THE INTRACELLULAR COMPONENTS OF SKELETAL MUSCLE

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INTRODUCTION

The electron microscope studies of muscle so far reported have been concerned mainly with the structural organisation of the tissue. In these investigations the material available has either been limited or has undergone considerable chemical modification during its preparation, and parallel biochemical examination has not been carried out. For a real understanding of the workings of the cell, electron microscope investigations make a most valuable supplement to biochemical studies of isolated, morphologically intact cell components. The work described in this paper is an electron microscope investigation complementary to preliminary studies of the biochemical organisation of intact components of the muscle cell (PERRY[1,2]). It includes details of the fine structure of myofibrils isolated from fresh and rigor muscle and a study of the effect of adenosine-triphosphate (ATP) on these intracellular components. In addition sarcoplasmic constituents, nuclei and granules are characterised.

METHODS

Preparation of myofibrils

Myofibrils were prepared as described by PERRY[1]. Briefly the method involves cutting sections of frozen rabbit psoas muscle, suspending the slices in a large volume of 0.08 *M* borate buffer, pH 7.1, and treating them for a few hours at 0° C with a purified collagenase preparation obtained from *Cl. welchii*. The enzyme-treated sections were broken up mechanically in a small Waring Blendor-type homogeniser and the myofibrils subsequently separated and washed by differential centrifugation. Myofibrils prepared in this way were enzymically active and remained structurally intact in the borate buffer medium. In solutions of higher ionic strength (0.5–1.0 *M* KCl) the fine structure did not survive as most of the protein went into solution.

Preparation of granules

Muscle from the hind legs of a freshly killed rat or rabbit was minced and homogenized with four volumes of 30% sucrose (w/v); occasionally the homogenization was carried out in 0.08 *M* borate buffer, pH 7.1. Centrifugation for 20 mins at 600 *g* brought down the myofibrils and general cell debris leaving a turbid supernatant from which the particulate components of the sarcoplasm were isolated by high speed centrifugation as described in the preceding paper[2].

References p. 498.

ATP treatment of myofibrils

ATP was prepared as the barium salt by Mr E. J. MORGAN and converted to the sodium salt as previously described[1]. Excess ATP was removed from the myofibril suspensions by centrifuging down the myofibrils and resuspending in the control medium which contained 0.08 *M* borate buffer, pH 7.1, and 0.0013 *M* magnesium chloride. After two such washings the myofibrils remained apparently unmodified in the shortened form.

Preparation of specimens and electron microscope examination

The electron microscopes employed in this investigation were the Siemens instrument and the R.C.A. Type E.M.B. Both microscopes were fitted with biassed electron guns, and objective apertures ranging from 40 μ to 70 μ in diameter were used. In order to photograph structural details in small particles and nuclei it was often necessary to raise the high tension voltage to 90 kV and 100 kV. Although such high voltages were used some of the particles remained quite dense to the electron beam.

Immediately a preparation was completed, material for examination was mounted on nitrocellulose films supported by standard Kodak copper grids. Myofibrils were fixed by exposing a drop of the freshly mounted suspension to formalin vapour. After 3–5 mins the grids were removed from the formalin, allowed to dry down in the air, and the salt washed out. No electron stain was used. In a similar way granules were fixed and stained by exposure to osmic acid vapour for 3 mins. Except where otherwise stated, specimens were shadowed with gold-palladium at an angle of 45°, employing the usual evaporating techniques.

RESULTS

Myofibrils

Most of the preparations examined in this investigation were obtained by sectioning fresh muscle with the freezing microtome. It has long been known that the freezing of muscle causes a slight contraction (DUBUISSON[3]), and many of the myofibrils isolated were in the partially contracted state. Consequently, in practically all cases in which the I and A bands could be identified with certainty, the A band, that is the band containing the electron-dense material, was longer than the I band. Figs. 1 and 2 are examples of myofibrils isolated from fresh muscle and indicate that their fine structure has survived the mild digestion with collagenase, the function of which was to weaken the muscle cell so that it could be readily broken up into its components. Superficial optical microscope examination of the collagenase-treated sections failed to reveal any obvious modification of structure.

Occasionally the myofibrils were isolated as bundles of two or three. Fig. 1 shows a double myofibril which has split into two components at one end whereas at the other there is no evidence of a differentiation in structure along the line at which the compound myofibril will ultimately split. When fresh muscle was used as starting material the A band substance appeared to be removed from the myofibril by washing; in many preparations, all of which had been centrifuged and resuspended in borate buffer three times, the A band substance was often very faint or altogether indistinguishable. A typical example of a myofibril from which the bands have been washed is shown in Fig. 3. ROZSA *et al.*[4] have also reported the washing out of the bands and suggest that the myofibril consists of longitudinal filaments overlaid and perhaps interpenetrated by a structureless material whose distribution determines the banded structure of muscle.

Preparations in which the A and I bands were no longer distinguishable did, however, invariably show one band (Figs. 3 and 6) which appeared to be made of a more insoluble material closely bound to the structure of the myofibril. This band could represent either the M line or the Z membrane, but the latter is considered to be more likely as previous electron microscope studies have shown it to be the more regularly observable feature of the myofibril.

References p. 498.

