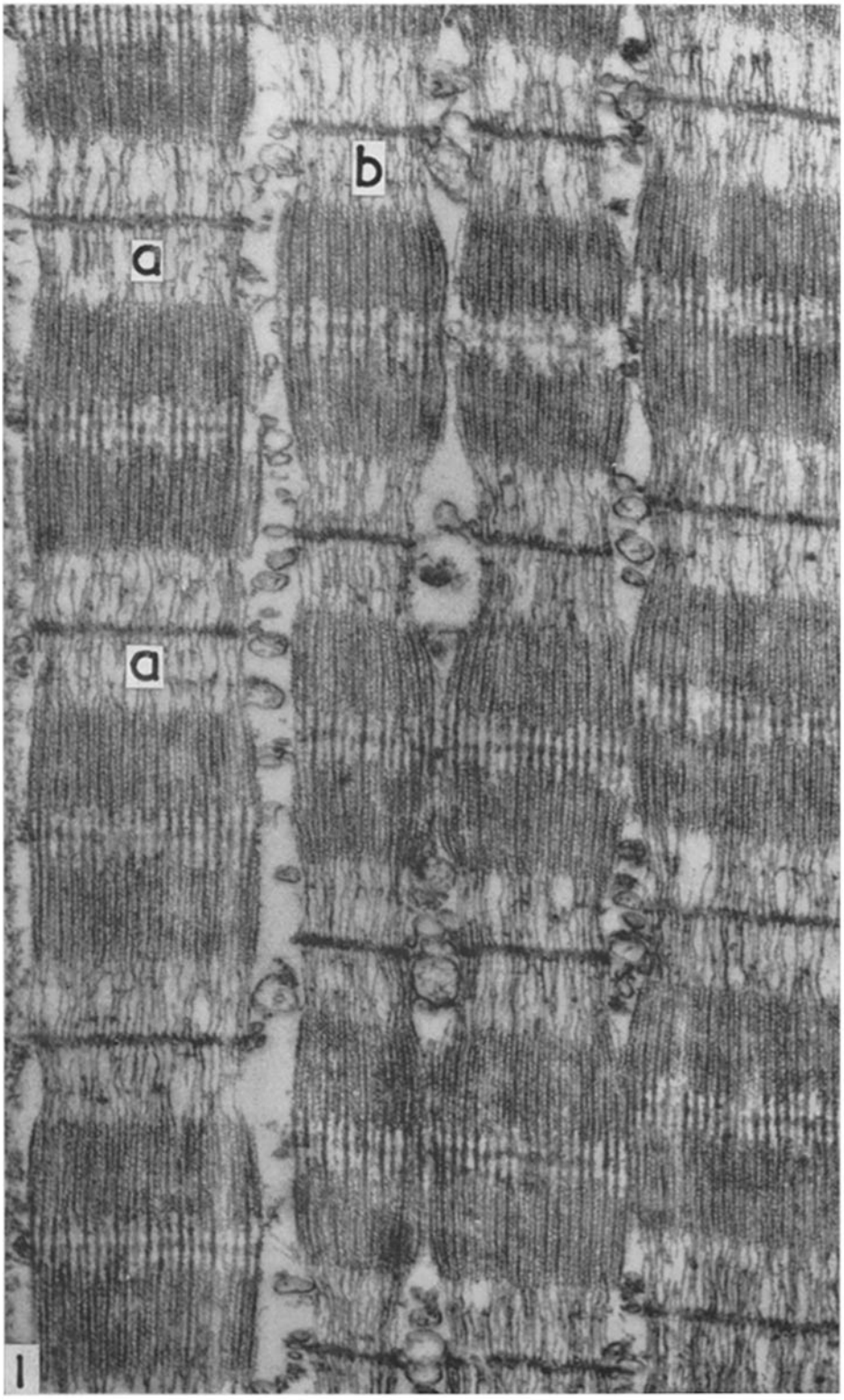
(*2*) Due to the change in the dimensions of the sections during the cutting process, an effect which is very marked when extremely thin sections are involved, the scale of the structure along the axis of the muscle is considerably foreshortened. Dimensions at right angles to the filaments are, however, largely unaffected.

(*3*) The lower magnification photographs were taken at an initial magnification of 8,000, the high magnification ones at 40,000, and the very high magnification photograph (Fig. 27) at an initial magnification of 160,000.

(*4*) Except where otherwise stated (Figs. 25 and 26), the material used was glycerinated psoas muscle.

PLATE 206

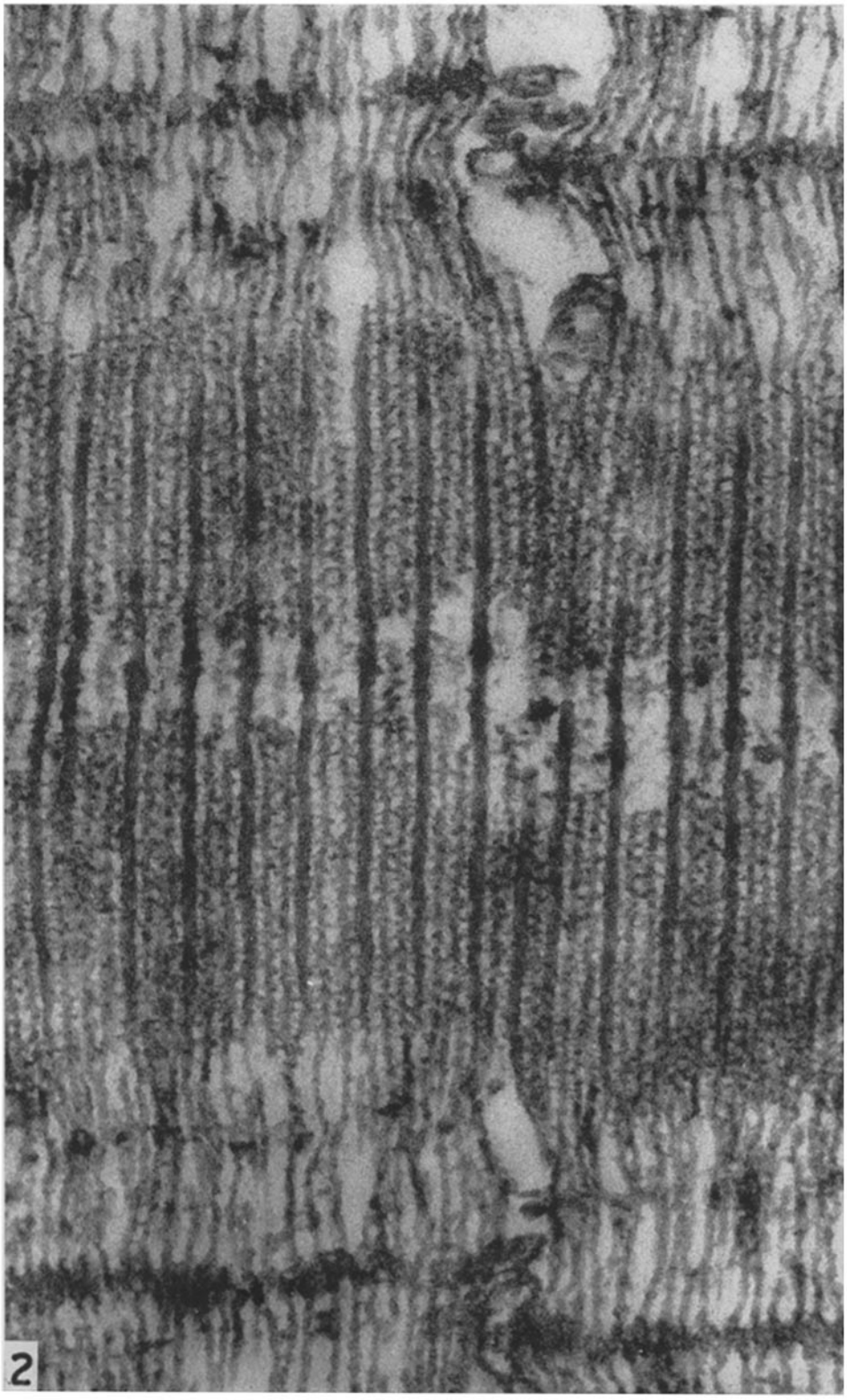
FIG. 1. Low power general view of fairly well oriented thin section through a number of striated myofibrils. Examples of sections parallel to the $10\bar{1}0$ lattice planes are marked *a*, and of sections parallel to the $11\bar{2}0$ lattice planes, *b*. Magnification, 60,000.



