Phil. Trans. R. Soc. Lond. B. **265**, 203–212 (1973) [203]
Printed in Great Britain

The assembly of ribbon-shaped structures in low ionic strength extracts obtained from vertebrate smooth muscle

By A. Sobieszek and J. V. Small
Biophysics Institute, Aarhus University, 8000 *Aarhus C, Denmark*

[Plates 20 to 22]

In actomyosin extracts from smooth muscle obtained at low ionic strength, an assembly of protein into long ribbon-shaped elements is observed to take place. These ribbons which range up to about 100 nm in width and up to many micrometres in length exhibit a strong repeat period of about 5.6 nm. Optical diffraction analysis shows that they possess a long repeat of 39.1 nm $\pm$ 0.4 nm. Tropomyosin purified from vertebrate smooth muscle can be induced to form the same ribbon-shaped elements. On removal of salt from solution the ribbons dissociate into fine filaments of average diameter about 8 nm which show subfilaments of about 2 to 3 nm diameter.

In crude preparations the ribbons occur in solution together with myosin. If such preparations are left to stand for several days, ribbons may be found that show a visible 14 nm period which appears to arise from the presence of a regular arrangement of projections.

Smooth muscle myosin alone assembles into cylindrical filaments which exhibit a regular arrangement of projections along their entire length, indicating an absence of polarity.

These results indicate, as have those recently obtained from section material, that the myosin-containing component of vertebrate smooth muscle contains a protein that forms the core of the filament, which is responsible for its ribbon-like shape and which probably determines the polarity of the attached myosin molecules. It is proposed that this protein is tropomyosin.

I. Introduction

Although the basic features of the mechanism of muscular contraction for vertebrate striated muscle are now well known (see reviews by Huxley 1969, 1971) the situation as concerns other contractile systems is far from settled. In particular, the structure of the contractile apparatus of mammalian smooth muscle is at present very controversial (see Small & Squire 1972) differences in opinion now being focused on the form of the myosin-containing filaments whose presence *in vivo* has recently been established by X-ray diffraction studies (Lowy, Poulsen & Vibert 1970).

In this laboratory, initial investigations by electron microscopy of fixed and embedded mammalian smooth muscle, coupled with X-ray 'controls' on the fixation procedure, provided evidence for the existence of myosin *in vivo* in long ribbon-shaped structures (Lowy & Small 1970). More recent studies (Small & Squire 1972) have extended this evidence for myosin containing 'ribbons' existing *in vivo* and have provided information about the molecular structure of these ribbons. Thus, it has been shown that they are made up from a ribbon-shaped core of non-myosin material which is sandwiched between regular surface lattices of myosin molecules.

In view of the problems associated with the preservation of the myosin-containing filaments by chemical fixation (see, for example, Pease 1968; Small & Squire 1972; Shoenberg 1973) experiments were also carried out to isolate the contractile elements from the cells and study them by the negative staining technique. Initial studies in this direction (Sobieszek 1972*a*) showed that long structures with a characteristic ribbon-like shape could indeed be observed in actomyosin extracts from smooth muscle obtained at low ionic strength. From the nature of

these structures (see Results) it became apparent that they were most likely composed of tropomyosin, a protein found in exceptionally large amounts in vertebrate smooth muscle (Sheng & Tsao 1955) and which is known to be present in extracts obtained at low ionic strength (Hamoir & Laszt 1962; Schirmer 1965; Rüegg, Strassner & Schirmer 1965).

In this paper we describe these structures and, in addition, we present some preliminary results which indicate that such ribbons of tropomyosin may form the 'core' of the myosin ribbons which has been identified in thin sectioned material.

2. Materials and methods

(*a*) *Fractions obtained at low ionic strength*

The muscles used were the chicken gizzard and the taenia coli and vas deferens of the guinea-pig.

Fresh muscles were efficiently fragmented at room temperature in a small volume of a 'wash solution' (w.s.: 0.06 mol/l KCl, 10^{-4} mol/l $MgCl_2$, 10^{-3} mol/l cysteine in 0.117 mol/l histidine buffer pH 6.8 to 7.0) either by crushing muscle pieces in a simple mortar-pestle-like apparatus (taenia coli and vas deferens) or by mincing in a meat grinder followed by homogenization in w.s. (chicken gizzard). The resulting suspension was washed in a large volume of w.s. and the tissue collected by centrifugation (1600 *g*, 45 min). Washing was repeated with at least two changes of w.s., and in the case of gizzard until the tissue was visibly free from blood. The washed muscle was then suspended in w.s., approximately 5 ml/g muscle (about 30 ml/g for taenia coli), containing additionally 1 to 2 mmol/l ATP, the suspension stirred for 1 to 30 min (at 2 °C or room temperature) and then centrifuged at 1600 *g* for 5 min. The supernatant so obtained, 'crude actomyosin', was then centrifuged for 12 h at 45000 *g* (2 °C) to remove most of the actin, and the supernatant used for electron microscopy. Because this latter fraction is rich in myosin (Sobieszek 1972*b*) we shall refer to it as the 'crude myosin fraction', c.m.f. The fractions obtained were stored in the cold room (about 3 °C).

(*b*) *Preparation of tropomyosin*

Tropomyosin was prepared from gizzard essentially according to the method of Bailey (1948) using the muscle residue remaining after extraction of actomyosin (see § 2*a*). The purified protein was dissolved in, and dialysed against w.s.

