STUDIES ON THE CROSS-STRIATION OF THE INDIRECT FLIGHT MYOFIBRILS OF THE BLOWFLY CALLIPHORA

By JEAN HANSON, Ph.D.

(From the Medical Research Council Biophysics Research Unit, Wheatstone Physics Laboratory, King's College, London)

Plates 179 to 182

(Received for publication, June 15, 1956)

INTRODUCTION

The muscles which drive the wings of flies are called the indirect flight muscles because they are inserted on the thoracic wall and not on the wings; by an interesting mechanism which Boettiger and Furshpan (3) have described, very small deformations of the thoracic wall result in strokes of the wings, up and down. These muscles occupy most of the thoracic cavity. The direct flight muscles are smaller and they carry out minor adjustments in the positions of the wings.

Myofibrils are the contractile elements of muscle fibres, and in striped muscles the cross-striation is a property of the fibrils. Studies on this striation in the fibrils of the rabbit's psoas muscle (8, 18) and studies on the arrangement of filaments in these fibrils (15–17) have led to a conception of the mechanism of muscular contraction (Hanson and Huxley (9)) which, if it is correct, should probably apply to all striped muscles. During these studies some parallel investigations have been made on the indirect flight myofibrils of the blowfly *Calliphora* which for several reasons are an interesting choice for comparison with rabbit fibrils. The contractions of these flight muscles are nearly isometric (3, 4, 25) and very rapid; they can maintain a rate of 300 contractions per second, and the rhythm is of myogenic origin (25, 26). Thus, comparative studies may possibly distinguish some of the fundamental features common to all striped muscles from features which are functional or phylogenetic specialisations. These insect muscles have other advantages, because their fibrils are large and easily isolated.

Two quite different explanations can be suggested to account for the cross-striation of myofibrils and for the changes that are always observed in the pattern as the fibril changes its length. One of these theories supposes that the fibril consists of contractile filaments between which are other materials differentiating the bands, and that some of these materials migrate along the fibril as the filaments contract. The other theory supposes that the fibril consists of two different kinds of filaments which overlap in some regions; contraction is brought about by the movement of filaments of one kind alongside

filaments of the other kind. This second theory (9, 14, 17, 18) is supported by the results of detailed studies on the cross-striation in vertebrate skeletal muscles (14, 18) and especially by the results of studies by low-angle x-ray diffraction (15, 16) and electron microscopy (17) on the arrangement of filaments in the fibrils. The first theory has often been suggested, and recently it has again been put forward by Hodge (13) to account for the features he observed in the cross-striation of the indirect flight myofibrils of the blowfly *Lucilia*, and to account for the results of his electron microscopical studies on these fibrils. These two theories concern fundamental aspects of the mechanism of muscular contraction, and it seems rather unlikely that both are correct, each for its own type of muscle.

Rabbit myofibrils (24), and presumably also the fibrils of insects, consist almost entirely of proteins, amongst which myosin and actin are of outstanding importance, for they are present in large amounts and can form contractile systems *in vitro*. Actomyosin has been extracted from the thoracic muscles of locusts and grasshoppers (5) but no studies on the proteins of dipteran flight muscles appear to have been published. There is much evidence that the main features of the cross-striation in a rabbit fibril are due to the distribution of myosin and actin (8–11, 19). Thus, investigations on the structural and chemical basis of the cross-striation can make important contributions towards an understanding of the mechanism of muscular contraction, and the research on the indirect flight myofibrils of *Calliphora* is directed towards this end. The results reported in this paper concern the features of the cross-striation visible in the light microscope, and the changes taking place in the striation as the fibril changes its length or when it is treated with solutions that extract components from it.

PREVIOUS WORK

Some of the earliest studies on the cytology of striped muscles were made on the flight myofibrils of insects. All the details of the patterns of cross-striation as seen in the ordinary light microscope or in polarised light were described, but no generally accepted or well established conclusions emerged about the precise manner in which the pattern changes on contraction or extension, *in vivo* or *in vitro*. These earlier studies have been reviewed elsewhere (1, 2, 20, 21, 29). Advances in muscle biochemistry (28) made it possible to induce contraction in isolated fibrils under the microscope (6), and this technique has recently been used by Hodge (13) in studies on the cross-striation of myofibrils from the indirect flight muscles of the blowfly *Lucilia cuprina*, of *Musca*, and of other insects, observed in a phase contrast microsope and in polarised light. He has described the fibrils isolated from fresh and from glycerol-extracted muscles and the changes they undergo when treated with adenosinetriphosphate in order to make them contract; he also tried, without success, to extract components from them by methods which remove myosin from rabbit fibrils. The studies on *Calliphora* fibrils which are the subject of the present paper have given results

which differ in their most important aspects from those of Hodge, so that it has been decided to present a complete account even though in part it repeats the descriptions given by Hodge.

Material and Methods

Nearly all the observations described in this paper were made on unfixed isolated myofibrils in a phase contrast microscope or in polarised light. Photographs for measurement were taken with an objective of numerical aperture 1.3 on micro-file film at magnifications between 350 and 400, and a fine grain developer was used.

Large blowfly maggots, *Calliphora erythrocephala* Mg. and *C. vomitoria* L., were obtained from a dealer. Both species gave the same results. The flies, which were fed on sugar and raw meat, were used during the first few weeks after they had emerged. The thorax was cut out of a living fly and bisected longitudinally. A group of muscle fibres from either the dorsal muscles or the dorsoventral muscles (27) was very gently teased with fine needles in a drop of "standard salt solution" on a microscope slide; many isolated fibrils and sarcosomes were released. The composition of standard salt solution was 0.1 M potassium chloride, 0.0067 M phosphate buffer, pH 7.0.

Many useful studies on mammalian muscles have been made on glycerol-extracted material prepared by a method (28) which removes water-soluble components but leaves the contractile apparatus apparently undamaged; experiments can then be performed in the absence of complicating factors (28, 31). In order to prepare glycerol-extracted *Calliphora* fibrils, the bisected thoraces were immersed at 4°C. in a mixture of 50 volumes of glycerol, 40 volumes of water, and 10 volumes of 0.067 M phosphate buffer, pH 7.0. The glycerol was freed from traces of heavy metals by filtering it, together with the water, through a column of ion-exchange resin, amberlite IR 120 H, before adding the buffer. At the end of 24 hours the medium was changed, and after another 24 hours at 4°C. the material was stored at −20°C. and was not used until it had been kept for at least 2 weeks. Groups of muscle fibres were then dissected out of the thorax and equilibrated at 4°C. with dilute glycerol, and then with standard salt solution; fibrils were isolated by gently teasing the muscles.

Solutions of ATP[1] were made from the sodium salt (commercial preparations) which was dissolved in water, neutralised with sodium hydroxide, and then diluted with standard salt solution. "Crude muscle extract" was made by teasing fresh thoracic muscle very roughly into a small volume of standard salt solution (about 4 ml. for one fly) so that sarcosomes were released in large quantities. Neither calcium nor magnesium was added to the extract or to the ATP solutions.

All solutions were made with water freed from heavy metals either by distillation or by ion-exchange resins.

