AN ELECTRON MICROSCOPE AND X-RAY STUDY OF THE SYNAERESIS OF ACTOMYOSIN

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INTRODUCTORY

An earlier electron microscope and X-ray study[1] of the muscle proteins myosin and actin was concerned with their mode of interaction in 0.5 M KCl solution, an ionic strength which ensures that both proteins and the complex they form (actomyosin) are in the sol state. Mixing solutions of the two proteins at this ionic strength results in a great increase in viscosity[2, 3], and the electron microscope reveals that this is due to the formation of network structures of anastomosed filaments. On addition of a small amount of adenosine triphosphate (ATP), the viscosity falls and the network structure is no longer evident.

A phenomenon that is apparently more directly relevant to the mechanism of muscular contraction may be brought about in much weaker salt solution, *viz.*, 0.05 M KCl. If a sol of actomyosin is squirted through a capillary tube into this weak salt solution, the complex is precipitated as a thread of protein gel, in which state the fibrillar proteins probably exist under approximately the ionic conditions prevailing within the muscle cell (0.17 M[4]). SZENT-GYÖRGYI and his collaborators[3] made the striking observation that in the presence of the order of 0.0005 M $MgCl_2$ and 0.1% ATP such a thread undergoes an intense and rapid shrinkage ('contraction'), an effect which has been made the centre-piece of a new theory of muscular contraction. It is of prime importance in relation to the soundness or otherwise of this theory to determine exactly what happens in such a system.

In contradistinction to the contraction of muscle, the shrinkage of actomyosin gel in the presence of ATP takes place uniformly in all directions; it is a synaeresis whereby much of the water is thrown out of the gel, leaving a dense mass in which the protein concentration is about 50% compared with the 2–3% in the original actomyosin. It is important to distinguish between the effect in the higher salt concentration and that with which we are concerned here, *i.e.*, in 0.05 M KCl. Presumably under both ionic conditions the first action of ATP is to break down actomyosin, but only at the lower ionic strength is this followed by pronounced synaeresis.

Synaeresis can conceivably come about in one or both of two ways: either by a coming-together and de-solvation of polar groups *in one and the same molecule* (intra-

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molecular synaeresis), or by combination between polar groups of *neighbouring molecules* (intermolecular synaeresis). If therefore it could be shown that the ATP-synaeresis of actomyosin is an *intra*molecular phenomenon, the effect would link up directly with much X-ray and related evidence from which it has been concluded that muscular contraction rests ultimately on a shortening of the myosin chains by intramolecular folding[5].

ELECTRON MICROSCOPE

Preparation of actomyosin (myosin-B)

Threads of actomyosin made by mixing various proportions of actin[2] and myosin[6] prepared independently did not show such pronounced synaeresis as those made from actomyosin extracted as such from minced muscle (SZENT-GYÖRGYI's myosin-B[7]). Myosin-B is a crude extract, and it was made by leaving minced rabbit muscle for 24 hours in three times its volume of an alkaline salt solution (0.5 M KCl, 0.01 M Na_2CO_3, and 0.04 M $NaHCO_3$). The resulting viscous mass was then diluted with 2 or 3 more volumes of the same salt solution in order to facilitate removal of the insoluble stroma by centrifugation. The supernatant fluid was filtered through a tight pad of glass wool, and the viscous filtrate was used for both the electron microscope and X-ray investigations.

Purification of myosin-B

Myosin-B was purified by precipitating with 10 volumes of water, centrifuging, and then dissolving the precipitate by adding sufficient solid KCl to bring the final concentration to 0.05 M. This precipitation procedure was carried out three times.

Preparation of actomyosin suspensions for electron microscope examination

Direct examination in the electron microscope of actomyosin filaments (0.2 to 0.5 mm thick and 2 to 3 mm long, for example) is hardly practicable, hence it was decided to study the synaeresis of a dilute suspension such as is obtained when ATP is added to actomyosin dispersed in a chemical environment identical with that used for the thread experiments.

The control suspensions of actomyosin gel were obtained by diluting 0.5 ml myosin-B with 4.5 ml distilled water, whereas the synaeresed forms were made by adding 0.5 ml of a neutral solution containing 0.005 M $MgCl_2$ and 0.012 M ATP to a suspension of actomyosin (0.5 ml myosin-B plus 4.0 ml distilled water). The synaeresed gel settled quickly to the bottom of the tube as a compact granular superprecipitate, whilst the control gel was slow in settling. In order to obtain concentrations suitable for electron microscope examination, the control and synaeresed forms were diluted, as required, with the appropriate media. Such diluted suspensions were used immediately for electron microscope examination, and p_H's were recorded using the glass electrode. Drops of the suspensions were placed on filmed specimen grids, and to avoid aggregation of the proteins a thin layer was formed by immediately draining the drop with filter paper. Such thin layers dry very quickly and the chances of material aggregation are greatly reduced. To ensure complete fixation the dried film preparations were exposed to the vapour of osmic acid (1% solution) for about five minutes, after which they were washed on the surface of distilled water to remove the salt. Finally the dried films were shadow-cast with chromium following the vacuum technique of WILLIAMS AND WYCKOFF[8].

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Results

Figs 1–4 show the appearance of material in the control suspension of actomyosin gel, *i.e.*, in the unsynaeresed form (0.05 M KCl, p_H 7). The structure is essentially a dense tangled network, very similar to that obtained in our previous study[1] of actomyosin prepared by mixing approximate physiological proportions of actin and myosin solutions in 0.5 M KCl. By further diluting the suspensions used for electron microscope preparation, attempts were made to open out the network and increase the dispersion of individual fibres. Fig. 4 is a typical example of the results obtained with a suspension ten times as dilute as that used for Figs 1, 2 and 3; it indicates, what indeed our earlier experiments have shown already, that the network structure is a property of the actomyosin complex as such rather than an aggregation effect obtained with more concentrated suspensions.

Myosin-B which has been precipitated three times gives the appearance of a network very similar to that obtained with the crude preparation, but the individual filaments appear sharper and the whole structure looks cleaner, as though some component other than actomyosin had been removed.

Figs 5–14 show the appearance of the synaeresed actomyosin, *i.e.*, the 'contracted' form. The dense network characteristic of unsynaeresed actomyosin is no longer apparent, and from the variety of fields illustrated it is possible to infer the probable sequence of events which finally results in the compact synaeresed gel. The dominating impression is one of a disruption and opening-out of the actomyosin network, with the production of small linear fibrils which then form more robust and dense fibres by lateral aggregation. These small fibrils, the first result of adding ATP, are clearly shown in Figs 6 and 7 and are similar in appearance to the fibrils found in actin and myosin preparations by JAKUS AND HALL[9], although it is not yet possible from electron microscope studies alone to say that they are actually actin and myosin. Their pronounced tendency to aggregate side to side, and thereby build up more robust fibres containing much less water than the unsynaeresed gel, is illustrated in Figs 8–14.

X-RAYS

A number of X-ray photographs were taken of actomyosin (myosin-B) prepared as described above, at various moisture contents and both as powders and thin films. Figs 15 and 16 are typical of the results obtained with preparations photographed while still moist, Fig. 15 representing the state of the gel before synaeresis and Fig. 16 after synaeresis. Both diagrams are of the familiar α-type (plus a halo arising from the fluid contained in the specimens), and according to our experience of such photographs it is not possible to point to any really *fundamental* difference between the two. There is in fact a difference of a secondary kind in the sense that, after synaeresis, there is a certain clarification and the edge of the characteristic α-reflection at 5.1 A. is better defined, but in order to account for such a change it would probably be sufficient to postulate only a side-to-side aggregation; the inference would be that improved definition arises from larger or better-defined diffracting units.