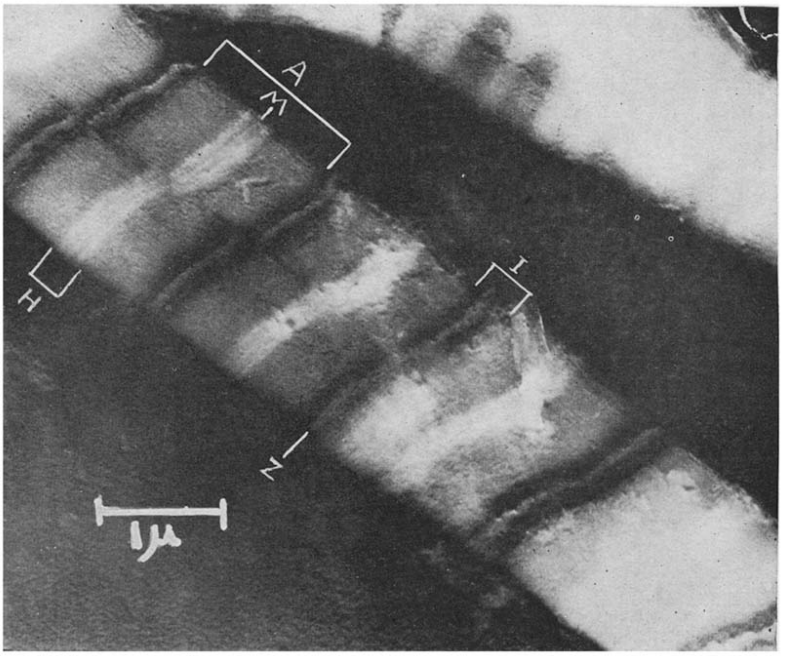

Fig. 1. Preparation 20 x. Double myofibril from rabbit psoas muscle

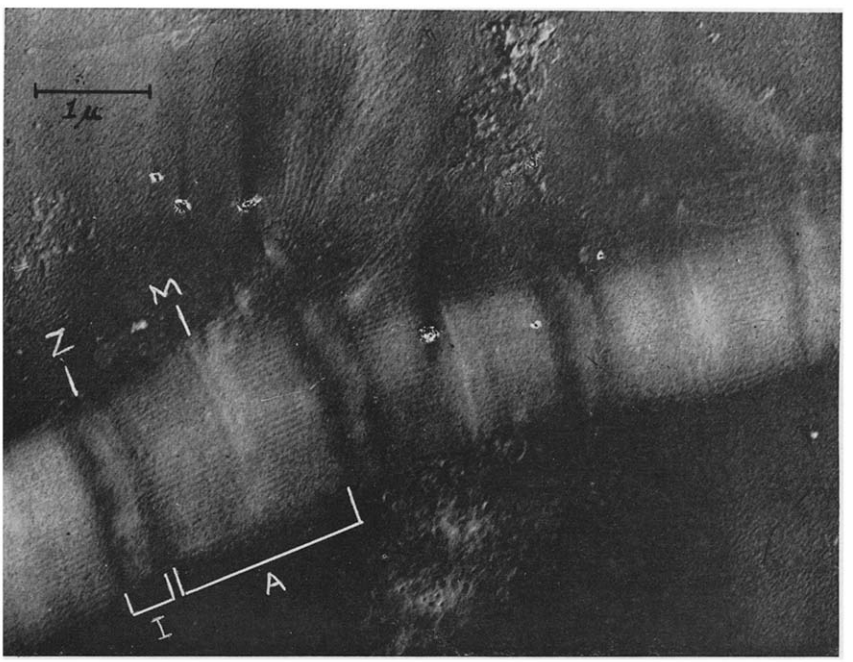

Fig. 2. Preparation 26a. Splayed out myofibril from the suspension (in 0.08 *M* borate containing 0.0014 *M* $MgCl_2$) used as a control to the ATP-treated myofibrils shown in Figs. 7 and 8

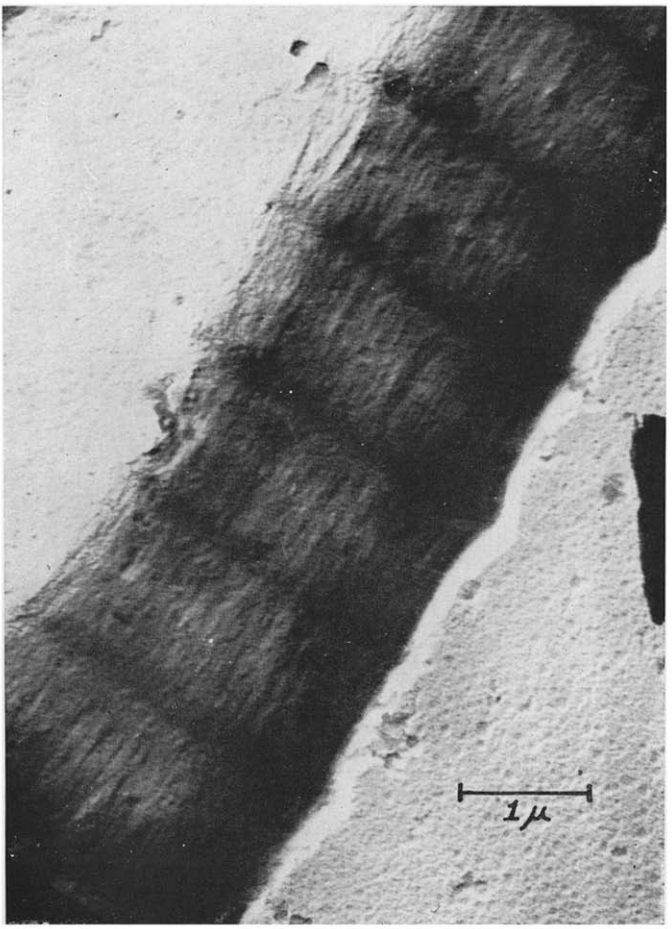

Fig. 3. Preparation 22A. Myofibril with very faint A bands. Shadow indicating maximum thickness at the Z band. Negative not reversed

Myofibrils from muscle allowed to go into rigor mortis by allowing to stand overnight at 4° C seemed to retain their banded structure more readily than those prepared from fresh muscle. This could be due to the mild fixative action of the lactic acid produced *in situ* by the glycolysis accompanying the onset of rigor. On several occasions it was noted that fine structure of muscle components was more readily preserved in preparations made from rigor mortis muscle. In the rigor mortis preparation shown in Fig. 4, the M line and the so-called H sub-lines (DRAPER AND HODGE[5]) are very striking, and both here and in Fig. 1 this group of lines appears as the most dense feature in the myofibril.

It is a general observation that the I band is less robust than the A band and that myofibrils tend to break in this region or at the Z band. This peculiarity is very pronounced in Fig. 4, and suggests that in the extremely short I band of the rigor mortis myofibril there is a structural weakness, which may be due to the manner in which the protein molecules within the filaments are oriented at this point. It is possible on

References p. 498.