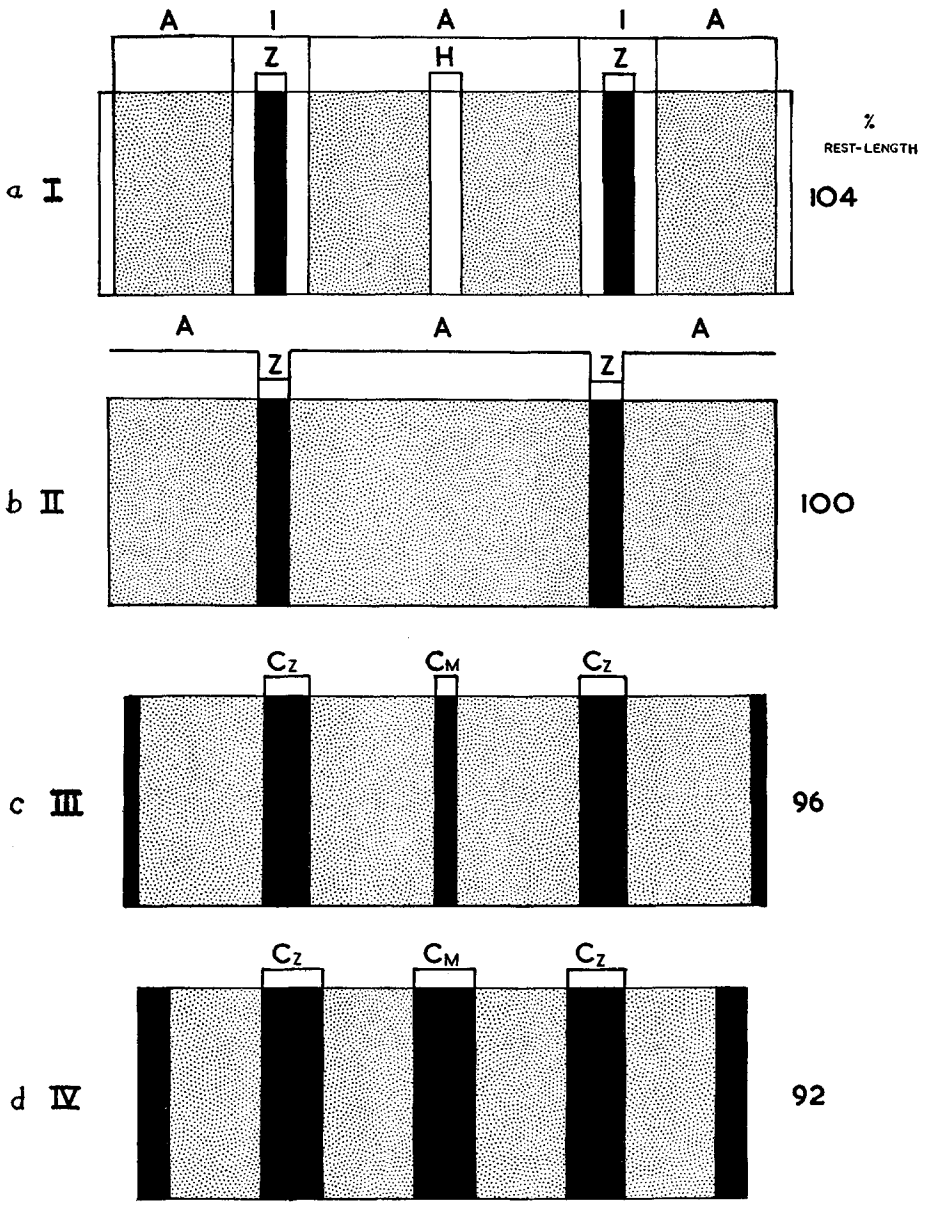
The effects of various treatments on the isolated fibrils were observed directly; a drop of the experimental or control solution was placed at one side of the coverslip and drawn through the preparation by filter paper at the other side.

RESULTS

1. Sarcomere Lengths and Band Patterns in Fibrils Isolated from Living Flies:

When a group of muscle fibres cut out of the thorax of a living fly is very gently teased with fine needles in a drop of standard salt solution, the fibrils which are isolated have band patterns of four main types, illustrated in Text-fig. 1 *a* to *d*; these will be called types I to IV.

[1] ATP, adenosinetriphosphate.



TEXT-FIG. 1. Diagrams illustrating the manner in which the pattern of cross-striation changes with fibril length. Over the range from about 98 to 104 per cent rest-length the process is reversible. All types of fibrils (I to IV) are found in preparations made from fresh muscles. No attempt has been made to represent optical density accurately in these diagrams.

In type I fibrils (Text-fig. 1 *a* and Fig. 1) the sarcomere is bounded by Z lines each of which lies in the middle of a very short band of low density. The rest of the sarcomere has a higher density except for a short zone in the centre. For reasons which will become apparent later, it is considered that these various bands are equivalent to the bands identified in a rest-length rabbit fibril, and they will therefore be given the same names; thus the region of low density in the middle of the sarcomere is an H zone and the bands of low density bisected by Z lines are I bands; the long band of high density, in the middle of which lies the H zone, is the A band. In polarised light the A band is birefringent and the Z lines are isotropic; the H zones and I bands are not clearly differentiated (Fig. 1). In a typical fibril of this type, with a sarcomere 3.6 μ long, the A band is about 3.0 μ long, the H zone about 0.3 μ, and each I band (from A to A) about 0.6 μ. Some of these measurements are of course only very approximate.

In type II fibrils (Text-fig. 1 *b* and Fig. 4 *a*) only the Z lines are clearly distinguishable: the rest of the sarcomere is uniformly dense and there are no well defined I bands or H zones. The Z lines are isotropic and the rest of the sarcomere anisotropic.

In the commonest type of fibril (type III) found in these preparations (Text-fig. 1 *c* and Fig. 2) a single dense line stands out in the centre of the sarcomere, but is not as conspicuous as the lines bounding the sarcomere. Using the names introduced by Hodge (13) the latter are called C_z lines and the former a C_m line, because they are bands formed in a contracted fibril around the Z and M lines. The C_z and C_m lines can easily be distinguished from each other by examining the fibril in polarised light; the C_z lines appear isotropic and the C_m lines anisotropic (Fig. 2).

Other fibrils (type IV) in these preparations (Text-fig. 1 *d* and Fig. 3) have thicker C_z lines and the C_m lines are longer.

When the fibrils in these preparations are made to contract under the microscope, the sequence of changes observed in the band pattern is the same as the sequence illustrated in Text-fig. 1 *a* to *d*, and a shortening of about 4 per cent accompanies each change from one main type of band pattern to the next. Although sarcomere length and band pattern are strictly correlated in any individual fibril, there is considerable variation in sarcomere length between different fibrils of the same band pattern; for example, type I fibrils have sarcomere lengths ranging from 3.2 μ to 3.7 μ, the length of every band in the sarcomere varying with its total length. These variations in size have been observed not only between different flies, but also between different fibrils in one muscle. Although it is not known whether sarcomere length changes with age, it has been noticed that fibril diameter is much less in young flies, during the 1st week after emerging from the pupa, than in older flies.

2. The Effects of Glycerol Extraction on the Band Pattern:

Nearly all the fibrils isolated from glycerol-extracted fibres have the type I pattern of cross-striation; a few are of types II and III, but none of type IV. Thus they contrast sharply with fresh fibrils amongst which type III predominates. The muscle fibres were usually treated with glycerol while they were attached to the walls of the bisected thorax. However, the same result was obtained when the fibres were cut out of the thorax before treatment, and it was also found that the predominant band pattern changes from type III to type I in suspensions of fresh fibrils isolated in standard salt solution and then mixed with glycerol and left in the refrigerator overnight.

3. The Effects of ATP and of Crude Muscle Extract on Sarcomere Lengths and Band Patterns:

For reasons which are not completely understood, ATP solutions often had no visible effects on either glycerol-extracted or fresh fibrils, although when tested on glycerol-extracted rabbit psoas fibrils the same solutions always induced contraction. The purpose of the experiments with ATP was to cause contraction in order to observe structural changes, and this was achieved without difficulty by using crude muscle extracts which also made glycerol-extracted rabbit fibrils contract. Since this paper is not concerned with nucleotide metabolism, no details will be given of experiments in which ATP solutions were tested under various conditions, and for the same reason no attempt will be made here to examine how crude muscle extracts produce their effects on fibril length.

(*a*) *The Different Responses of Fresh and Glycerol-Extracted Fibrils.*—Glycerol-extracted fibrils always contracted when they were treated with crude muscle extract, and when they showed any response to ATP it was a contraction. Fresh fibrils, on the other hand, either elongated or contracted, depending on various circumstances. During treatment with freshly made crude muscle extract or, sometimes, with ATP, type II fibrils changed to type I; they acquired H zones and I bands and lengthened by about 4 per cent (Figs. 4 *a* and *b*); other types of fibrils were apparently unaffected, although fibrils intermediate between types II and III also elongated. When irrigation of the preparation was stopped, all types of fibrils began to contract, except type IV fibrils, which are already much contracted; but if treatment was resumed, contraction ceased and type II fibrils again elongated. After a few cycles of elongation and contraction (Fig. 4) the fibrils irreversibly contracted. Only freshly made extracts had these effects; after about 45 minutes at room temperature they failed to cause elongation, but their ability to make fibrils contract persisted for several hours. In a few experiments in which newly emerged flies were used it was found that extracts of their muscles always induced contraction; none of the fibrils elongated.

It has been shown (Hanson (7)) that elongation is not a passive process brought about by a pull exerted by other fibrils or other sarcomeres; it appears to be a process effected by the sarcomeres themselves.

(*b*) *The Changes Taking Place during Contraction.*—The changes in sarcomere length and band pattern during contraction have been studied by observing and photographing individual fibrils treated under the microscope. Both fresh and glycerol-extracted material gave the same results.

In the first stage of contraction the fibril changes from type I to type II; the H zones and I bands disappear (Text-fig. 1 *a* and *b* and Figs. 4 and 5); the sarcomere shortens by about 4 per cent and the density of the A band is not appreciably changed. The disappearance of the H zone is very obvious, but photographs of type II fibrils (Fig. 5 *b*) often show indistinct zones next to the Z lines which may be imperfectly resolved short I bands. As shortening continues, C_m lines appear where the H zones disappeared, and the Z lines begin to thicken to form C_z lines (Text-figs. 1 *b* and *c* and Figs. 5 and 6). When the C_z and C_m lines are well developed (type III), the sarcomere has shortened to about 92 per cent of its original length, and its density (apart from that of the C_z and C_m lines) often appears slightly lower than that of the original A band. As contraction continues, the density of the sarcomere between the C_m and C_z lines decreases, the diameter of all regions continues to increase, and more material appears to accumulate in the C_z and C_m lines (Text-figs. 1 *c* and *d* and Fig. 9). Contraction ceases when the fibril has shortened by about 12 per cent.