(*c*) *Negative staining and electron microscopy*

Negative staining was performed according to the method described by Huxley (Moore, Huxley & De Rosier 1970) using uranyl acetate and carbon-coated 400-mesh grids.

In order to define the repeat periods on the ribbons (see Results) as precisely as possible, paramyosin filaments obtained from a molluscan muscle, the anterior byssus retractor muscle of *Mytilus edulis* (a.b.r.m.), were used as a standard. These filaments, prepared in a manner similar to that described by Szent-Györgyi, Cohen & Kendrick-Jones (1971) were suspended in w.s. and added to the ribbons on the same grid. Micrographs were then taken, at a primary magnification of 40000 for which images of paramyosin filaments and ribbons could be recorded on the same plate (figure 3 *m*, *r* and *s*). Negative masks were prepared from these micrographs and the periods on adjacent paramyosin filaments and ribbons compared using an optical diffractometer (Klug & Berger 1964).

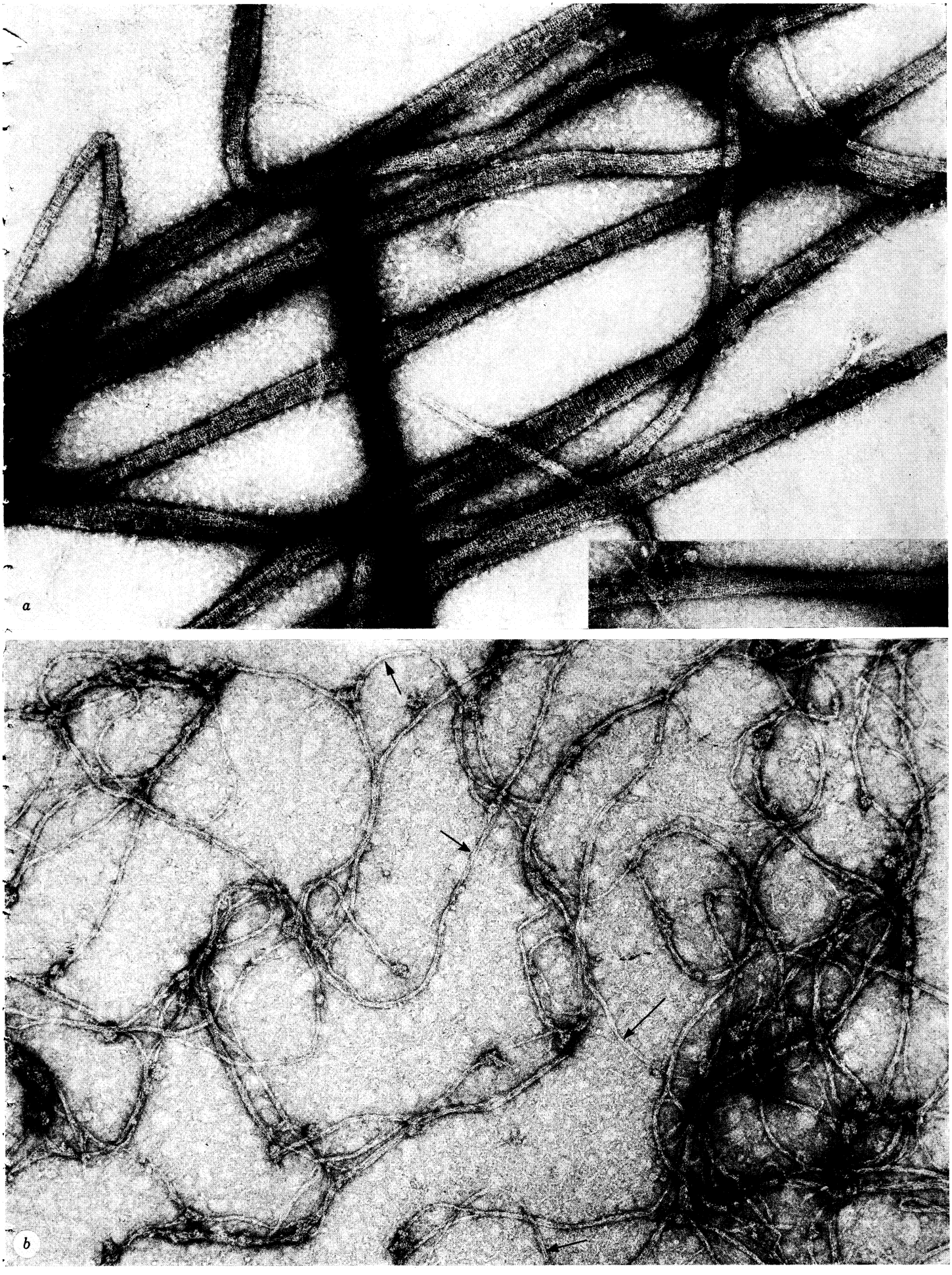

FIGURE 1. (*a*) Partially purified ribbons obtained from chicken gizzard (see Methods). Inset: micrograph illustrating the ribbon-like shape of these structures. (Magnifications of both, × 90000.) (*b*) Preparation obtained by dialysing ribbons such as those shown in (*a*) against distilled water. The ribbons have dissociated into fine filaments (see text). At the arrows subfilaments can be seen. (Magn. × 110000.)