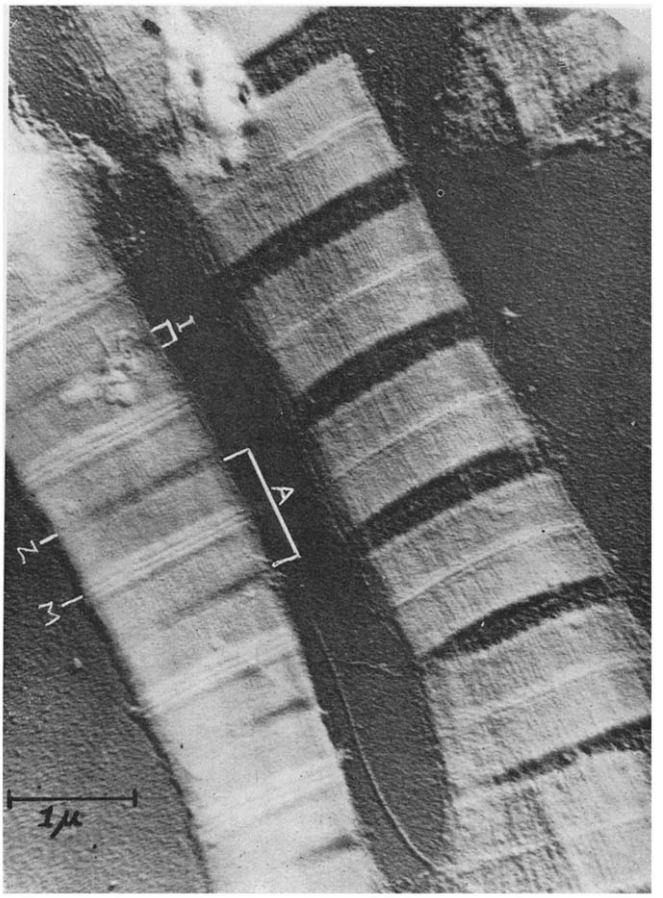

Fig. 4. Preparation 36. Myofibrils from muscle in rigor mortis. Chromium shadowed at 25°

the other hand, however, that the weakness could arise from the absence of the A substance which acts as a cementing material giving a greater strength to the A band.

As Figs. 1 and 2 clearly show, in partially contracted myofibrils the filaments are arranged parallel to one another with extreme precision. Single myofibrils varied in width from 0.8 μ to 1.8 μ depending on how splayed out they became during the preparation of the specimen, and on an average 37 (range 32–45) filaments could be counted in each. Filaments from both normal and rigor mortis preparations varied somewhat in width, but if an allowance for the space between the filaments is made, the above figures give an average filament width of about 200 A. There is also a marked regular periodicity along the filament axis which does not disappear even though the normal muscle bands are no longer recognisable. This periodicity is such that the filaments appear to be composed of a string of globules which are arranged in parallel filaments so that the general effect is one of fine transverse striations such as have been demonstrated by the micro-incineration technique of DRAPER AND HODGE[6]. In a number of preparations (Figs. 2 and 4) the filaments show evidence of a helical structure similar to that described by BENNETT[7], but as this appearance was not regularly obtained we

References p. 498.

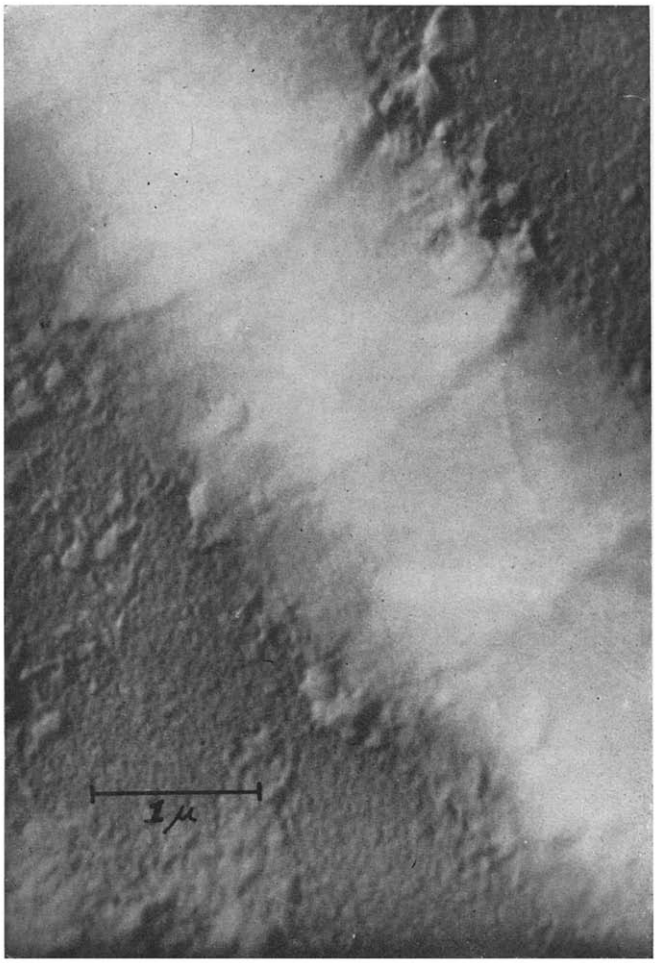

Fig. 5. Preparation 35. Myofibril showing elliptical Z band

feel that in our investigation it may be an artefact due to slight astigmatism in the microscope, the directional effect of shadowing, or to the grain of the film supporting the specimen.

PEASE AND BAKER[8], and DRAPER AND HODGE[5], have produced evidence for a hollow tube-like myofibril, whereas other workers, *e.g.*, MORGAN *et al.*[9], and ROZSA *et al.*[4] are unable to support this conclusion. The observations made on collagenase-prepared material, which differs from that used for the investigations mentioned above in that it has been washed completely free of sarcoplasm, suggest that although the myofibril drys down on the grid as a thin ribbon, in the muscle cell it may in fact exist as a hollow tube or at least one loosely packed with filaments. Fig. 5 shows a myofibril along whose length a shearing force has been fortuitously applied, with the result that the Z band appears elliptical, as would be expected if the latter girdled the myofibril. Examination of Fig. 6 indicates that when the filaments splay out another layer of filaments is disclosed beneath, and the Z band becomes considerably thinner. These observations fit

References p. 498.