The length of the A band in a type I sarcomere cannot be measured accurately because the I bands are not adequately resolved. Therefore it is not possible to determine with any certainty whether the length of the A band stays constant until C_z lines are formed.

4. The Band Pattern in the Intact Fly:

Flies were exposed for 2 or 3 hours to the vapour of osmium tetroxide in the expectation that the fibrils might be fixed with their cross-striations as they were in life, since it is known that the tracheoles penetrate amongst the fibrils and sarcosomes (13, 32). The flies died with their wings folded back along the abdomen, but even after 3 hours, pressure on the thorax could make the wings move in the usual way (3) into either of the two extreme positions reached during flight; they stayed either up or down until forced into the other position. When the thorax was opened it was found that the muscles had become brown in colour. The fibrils were isolated from the indirect flight muscles in the usual way and it was noticed that numerous sarcosomes clung to them; thus the fixative had reached and affected the sarcoplasm. Nearly all the fibrils were of type II; a few were of type I or intermediate between types II and III, but none was of type IV. They were unaffected by treatment with crude muscle extract.

Other flies were narcotised with carbon tetrachloride vapour (3) until the wings became locked either up or down. They were then exposed to osmium tetroxide vapour; the position of the wings did not change. Fibrils isolated from the indirect flight muscles were again nearly all of type II; both the dorsal muscles and the dorsoventral muscles gave the same result.

5. Stretched Fibrils:

Fresh muscle fibres attached to the skeleton were found to be inextensible, but their fibrils could be stretched, irreversibly, by pulling them with needles while they were being isolated. If care was taken to tease the fibres as gently as possible no stretched fibrils were produced. Glycerol-extracted fibres are also inextensible; their fibrils can be irreversibly stretched in the same way as fresh fibrils. Many of the fibrils isolated by homogenising either type of material in a Waring blendor were found to be slightly stretched.

It is unusual to find a fibril with every sarcomere extended (Fig. 7); more often, most of the sarcomeres are unaffected, and those that have been stretched vary considerably in length. A stretched sarcomere can always be recognised by the presence of a band of exceptionally low density next to either or both of its two Z lines (Fig. 8). When two adjoining sarcomeres have both been stretched, the Z line they share is usually distorted, and the bands of low density on either side of it are very often of unequal length (Figs. 8 and 11). The presence of C_m lines in many stretched sarcomeres (Fig. 9) indicates that the bands of low density next to the Z lines are not extended I bands, for fibrils with C_m lines (types III and IV) do not have I bands. When fibrils of type I have been stretched, the H zone is still of normal length (Fig. 10). The extensible regions of the sarcomere in any type of fibril appear to be strictly localised near to the Z lines; these regions in a stretched sarcomere will be referred to as "stretched zones." Although their density is very low their birefringence is considerable (Fig. 11).

When stretched fibrils are treated with crude muscle extract they behave abnormally. No C_z line is formed on a Z line between two stretched zones, although a half C_z line is developed where a Z line had an I band on one side of it and a stretched zone on the other side (Fig. 10). In a sarcomere with two stretched zones, one at either end, the dense part of the sarcomere shortens and develops a C_m line in place of an H zone, and the stretched zones are further extended (Figs. 9 and 10).

6. Extraction and Reconstitution Experiments:

Both fresh and glycerol-extracted fibrils have been treated under the microscope with solutions which, in the case of rabbit fibrils, are known to extract myosin, actin, and other proteins. The effects of these solutions on the structure

of the insect sarcomere have been investigated, but no attempt has yet been made to identify the extracted components. The same results have been obtained with both fresh and glycerol-extracted material.

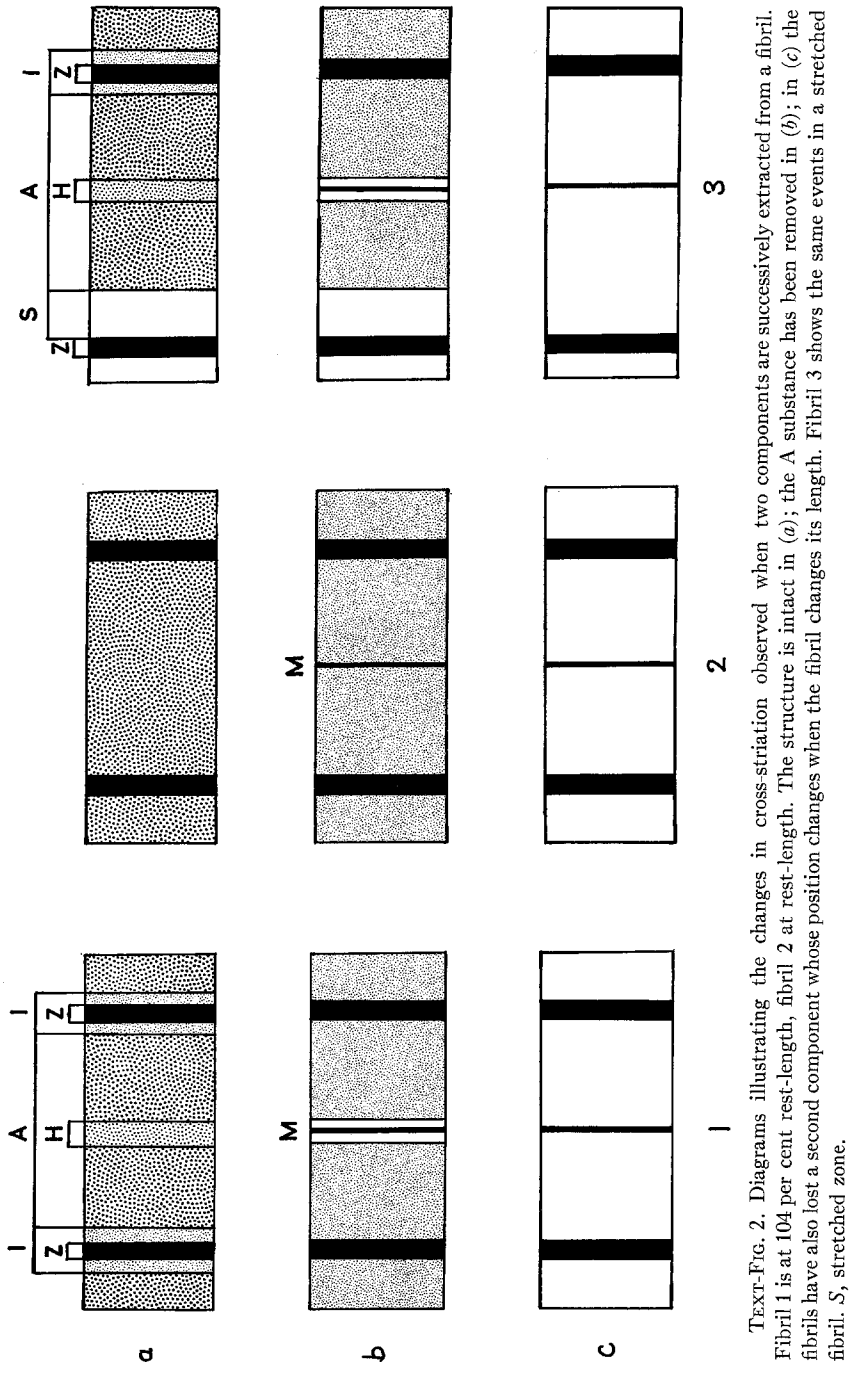
Hypertonic potassium chloride solutions at pH 6.5 or 7.0 extract the A substance[2] completely, provided that the total ionic strength is higher than about 0.7; extraction proceeds slowly and often takes as long as 10 minutes to complete. These solutions have the same effect whether or not a plasticiser (ATP or pyrophosphate in the presence of magnesium ions) is included. If the total ionic strength is lower than 0.7, little or none of the A substance is removed, even in the presence of a plasticiser. Thus Guba-Straub solution (0.3 M KCl, 0.15 M phosphate buffer, pH 6.5) with ATP and magnesium is ineffective; and a solution containing 0.47 M KCl, 0.01 M pyrophosphate, 0.1 M phosphate buffer, pH 6.4 (Hasselbach and Schneider (12)) with 10^{-3} M $MgCl_2$ is also ineffective. Efficient extraction is achieved by using a modified Hasselbach-Schneider solution in which the concentration of potassium chloride is raised to 0.6 M and Mg^{++} is included; most of the observations reported here have been made on fibrils treated with this solution.