DISCUSSION

The appearance of actomyosin in the electron microscope is essentially the same whether it is examined in the gel or in the sol state; in both cases the picture is of a

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network of anastomosed protein filaments. The disruptive action of ATP on this structure is such as would be predicted from biochemical studies[10] of the interaction of myosin and actin in 0.5 M KCl, which have shown that the same SH groups in the myosin molecule are essential both for ATP-ase activity and for the formation of actomyosin. By virtue of these SH groups myosin can form a complex either with ATP or with actin, depending on whether it acts as an enzyme and splits ATP or whether it interacts with the protein to form actomyosin. The affinity of the enzyme for substrate is much greater than for protein, so that when ATP is added to actomyosin the actin is displaced, with a consequent change in the colloid properties of the system. It is only when all the ATP has been split that the viscosity of the sol increases as a result of renewed interaction between myosin and actin.

It is to the change subsequent to this colloidal effect, *i.e.*, to the synaeresis obtained in 0.05 M KCl rather than to the decrease in viscosity in 0.5 M KCl, that we must look for a possible *in vitro* counterpart of myofibrillar contraction. The *isodimensional* shrinkage of actomyosin threads is not in itself an objection to SZENT-GYÖRGYI'S view, because the filaments in such threads are in random orientation; what has to be shown is that an actomyosin system oriented as in the muscle cell would, by a process of alternate dehydration and rehydration, reproduce the mechanical properties of the cell. Any such theory, however, at once faces difficulties in accounting for the great rapidity with which certain muscles contract and relax; for example, in *Decticus* thoracic muscle at 24° C fusion of contractures does not take place until a stimulation frequency of 100 per second is reached[11]. At room temperature synthetic actomyosin threads shrink within a few seconds and would presumably show a much more rapid change if they were of the dimensions of the filaments within the myofibril: rehydration of actomyosin that has undergone synaeresis cannot, however, take place at the same speed[3] – in fact it has yet to be demonstrated that the synaeresis of actomyosin threads is as reversible as the contraction of muscle.

Apart from these more speculative points, very real difficulties arise when the behaviour of loaded actomyosin threads is considered[12]. When treated with ATP they *increase* in length, *i.e.*, their tensile strength decreases, whereas a fundamental property of stimulated muscle is to shorten under tension. In terms of micellar structure, the dissociation of actomyosin destroys the network of anastomosed filaments on which the tensile strength of the thread depends.

After synaeresis with ATP, actomyosin bears some resemblance to the products of certain types of proteindenaturation – dense, coherent precipitates in which the loss of solubility is due to a disorganisation into multiple irregularly coiled-up groupings and, in the last resort, to the formation of chain-bundles in the β-configuration. Heat-denaturation in particular brings about changes of this kind, and they are revealed in the electron microscope by a tendency to 'clumping', and in X-ray photographs by the appearance of the well-known β-diagram. No such effects are observed after the synaeresis of actomyosin, where aggregation must arise because (in 0.05 M KCl and in the presence of small amounts of ATP) these proteins are in fact topochemically disposed to form a dense dehydrated structure. If the synaeresis were due solely to the elimination of water as a consequence of a folding of individual chains within the protein filaments (intramolecular synaeresis), we might expect a condensation of the network and probably 'clumping' too – the components would become both thicker *and closer together*; but if the action of ATP is to induce side-to-side synaeresis, we should expect a thickening

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of the components *plus a separation*, which is what is actually observed. The most reasonable inference, therefore, from the electron microscope observations is that the main phenomenon consists of an intermolecular synaeresis.

The electron microscope observations alone do not, however, exclude the possibility that an initial shortening and thickening of the threads of the network takes place before the onset of side-to-side aggregation. The evidence on this point comes from the X-ray results, from which it appears that there is no deep-seated difference between actomyosin before and after synaeresis: no change is apparent in the state of intramolecular folding but only in the state of aggregation. The X-ray and electron microscope observations reinforce each other in the conclusion that the ATP-synaeresis of actomyosin is essentially a side-to-side building of larger aggregates.

It must be confessed that it is with a sense of disappointment that we report the results of these investigations because, as pointed out above, it would have been an important step forward to have been able to establish a clear connection between the findings of SZENT-GYÖRGYI and his school and the results of X-ray studies of muscle and the k-m-e-f group in general. Since in an actual muscle both myosin and actin are known to lie lengthways[13], *i.e.*, parallel to the direction of contraction, the synaeresis of actomyosin *in vitro* represents a drawing-together in a direction at right angles to the macroscopic change observed *in vivo*; and therefore, for the present at least, it does not seem possible from electron microscope and X-ray studies to trace any direct relation between the two phenomena.

Although the evidence presented above does not support the view that synaeresis of actomyosin is the process underlying the act, itself, of muscular contraction, yet the interaction of myosin and actin must have some deep significance in this respect. It is prevented only by ATP, the substance which modern theories of muscular activity use to bridge the gap between the chemical and mechanical events. As the compound in which the chemical energy of carbohydrate metabolism is concentrated, ATP is considered to supply the energy required by the contractile mechanism. It has yet to be shown that actomyosin is in fact 'energised' by ATP; on the contrary, by dissociating actomyosin, followed by side-to-side aggregation, it would appear to promote a state of minimum energy. In this effect, however, ATP holds the unique position of being able to modify profoundly the physical properties of the enzyme, myosin, whose substrate it is.

One final point concerns the remarkable compactness of the product resulting from the ATP-synaeresis of actomyosin. When ATP dissociates actomyosin in 0.5 M KCl, the salt concentration is great enough to keep the liberated components in solution and there is a sudden great fall in viscosity, but at low salt concentration (0.05 M KCl) the now independent myosin and actin particles undergo a side-to-side synaeresis which shows itself as the 'superprecipitation' of a suspension or the 'contraction' of a thread. The reason why the superprecipitate has such an abnormally high protein content (of the order of 50%) as compared with what can be achieved (only a few per cent) by precipitating myosin and actin in more familiar ways would appear to be that when ATP dissociates the two components, they are liberated in a temporarily 'active' state leading to a much more rapid and thorough side-to-side aggregation than is normally feasible.

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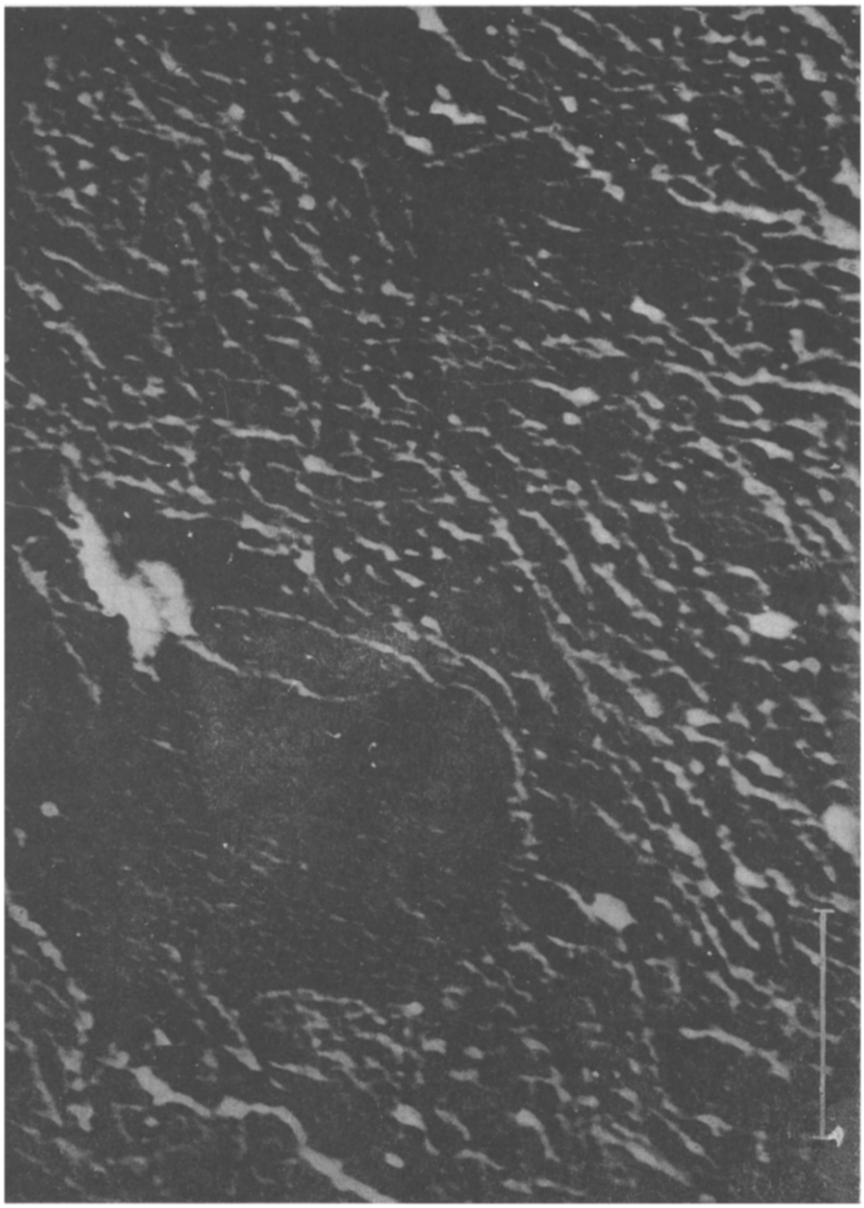