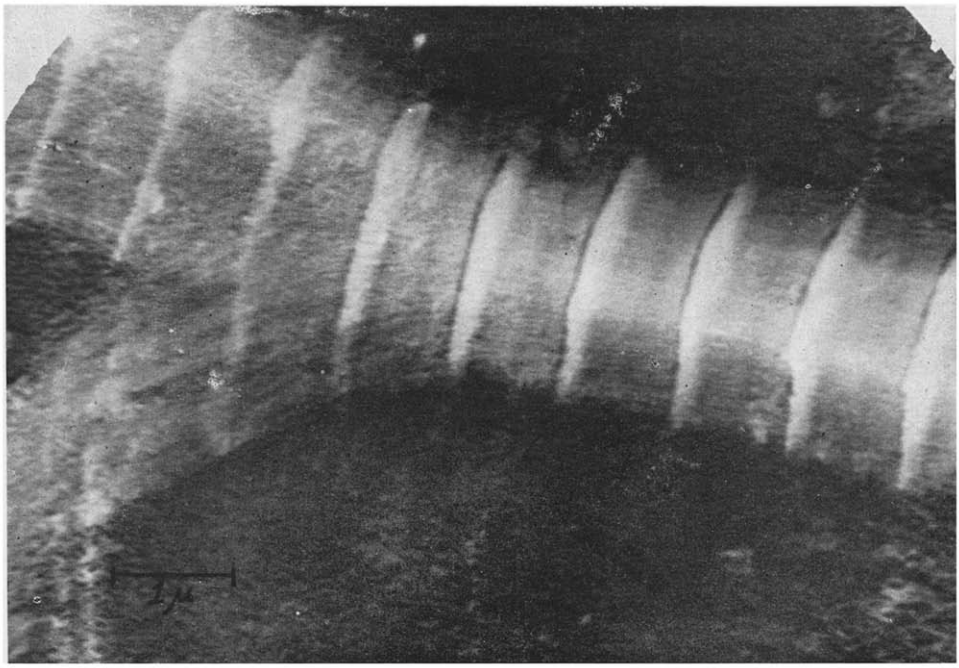

Fig. 6. Preparation 24A. Splayed out myofibril with underlying filaments

in well with the concept of the myofibril as a hollow tube consisting of protein filaments held in position by the Z band, the whole drying down on the electron microscope grid as a thin ribbon of apparently even thickness.

*ATP and the myofibril**

If a suspension of freshly prepared myofibrils is treated with 0.00013 *M* ATP in the presence of 0.0013 *M* magnesium chloride, the individual myofibrils shorten and the suspension settles rapidly. Such ATP-treated myofibrils show a concertina-like structure in the electron microscope (Fig. 7). The original bands are replaced by one rather diffuse band, the spacing of which is about 1/3 to 1/4 of the sarcomere length found in the control myofibrils when they have been dispersed in the same medium without ATP. The shortened myofibrils shown in Figs. 7 and 8 should be compared with those of Fig. 2 which came from the same preparation and were not treated with ATP. It is not possible to decide which of the original bands corresponds to the diffuse band in the ATP-treated myofibril, but it is obvious that the filaments have shortened longitudinally. Intact filaments are not readily distinguished in the shortened myofibril, yet there is a vague suggestion of longitudinal elements at the sides of the myofibril shown in Fig. 7. More definite structures still showing periodicity can be seen at the end of the shortened myofibrillar clump in Fig. 10.

This shortening effect of ATP can also be readily demonstrated with myofibrils prepared from muscle which has been allowed to go into rigor mortis by keeping it for 24 hours at 4° C. Such muscle is slightly shortened, quite stiff, and no longer contractile.

* Some aspects of this work were presented at the Birmingham Meeting of the British Association in September 1950. See *Nature*, 166 (1950) 591.

References p. 498.

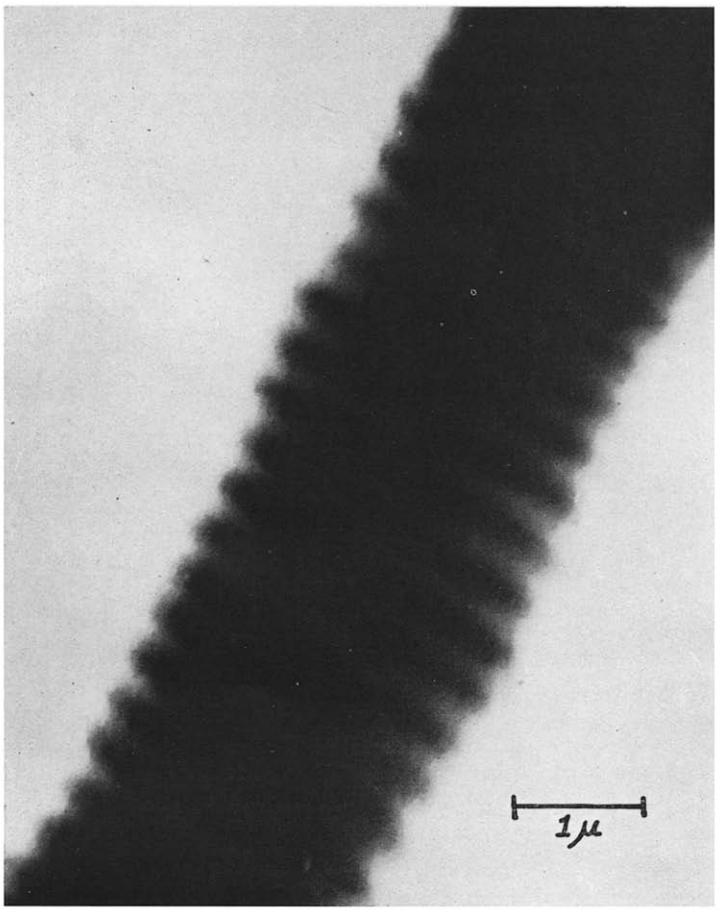

Fig. 7. Preparation 26a. Myofibril treated with 0.00013 *M* ATP. Negative not reversed

Granular fraction

The granular fractions obtained by high speed centrifugation of the turbid supernatant which remains when the myofibrils and cell debris have been removed from a muscle homogenate, presumably correspond to the mitochondrial and microsome fractions which have been prepared from other tissues. Granules from the rat were investigated in some detail as they are more plentiful in muscle from this animal than from the rabbit. Also, if 30% sucrose was used as the medium in which they were isolated, rat muscle granules provided better material for electron microscope examination. In 0.08 *M* borate the granules readily agglutinated and lost their original form as is indicated in Fig. 9. Like all cytoplasm, rat sarcoplasm contains fat globules which rose to the surface of the liquid during the high speed centrifugation. These were removed whilst the granules of lipoprotein nature studied in this investigation collected at the bottom of the centrifuge tube as a translucent, faintly yellow pellet.