Extraction of the A substance from a type I fibril reduces the density of the A band until there is no longer any distinction between A bands and I bands; the density of the H zone is also reduced, and an M line is unmasked in the middle of it; the sarcomere is now of uniformly low density apart from the H zone, which has an even lower density, and the dark Z and M lines (Figs. 11 and 12). Type II fibrils differ from type I fibrils after extraction only in the absence of an H zone; the sarcomere, apart from M and Z lines, is of uniform density. Fibrils of type III resemble those of type II after the A substance has been removed. In stretched fibrils there remains an obvious difference between the very pale stretched zones and the adjacent darker parts of the sarcomere (Fig. 11). Text-fig. 2 illustrates some of these changes. Removal of the A substance considerably reduces the birefringence of the A band.

During treatment with extracting solutions, the A substance begins to be removed first from the centre of the sarcomere. If the process is arrested at this stage, the fibril is still capable of responding to crude muscle extract by forming strong C_z and C_m lines, although it may not shorten (Fig. 13). After all the A substance has been removed the fibril no longer responds in any way to crude muscle extract.

Extraction of the A substance can also be achieved by lowering the pH to about 5.0 even at isotonic ionic strength. This was first noticed when an unbuffered dilute ATP solution was given to fibrils during studies on contraction; they rapidly shrank, and after the ATP was washed out and the original diameter restored, it was found that material had been removed from the

[2] The A substance is the material characterising the A band in a striped fibril and making it denser and more birefringent than the I band.



TEXT-FIG. 2. Diagrams illustrating the changes in cross-striation observed when two components are successively extracted from a fibril. Fibril 1 is at 104 per cent rest-length, fibril 2 at rest-length. The structure is intact in (*a*); the A substance has been removed in (*b*); in (*c*) the fibrils have also lost a second component whose position changes when the fibril changes its length. Fibril 3 shows the same events in a stretched fibril. *S*, stretched zone.

centre of the sarcomere (Fig. 14). Longer treatment at low pH extracts all the A substance.

It was observed that frequently the Z lines and occasionally the M lines became thicker during the course of extraction of the A substance (Figs. 11 and 12). This effect was obtained with any of the extracting solutions, but it did not always occur.

When fibrils from which the A substance had been removed were treated with a solution containing 0.6 M potassium iodide and 0.006 M sodium thiosulfate (pH 5.5), their density decreased until only the Z and M lines and a very pale backbone remained (Fig. 12 *b* and *c*). Both stretched zones and H zones were now indistinguishable from the rest of the sarcomere.

Fibrils from which the A substance had been removed completely were washed with standard salt solution and then treated with a solution of pure rabbit myosin, which was taken up by all regions of the sarcomere with the exceptions of the H zones and of stretched zones. The fibrils did not become as dense as they originally were, but a considerable increase in density was nearly always observed (Fig. 15 *b* and *c*). The fibrils were then washed with standard salt solution and treated with ATP or with crude muscle extract. Very thick contraction bands were formed around the Z lines (Figs. 15 *d* and 16 *b*); in some fibrils, where exceptionally thick contraction bands were formed (Fig. 16), very little material was left in the rest of the sarcomere. There was no obvious shortening of the sarcomere during this abnormal kind of contraction. Where stretched zones were present on both sides of the Z lines in reconstituted sarcomeres, no contraction bands were formed and no changes were observed in the other parts of the sarcomeres (Fig. 15).

DISCUSSION

1. Band Patterns in the Intact Living Fly:

Nearly all the fibrils isolated from muscles fixed by killing the flies in the vapour of osmium tetroxide were of type II. The fixative certainly reached the fibrils, but they might have been made to contract or to elongate before it stabilised their lengths. The observation that the wings of the fly assumed the resting position immediately on exposure to osmium tetroxide and remained in that position indicates that none of the muscles either contracted or was stretched by its antagonist, for even a slight contraction causes the wings to move, and persistence of tension locks them either up or down (3); it was shown that the fixative had not affected the ability of the skeletal components—to which the indirect flight muscles are attached—to move the wings. Any elongation of the fibrils before they were fixed would presumably have made them bend, but no bent fibrils were found in the fixed muscles. It can be concluded, therefore, that fibrils in the body at rest-length have the type II band

pattern in which the Z lines nearly touch the A band and there is neither an H zone nor a C_m line in the centre of the sarcomere (Text-fig. 1 *b* and Figs. 4 *a* and *c* and 5 *b*).

It is known that very slight contractions of these indirect flight muscles are sufficient to deform the skeletal structures whose movements result in the wing strokes (3). In bumblebees, for example (4), the muscles shorten by only about 2 per cent, and the degree of contraction in *Calliphora* is probably about the same; the tymbal muscles of the cicada *Platypleura capitata* which function in a similar manner shorten by 1.5 per cent (26). Thus the changes in band pattern taking place during flight must be very slight; probably when the muscle contracts the Z lines are just brought into contact with the ends of the A band, but neither a C_m line nor C_z lines develop; when the muscle is stretched by its antagonist, short I bands and a short H zone probably appear.

Fibrils isolated from freshly excised muscles have band patterns (Text-fig. 1) indicating that some of them (type I) have elongated by about 4 per cent during isolation and others (types III and IV) have contracted by various amounts down to about 92 per cent rest-length. Glycerol-extracted fibrils appear to have elongated by about 4 per cent.

2. Factors Influencing Sarcomere Length:

It has been found that the isolated fibrils can be induced either to elongate or to contract, and although the factors determining in which direction the length will change are not understood, it will be useful to summarise some of these initial observations and to define the questions that have to be answered by further experiments.

(*a*) Elongated fibrils (104 per cent rest-length) are rarely found in the intact thorax, but are common in preparations of fibrils isolated from fresh muscles. Apparently they elongated at some stage after the thorax was opened. Many of these elongated fibrils contract soon after they have been put under the microscope, but some of them remain extended.

(*b*) Rest-length fibrils in these preparations elongate while they are being treated with fresh crude muscle extract or with ATP, and contract when treatment ceases; they can then be made to elongate again, but after a few cycles of length changes they remain contracted. Extended fibrils (104 per cent rest-length) in these preparations contract when treatment ceases and remain contracted.

(*c*) Elongation occurs at some stage during the preparation of glycerol-extracted material and persists, but on treatment with fresh crude muscle extract or ATP the fibrils contract and remain contracted.

These observations, which are not immediately relevant to the main theme of the work reported in this paper, raise two important questions: what factors determine whether a fibril will contract or extend, and what processes stabilise

the fibril in either the contracted or the extended configuration or at rest-length? It is premature to attempt to answer these questions, but by analogy with rabbit muscle (30) it is supposed that the concentration of ATP at specific sites within the fibril determines the type of length change it will undergo, and that this concentration is controlled by relaxing factors; thus when the fibril has a high concentration of ATP it relaxes, and when this falls below a certain threshold it contracts. It is much more difficult to put forward any explanation of why fibrils sometimes remain extended while their ATP content is likely to be diminishing, for example during glycerol extraction.

Crude muscle extracts presumably contain ATP which may be present in higher concentration in fresh extracts—inducing elongation—than in extracts that have been kept for some time, or in extracts made from the muscles of young flies. It is relevant that sarcosomes from *Calliphora* during the 1st week of adult life have submaximal oxidative phosphorylation activity (23); and in *Phormia* the cytochrome content and the size of the sarcosomes increase during the 1st week, while the frequency of wing beat is less than in adult flies (22). It has also been noticed in the present investigations that these young flies have thinner myofibrils than older flies.

3. Comparison with Rabbit Myofibrils:

It will be profitable briefly to compare the results described in this paper with those obtained by the parallel studies made by this author and H. E. Huxley on the cross-striation of rabbit psoas myofibrils (8–10, 18, 19). It seems very probable that fundamentally all striated fibrils are alike; any conclusions drawn from comparative studies should contribute towards an understanding of the mechanism of muscular contraction.

(*a*) *Changes in Band Pattern during Contraction and Elongation.*—The changes observed in the cross-striation of the sarcomere as it shortens are the same in both rabbit and insect fibrils. The I bands disappear, bringing the Z lines into contact with the ends of the A band, and contraction bands (C_z lines) then form around the Z lines as shortening continues; while the I bands are disappearing the H zone also disappears, and a C_m line develops in its place. Measurements made on rabbit fibrils showed that until C_z lines develop, sarcomere shortening is achieved by I band shortening and does not involve any resolvable change in A band length.

The insect sarcomere, even when it is extended (104 per cent), has shorter I bands than the rabbit sarcomere, and it has not been possible to make sufficiently accurate measurements of the lengths of A and I bands; however, the finding that the H zone closes up while the I bands are disappearing is an indication that, as in the rabbit, the I bands are being retracted into the A band and filling up the H zone; other evidence is discussed later.