(*Facing p.* 204)

FIGURE 2. Long ribbon-shaped structures observed in preparations obtained from different muscles. (*a*) to (*f*), chicken gizzard, c.m.f. with added 50 mmol/l $CaCl_2$; (*g*) to (*i*), vas deferens, c.m.f.; (*j*), (*k*), taenia coli, c.m.f. (Magnification range from $\times 80000$ to $\times 150000$; finest period 5.6 nm.)

Electron micrographs were taken on a Siemens Elmiskop 101 operating at 80 kV with a 50 μm objective aperture.

3. Results

(*a*) *Ribbon-shaped structures and tropomyosin*

As might be expected from the centrifugation conditions, no large filamentous structures are observed in the c.m.f. directly following centrifugation. However, interesting changes occur when this fraction is left to stand in the cold for one or more days. Thus, a dramatic assembly of proteins into primarily two distinct types of highly ordered filamentous structures is seen to take place.

The first type of filamentous aggregate occurs as long, ribbon-shaped structures (figure 1*a*) which commonly form an extensive interlacing network, but which also occur as individual filaments of many micrometres in length (figure 2). These ribbons are often very wide, ranging up to about 100 nm in width. Their ribbon-like shape may be inferred from their general staining properties, especially when they are wide, and also from images such as shown in figure 1*a* (inset) which are consistent with a ribbon twisting about its long axis through 180°. A characteristic feature of these ribbons is the presence of a prominent transverse striation of period about 5.6 nm (figures 1*a* and 2). Sometimes this period is not seen and the ribbons exhibit a very smooth appearance which may be attributed either to a certain degree of molecular disorder or to inadequate staining.

By the addition of $CaCl_2$ (50 mmol/l) to the c.m.f. directly following centrifugation the ribbons may be induced to form more quickly, within 1 to 3 h. Under these conditions the myosin and any remaining actin is precipitated and may be removed from solution by centrifugation (25000*g*, 30 min). The ribbons may then be partially purified by precipitation with ethanol (7 to 12%) and suspension in w.s. or w.s. containing a higher concentration of KCl. In this respect we have found that KCl concentrations of up to 0.4 mol/l do not seem to affect the dimensions of the ribbons, although under all conditions in this buffer a large amount of the material is evidently in a relatively disaggregated state, as indicated by the presence of a fibrillar background on the carbon film.

Optical diffraction patterns obtained from the ribbons showed the presence of several meridional reflexions (figure 3*l* and *o*). By comparison with paramyosin filaments (see § 2*c*) the spacings of the prominent reflexions had values 4.9, 5.6, 6.5 and 13.0 nm and could be indexed on a long repeat of about 40 nm (39.1 nm ± 0.4 nm).

The presence of a repeat period on the ribbons of about 40 nm and also the fact that they were soluble in water suggested that they were composed of tropomyosin (see Discussion). This was confirmed by the observation that tropomyosin extracted and purified by the conventional procedure (see § 2*b*) could be induced to assemble into the same ribbon-shaped structures (figure 3 *r*, *s*, *x* and *y*) as well as thicker structures exhibiting the same striation pattern (figure 3 *v*, *w* and *z*). This could be achieved by dialysis of a solution of tropomyosin in w.s. against the same solution containing 50 mmol/l $CaCl_2$. Optical diffraction patterns from these structures showed the same meridional reflexions (figure 3 *q* and *t*) as observed for the ribbons but with a slightly different intensity distribution. The reason for these differences in the intensities of the reflexions have yet to be established.

(*b*) *Myosin filaments*

A second filament type (Sobieszek 1972*b*) apart from the ribbons is observed to form in the crude myosin fraction. This occurs as relatively short, tapered filaments which bear a regular arrangement of projections along their entire length and which give rise to an axial repeat period of about 14 nm (figure 3*d* to *h*). Experiments in which these filaments have been purified from the crude myosin fraction have shown that they are composed predominantly, if not entirely of myosin and that under suitable conditions they may be induced to grow up to several micrometres in length (figure 3*d* and *e*). The striking feature of these filaments is that they do not appear to possess bipolarity, that is there is no central 'bare zone' (Huxley 1963).

(*c*) *Other observations on the ribbons*

Two further observations have been made which have given an indication of the relation of the ribbon structures described above to the contractile elements of the smooth muscle cell (Small & Squire 1972).

If a suspension of ribbons in w.s. is dialysed against distilled water the ribbons dissociate into finer filaments (figure 1*b*) which exhibit subfilaments of about 2 to 3 nm diameter. In such preparations filaments of about 8 nm in width are commonly observed and these may be seen to consist of two parallel subfilaments (figure 1*b*). Such latter filaments exhibit the same dimension and subfilaments as demonstrated for the intermediate sized 'lentofilaments' (Small & Squire 1972) observed in sections of smooth muscle and which are derived from a dissociation of the core of the myosin ribbons.

Description of plate 22

FIGURE 3. (*a*), (*c*) Ribbons observed in c.m.f. from chicken gizzard after about 1 to 2 weeks of standing at 3 °C, and which show a repeat along part of their length of about 14 nm (see also (*j*) and (*k*)).