Figs 1, 2 and 3. Appearance of a suspension of myosin-B (actomyosin) dried on a collodion film. Chromium-shadowed. (Figs 1 and 2 pH 7.3; Fig. 3, pH 7.0).

Fig. 2

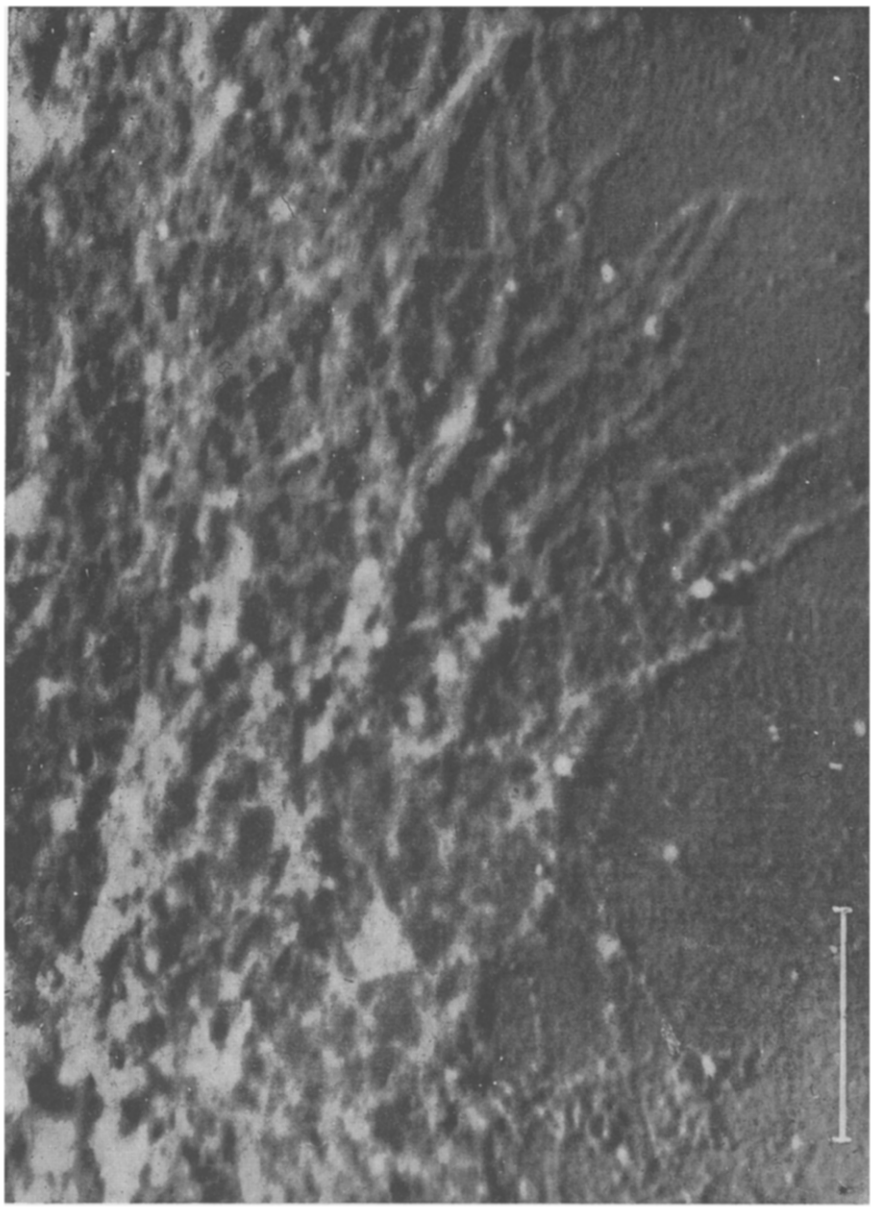


Fig. 3

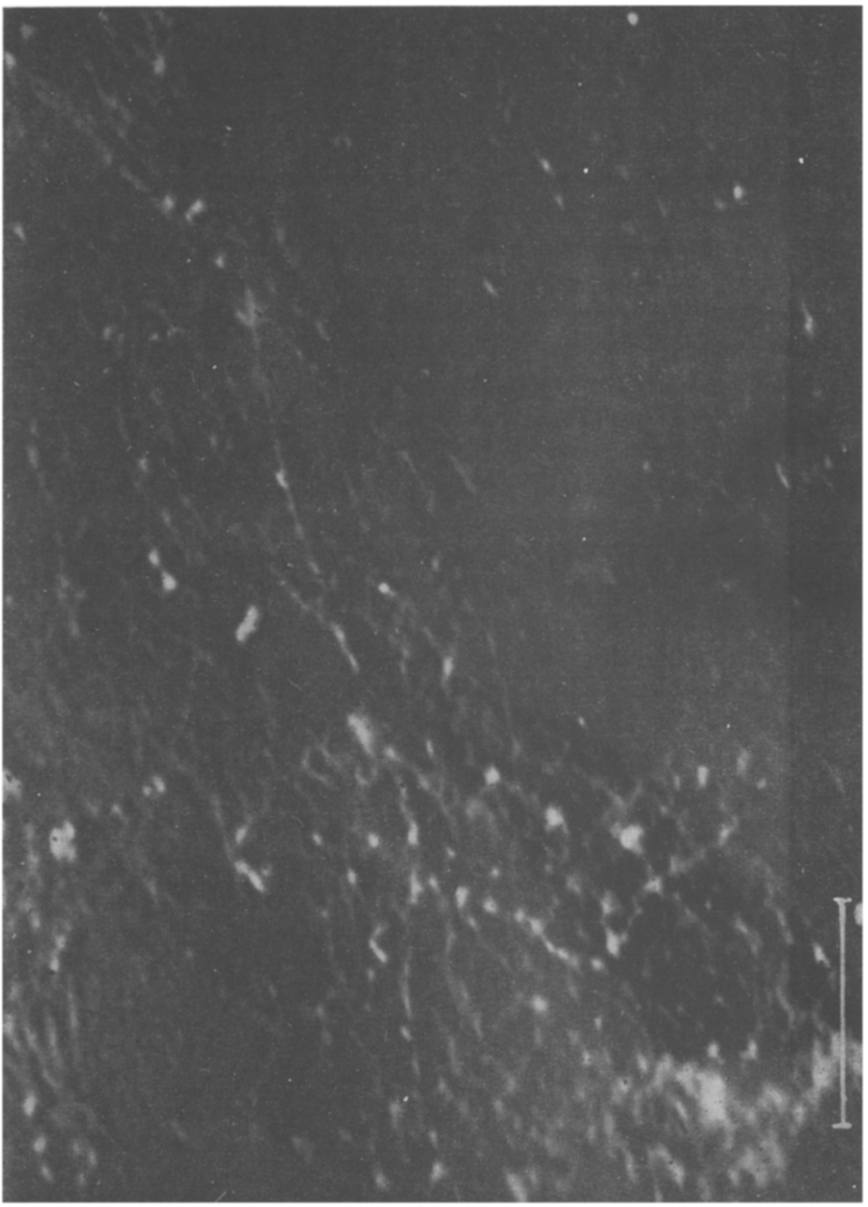


Fig. 4. Appearance of a suspension of myosin-B, ten times more dilute than for Figs 1, 2 and 3, dried on a collodion film. Chromium-shadowed. (PH 7.3).