(Huxley: Filaments in cross-striated muscle)

PLATE 207

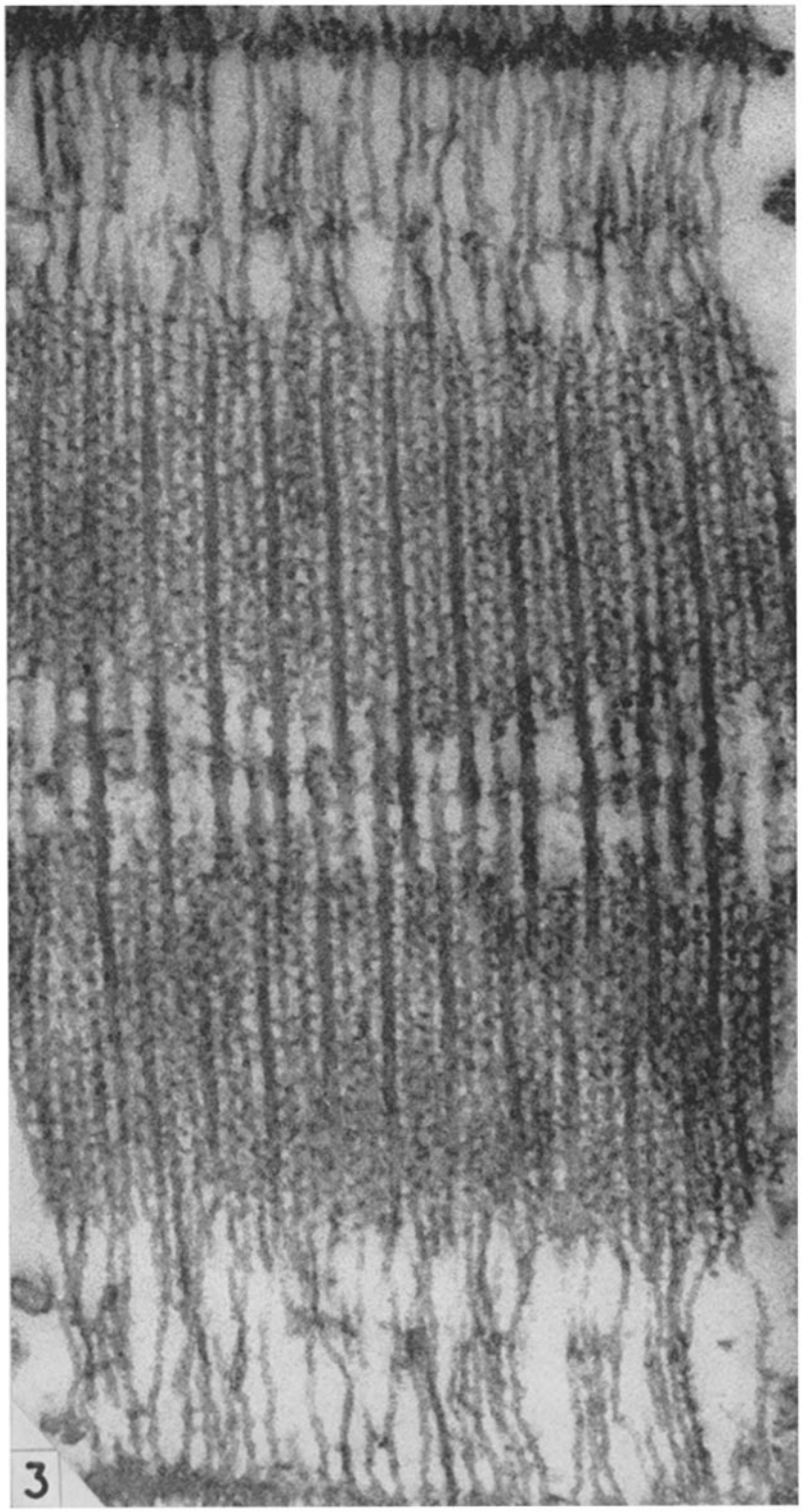
FIG. 2. Section through one sarcomere, parallel to $11\bar{2}0$ planes, showing primary filaments, with large interfilament spacing (about 500 A), and pairs of secondary filaments in between them. The interruption of the secondary filaments at the edges of the H zone is readily visible. The characteristic tapering of the primary filaments at the ends of the A bands is seen quite generally in this and other pictures, and seems to occur independently of effects caused by the filaments passing out of the section. The cross-bridges between primary and secondary filaments can also be seen and counted. The tapering of the primary filaments seems to occur over the length occupied by the last four to six bridges, *i.e.* the last 1600 to 2400 A of the filament. The primary filaments are somewhat thickened in the H zone, elsewhere their diameter is 110 to 120 A; the diameter of the secondary filaments is 50 to 60 A. Magnification, 175,000.



(Huxley: Filaments in cross-striated muscle)

PLATE 208

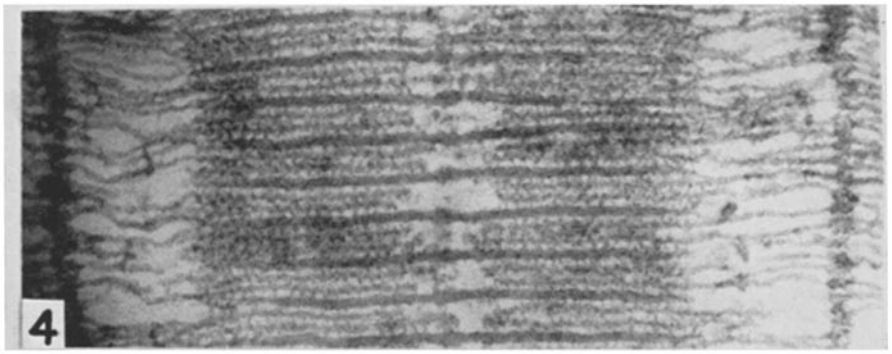
FIG. 3. Section through one sarcomere, almost parallel to $11\bar{2}0$ plane. The filaments run at an angle of about 1° to the plane of the section, and near the centre of the sarcomere, the section passes from one layer of the lattice to the next. The substantially poorer orientation of the filaments in the I bands, as compared with the A bands, is apparent. However, the order is sufficiently good for one to see that the secondary filaments in the I bands do not have projections on them. Magnification, 195,000.