(*b*) Enlargement from (*a*) showing fine 5.6 nm periodicity at end of ribbon. (Magnifications: (*a*) × 80000; (*b*) and (*c*), × 108000.)

(*d*) to (*h*) Self-assembled filaments of smooth muscle myosin. Note constant 14 nm periodicity (see (*i*)) arising from the regular arrangement of projections and which may be observed along the entire length of the filaments.

(*d*), (*e*) Myosin filaments formed from purified chicken gizzard myosin (Sobieszek & Small 1972). (Magnifications: (*d*) × 69000; (*e*) × 61000.)

(*f*) to (*h*) Myosin filaments observed in c.m.f. obtained from vas deferens. (Magnification of each, × 90000.)

(*i*) Optical diffraction pattern from myosin filament shown in (*h*). Reflexions occur at 14.4 and 7.2 nm.

(*j*), (*k*) Optical diffraction patterns from ribbons shown in (*a*) and (*c*) respectively. Both show a reflexion at 14.4 nm. Pattern from (*c*) also shows a reflexion of 13.0 nm, characteristic of the tropomyosin ribbons (see (*l*), (*o*), (*q*) and (*t*)).

(*l*) Optical diffraction pattern from ribbon shown in figure 2(*k*).

(*m*) to (*u*) Calibration of the spacing exhibited by the ribbons using the constant 14.5 nm period of paramyosin filaments as a standard (see Methods). Chicken gizzard.

(*n*), (*o*) Optical diffraction patterns from the paramyosin filament and the ribbon shown in (*m*), respectively.

(*p*), (*q*) Patterns obtained from filaments shown in (*r*).

(*t*), (*u*) Patterns obtained from filaments shown in (*s*).

(*m*) Ribbon (right) from partially purified preparation (see text § 3*a*) together with a paramyosin filament (left).

(*r*), (*s*) Ribbons formed from purified chicken gizzard tropomyosin (on right side of micrographs) together with paramyosin filaments (on left side of micrographs). (Magnifications of (*m*), (*r*) and (*s*), × 88000.)

(*v*) to (*z*) Aggregates formed from purified chicken gizzard tropomyosin in the presence of calcium 50 mmol/l) and in w.s. (Magnifications: (*v*), (*w*), (*z*) × 71000; (*x*) × 110000; (*y*) × 95000.)