The majority of the granules ranged in diameter from 300 A to 5000 A, whilst occasionally larger particles, some with diameters approaching 10,000 A, were seen. On

References p. 498.

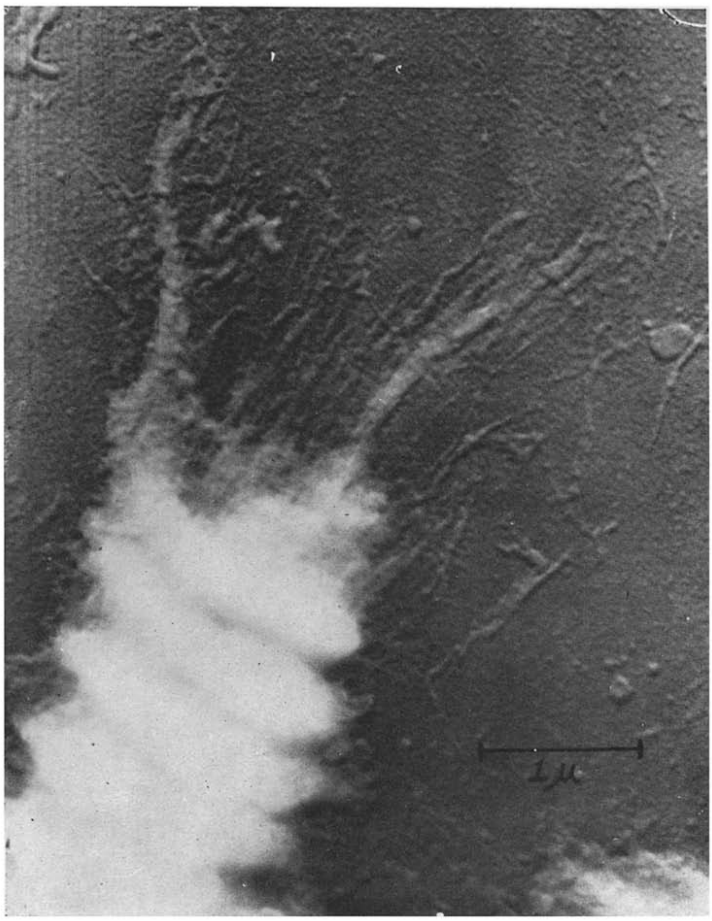

Fig. 8. Preparation 26a. Splayed out end of myofibril treated with 0.00013 *M* ATP

examination in the electron microscope abundant fine granular material could be observed in the supernatant obtained after centrifuging the original sucrose extract 150 min at 16,000 *g*. Some fractionation of the particles could be obtained by single centrifugations at different speeds but, as Figs. 10–13 show, any one fraction contains a wide range of particle sizes. The smaller granules were very abundant and contaminated the heavier fractions, even after resuspending in fresh sucrose solution and centrifuging.

In Figs. 12 and 13 the larger granules show some internal structure; the dense material inside the particle appears to be withdrawing from an external membrane. In view of the fact that the sucrose medium is hypertonic to normal tissue fluids, this might be interpreted as a kind of plasmolysis and suggests the existence of a semipermeable membrane. Fig. 11 gives still further evidence of a membrane structure for here the granules appear rather like hollow spheres which have collapsed.

Frequently some of the larger granules were seen with smaller particles inside and attached to their outer edges. Bearing in mind that these associations could be fortuitous,

References p. 498.

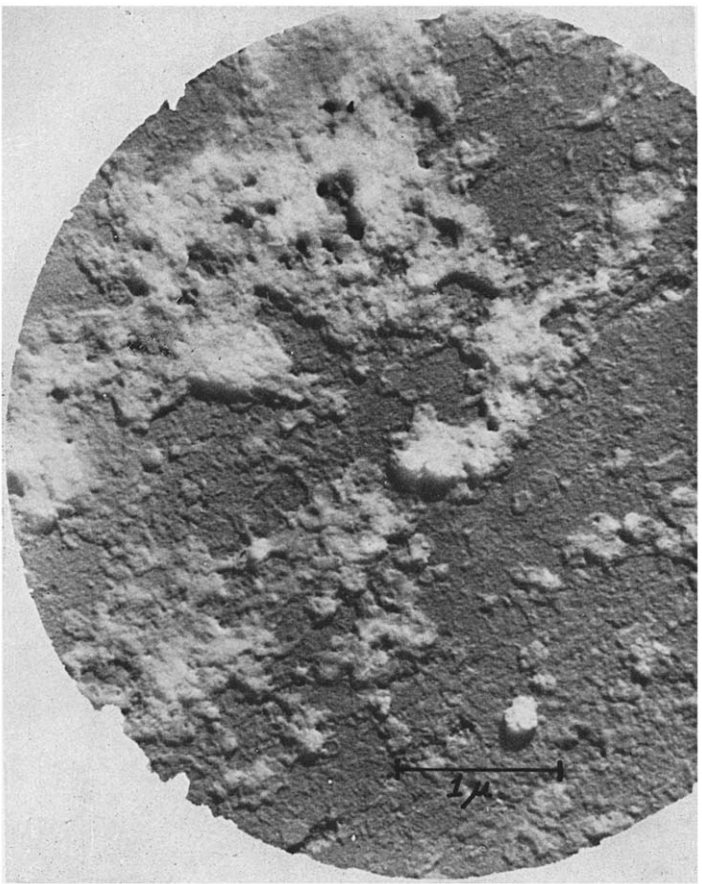

Fig. 9. Preparation 7RB. Agglutinated granules prepared from rabbit muscle by centrifugation of turbid supernatant for 20 mins at 14,000 *g* followed by resuspension and 35 mins at 14,000 *g*. 0.08 *M* borate buffer pH 7.1

it is interesting to speculate on the possibility that the smaller granules might arise from the larger by some sort of self-duplicating mechanism.