In the insect sarcomere at rest-length (type II fibril) the I bands are either

absent or so short that they are not clearly resolved; even at 104 per cent rest-length (type I fibril) the A band is at least five times as long as one I band (A to A). In the rabbit sarcomere at rest-length the I band is about half as long as the A band. This difference between the two kinds of muscle may be related to the difference in their ability to shorten *in vivo* or *in vitro*. The insect fibril shortens by about 2 per cent during flight and by not more than about 8 per cent when it has been isolated from the body; the isolated rabbit psoas fibril can contract by as much as 70 per cent, and even in the body its length changes are considerable. It may be suggested that in either type of muscle, and perhaps in all striped muscles, the lengths of A and I bands are designed so that on maximal normal contraction the I bands are completely retracted into the A bands, but no shortening of the A bands need occur. This would be an efficient arrangement in a sarcomere where shortening was brought about by movements of filaments relative to each other, and where the sites of enzyme activity controlling the supply of energy for contraction lay along filaments whose shape need not be grossly disturbed as the sarcomere changed its length.

When these insect fibrils, either fresh or glycerol-extracted, are passively stretched they behave like rabbit fibrils stretched while they are in rigor or while they are exerting tension: they "give" at sites alongside the Z lines, and no changes are visible in other parts of the sarcomere; in particular, H zones do not elongate and C_m lines do not disappear. On the other hand, the changes in the band pattern observed in fresh fibrils during the "active" elongation induced by crude muscle extracts are the reverse of the changes occurring during contraction, and are entirely comparable to the changes taking place in a rabbit fibril stretched while it is in a relaxed (plastic) state; the C_m line gives way to an H zone and the I bands appear and elongate. Although all these aspects of the behaviour of the cross-striation during elongation of the sarcomere suggest that rabbit and insect sarcomeres have the same type of structure, they also reveal that a fresh insect fibril is perhaps not in the relaxed state; the fresh muscle itself is inextensible (unlike rabbit muscle which retains its plasticity for some time after death) and the fibrils can only be stretched by an irreversible process which results in the same changes in band pattern as those observed in a rabbit fibril pulled out while it is exerting tension or is in rigor.

(*b*) *Results of Extraction and Reconstitution Experiments.*—The effect of removal of the A substance on the appearance of the sarcomere is the same for the insect fibril as for the rabbit fibril: the site of the A band becomes indistinguishable from the I bands; H zones come to look like gaps in the fibril because their optical density is now so low; in shortened fibrils without H zones there is no "gap" in the extracted sarcomere which, apart from Z and M lines, is of uniform density. In fact, after the A substance has been removed, rabbit and insect fibrils very closely resemble each other. If they are now treated with potassium iodide to remove more material there is a further drop in density until the H

gaps are no longer differentiated from the rest of the sarcomere; the Z and M lines persist. Thus three main components of the sarcomeres of both rabbits and insects can be characterised: (*a*) the A substance which is located in the A band and certainly in the rabbit, probably also in the insect, does not change its position when the sarcomere changes its length; (*b*) a "backbone" with Z and M lines; (*c*) a third component extending in long sarcomeres from the Z lines to the borders of the H zone, but moving inwards to fill up the H zone when the fibril shortens. Component (*a*) in the rabbit sarcomere is considered to be myosin (9, 10, 19) for many reasons including the following: firstly, the material extracted from isolated washed fibrils treated *en masse* (by procedures which selectively and completely extract myosin from whole muscle and, under the microscope, remove the A substance from isolated fibrils) consists almost entirely of myosin; secondly, the quantity of material extracted from fibrils *en masse* agrees with the quantity of material extracted by the same procedures from individual sarcomeres "weighed" by interference microscopy, and nearly all of this material is the A substance; thirdly, the quantity of myosin in the extract agrees with the quantity of A substance in the fibrils. The extracting solutions used in these studies on rabbit fibrils remove the A substance from insect fibrils and at the same time abolish their contractility. Thus it appears very probable that component (*a*) contains the myosin of the insect fibril. Similar evidence suggests that actin is located in component (*c*), and the finding that this component will capture rabbit myosin and form with it a contractile system is additional evidence. Component (*b*) is the stroma of the fibril. Confirmation of these suggestions awaits investigations on the properties of the materials extracted from the insect sarcomere.

The A substance is less easily extractable from the sarcomere of the insect than from that of the rabbit: the process takes longer, partly, no doubt, because the fibril is thicker, and a higher ionic strength is needed. It has also been found that a potassium chloride solution of sufficiently high ionic strength (0.75, for example), in the absence of a plasticiser (ATP or pyrophosphate), will remove the A substance completely from an insect sarcomere but leaves a large part of it behind in a rabbit sarcomere. Thus there appear to be differences in the mode of attachment of the A substance to other components in the sarcomere. There is, unfortunately, no information about the properties of the structural proteins of dipteran flight muscles; the studies of Gilmour and Calaby (5) on locust and grasshopper thoracic muscles are probably not relevant, because these muscles are relatively unspecialised and may be very different.

4. Does the A Substance Migrate?

The results described in this paper indicate that the A substance does not change its position when the sarcomere changes its length. Hodge (13), how-

ever, has put forward evidence that in the indirect flight myofibrils of another blowfly, *Lucilia cuprina*, the A substance migrates along the fibril axis; his observations and conclusions must now be discussed.

Firstly, he pointed out that in fresh fibrils with well developed C_z lines, and in glycerol-extracted fibrils after ATP-induced contraction, the optical density of the rest of the sarcomere appears lower than it is in less contracted fibrils without C_z lines or with thin C_z lines. This finding, which is in agreement with the observations reported here, cannot immediately be accepted as evidence for the migration of the A substance from the A bands to the Z lines, because it could equally well be accounted for by a model (as suggested for rabbit psoas fibrils—(9 and 18)) in which the main filaments fold up at the Z lines while remaining straight in the rest of the sarcomere: if the volume of the fibril remains constant while it shortens, one might therefore expect to observe a decrease in its optical density between the C_z lines.

Secondly, in experiments on glycerol-extracted fibrils treated with ATP under the microscope, Hodge observed a number of very interesting changes in the band pattern, which clearly suggested that the A substance was leaving the A bands and accumulating on the Z lines. For instance, when the fibril was restrained from shortening appreciably, the Z lines could become very much thicker and the A bands paler, in spite of the fact that I bands persisted. In such fibrils, the decrease in A band density, sometimes particularly in the centre of that band, was very striking. Phenomena like these have never been observed in *Calliphora* fibrils, glycerol-extracted or fresh, during treatment with ATP (provided that the pH was controlled at between 6.5 and 7.0), even when the fibril was unable to shorten by more than about 4 per cent. However, it must be pointed out that changes in band pattern closely similar to those described by Hodge have been observed under conditions when the A substance was being extracted: in particular, the Z lines often thickened, presumably because they captured some of the extracted A substance; and it has been found that extraction begins in the middle of the A band before it obviously affects the two ends of that band.

5. Conclusions:

Although this is not a suitable place for a discussion of the structure of cross-striated myofibrils and the probable mechanism of their contraction, a few final comments must be made about the relevance to these problems of the results reported in this paper. One of the chief questions concerns the arrangement of the filaments in the sarcomere and their behaviour while the length of the fibril is changing. Direct and essential information can be given by electron microscopy, but it is not easy to interpret and evaluate. Although the resolution of the light microscope is severely limited, it has the advantage that observations and experiments can be made on myofibrils while they are behaving in a life-like manner.

Hodge (13) has concluded from electron micrographs of thin sections through the indirect flight myofibrils of flies that there is a single set of myofilaments continuous throughout the length of the fibril; if this is so, then these filaments must shorten (or fold) during contraction; the changes observed in the light microscope in the pattern of cross-striation could be accounted for if materials migrated along the fibril axis, and Hodge has produced evidence that the A substance moves in this way. Huxley has concluded from electron micrographs of sectioned vertebrate skeletal myofibrils (17) and from low-angle x-ray diffraction data obtained on similar muscles (15, 16) that there are two types of filaments in the sarcomere, one of which is confined to the A bands, and indeed characterises these bands, whilst the other extends from the Z lines, through the I bands, and as far as the borders of the H zone, interdigitating with the A band filaments. A model based on data obtained by electron microscopy, x-ray diffraction, and experiments performed under the light microscope (8–10, 14–19) has been put forward by Hanson and Huxley (9 and 18) to describe how the sarcomere changes its length. The chief feature of this model is that length changes are brought about by movements of filaments relative to each other along the fibril axis. This model accounts for the features observed in the cross-striation not only of vertebrate skeletal myofibrils but also of the indirect flight myofibrils of *Calliphora*; it is consistent with all the results described in this paper.