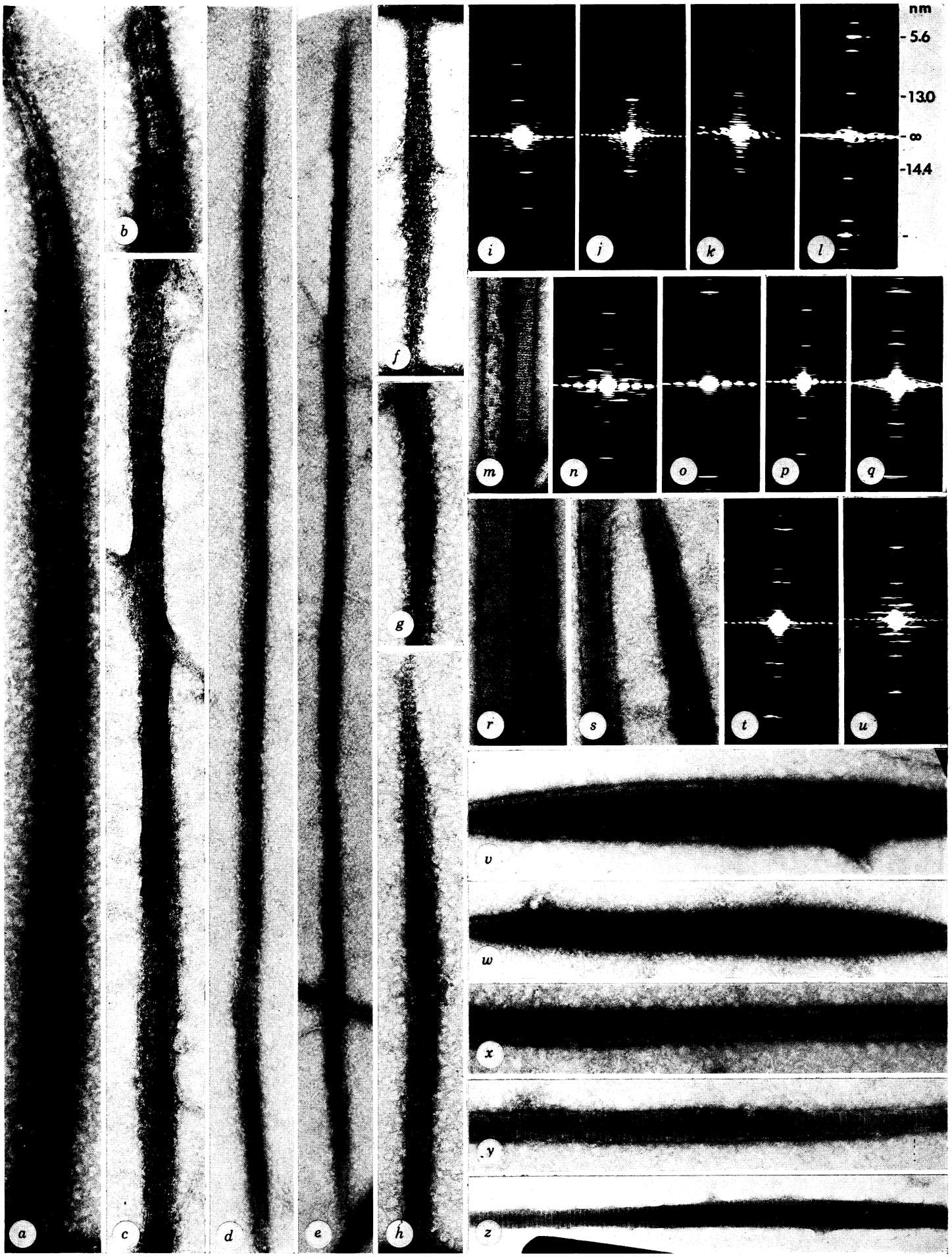

FIGURE 3. For legend see facing page.

Facing p. 206)

Secondly, on investigation of the c.m.f. after a week or more of standing in the cold it was found that many ribbons invariably exhibited a rough, irregular surface which effectively obscured the fine, 5.6 nm periodicity. This was apparently due to the addition of extra material on the ribbon surfaces. In some instances a regular period of about 14 nm was observed on these ribbons (figure 3*a* and *c*), the same period as detected on the myosin ribbons observed in thin sections (see Discussion), and this appeared to arise from this extra material. On some parts of the same ribbons the fine 5.6 nm period could also be observed (figure 3*b*). In thin sections of smooth muscle a period of about 14 nm has been identified on the surface of the myosin ribbons (Small & Squire 1972) and was shown to arise from a regular arrangement of projections, or cross-bridges, which were presumed to represent the heads of myosin molecules. In consequence, the most likely interpretation of the present results is that the 14 nm repeat (figure 3*a* and *c*) arises from an orderly association of myosin molecules with the tropomyosin ribbons. This interpretation is reinforced from optical diffraction patterns from these apparently composite structures (figure 3*j* and *k*) which have shown not only a reflexion at about 14 nm, characteristic of myosin, but also one at 13 nm characteristic of the tropomyosin ribbons (figure 3*k*).

4. Discussion

Previous attempts (Shoenberg 1965, 1969; Nonomura 1968; Panner & Honig 1967; Kelly & Rice 1968) to isolate the contractile elements from vertebrate smooth muscle have all failed to show the presence of relatively large structures that could be presumed to contain myosin. Instead, at most only very short filaments have been seen of similar appearance to those that may be synthesized from purified smooth muscle myosin (Kaminer 1969). In view of the high solubility of smooth muscle actomyosin at low ionic strength (see review by Needham & Shoenberg 1968) these results were perhaps not surprising. In the same way we have also been unable to satisfactorily isolate large myosin-containing structures directly from vertebrate smooth muscle. We have observed instead, the assembly of structural proteins into highly ordered filamentous structures whose relation to the *in vivo* contractile elements may only be inferred with reference to other data.

From the investigations of ultrathin sections of vertebrate smooth muscle it has been deduced that myosin exists *in vivo* in long, ribbon-shaped structures and that these structures possess a ribbon-shaped core of non-myosin material (see Introduction). It was therefore of considerable interest to find that a protein in the smooth muscle cell could assemble into structures of a form closely similar to the ribbons seen in sections.

It is clear that the ribbons we present here are composed of smooth muscle tropomyosin. They exhibit the repeat periodicity of about 40 nm characteristic of tropomyosin from various sources, including smooth muscle (Tsao *et al.* 1965; Cohen & Longley 1966; Caspar, Cohen & Longley 1969; Millward & Woods 1970), they are water-soluble and they are identical in appearance to structures that may be formed from purified tropomyosin preparations from gizzard.

In view of the compelling evidence from experiments on striated muscle that tropomyosin is associated with the actin filaments and forms part of the relaxing protein system (see review by Ebashi & Endo 1968; Hartshorne & Mueller 1967; Hartshorne, Theiner & Mueller 1969; Schaub & Perry 1969; Spudich & Watt 1971) it may appear extravagant to claim an association of this protein with myosin in smooth muscle. However, we have very few data at present

on the relaxing protein system of vertebrate smooth muscle (Carsten 1968, 1971; Ebashi *et al.* 1966) and in the context of recent studies on molluscan muscles (Kendrick-Jones, Lehman & Szent-Györgyi 1970) the type and specific location of these proteins in smooth muscle, either to actin or to myosin must await further investigations (see also below).

In any case the amount of tropomyosin in vertebrate smooth muscle (Sheng & Tsao 1955) is extremely high in relation to the actomyosin content, present estimates on different muscles indicating a factor of 4 to 40 times as much tropomyosin in relation to actomyosin as compared to rabbit skeletal muscle (see review by Rüegg 1971). It is further interesting to note (Rüegg 1971) that the ratio of tropomyosin to actomyosin in gizzard muscle is comparable to the ratio of paramyosin to actomyosin in molluscan muscles in which the paramyosin forms the core of the myosin filaments (Hanson & Lowy 1961, 1964; Szent-Györgyi *et al.* 1971).