Nuclear fraction

Nuclei are conspicuous components of the muscle cell, and during routine optical microscope examination of crude myofibrillar preparations, they could often be seen attached to single myofibrils. In the purified washed myofibril preparations nuclei were infrequently encountered during the course of electron microscope examination. Usually these were dense bodies varying considerably in size and no detail could be distinguished. On occasions only the membrane survived.

In one preparation of myofibrils from rigor mortis muscle which contained more nuclei than was usual, several nucleus-like bodies were observed which showed remarkable structural detail (see Fig. 14). In view of their size and the similarity of the radial markings with those seen in other nuclei investigated in this laboratory, these structures have been provisionally identified as nuclei.

References p. 498.

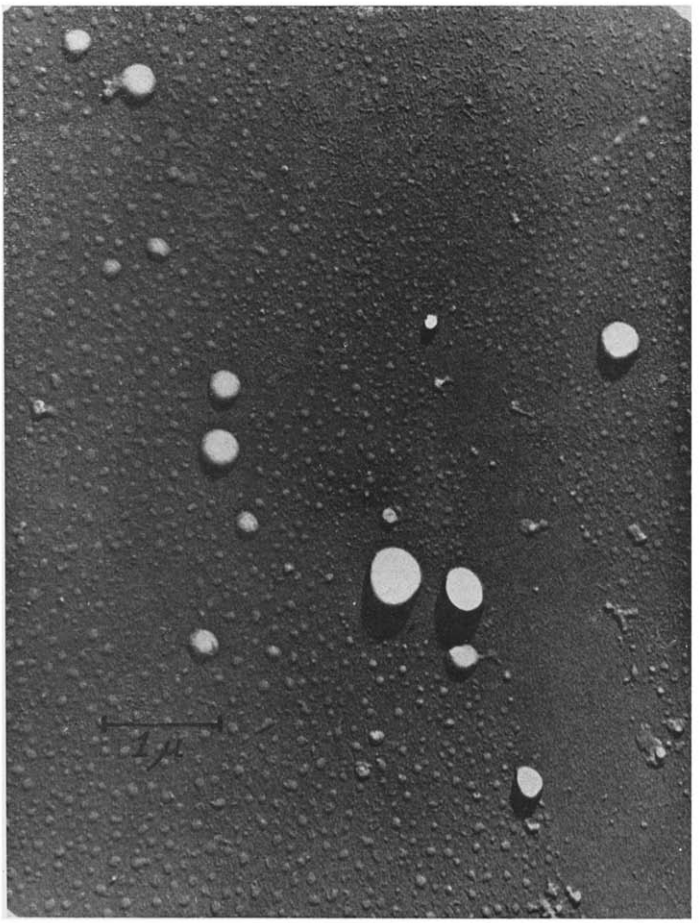

Fig. 10. Preparation 11. Rat muscle granules isolated in 30% sucrose. Light fraction sedimenting after 60 mins at 19,000 *g*

DISCUSSION

The demonstration that actomyosin can be extracted from isolated myofibrils[1], of which it forms the main component, only confirms what has been deduced by indirect means of the localisation of this complex in the muscle cell. Actin and myosin together make up such a large proportion of the myofibril that its main structural components, the filaments, must be largely composed of these two fibrous proteins. In addition, the type of association between the two proteins must be similar to that which exists in the extracted actomyosin gel, for both myofibril and extracted gel undergo profound change with ATP in the correct ionic environment. The marked difference between these two systems is one of organisation; in the myofibril the filaments are precisely oriented along the axis, whereas in the gel they are randomly arranged to form an anastomosed network structure; hence whereas threads made from the extracted actomyosin shorten isodimensionally, so far as can be judged, shortening of the myofibrils is confined to the longitudinal axis.

References p. 498.

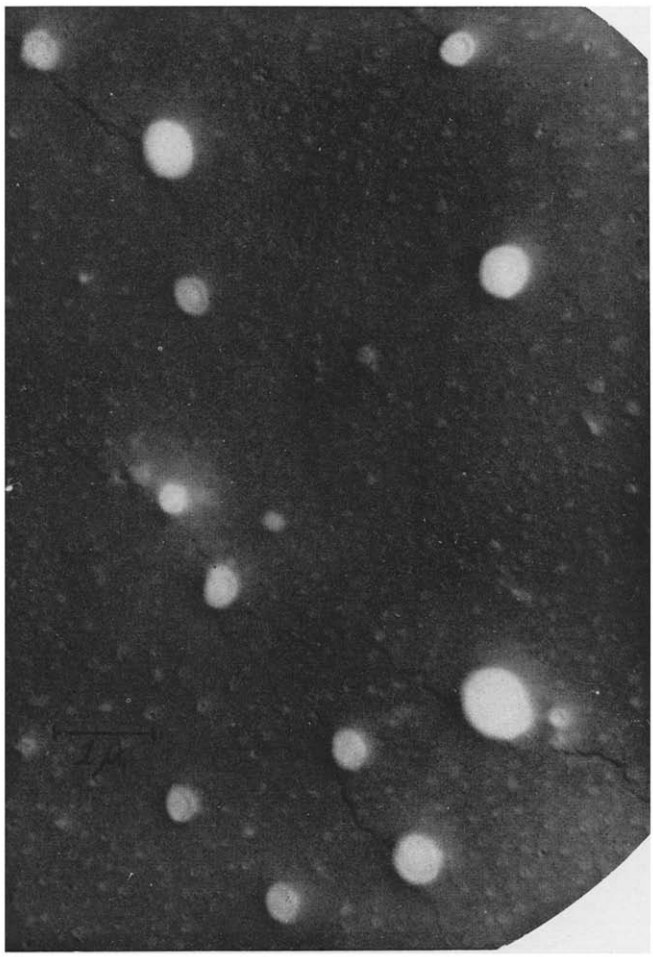

Fig. 11. Preparation 11. Rat muscle granules isolated in 30% sucrose. Heavy fraction sedimenting after 10 mins at 1000 *g*

Although it is difficult to recognise the individual filaments in the electron microscope after the myofibril has been treated with ATP, it is an inescapable conclusion that these component filaments themselves have shortened. The dimensions of the shortening unit, 200 A in width, strongly suggest that the change in length is of intramolecular origin. From the electron microscope study of the effect of ATP on the isolated actomyosin gel (PERRY *et al.*[10]), it was not possible to say definitely whether the fine anastomosed protein threads which made up the gel structure had shortened, but in this system under the influence of ATP the gel structure broke down and finally reaggregated to give a more dense compact mass.