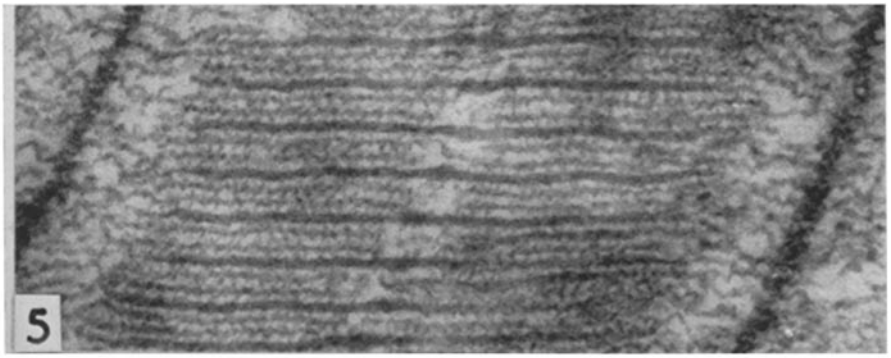


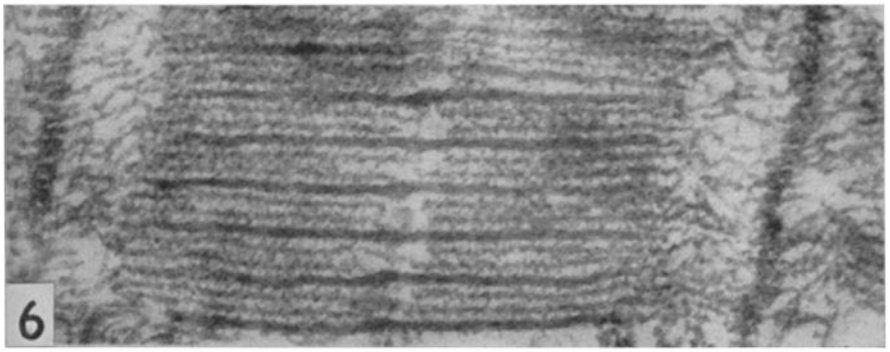
(Huxley: Filaments in cross-striated muscle)

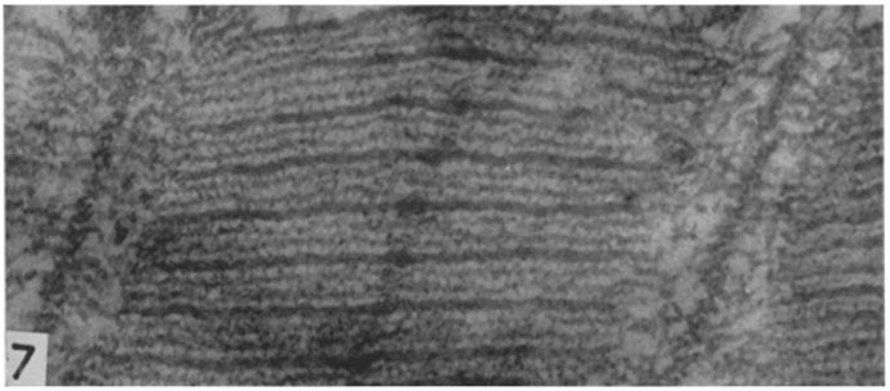
PLATE 209

FIGS. 4 to 7. Sections through sarcomeres in $11\bar{2}0$ direction, showing variation in length of gap between ends of secondary filaments in centre of A bands at different sarcomere lengths. Fig. 4 shows a slightly stretched sarcomere; Fig. 7 a slightly contracted one. Magnification, 100,000.





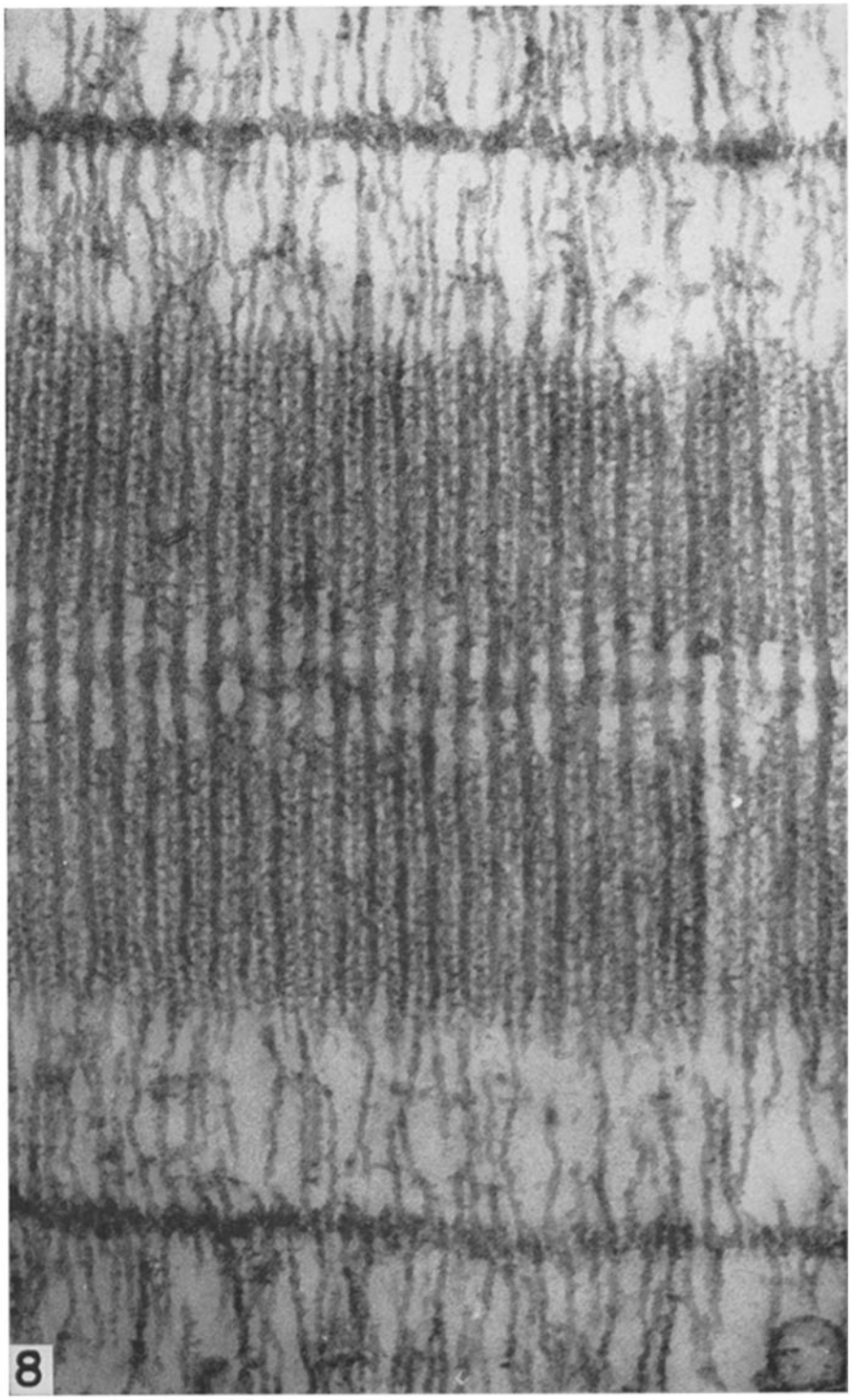




(Huxley: Filaments in cross-striated muscle)