The previous estimate (Small & Squire 1972) that the increased amount of actin in relation to myosin in vertebrate smooth muscle could account for the larger amount of tropomyosin (if complexed to actin) in this tissue were based on tropomyosin yields which were apparently rather low. We therefore consider that the amount of tropomyosin is in excess of the quantity which could hypothetically be complexed with the actin filaments. From the results of Sparrow, Maxwell, Rüegg & Bohr (1970), in fact, it would seem that smooth muscle tropomyosin is not a primary requirement for Ca^{2+} sensitivity since they have purified smooth muscle actomyosin which is Ca^{2+} sensitive under conditions for which the bulk of the tropomyosin is apparently discarded at an early stage of purification. This result may simply reflect the presence of two (or more) tropomyosin-like proteins, one that is associated with the myosin and the other with the actin component, a possibility that will require further investigation.

The existence of a backbone structure in the filaments of vertebrate smooth muscles is independently suggested by the observation on the nature of assembly of purified smooth muscle myosin. The cylindrical filaments formed from this myosin do not appear to possess any polarity. Projections, or cross-bridges, arranged in a regular manner may be detected along the entire filament length so that there is no central 'bare zone'. Thus, rather than being formed from the parallel association of molecules in each half of the filament and an antiparallel association at the centre (Huxley 1963) these smooth muscle myosin filaments appear to be made up from the assembly of antiparallel myosin dimers (Sobieszek 1972*b*). The existence of a core component in the *in vivo* myosin structures would therefore appear necessary to determine the polarity of the myosin in the contractile element, in the same way that the polarity of myosin molecules on the surface of paramyosin filaments of molluscs is probably determined by the underlying polarity of the paramyosin core (Szent-Györgyi *et al.* 1971).

From longitudinal sections of vertebrate smooth muscle, evidence has been obtained which indicates that the myosin ribbons inside the cells have 'face polarity' (Small & Squire 1972), i.e. molecules on opposite faces are polarized in opposite directions. If the core of the filament is to determine this polarity (see also Small & Squire 1972) then this would require a core with a reversed polarity of molecules on one face with respect to the other. Since smooth muscle tropomyosin may form either polar, or bipolar aggregates (Millward & Woods 1970; Cohen & Longley 1966) we may presume that such an arrangement is possible for the tropomyosin ribbons. In the earlier study (Small & Squire 1972) we attributed a repeat of 43 nm to the core of the ribbons which differs from the 40 nm repeat found in the present study for the ribbons of tropomyosin. The reflexions deduced as arising from the ribbon backbone in diffraction patterns obtained from section material are, however, generally rather weak. In

consequence, the number of patterns in which these reflexions were seen were relatively small and the apparent difference in spacings may therefore not be significant.

From the similarity in size and the abundance of the tropomyosin ribbons described here to those observed in section material it was immediately tempting to conclude that the two structures were identical. Although by no means conclusive, the evidence thus far obtained suggests that this is the case.

The investigation of thin sections of vertebrate smooth muscle has shown that the disaggregation of the myosin ribbons gives rise to small, regularly sized filaments which are evidently not myosin (Small & Squire 1972) and which appear to make up the backbone or core of the ribbons. These small filaments, which have been termed '100 Å filaments' (Uehara, Campbell & Burnstock 1971) are square in cross-section, about 7.5 nm across and are composed of four subfilaments each of about 3.5 nm diameter (Small & Squire 1972). The dissociation of the tropomyosin ribbons described here into fine filaments of similar dimensions to those observed in sections is consistent with this protein forming the core component of the myosin ribbons.

The further observation of ribbons showing a repeat of about 14 nm, the same repeat as previously identified in thin section material, and characteristic of myosin, is extremely suggestive of an orderly interaction of myosin molecules with the ribbons of tropomyosin. Such two component structures have, as yet only been identified in aged preparations and the number on which we have recognized some degree of order are so far rather few. However, the detection in optical diffraction patterns of reflexions at both 13 and 14 nm from the same ribbon indicate very strongly that such an orderly interaction can take place. The conditions under which the interaction can be controlled have yet to be determined.

In conclusion, the finding of ribbon-shaped structures in extracts from smooth muscle which appear to be capable of interaction with myosin adds further weight to the suggestion that myosin-containing ribbon-shaped elements exist *in vivo* in vertebrate smooth muscle. Our further work will be directed towards further elucidating the relation of the ribbons described here to the *in vivo* filaments and defining their structural details more precisely.

We are grateful to Dr S. Magnusson for valuable discussions during the course of this study. We thank Mrs L. Nychel for valuable technical assistance and Mr P. Boldsen and Mrs I. Lunde for their help with photography. The Siemens 101 electron microscope was purchased from a grant from the Danish Statens Naturvidenskabelige Forskningsråd (S.N.F.). One of us (A.S.) was supported during the early part of this study by a grant from the Danish Statens Lægevidenskabelige Forskningsråd (grant number J. 512 to 674). We also acknowledge the generosity of Nordjysk Andels-Fjerkræslagteri in Randers for a constant supply of chicken gizzards.

References (Sobieszek & Small)

Bailey, K. 1948 *Biochem. J.* **43**, 271–279.
Carsten, M. E. 1968 *Biochemistry* **7**, 960–967.
Caspar, D. L. D., Cohen, D. & Longley, W. 1969 *J. molec. Biol.* **41**, 87–107.
Cohen, C. & Longley, W. 1966 *Science, N.Y.* **152**, 794–796.
Ebashi, S. & Endo, M. 1968 *Prog. biophys. molec. Biol.* **18**, 123–185.
Ebashi, S., Iwakura, H., Nakajima, H., Nakamura, R. & Ooi, Y. 1966 *Biochem. Z.* **345**, 201–211.
Hamoir, G. & Laszt, L. 1962 *Nature, Lond.* **193**, 682–684.
Hanson, J. & Lowy, J. 1961 *Proc. R. Soc. Lond.* B **154**, 173–196.