The physical change which ATP induces in the filament indicates that it contains both actin and myosin, although from its periodicity the filament shows more resemblance to polymerised actin itself[11]. It is not easy to visualize how the myofibrillar protein components, each with their own characteristic molecular form, are arranged within a filament of some 200 A in width.

References p. 498.

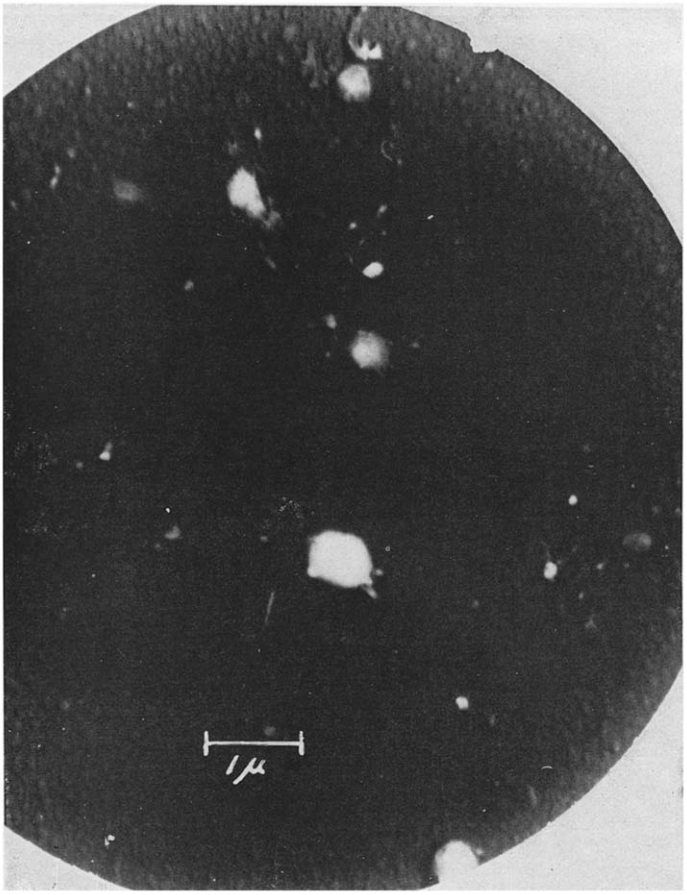

Fig. 12. Preparation 12. Rat muscle granules remaining in the turbid supernatant obtained after the whole muscle homogenate in 30% sucrose was centrifuged for 20 mins at 600 *g*

With the exception of WEBER's[12] early studies, the relation of the myofibril to the sarcoplasm has been somewhat neglected. The sarcoplasm contains the glycolytic enzymes whose function is to provide an anaerobic supply of ATP, whilst this compound is produced in much larger amounts by the oxidative systems presumed to be localised, by analogy with other cells, in the larger lipoprotein granules. The relation of the myofibrils and the granules to the oxidative systems of muscle is now under investigation.

It is extremely likely that at some phase of the contractile process ATP must have free access to the filaments, for quite apart from inducing a physical change in the myofibril, it is this latter structure which is the main ATP-splitting system in the muscle cell[2]. ATP is not always freely available to the myofibril, because when it is, for example as in thaw rigor, the whole muscle shortens to a third or a quarter of its original length, due to the *in situ* shortening of the myofibrils. Such uncontrolled *in situ* action of ATP only results when the muscle cytoplasm has been disorganised by freezing and subsequent thawing[13]. In rigor mortis, however, the intracellular ATP does not act on the myofibril in this drastic manner, for in this condition muscle shortens only slightly and

References p. 498.

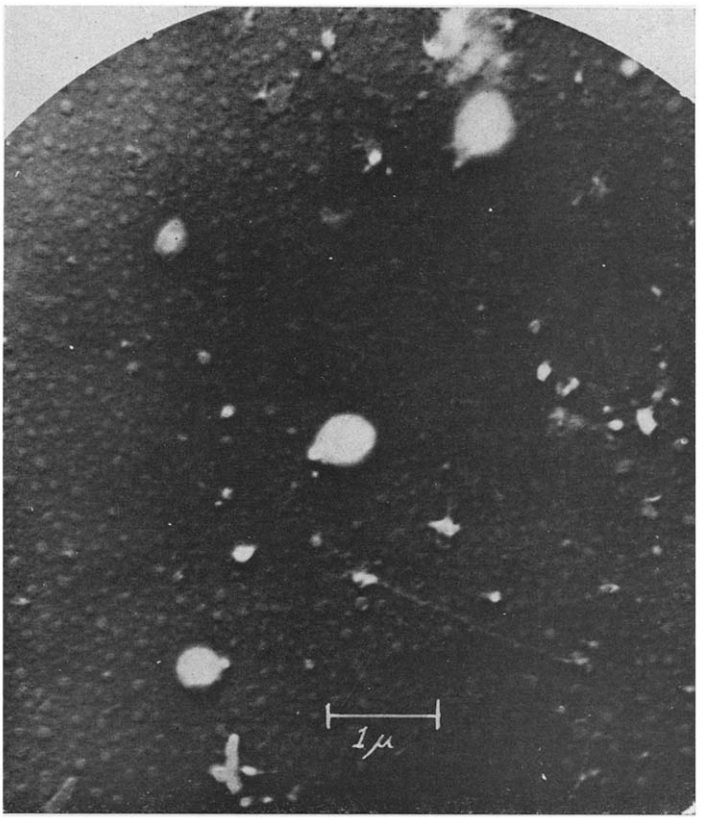

Fig. 13. Preparation 12. As for Fig. 12.

myofibrils isolated from it will still shorten further to a marked extent in the presence of ATP. This must mean that in rigor muscle ATP can be broken down in its "localised" state and before the cell is sufficiently disorganised to allow it to act appreciably in a manner comparable to that in the thaw rigor muscle. It could well be that the ATPase localised in the granules (PERRY[2]) is mainly responsible for the breakdown of ATP in the rigor muscle, whilst the specialised active ATPase of the myofibril is concerned with splitting in the contraction-relaxation cycle.