PLATE 210

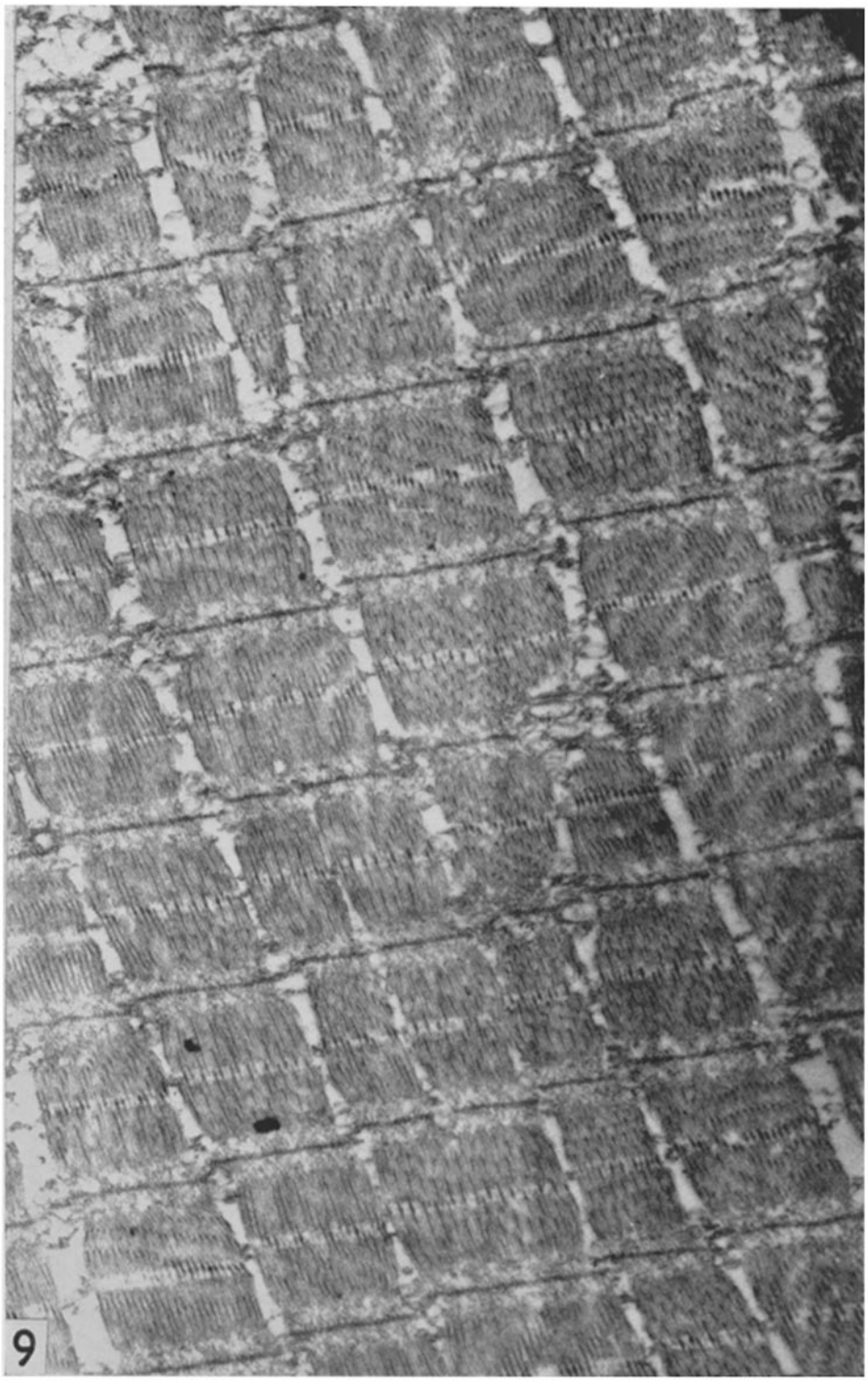
FIG. 8. Section through one sarcomere, parallel to $10\bar{1}0$ planes. A simple alternation of primary and secondary filaments is seen, the secondary filaments terminating at the edges of the H zone. The secondary filaments appear thicker than in Figs. 2 and 3 because in general they will really consist of two superposed secondary filaments in the plane of the section, as shown in Text-fig. 1. Magnification, 150,000.



(Huxley: Filaments in cross-striated muscle)

PLATE 211

FIG. 9. Low power picture of oblique section through a number of myofibrils. The filaments run at an angle of about 5 to 10° to the plane of the section, and an interesting variety of patterns is seen; the hexagonal lattice of filaments in any given sarcomere may be rotated or twisted slightly relative to that in the adjoining one. The absence of secondary filaments from the H zones may be verified in this type of section. Magnification, 25,000.

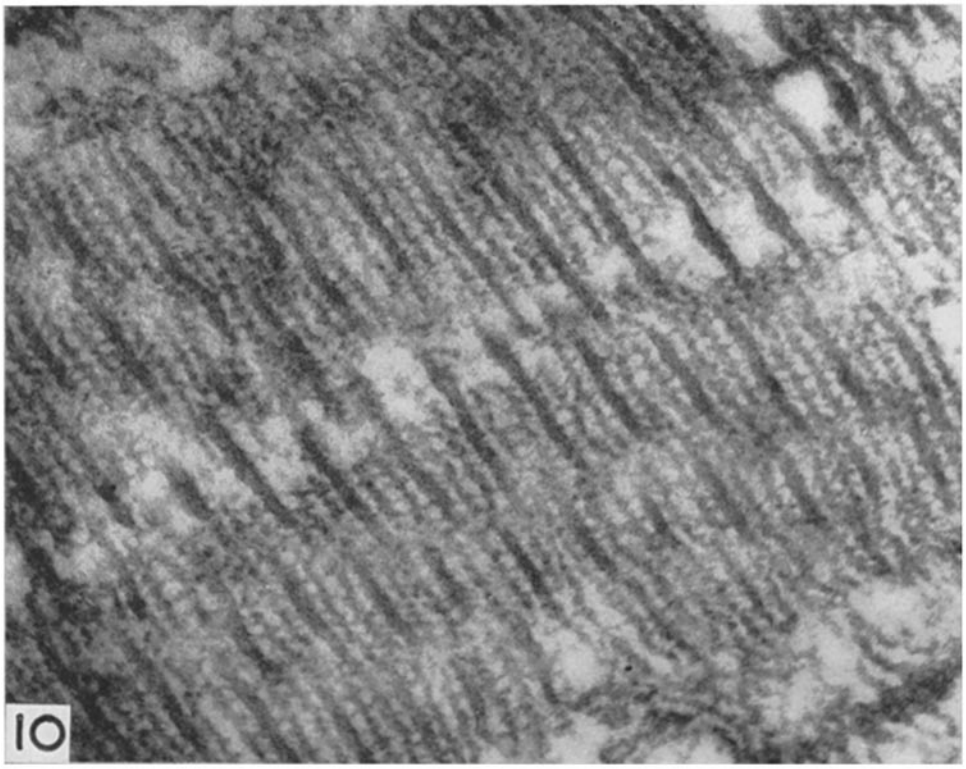


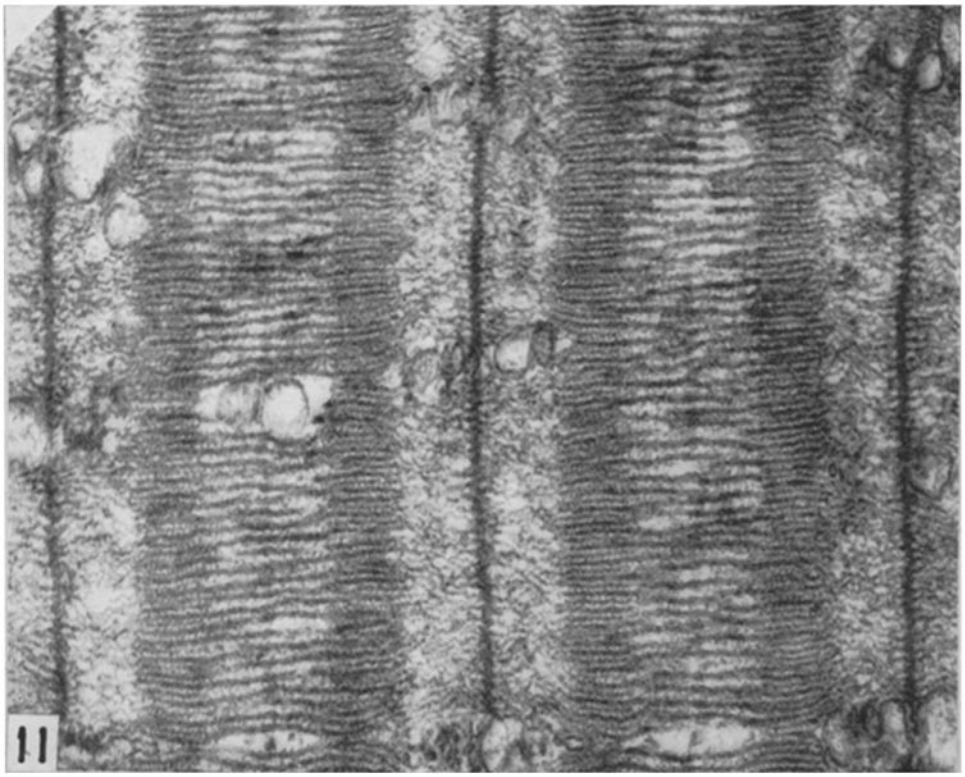
(Huxley: Filaments in cross-striated muscle)

PLATE 212

FIG. 10. Oblique section through one sarcomere. The thicker, primary filaments in successive layers occur midway between the filaments in the preceding layer. The pair of secondary filaments between each pair of primary filaments are located in the same plane as the primary filaments, and leave the section simultaneously; in the H zone, only the primary filaments are visible. Magnification, 150,000.