Hanson, J. & Lowy, J. 1964 In *Biochemistry of muscle contraction* (ed. J. Gergely), p. 400–409. Boston: Little, Brown.
Hartshorne, D. J. & Mueller, H. 1967 *J. biol. Chem.* **242**, 3089–3092.
Hartshorne, D. J., Theiner, M. & Mueller, H. 1969 *Biochim. biophys. Acta* **175**, 320–330.
Huxley, H. E. 1963 *J. molec. Biol.* **7**, 281–308.
Huxley, H. E. 1969 *Science, N.Y.* **164**, 1356–1366.
Huxley, H. E. 1971 *Proc. R. Soc. Lond.* B **178**, 131–149.
Kaminer, B. 1969 *J. molec. Biol.* **39**, 257–264.
Kelly, R. V. & Rice, R. V. 1968 *J. Cell. Biol.* **37**, 105–130.
Kendrick-Jones, J., Lehman, W. & Szent-Györgyi, A. G. 1970 *J. molec. Biol.* **54**, 313–326.
Klug, A. & Berger, J. E. 1964 *J. molec. Biol.* **10**, 565–569.
Lowy, J., Poulsen, F. R. & Vibert, P. J. 1970 *Nature, Lond.* **225**, 1053.
Lowy, J. & Small, J. V. 1970 *Nature, Lond.* **227**, 46–51.
Millward, G. R. & Woods, E. F. 1970 *J. molec. Biol.* **52**, 585–588.
Moore, P. B., Huxley, H. E. & DeRosier, D. J. 1970 *J. molec. Biol.* **50**, 279–295.
Needham, D. M. & Shoenberg, C. F. 1968 In *Handbook of physiology* (ed. C. F. Code & W. Heidel), § 6, vol. 4, 1793–1810. Washington: American Physiological Society.
Nonomura, Y. 1968 *J. Cell. Biol.* **39**, 741–745.
Panner, B. J. & Honig, C. R. 1967 *J. Cell. Biol.* **35**, 303–321.
Pease, D. C. 1968 *J. ultrastruct. Res.* **23**, 280–303.
Rüegg, J. C. 1971 *Physiol. Rev.* **51**, 201–248.
Rüegg, J. C., Strassner, E. & Schirmer, R. H. 1965 *Biochem. Z.* **343**, 70–85.
Schaub, M. C. & Perry, S. V. 1969 *Biochem. J.* **115**, 993–1004.
Schirmer, R. H. 1965 *Biochem. Z.* **343**, 269–282.
Sheng, P. K. & Tsao, T. C. 1955 *Sci. sinica (Peking)* **4**, 157–175.
Shoenberg, C. F. 1965 *Nature, Lond.* **206**, 526.
Shoenberg, C. F. 1969 *Tissue & Cell* **1**, 83–96.
Shoenberg, C. F. 1972 *Phil. Trans. R. Soc. Lond.* **265**, 197–202 (this volume).
Small, J. V. & Squire, J. M. 1972 *J. molec. Biol.* **67**, 117–149.
Sobieszek, A. 1972*a* *J. ultrastruct. Res.* **38**, 208.
Sobieszek, A. 1972*b* *J. molec. Biol.* **70**, 741–744.
Sobieszek, A. & Small, J. V. 1972 Cold Spring Harb. Symp. on Quant. Biol. **37** (in the Press).
Sparrow, M. P., Maxwell, L. C., Rüegg, J. C. & Bohr, D. F. 1970 *Am. J. Physiol.* **219**, 1366–1372.
Spudich, J. A. & Watt, S. 1971 *J. biol. Chem.* **246**, 4866–4871.
Szent-Györgyi, A. G., Cohen, C. & Kendrick-Jones, J. 1971 *J. molec. Biol.* **56**, 239–258.
Tsao, T. C., Kung, T. H., Peng, C. M., Chang, Y. S. & Tsou, Y. S. 1965 *Sci. sinica (Peking)* **14**, 91–105.
Uehara, Y., Cambell, G. R. & Burnstock, G. 1971 *J. Cell Biol.* **50**, 484–497.

Discussion

A. P. Somlyo: The conclusion of Sobieszek & Small that the intermediate 10 nm filaments are composed of tropomyosin and are the backbone of the organized form of myosin in vertebrate smooth muscle is in serious conflict with the known and very different solubilities of the intermediate filaments and of the tropomyosin of smooth muscle. The tropomyosin preparation described by Dr Small here is a low ionic strength extract, whereas the intermediate filaments are among the most insoluble components of smooth muscle and remain intact even after high ionic strength extraction of the soluble myofibrillar proteins (Cooke & Chase (1971) *Expl Cell Res.* **66**, 417; Rice (1972) *Abstr. Cold Spring Harbor Symposium*). Also, the clearly circular, 10 nm diameter profiles of the intermediate filaments cannot possibly be subunits of dispersed thick filaments (15.5 nm in diameter and composed of smaller and more irregular subunits) that we consider to be the organized form of myosin in vertebrate smooth muscle, nor are they present in normal smooth muscle in numbers sufficient to account for this postulated role as a backbone of the organized myosin. In view of the great length (several micrometers) of the bundles of intermediate filaments, I would not rule out the possibility that some very long ribbons, interpreted by Drs Small & Squire and Professor Lowy to be organized myosin, may in fact be

longitudinal profiles of aggregated bundles of intermediate, rather than thick (myosin), filaments.