Other electron microscopical data and other models have also been suggested for vertebrate muscles, but they cannot be discussed here. Since the results on *Calliphora* myofibrils reported in this paper do not include any obtained by electron microscopy, no attempt will be made here to discuss the results of Hodge and others (reviewed in reference 13) concerning the fine structure of insect fibrils. However, any model suggested for the organisation and behaviour of a cross-striated myofibril must take into account all the features of the band pattern. The most significant information of this nature, derived from studies on both rabbit and insect fibrils, appears to be the following:

1. Shortening of the sarcomere until C_z lines form is accomplished by shortening of the I bands, the A bands staying at constant length. This was clearly shown by studies on rabbit fibrils, and it probably also holds true for insect fibrils, although the small dimensions of the I bands in this material have made it impossible to obtain sufficiently accurate measurements.

2. While the I bands are disappearing the H zone in the centre of the A band closes up. By studies on fibrils from which the A substance has been extracted, the disappearance of the H zone has been found to be due to a movement towards the middle of the sarcomere of a component which, in long sarcomeres, reaches from either Z line to the border of the H zone and then, as the sarcomere shortens, moves inwards, without itself shortening, to close up the H zone, and thus to bring the Z lines nearer together.

3. Two quite different types of changes in striation have been observed in lengthening sarcomeres. Under some conditions, the changes are the reverse of those seen during shortening, and in the rabbit there is evidence that this can only happen when actin-myosin links are absent. Alternatively, lengthening is not accompanied by any changes in the centre of the A band (H zones do not appear or do not lengthen); in the rabbit this type of lengthening is only observed under conditions in which actin and myosin are probably linked together.

4. Evidence has been given concerning the location of myosin and actin in different and well defined structural components within the sarcomere of rabbits or insects; any model accounting for the behaviour of the sarcomere during contraction or lengthening must take such evidence into consideration and must be consistent with the known properties of these two proteins.

SUMMARY

1. The cross-striation in the indirect flight myofibrils of *Calliphora* has been studied by phase contrast and polarised light microscopy. The band pattern at rest-length has been determined in flies killed in osmium tetroxide vapour while their wings remained in the resting position. All other observations have been made on unfixed fibrils. Although length changes *in situ* are probably very slight (about 2 per cent), isolated fibrils, by treatment with crude muscle extract or with ATP, can be induced to elongate to 104 per cent rest-length, or to shorten by 8 per cent but no more. Over the range 98 to 104 per cent rest-length, experimentally induced length changes are reversible. The fibrils can also be stretched beyond 104 per cent rest-length, but the process is irreversible. During the course of glycerol extraction the fibrils elongate to 104 per cent rest-length.

2. The changes in band pattern observed over the range 104 to 92 per cent rest-length are qualitatively the same as the changes observed over a wider range (about 130 to 40 per cent rest-length) in the skeletal myofibrils of rabbits. The earlier stages of shortening appear to be effected by retraction of the I bands into the A bands where they fill up the H zones. No evidence has been found that any changes in band pattern are due to a migration of the A substance.

3. Two components of the sarcomere can be extracted from it and a third component remains behind. These three components, which have also been demonstrated in skeletal myofibrils of the rabbit, where they behave in the same way, are: (*a*) the A substance which does not change its position as the fibril changes its length, and which can be extracted by the same procedures as remove myosin (shown elsewhere to be the A substance) from rabbit fibrils; (*b*) a material which extends from the Z lines to the borders of the H zone and which moves inwards during contraction and outwards during elongation; it can

capture rabbit myosin from solution and form with it a contractile system, and it is thought to be actin; (*c*) a "backbone" or stroma bearing Z and M lines.

4. Since all these features of the cross-striation are the same in the insect fibrils as in rabbit fibrils, it is considered very probable that the sarcomere is similarly organised in both types of muscle and contracts by essentially the same mechanism.

I am much indebted to Professor J. T. Randall and Dr. H. B. Fell for their encouragement of this research. It is a pleasure to acknowledge, with gratitude, my deep indebtedness to Dr. H. E. Huxley for many discussions of this work and for the stimulation and ideas derived from our collaborative studies on other muscles.

REFERENCES

1. Bennett, H. S., *Am. J. Physic. Med.*, 1955, **34,** 46.
2. Bennett, H. S., and Porter, K. R., *Am. J. Anat.*, 1953, **93,** 61.
3. Boettiger, E. G., and Furshpan, E., *Biol. Bull.*, 1952, **102,** 200.
4. Boettiger, E. G., and Furshpan, E., *Biol. Bull.*, 1954, **109,** 305.
5. Gilmour, D., and Calaby, J. H., *Enzymologia*, 1953, **16,** 23.
6. Hanson, J., *Nature*, 1952, **169,** 530.
7. Hanson, J., *Biochim. et Biophysica Acta*, 1956, **20,** 289.
8. Hanson, J., and Huxley, H. E., *Nature*, 1953, **172,** 530.
9. Hanson, J., and Huxley, H. E., *Symp. Soc. Exp. Biol.*, 1955, **9,** 228.
10. Hanson, J., and Huxley, H. E., *Biochim. et Biophysica Acta*, in press.
11. Hasselbach, W., *Z. Naturforsch.*, 1953, **8b,** 449.
12. Hasselbach, W., and Schneider, G., *Biochem. Z.*, 1951, **321,** 461.
13. Hodge, A. J., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 361.
14. Huxley, A. F., and Niedergerke, R., *Nature*, 1954, **173,** 971.
15. Huxley, H. E., *Faraday Soc. Discussions*, 1951, **11,** 148.
16. Huxley, H. E., *Proc. Roy. Soc., London, Series B*, 1953, **141,** 59.
17. Huxley, H. E., *Biochim. et Biophysica Acta*, 1953, **12,** 387.
18. Huxley, H. E., and Hanson, J., *Nature*, 1954, **173,** 971.
19. Huxley, H. E., and Hanson, J., *Biochim. et Biophysica Acta*, in press.
20. Jordan, H. E., *Am. J. Anat.*, 1920, **27,** 1.
21. Jordan, H. E., *Physiol. Rev.*, 1933, **13,** 301.
22. Levenbook, L., and Williams, C. M., *J. Gen. Physiol.*, 1956, **39,** 497.
23. Lewis, S. E., and Slater, E. C., *Biochem. J.*, 1954, **58,** 207.
24. Perry, S. V., *Symp. Soc. Exp. Biol.*, 1955, **9,** 203.
25. Pringle, J. W. S., *J. Physiol.*, 1949, **108,** 226.
26. Pringle, J. W. S., *J. Physiol.*, 1954, **124,** 269.
27. Ritter, W., *Smithsonian Inst. Pub., Misc. Collections*, 1912, **56,** No. 12.
28. Szent-Györgyi, A., Chemistry of Muscular Contraction, New York, Academic Press, Inc., 2nd edition, 1951.
29. Tiegs, O., *Phil. Tr. Roy. Soc. London, Series B*, 1955, **238,** 221.
30. Weber, H. H., *Biochim. et Biophysica Acta*, 1953, **12,** 150.
31. Weber, H. H., and Portzehl, H., *Advances Protein Chem.*, 1952, **7,** 161.
32. Wigglesworth, V., Insect Physiology, London, Methuen, 4th edition, 1950.

EXPLANATION OF PLATES[3]

Myofibrils isolated from the indirect flight muscles of *Calliphora* and photographed, unfixed, in a phase contrast microscope or, occasionally, in polarised light. All photographs are at the same magnification.

PLATE 179

FIG. 1. A fresh fibril, (*a*) in phase contrast, (*b*) in polarised light, showing H zones and I bands (type I—104 per cent rest-length).