FIG. 11. Stretched muscle, showing long H zones. Section approximately parallel to $10\bar{1}0$ planes. The secondary filaments can be seen to extend only a short distance into the A bands. The regularity in the arrangement of the filaments in the A bands of stretched specimens is always found to be lower than in rest-length fibrils; possibly the absence of secondary filaments from a long region in the center of the A band allows the primary filaments there, no longer cross-linked via the secondary filaments, to become disarranged. The specimen shown in this section was not held at a fixed length during fixation, and the I bands too are disorganised and somewhat shortened. Magnification, 45,000.

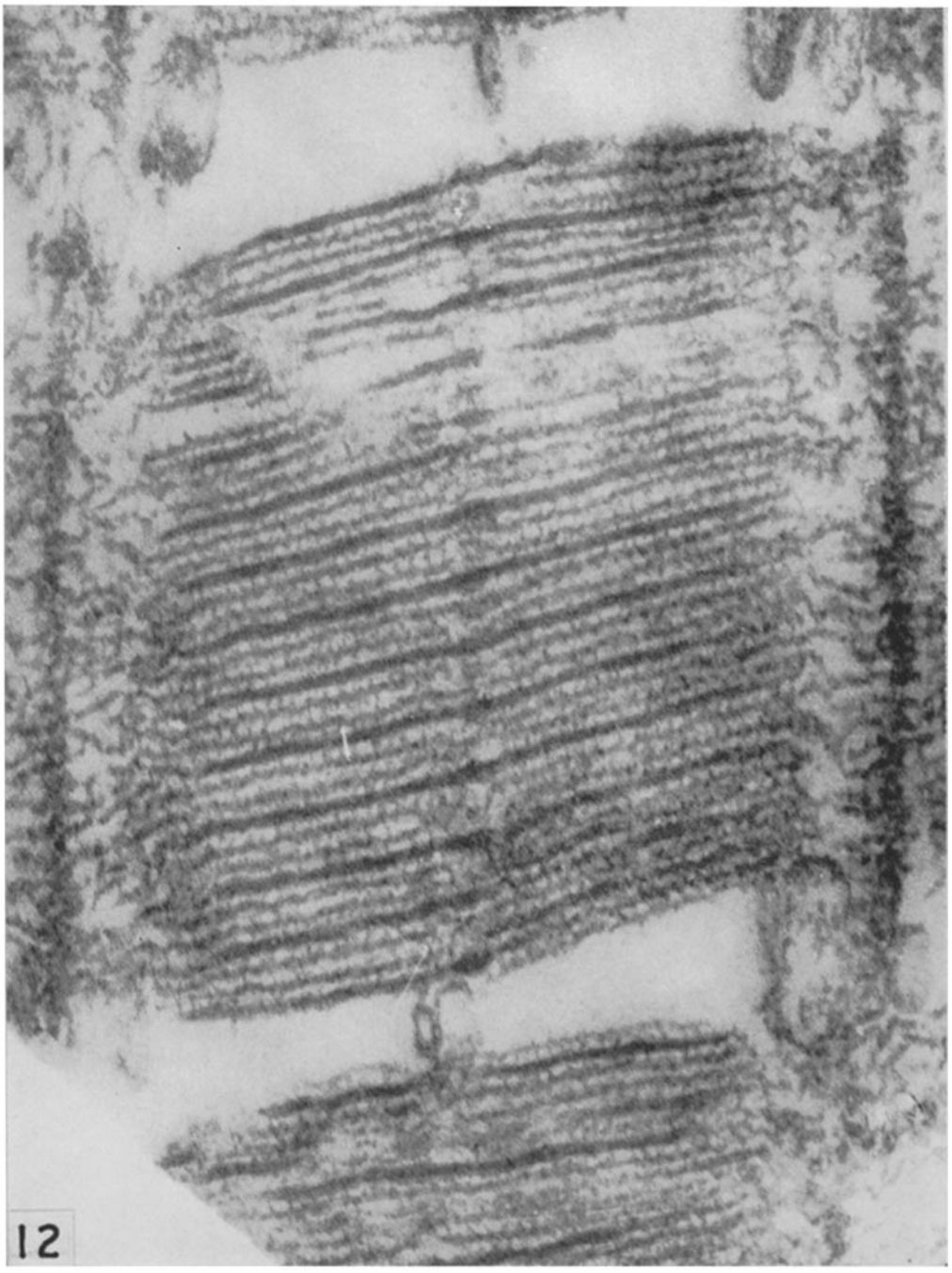




(Huxley: Filaments in cross-striated muscle)

PLATE 213

FIG. 12. Section through fibrils which were not held at a fixed length during fixation. Over most of the picture, the section is parallel to the $11\bar{2}0$ planes and shows the characteristic appearance of primary and secondary filaments. The secondary filaments in the I bands are considerably disoriented. In the lower part of the picture, the section passes through about four secondary filaments in between very widely spaced pairs of primary filaments. One secondary filament can be seen running along the outside of this lower fibril. Magnification, 150,000.