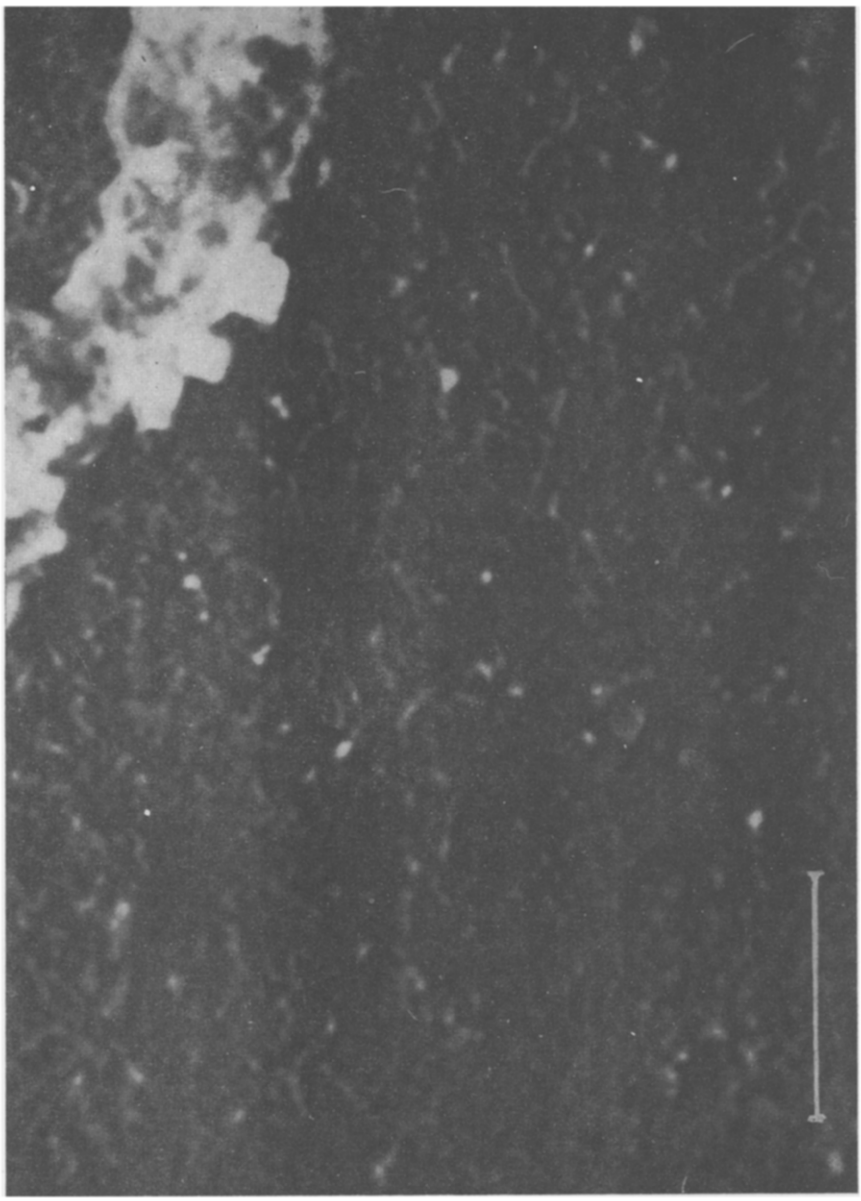
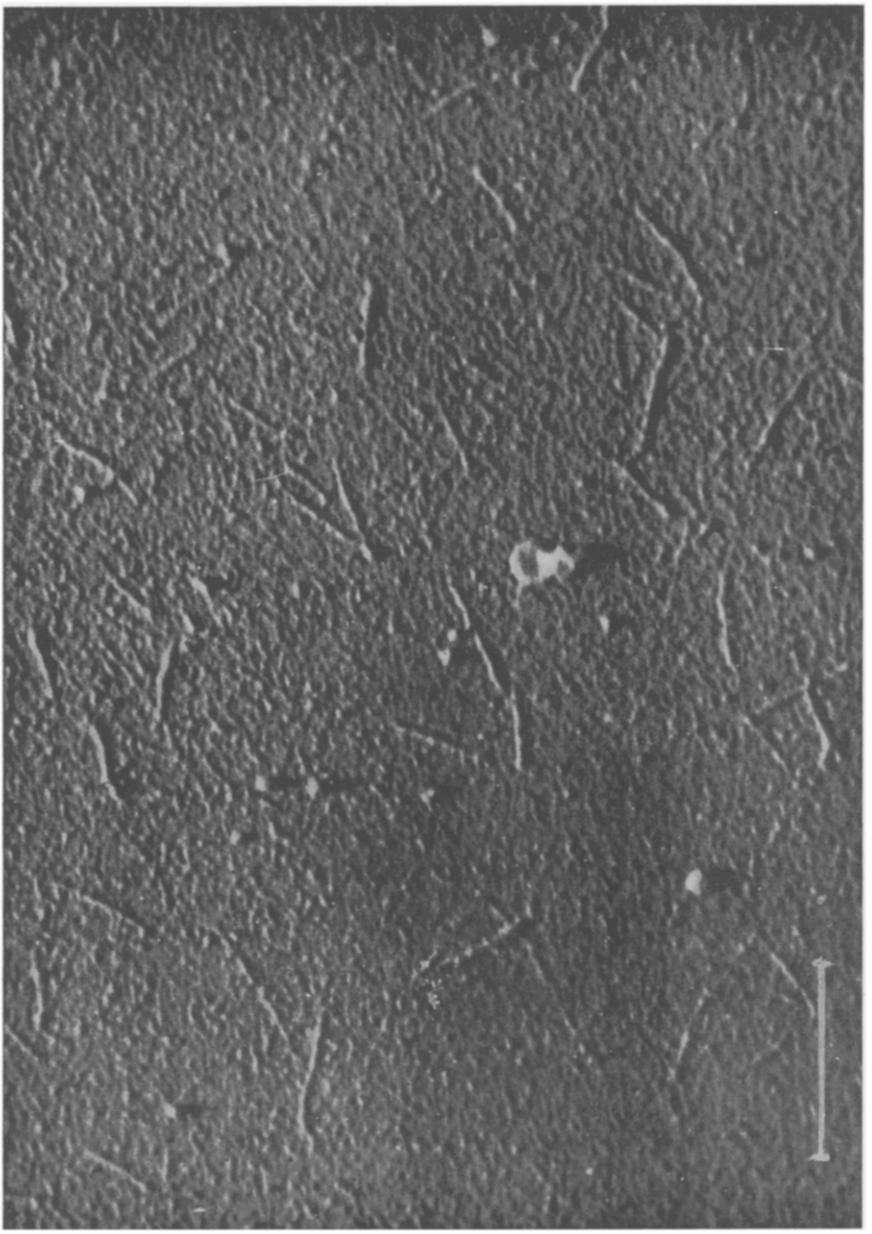


Fig. 5. Appearance of a 3-times purified suspension of myosin-B after synaeresis by addition of ATP. Dried on a collodion film. Chromium-shadowed. (pH 7.3).



Figs 6–14. Appearance of a suspension of myosin-B after synaeresis by addition of ATP. Dried on acollodion film. Chromium-shadowed. (pH 7.3).