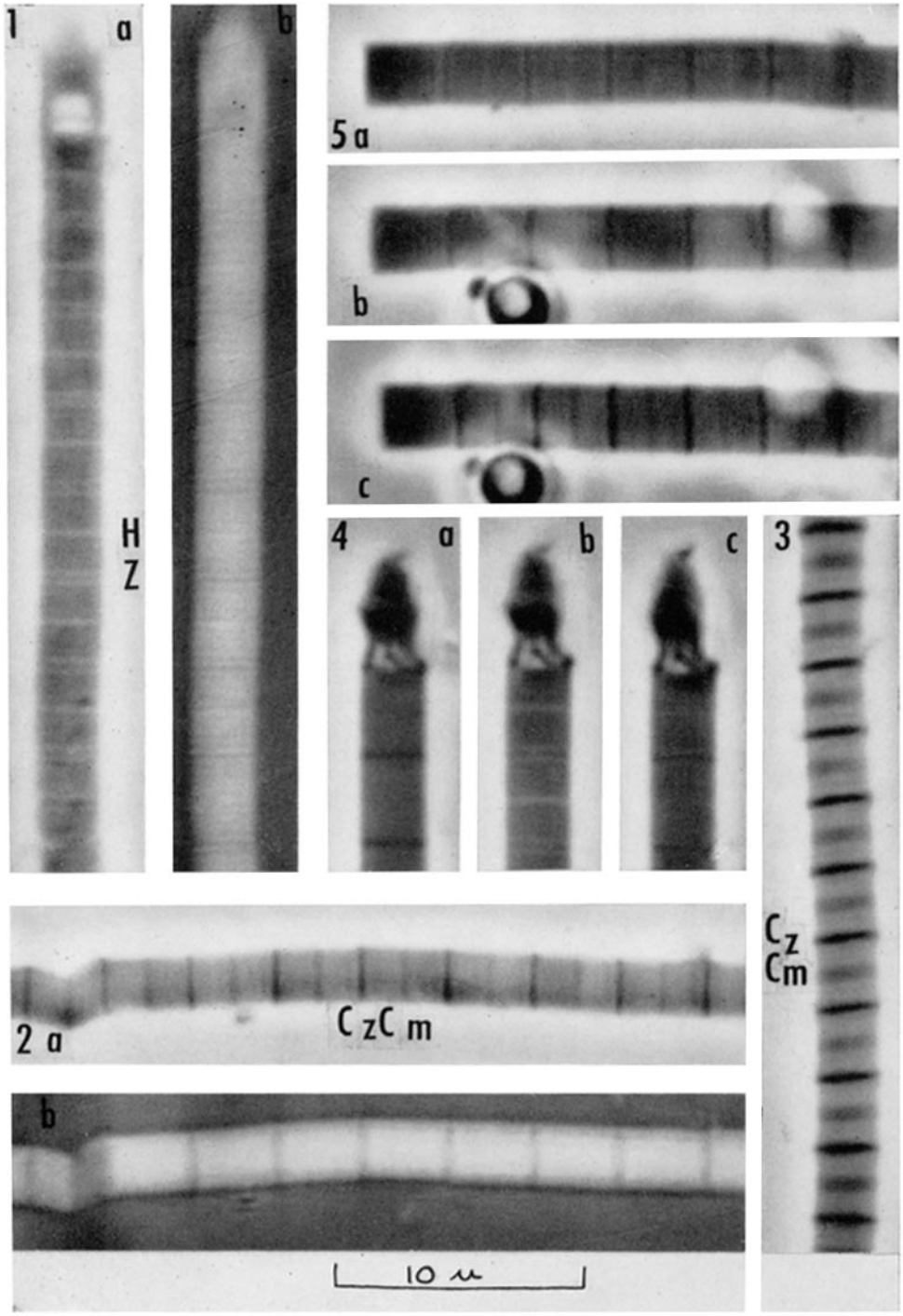
FIG. 2. A fresh fibril, (*a*) in phase contrast, (*b*) in polarised light, showing C_z and C_m lines (type III—96 per cent rest-length).

FIG. 3. A fresh fibril showing strongly developed C_z and C_m lines (type IV—92 per cent rest-length).

FIG. 4. A fresh fibril (*a*) (type II—about rest-length), which (*b*) elongated by about 4 per cent on treatment with crude muscle extract, acquiring H zones and I bands, then (*c*) shortened when treatment ceased.

FIG. 5. A fresh fibril (*a*), photographed at two stages (*b* and *c*) during contraction induced by crude muscle extract. The I bands and H zones disappeared and C_z and C_m lines developed.

[3] Some of these illustrations have already appeared elsewhere, and I am grateful to the editors concerned for permission to reproduce Fig. 2 from *Symposia of the Society for Experimental Biology*, and Figs. 4 and 6 from *Biochimica et Biophysica Acta*.



(Hanson: Cross-striation of blowfly myofibrils)

PLATE 180

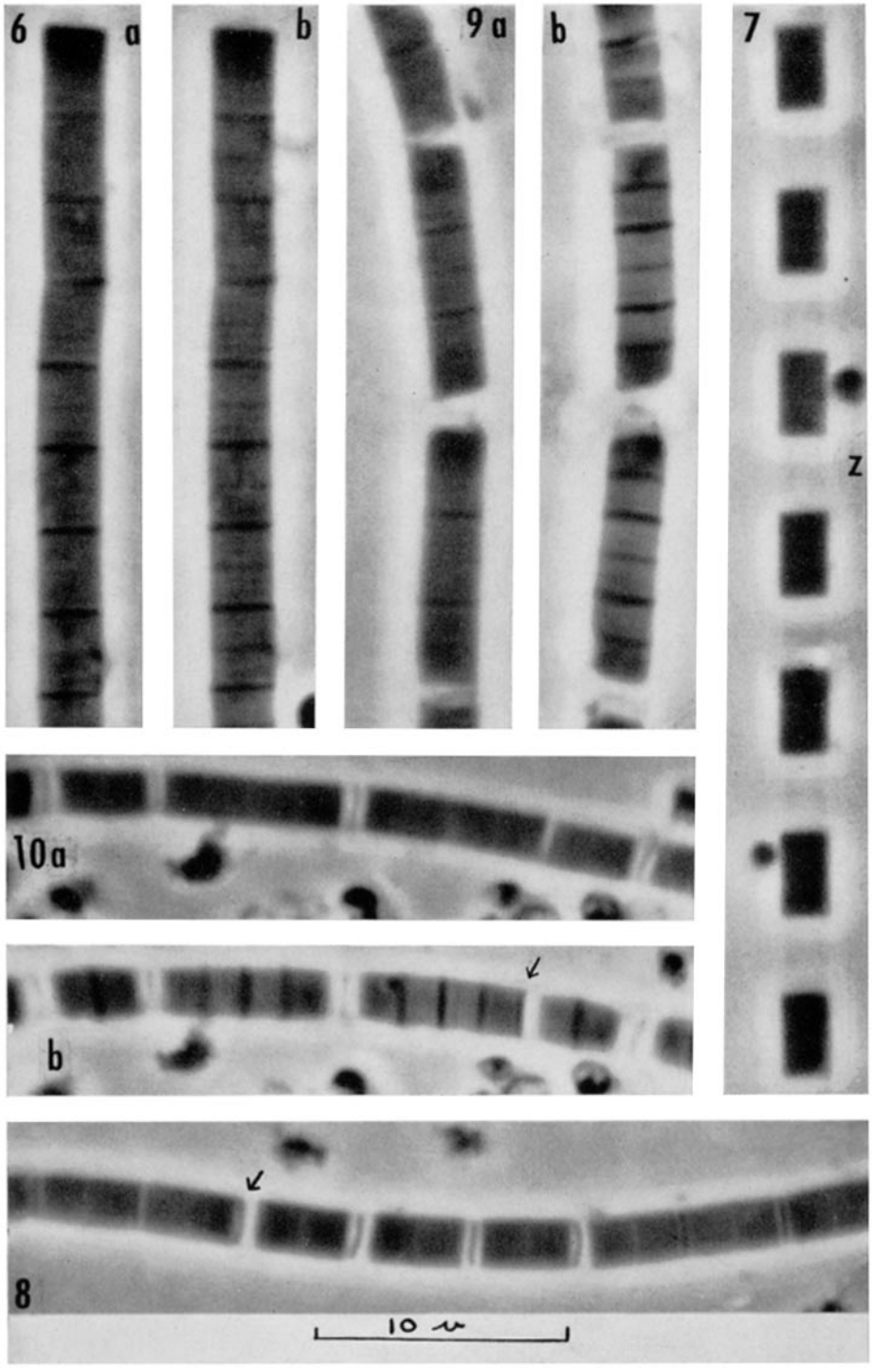
FIG. 6. A fresh fibril at about rest-length (*a*), and the same fibril (*b*) after a slight contraction induced by crude muscle extract, during which C_z and C_m lines developed.

FIG. 7. An excessively stretched fresh fibril showing a long stretched zone on either side of each Z line.

FIG. 8. A glycerol-extracted fibril (type I), showing stretched zones of various lengths next to some of the Z lines; one Z line (marked with an arrow) has an I band at one side and a stretched zone at the other side.

FIG. 9. A stretched fresh fibril (type III) photographed before (*a*) and after (*b*) contraction induced by crude muscle extract; the stretched zones were further extended during this process.

FIG. 10. A stretched glycerol-extracted fibril (type 1) photographed before (*a*) and after (*b*) contraction induced by crude muscle extract; no C_z lines formed around the Z lines bounded on both sides by stretched zones; an incomplete C_z line (marked with an arrow) formed on the Z line bounded on only one side by a stretched zone.