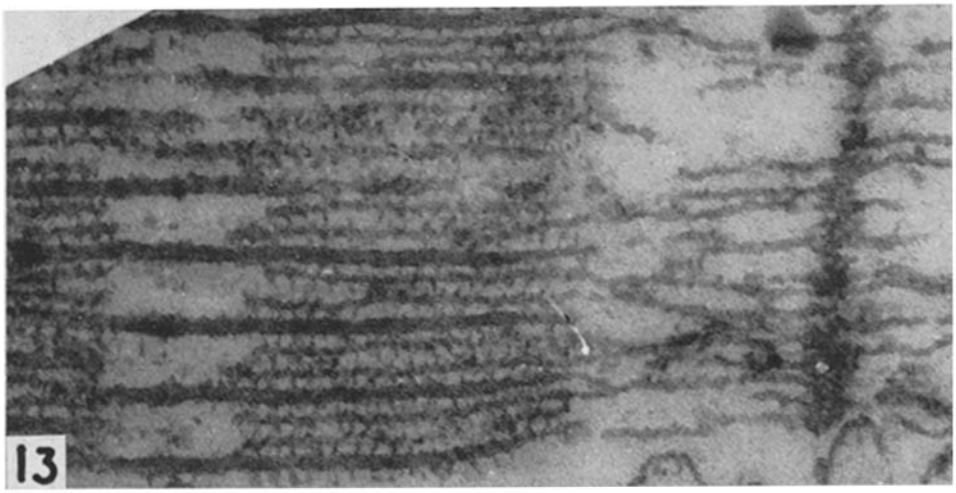


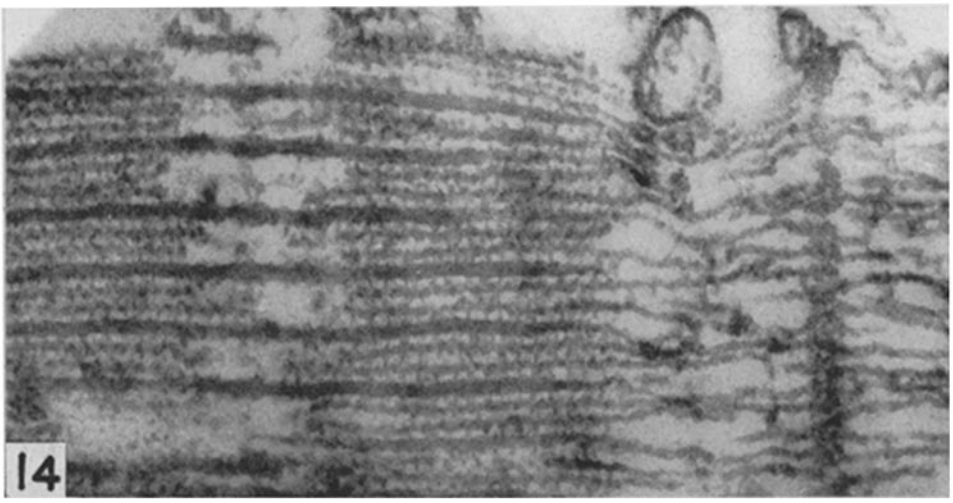
(Huxley: Filaments in cross-striated muscle)

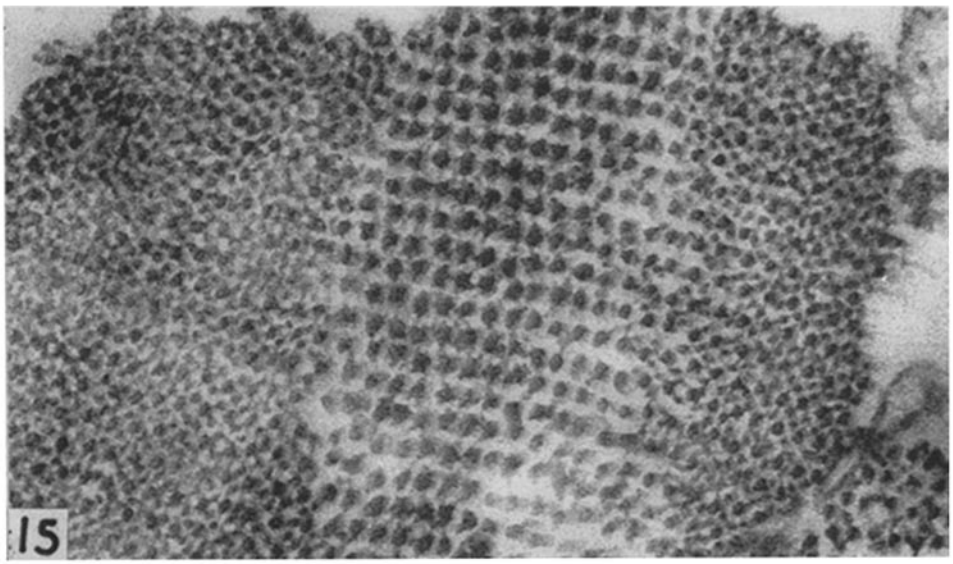
PLATE 214

FIGS. 13 and 14. Sections showing primary and secondary filaments at the A-I boundary and at the H-A boundary. The tapering of the thick filaments at the A-I boundary is readily seen, and in places they may be observed to terminate, while the secondary filaments continue on into the I bands. Because of the inherent lack of order at this boundary, however, this appearance cannot be seen in every case. Magnification, 150,000.

FIG. 15. Cross-sectional view of fibril in neighborhood of the H zone. The section has passed through the A band proper on the left and right hand sides of the picture, and through the H zone in the center of the picture. The rows of primary filaments in the H zone can be followed through into the A bands (particularly on the right hand side of the picture); at the H-A boundary, the array of secondary filaments around each of the primary ones makes its appearance. Magnification, 150,000.







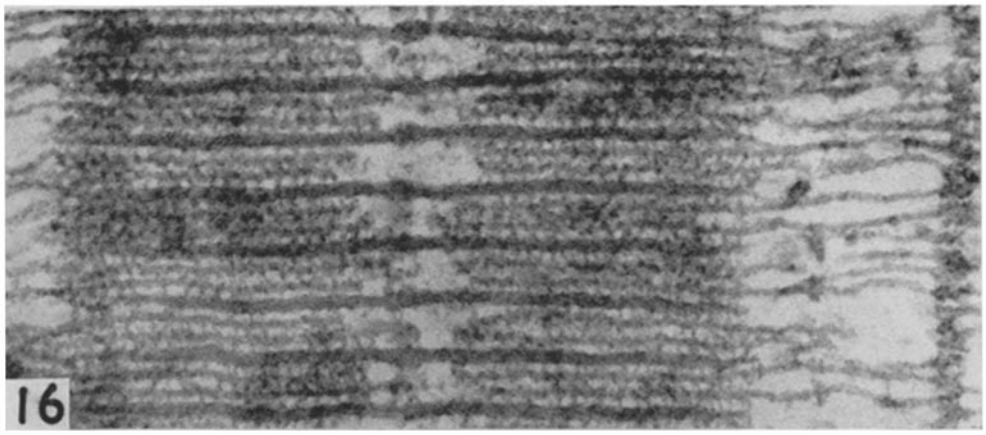
(Huxley: Filaments in cross-striated muscle)

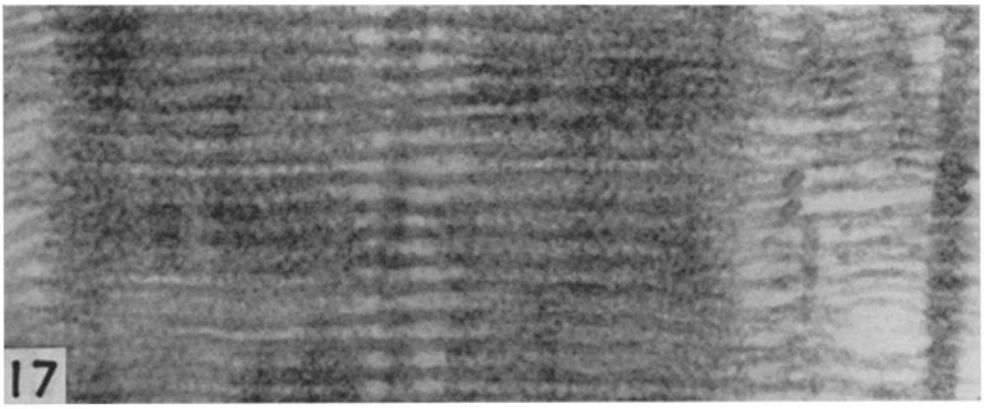
PLATE 215

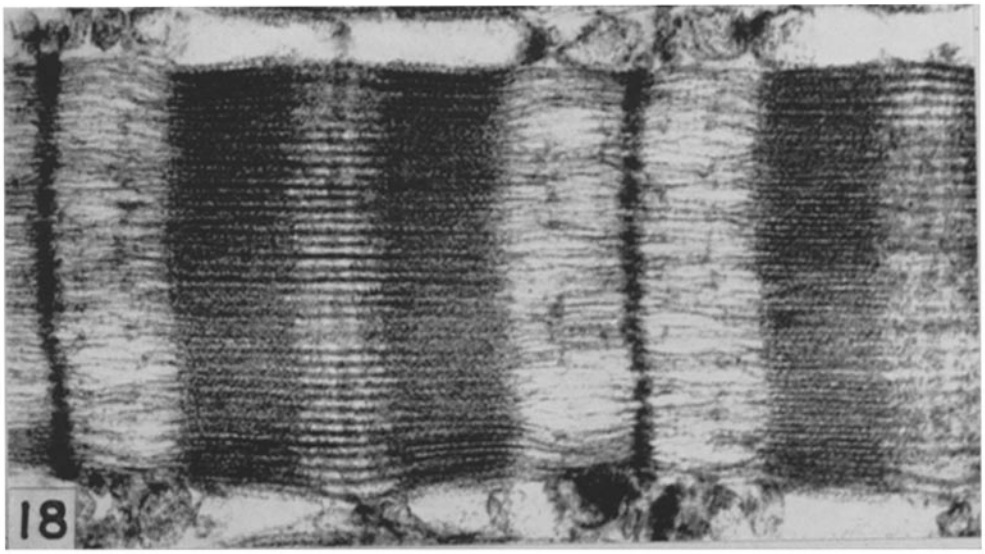
FIG. 16. Typical section through fibril parallel to $11\bar{2}0$ planes, section thickness probably about 100 to 150 A. Magnification, 150,000.