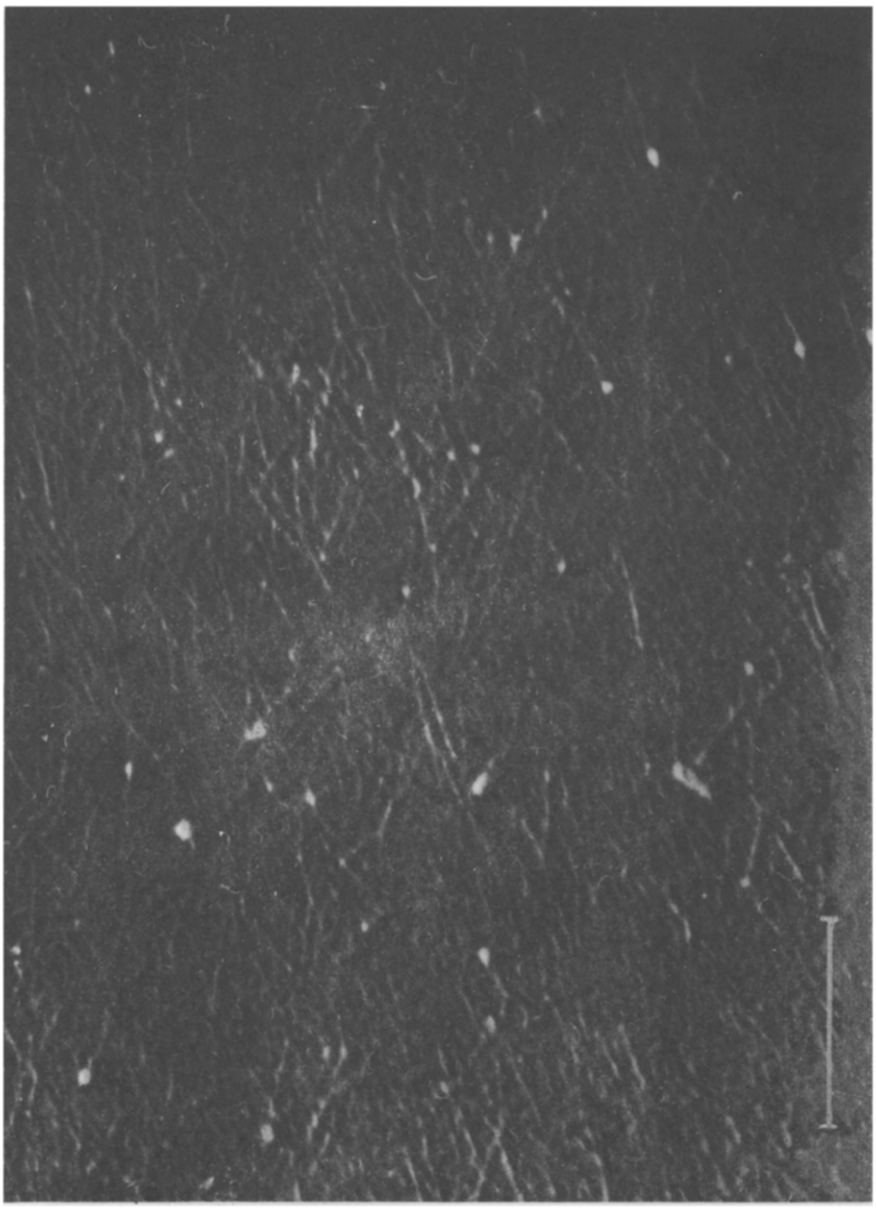


Fig. 7

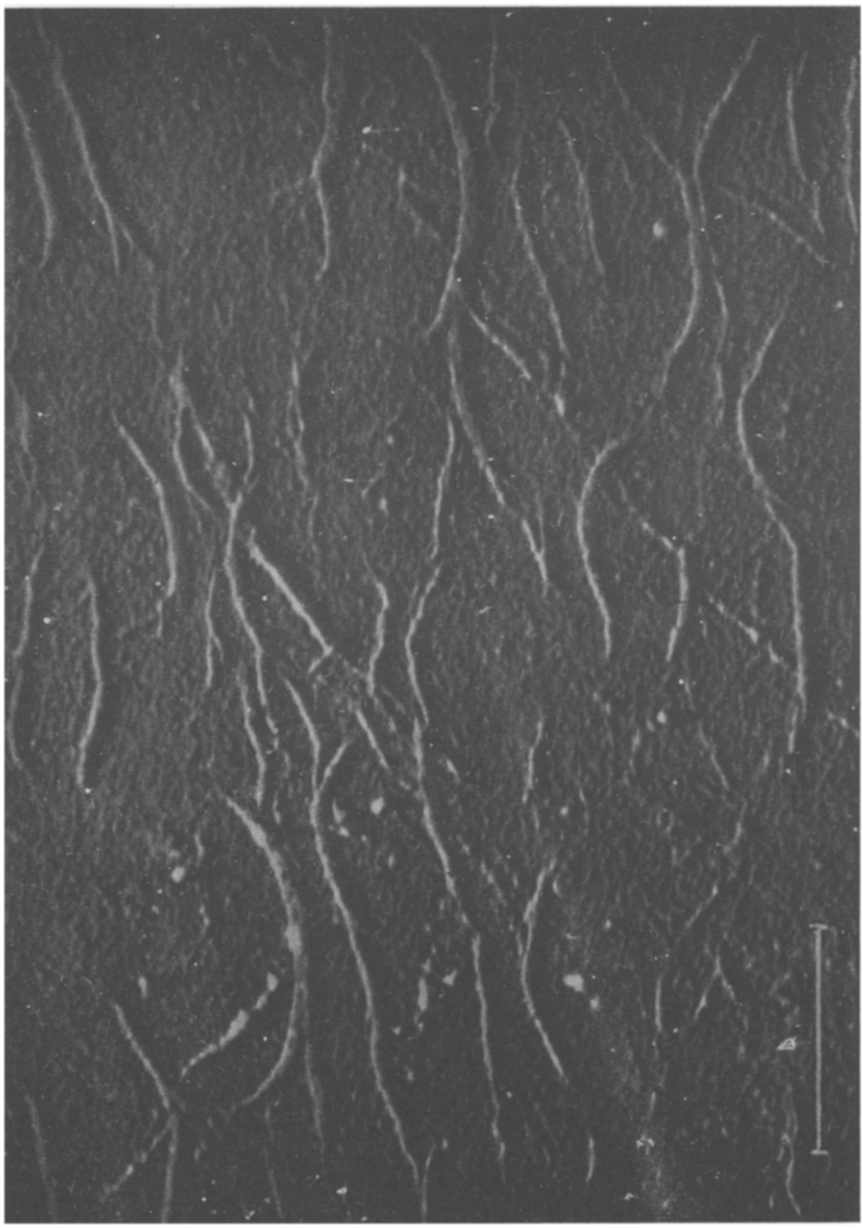


Fig. 8

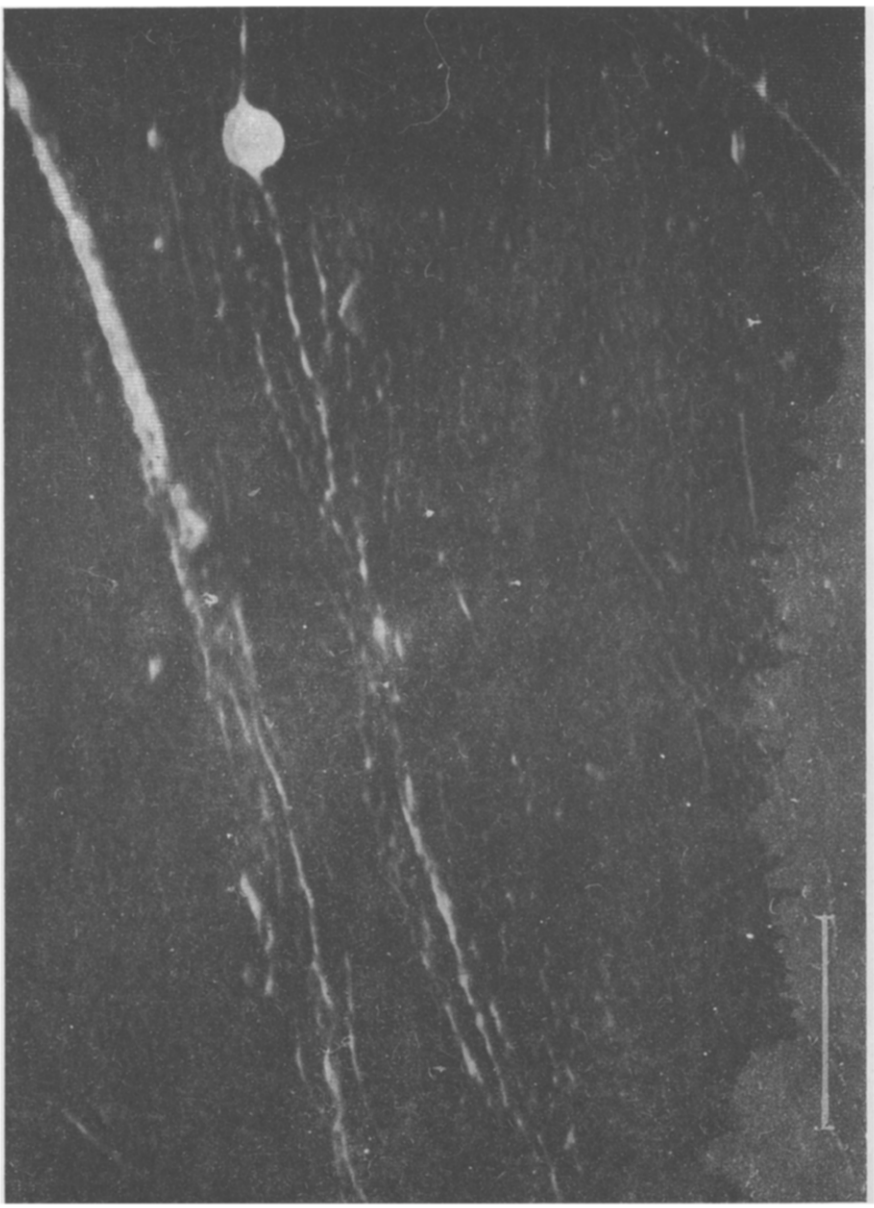


Fig. 9

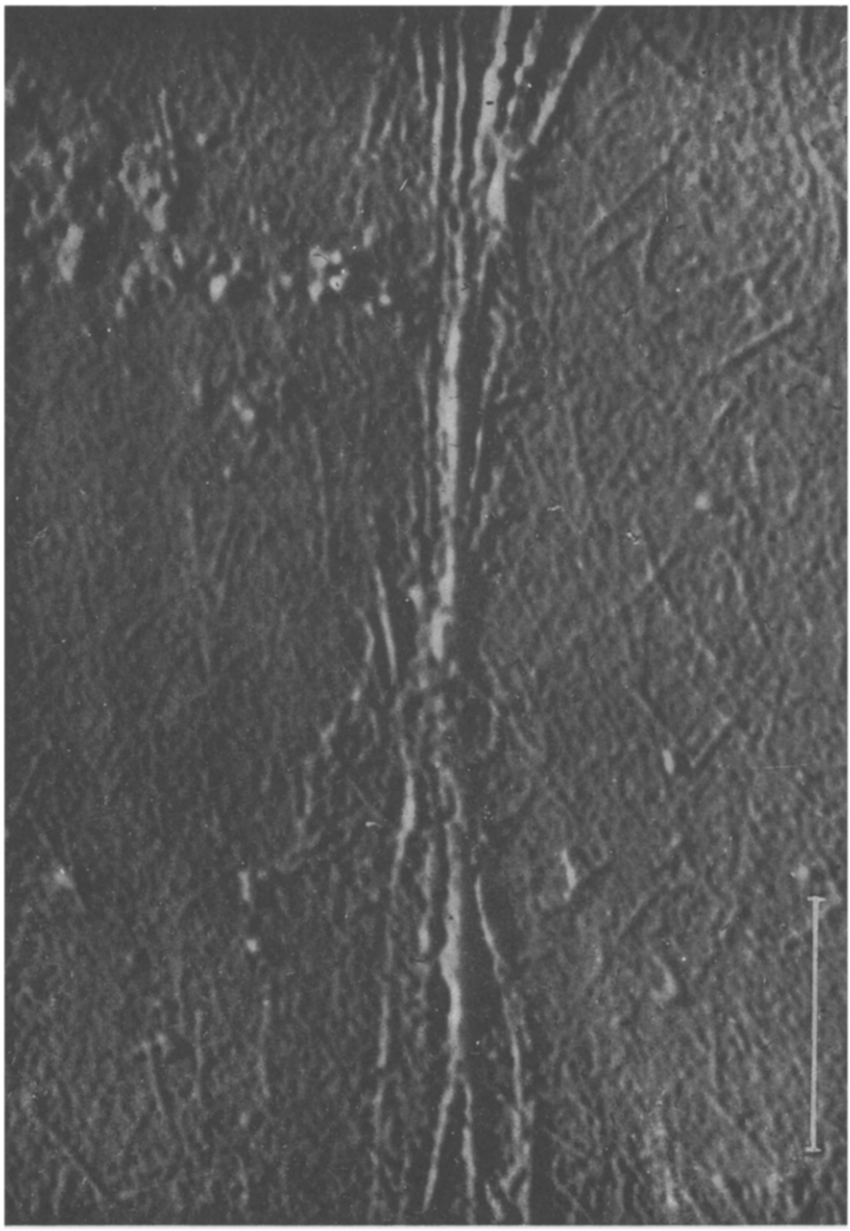


Fig. 10

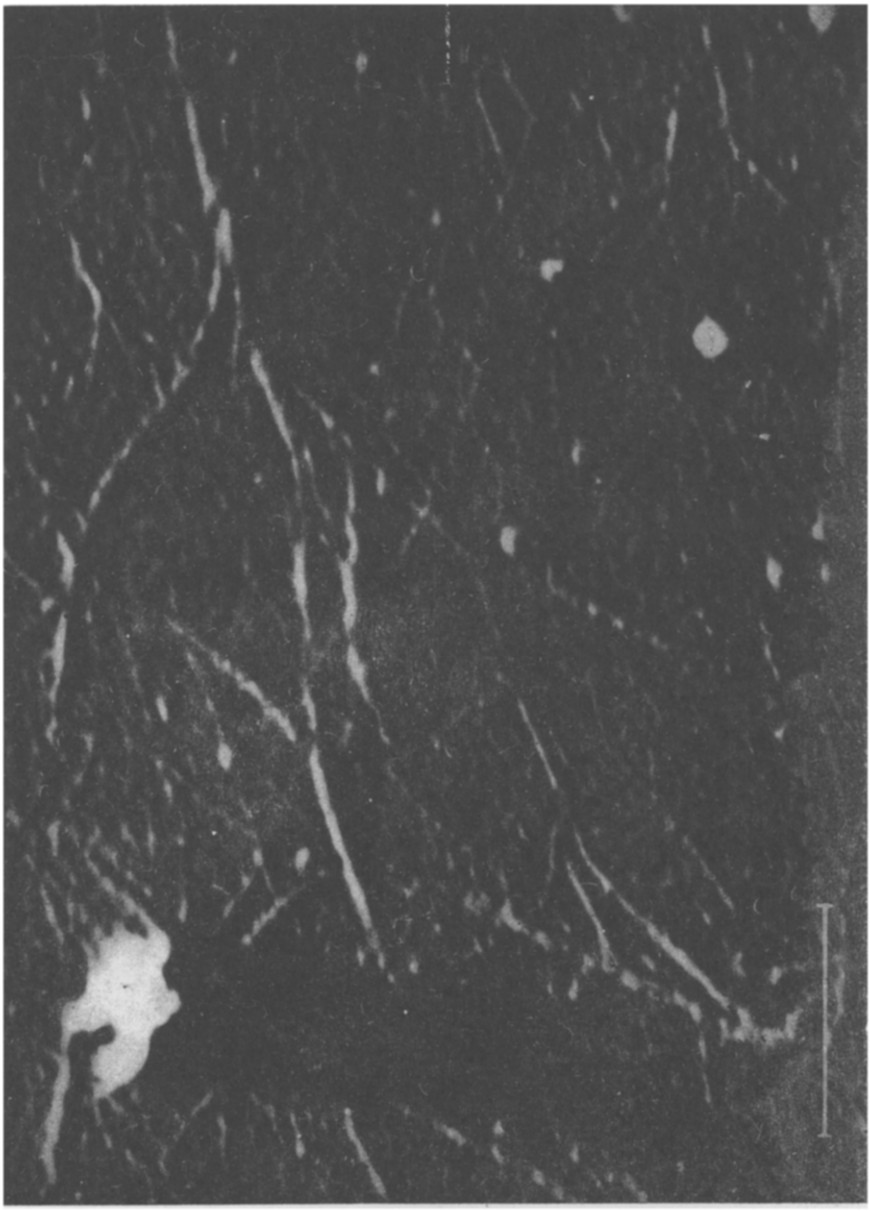


Fig. 11

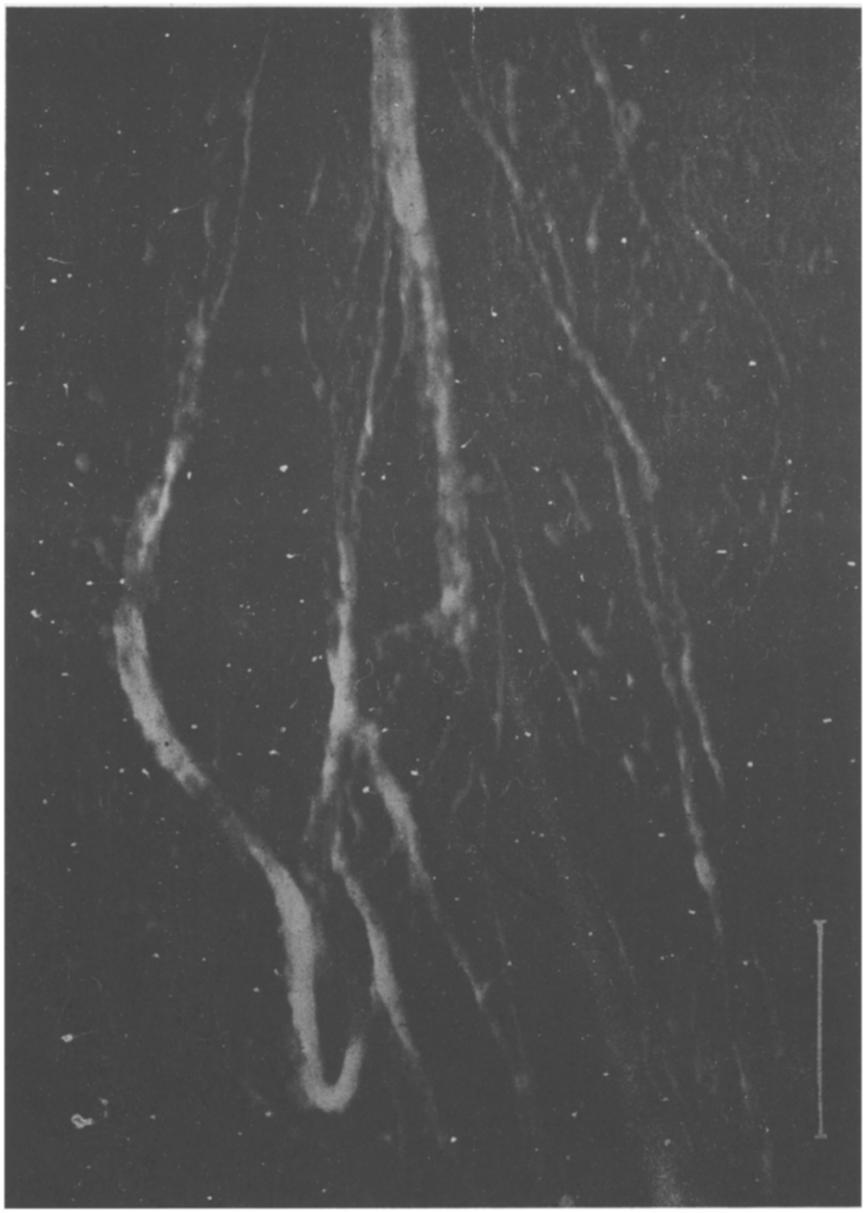


Fig. 12

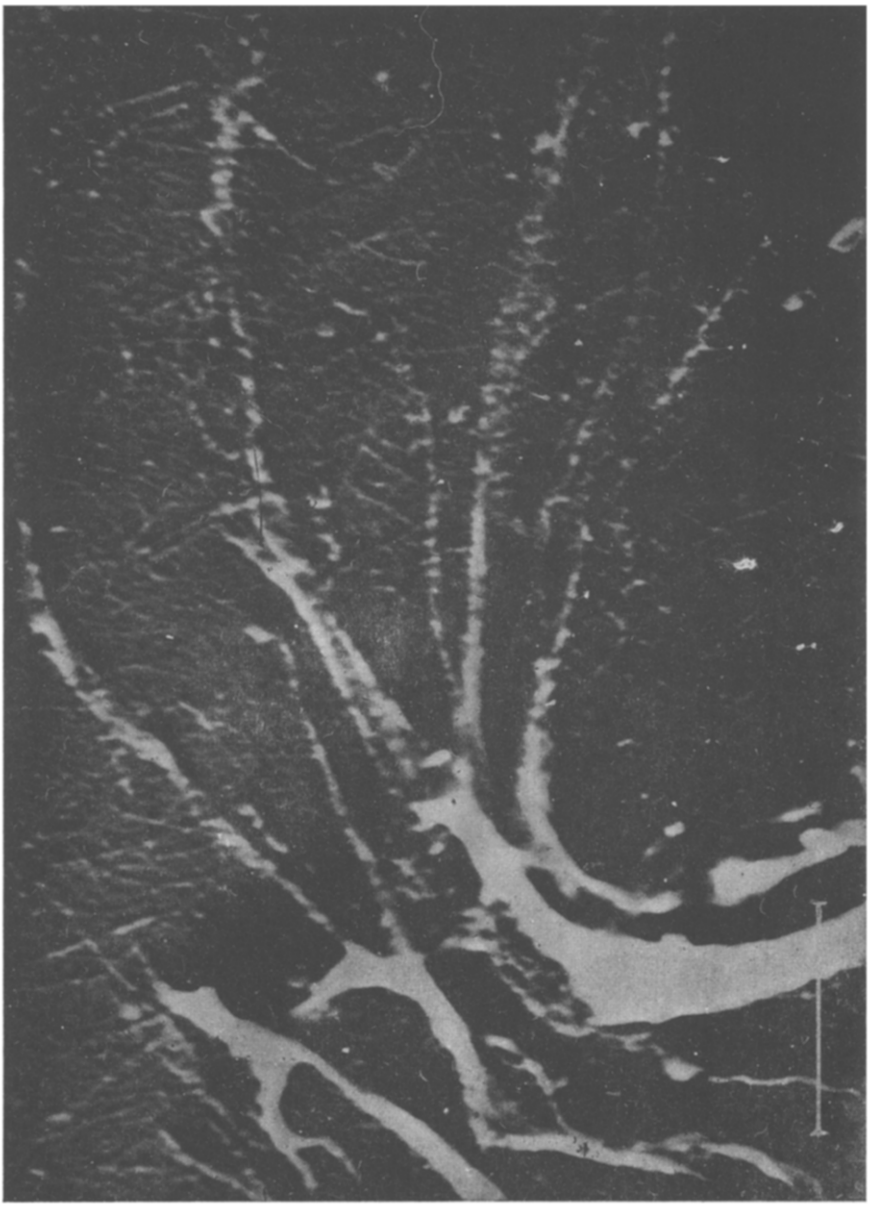


Fig. 13

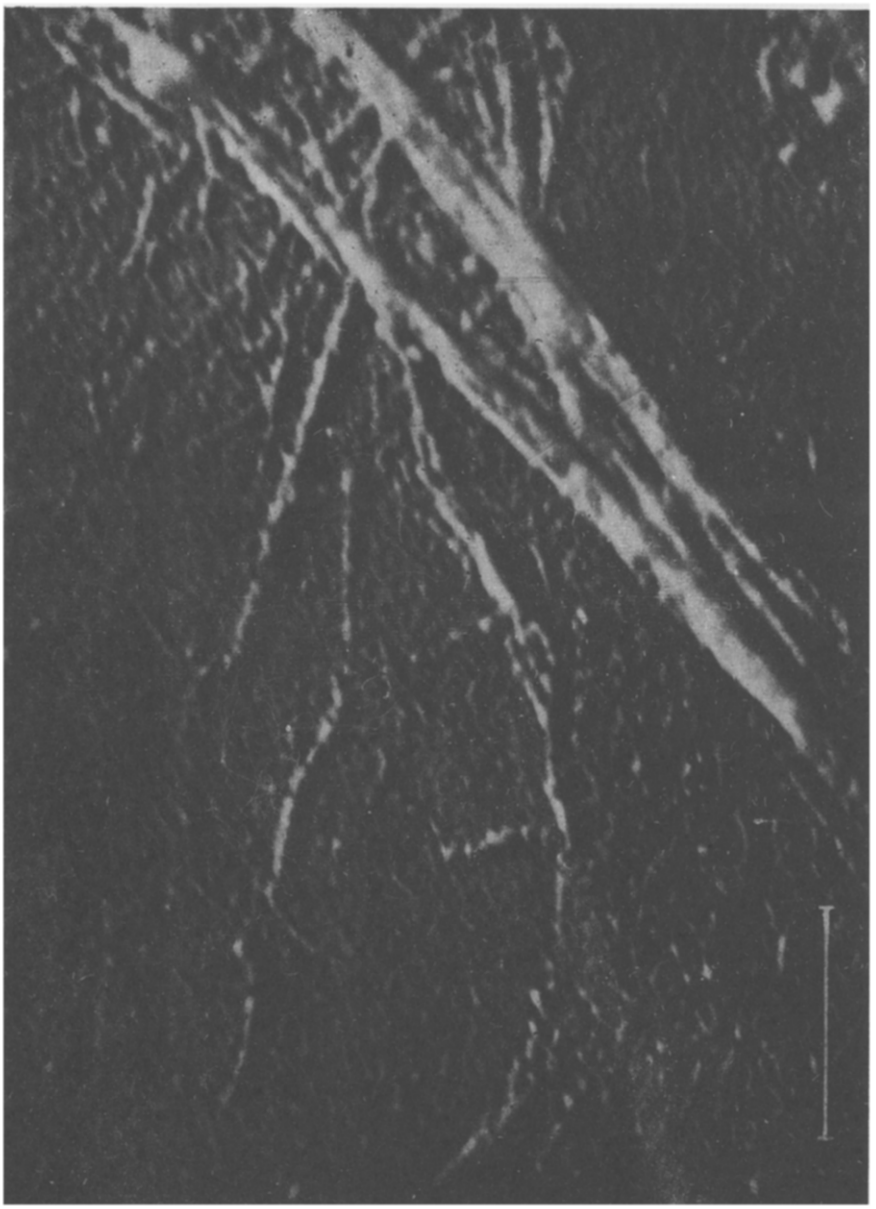


Fig. 14

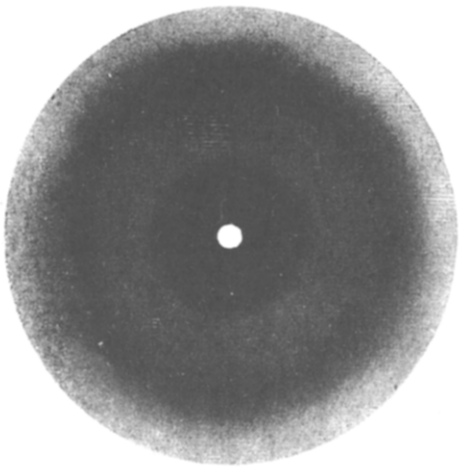


Fig. 15. X-ray powder photograph of a moist gel of myosin-B before synaeresis. Cu Kα. D $\approx$ 4 cm

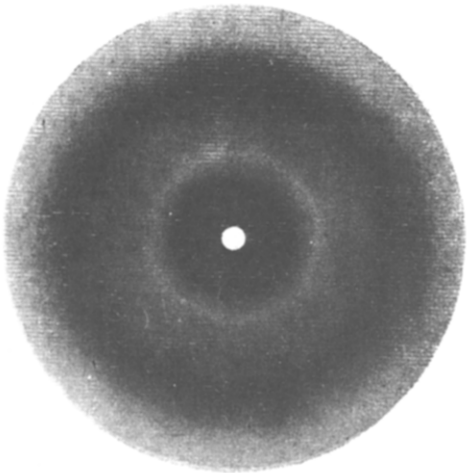


Fig. 16. X-ray powder photograph of a moist gel of myosin-B after synaeresis by addition of ATP. Cu Kα. D $\approx$ 4 cm

SUMMARY

1. The synaeresis of actomyosin in 0.05 M KCl in the presence of adenosine triphosphate (ATP) has been examined in the electron microscope and by X-ray diffraction .