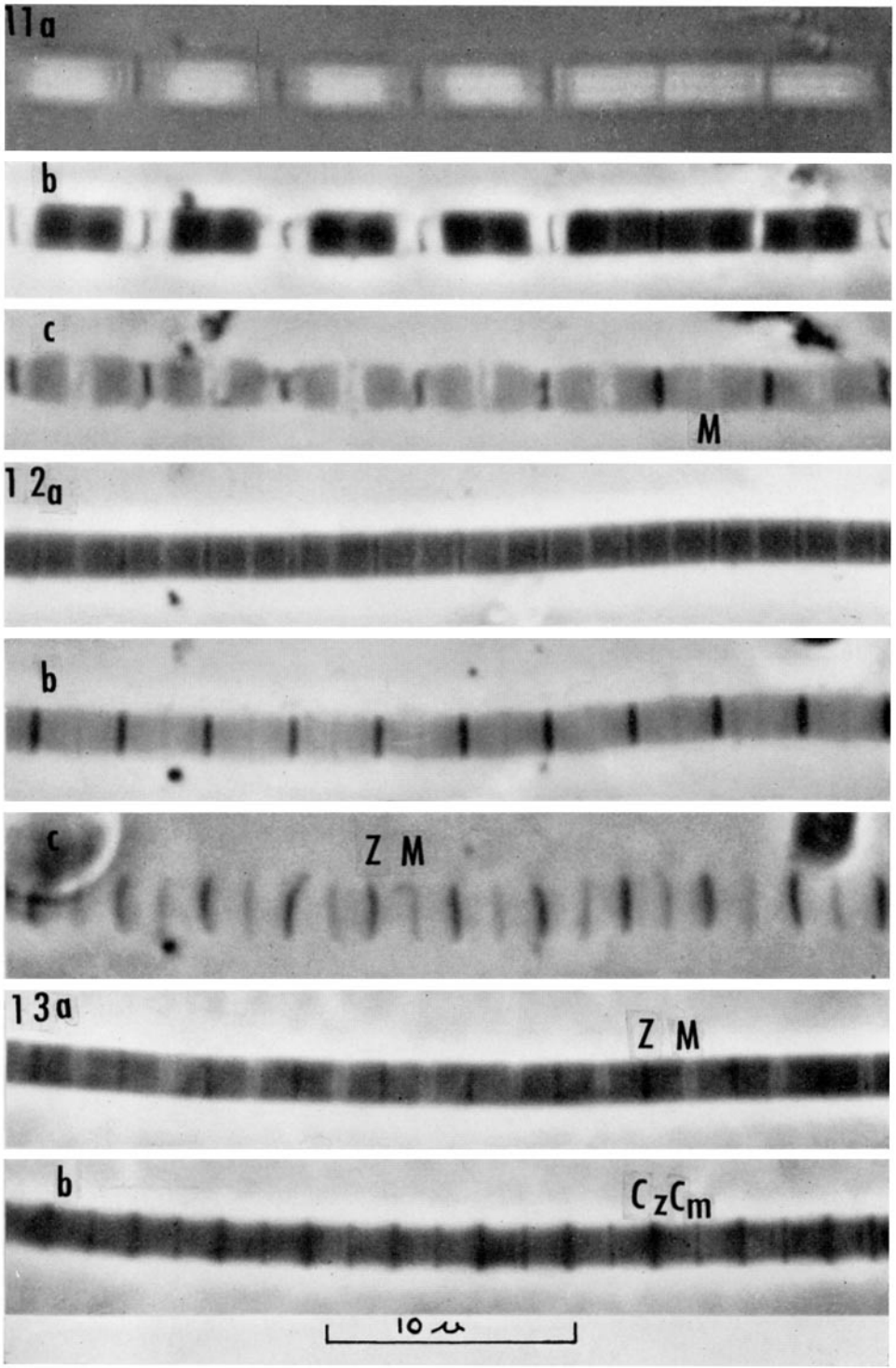
(Hanson: Cross-striation of blowfly myofibrils)

PLATE 181

FIG. 11. (*a*) and (*b*) show a stretched glycerol-extracted fibril in phase contrast (*b*) and in polarised light (*a*). (*c*) shows the same fibril after extraction of the A substance by modified Hasselbach-Schneider solution (see text). The H zones now have the same low density as the stretched zones, and there is an M line in the middle of each H zone.

FIG. 12. A fresh fibril (*a*), treated first with modified Hasselbach-Schneider solution (see text) to remove the A substance (*b*), and then with potassium iodide which leaves only the backbone and the Z and M lines (*c*).

FIG. 13. A fresh fibril which has been treated with a dilute acid ATP solution and lost some of the A substance from the centre of the sarcomere (*a*), and then with neutral ATP (*b*) which induced an atypical contraction.



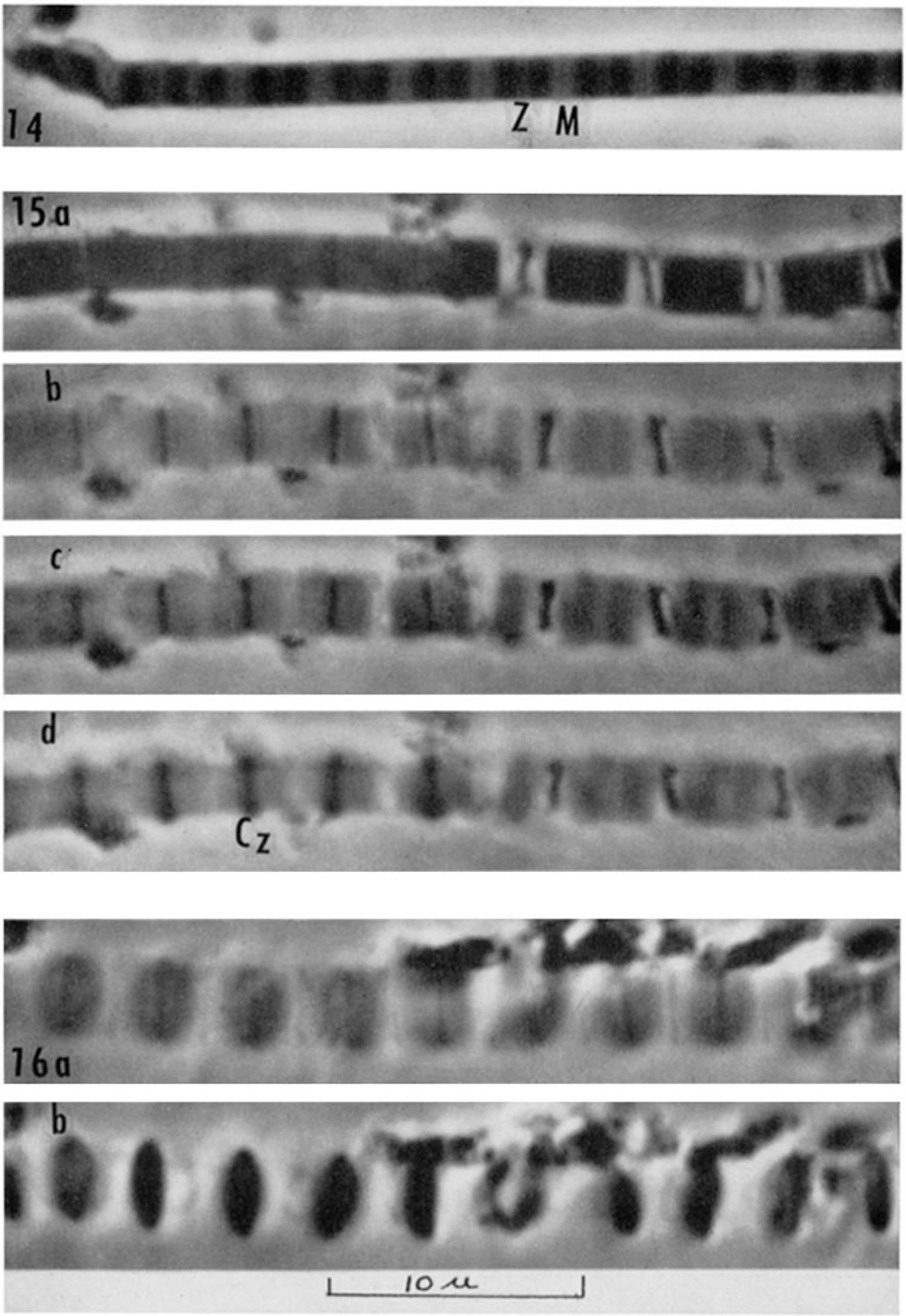
(Hanson: Cross-striation of blowfly myofibrils)

PLATE 182

FIG. 14. A fresh fibril which has been treated with a dilute acid ATP solution and lost some of the A substance from the centres and ends of the A bands.

FIG. 15. A glycerol-extracted fibril (type I) with stretched zones photographed intact (*a*), after extraction of the A substance (*b*), then after treatment with a solution of rabbit myosin (*c*), and lastly after a type of contraction induced by ATP (*d*) during which strong contraction bands formed on the Z lines except those bounded by stretched zones.

FIG. 16. A glycerol-extracted fibril (type I) which has been treated with rabbit myosin (*a*) after first removing its A substance. On giving ATP (*b*), strong contraction bands were formed.



(Hanson: Cross-striation of blowfly myofibrils)