FIG. 17. Same photograph, but doubly printed to imitate appearance of similarly oriented section about 300 A thick (*i.e.* containing two layers of primary filaments). It will be seen that the secondary filaments are now obscured.

FIG. 18. Thicker section (probably 600 to 700 A in thickness) through muscle fibrils. This is a section of conventional thickness (silver in color when viewed in reflected light); the secondary filaments cannot be distinguished. Magnification, 50,000.







(Huxley: Filaments in cross-striated muscle)

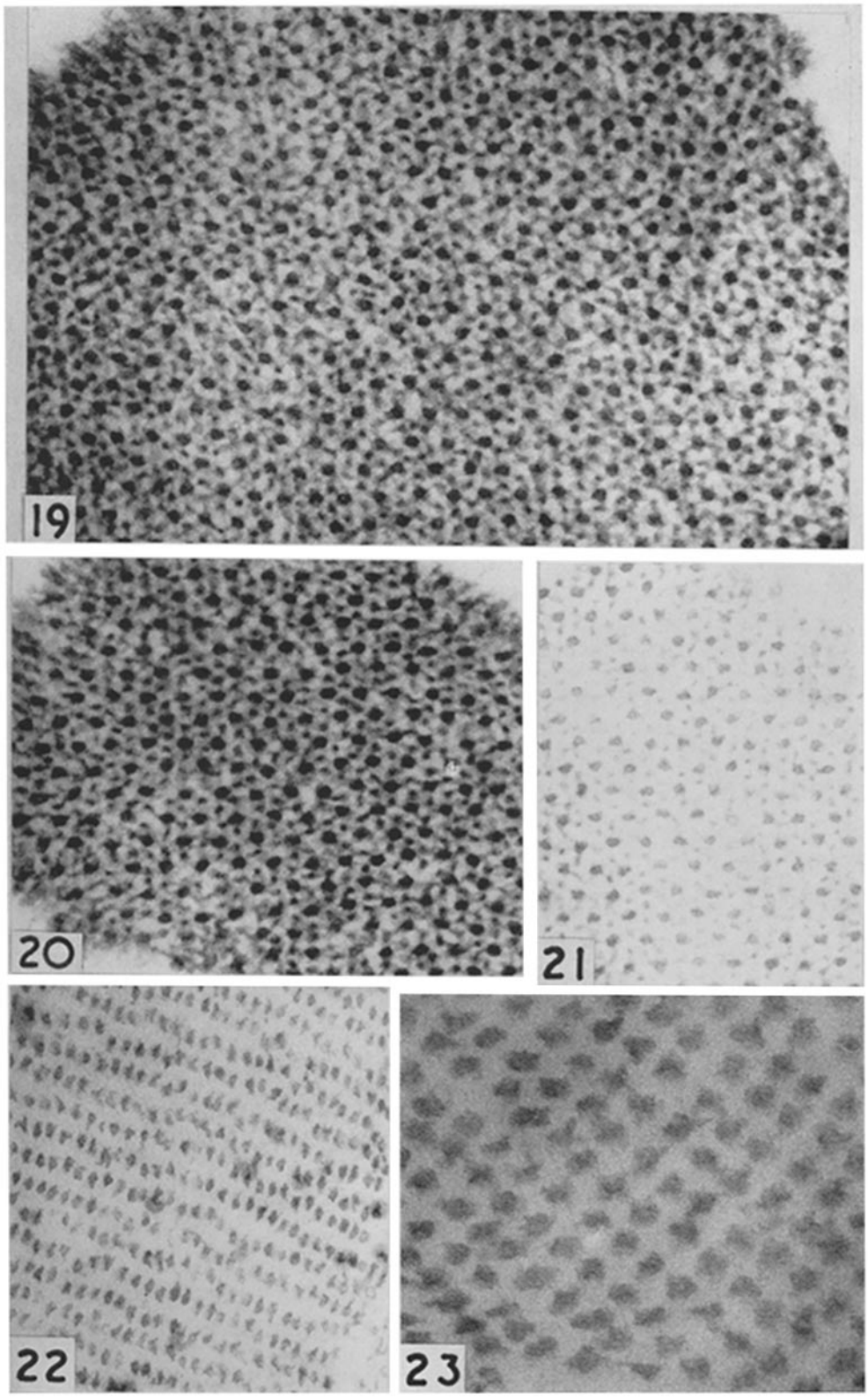
PLATE 216

FIGS. 19 and 20. Cross-sections through A band region, showing double hexagonal array of primary and secondary filaments; and the cross-bridges between them. Magnification, 150,000.

FIG. 21. Similar section, but printed with higher contrast to bring out the angular form of many of the filaments in cross-section. Magnification, 150,000.

FIG. 22. Very thin cross-section through an H zone, showing the pronounced non-circular appearance of the filaments in this region. Magnification, 100,000.

FIG. 23. Highly magnified view of an H zone, showing the triangular and other geometric forms assumed by the filaments in cross-section. Magnification, 200,000.



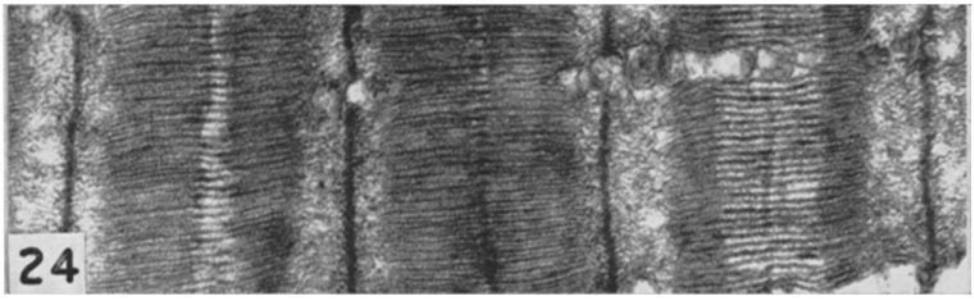
(Huxley: Filaments in cross-striated muscle)