2. The most reasonable interpretation of the combined results is that the primary dissociation of the actomyosin network by ATP is followed by the formation of more robust and dense fibres by a process of side-to-side aggregation, and that this effect takes place without any deep-seated change of molecular configuration. In other words, the synaeresis is an *intermolecular* drawing-together in a direction at right angles to the macroscopic change that occurs when muscle contracts. For the present, therefore, it does not seem possible from electron microscope and X-ray studies to trace any direct relation between the two phenomena.

3. The findings are discussed in connection with the theory of muscular contraction advanced by SZENT-GYÖRGYI and his school

RÉSUMÉ

1. La synérèse de l'actomyosine dans KCl 0.05 M, en présence de triphosphate d'adénosine (ATP) a été étudiée au microscope électronique et par la diffraction aux rayons X.

2. L'interprétation la plus plausible des résultats est que la dissociation initiale du réseau d'actomyosine par l'ATP est suivie de la formation de fibres résistantes et épaisses par accolement latéral, ce phénomène se faisant sans modification profonde de la structure moléculaire. Autrement dit, la synérèse est une sorte de contraction à angle droit avec la contraction macroscopique du muscle. La microscopie électronique et les études aux rayons X ne permettent donc pas actuellement de trouver de relations directes entre les deux phénomènes.

3. Les auteurs discutent ces résultats et leurs relations avec la théorie de la contraction musculaire proposée par SZENT-GYÖRGYI et ses collaborateurs.

ZUSAMMENFASSUNG

1. Die Synärese von Aktomyosin in 0.05 M KCl bei Anwesenheit von Adenosintriphosphat (ATP) wurde im Elektronenmikroskop und durch Röntgenstrahlendiffraktion untersucht.

2. Die vernünftigste Interpretation der kombinierten Resultate ist, dass nach der primären Dissoziation des Aktomyosinnetzwerks durch ATP die Bildung stärkerer und dichterer Fasern durch einen Seite-an Seite-Anlagerungsprozess erfolgt, und dass dieser Effekt ohne tiefliegende Veränderungen der Molekularkonfiguration geschieht. Mit anderen Worten: Die Synärese ist eine *intermolekulare* Zusammenziehung in einer Richtung in rechtem Winkel zur makroskopischen Veränderung, die bei der Muskelkontraktion auftritt. Im Augenblick scheint es daher nicht möglich zu sein aus Elek-

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tronenmikroskop- und Röntgenstrahldaten irgend eine direkte Beziehung zwischen beiden Erscheinungen abzuleiten.

3. Die Funde werden im Zusammenhang mit der von SZENT-GYÖRGYI und seiner Schule aufgestellten Theorie der Muskelkontraktion diskutiert.

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