Skeletal muscle, particularly that from the rabbit, does not appear to be as rich in granules as kidney or liver. Electron microscope examination of sucrose extracts suggests that there is no abrupt change in size of the particles, rather a gradation in size from the large granules, presumably mitochondria, to the smaller, presumably microsomes. Mitochondria respond osmotically to the ionic strength of the environment, swelling in hypotonic solution, and in water may reach the size of red blood cells before finally disintegrating (CLAUDE[14,15]). These effects suggest the presence of a semi-permeable membrane, and the recent work of DE DUVE[16] on the release of phosphatase from liver mitochondria can be explained on this basis. As yet there has been no clear demonstration of this membrane but the electron micrographs shown in Figs. 11 and 12 do very strongly suggest a peripheral structure to the larger granules found in muscle.

References p. 498.

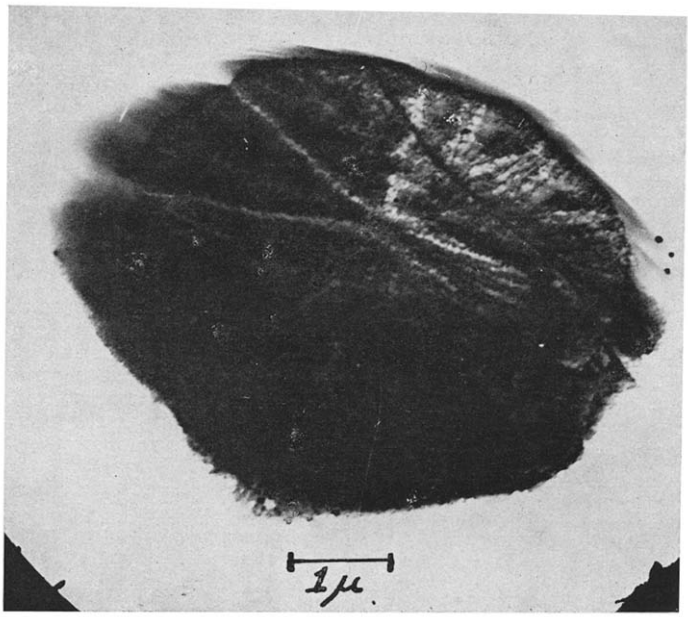

Fig. 14. Preparation 24B. Nuclear structure from rigor mortis myofibril preparation. Negative not reversed

In addition the granule contents appear to be drawn away from this membrane, as would be expected in the hypertonic sucrose medium.

In rabbit psoas muscle the granules are readily washed from the myofibrils and the methods used in the present investigation would not detect any regular localised attachment to the myofibril such as has been suggested by the careful histological studies of BULLARD[17].

We wish to express our thanks to Dr V. E. COSSLETT for his interest in this work and helpful advice during its progress.

SUMMARY

Myofibrils prepared free of sarcoplasm from rabbit psoas muscle by a method involving collagenase, showed detailed fine structure in the electron microscope. The filaments had an average width of 200 A and were extremely regularly arranged in parallel array in muscle which had been slightly contracted as a result of freezing. In many preparations bands other than the Z appeared to be leached out of the myofibril by washing in 0.08 *M* borate. In the presence of low concentrations of ATP, myofibrils from fresh and rigor muscle shortened to 1/3 or 1/4 of their original length and still retained considerable structural organisation.

Granules isolated from rat sarcoplasm by homogenization of the muscle in 30% sucrose solution have been characterised. These particles varied considerably in size, the majority ranging from 300 A to 5000 A in diameter. The largest of the granules appeared to have some internal structure and a limiting membrane could be clearly distinguished.

RÉSUMÉ

Des myofibrilles exemptes de sarcoplasme préparées à partir de muscle psoas de lapin par une méthode employant la collagénase montrèrent une superstructure détaillée au microscope électronique. Les filaments avaient une largeur moyenne de 200 A et montrèrent une disposition parallèle

References p. 498.

extrêmement régulière dans du muscle légèrement contracté par congélation. Dans plusieurs préparations des bandes autres que Z avaient disparu de la myofibrille par lavage dans le borate 0.08 *M*. En présence de faibles concentrations d'ATP des myofibrilles de muscle frais et à l'état de rigor se raccourcissèrent à 1/3 ou 1/4 de leur longueur originale tout en conservant une structure considérablement organisée.

Des granules isolées de sarcoplasme de rat par homogénisation du muscle dans une solution de saccharose à 30% ont été caractérisées. Ces particules variaient considérablement en taille, la majorité ayant des diamètres de 300 à 5000 A. Les plus grosses de ces granules montrèrent une structure interne et une membrane s'y distinguait clairement.

ZUSAMMENFASSUNG

Sarkoplasma-freie Myofibrillen, welche mit Hilfe einer Kollagenase verwendenden Methode aus Kaninchen-Psoas-Muskel hergestellt worden waren, zeigten im Elektronenmikroskop eine detaillierte Feinstruktur. Die Fasern hatten eine durchschnittliche Breite von 200 A und waren in Muskeln, welche durch Gefrieren leicht kontrahiert waren, ausserordentlich regelmässig parallel angeordnet. Es zeigte sich, dass in vielen Präparaten andere Banden als Z durch Waschen in 0.08 *M* Borat aus der Myofibrille verschwunden waren. In Gegenwart von ATP in niedriger Konzentration verkürzten sich Myofibrillen aus frischem und aus Rigor-Muskeln auf 1/3 oder 1/4 ihrer ursprünglichen Länge und zeigten noch eine weitgehend organisierte Struktur.

Körner wurden charakterisiert, welche aus Ratten-Sarkoplasma durch Homogenisieren des Muskels in 30%-iger Saccharose-Lösung isoliert worden waren. Diese Teilchen zeigten eine sehr unterschiedliche Grösse; der Durchmesser variierte meistens zwischen 300 und 5000 A. Die grössten der Körner zeigten eine gewisse innere Struktur und eine Membrane konnte deutlich unterschieden werden.

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