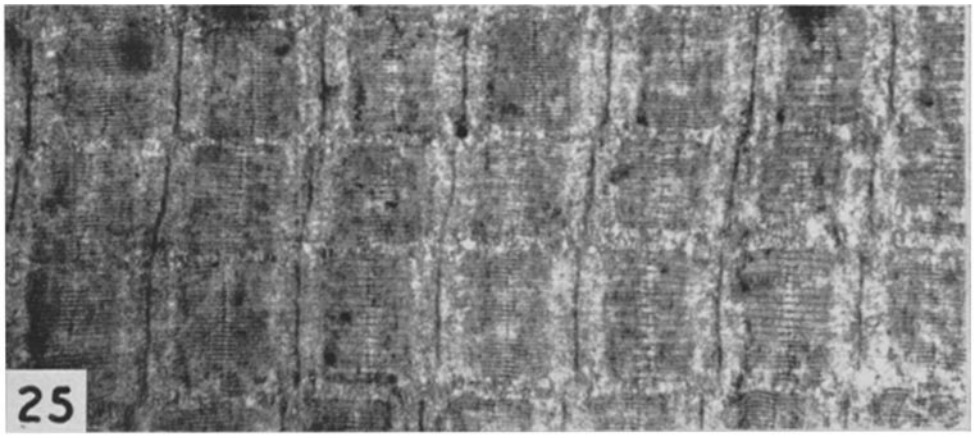
PLATE 217

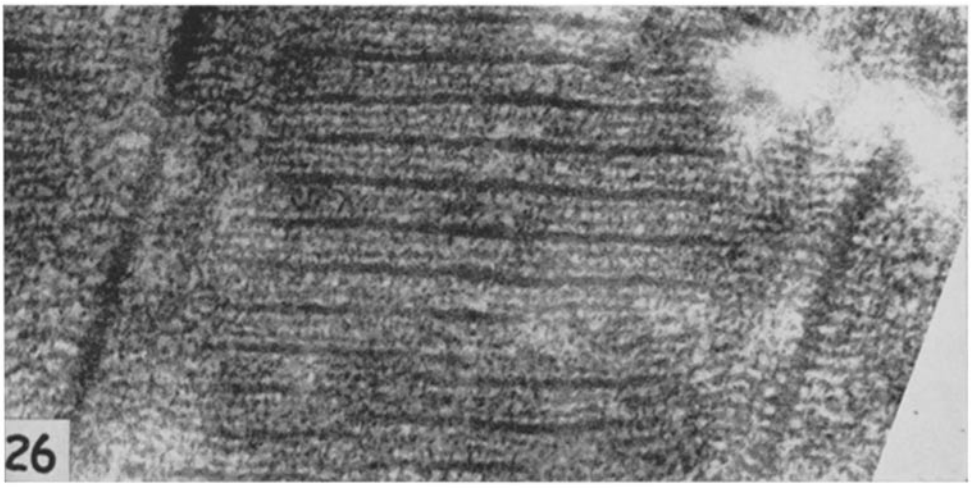
FIG. 24. Longitudinal section through adjacent contracted and stretched sarcomeres. Note the longer I bands and H zone in the stretched sarcomere; the withdrawal of the secondary filaments from the H region is well marked. Magnification, 35,000.

FIG. 25. Low power view of longitudinal section of rabbit psoas muscle fixed while in the living state (*i.e.* no glycerol treatment). The characteristic patterns of primary and secondary filaments can be seen just as in glycerinated materials, although the contrast and clarity of the picture is lower. Magnification, 20,000.

FIG. 26. Longitudinal section of fresh muscle (as in Fig. 25), cut parallel to $11\bar{2}0$ planes, showing pairs of secondary filaments in between the widely spaced pairs of primary filaments, in the same manner as in glycerinated material. Magnification, 100,000.



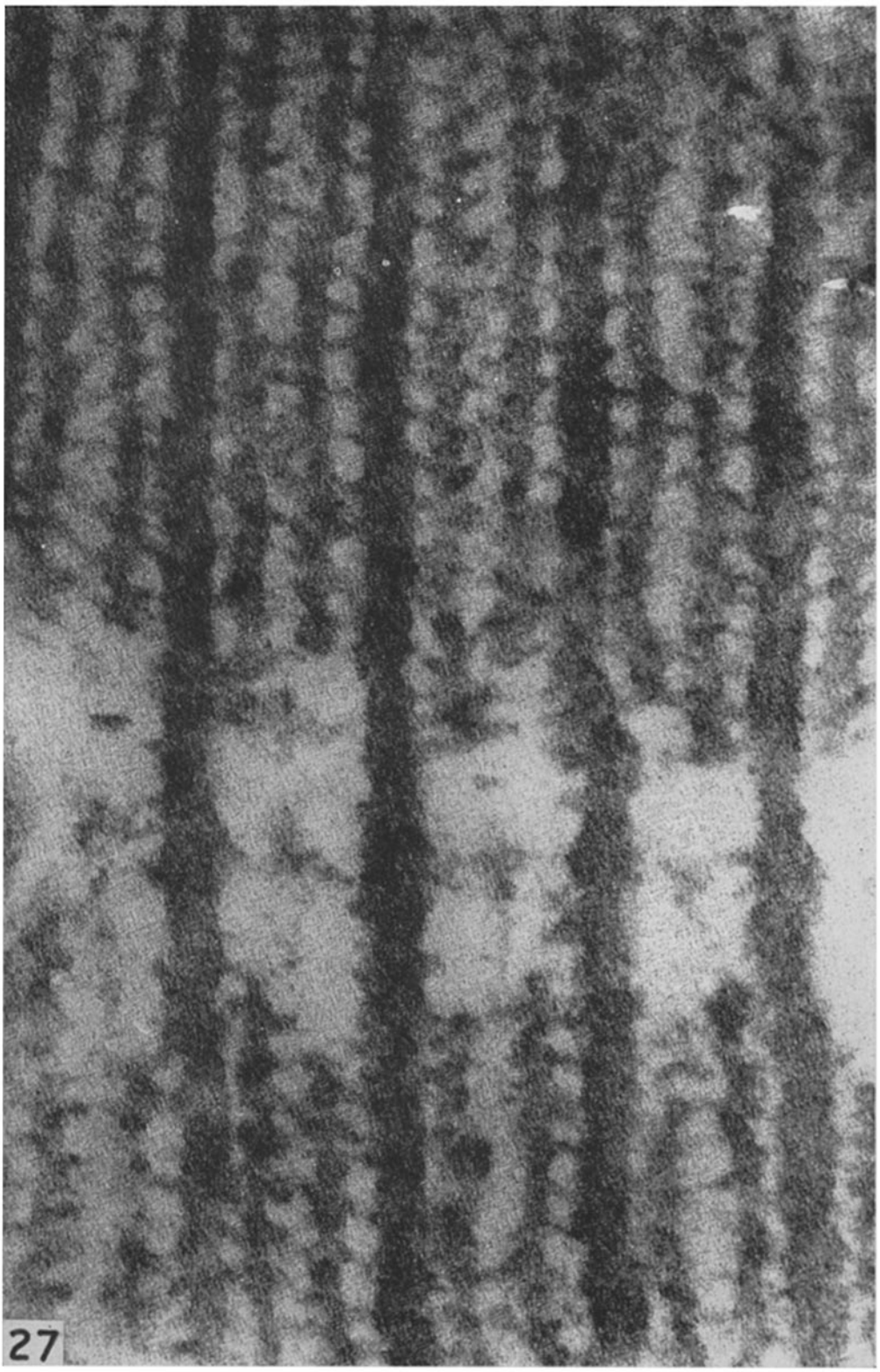




(Huxley: Filaments in cross-striated muscle)

PLATE 218

FIG. 27. Highly magnified view of central region of an A band, sectioned parallel to $11\bar{2}0$ planes, showing primary and secondary filaments and bridges between them. The termination of the secondary filaments at the H zone is readily seen. The initial magnification of this photograph was 150,000, and the instrumental resolution is substantially better than 10 A, judging from measurements on the background granularity due to phase-contrast effects very close to focus. The axial spacing between the bridges is greatly foreshortened by the change in section dimensions produced during the cutting of this very thin section. The appearance of bridges between pairs of secondary filaments is probably due to those bridges which extend out of the plane of the section to the primary filaments in the layers above and below. In many places, there are indications that the filaments have a helical structure. Magnification, 600,000.



(Huxley: Filaments in cross-striated muscle)