The presence of large groupings of very well organized actin filaments surrounded by ribbons in the micrographs of Dr Small raises another perplexing question. Certainly, many thin filaments in the centre of such groupings are completely isolated from any possible interaction with the ribbons, considered by Small, Lowy and Squire as the organized form of myosin. It is rather difficult to see the function of such thin filaments, isolated from myosin.

There are two further serious consequences of the model proposed by Professor Lowy, Dr Small and their co-workers. The first of these is that, in view of the greatly varying width and length of their ribbons, their model assumes that in vertebrate smooth muscle there is no length and width determining mechanism to regulate the shape and size of organized myosin. The second problem concerns the polarity of actin filaments implied in the ribbon model that assumes different polarities on the two faces of the ribbon and the absence of a possible ordering structure (dense body) analogous to the Z-line. According to this view, actin filaments throughout their entire length should have the same polarity on one side of the ribbon while having an opposite polarity to that of the actin filaments on the opposite side of the ribbon. This assumption implies a genetic control mechanism of determining thin filament polarity that would have to be rather different from that found in other muscles.

The preparatory techniques used by Dr Small and his associates, often including incubations at low temperatures for several hours and the use of hypertonic fixatives, are such that they might favour rather abnormal myosin–myosin and actin–actin interactions. We have reproduced the ribbons whenever aggregation due to cell shrinkage could be predicted from the osmometric properties of smooth muscle.

The X-ray pattern, as also indicated by Professor Lowy, could be produced by either the ribbon model proposed by his group, or by an alinement of two or more thick filaments in at least one lateral direction as suggested by us. The possibility of such alinement would be favoured if the amorphous gray zone, seen in our electron micrographs between three to four laterally alined, adjoining thick filaments, was in fact analogous to the M-substance.

J. V. Small: In reply to Professor Somlyo's remarks I shall be rather brief since a full discussion of virtually all the points he has raised may be found in a paper which is to be published very shortly (Small, J. V. & Squire, J. M. 1972 *J. molec. Biol.* **67**, 117).

As regards the nature of the '100 Å filaments' of vertebrate smooth muscle we should not forget that Dr Sobieszek and myself have shown that the ribbons observed in low ionic strength extracts obtained from smooth muscle may dissociate into finer filaments of various diameters, including some of about 10 nm diameter, and all of which appear to possess subfilaments of about the same dimension (2 to 3 nm diameter) as the subfilaments of the '100 Å filaments' that have been observed in section material. Furthermore, the fact that the material (tropomyosin) that makes up both the ribbons and these fine filaments is readily extracted together with actomyosin is consistent with the conclusion that it forms an integral part of the contractile apparatus. Whether the '100 Å filaments' found by us and those described by Drs Cooke and Chase and by Professor Rice are the same filaments remains to be established. We should only note here that if they are the same filaments then the apparent differences in solubility properties may simply result from the differences between the preparation procedures.

A. P. Somlyo: The paper by Small & Squire, referring to by Dr Small, claims that our

interpretation of the width of the 14.3 nm meridional X-ray reflexion being due to regular arrangement of the thick filaments 'has been shown to be erroneous'. The X-ray evidence presented by Professor Lowy at these meetings does not show this to be the case. The regularity of the distribution of thick filaments seen in our micrographs has not been shown to be absent from living muscle. Therefore, as pointed out to me by Dr K. Blasie (University of Pennsylvania), the possibility remains that the narrowing of the meridional reflexion in the equatorial direction is due to an interference function generated by the not strictly random arrangement of the diffracting units.

J. Lowy: As regards the X-ray evidence it might be as well to emphasize that at low temperatures or in hypertonic solutions, that is under conditions where both groups of investigators agree that myosin is present in the form of ribbon-like elements, the shape of the 14.3 nm reflexion is much the same as under conditions where Professor Somlyo and his colleagues claim that only round myosin filaments are present. This means that if the latter really exist, the lateral register of their 14.3 nm periodicity must be maintained by some structure equivalent to an M-line. We point out that such a structure is very likely to be distorted during the assumed aggregation of round filaments into ribbons and that this would upset the lateral register of the 14.3 nm periodicity and hence lead to a broadening of the 14.3 nm reflexion. However, from our studies there is no evidence for such a shape change and this supports our contention that the equatorial breadth of the 14.3 nm reflexion can be most straightforwardly interpreted in terms of the ribbon structure.

A. P. Somlyo: Whether significant distortion of the ordering structure occurs during shrinkage remains to be shown. The alinement of the cross bridges on the ribbon face, seen in Dr Small's electron micrographs, might be interpreted to indicate that such disordering does not occur. Furthermore, equatorial narrowing may occur even without precise lateral register of the 14.3 nm periodicity (see reply to Dr Small).

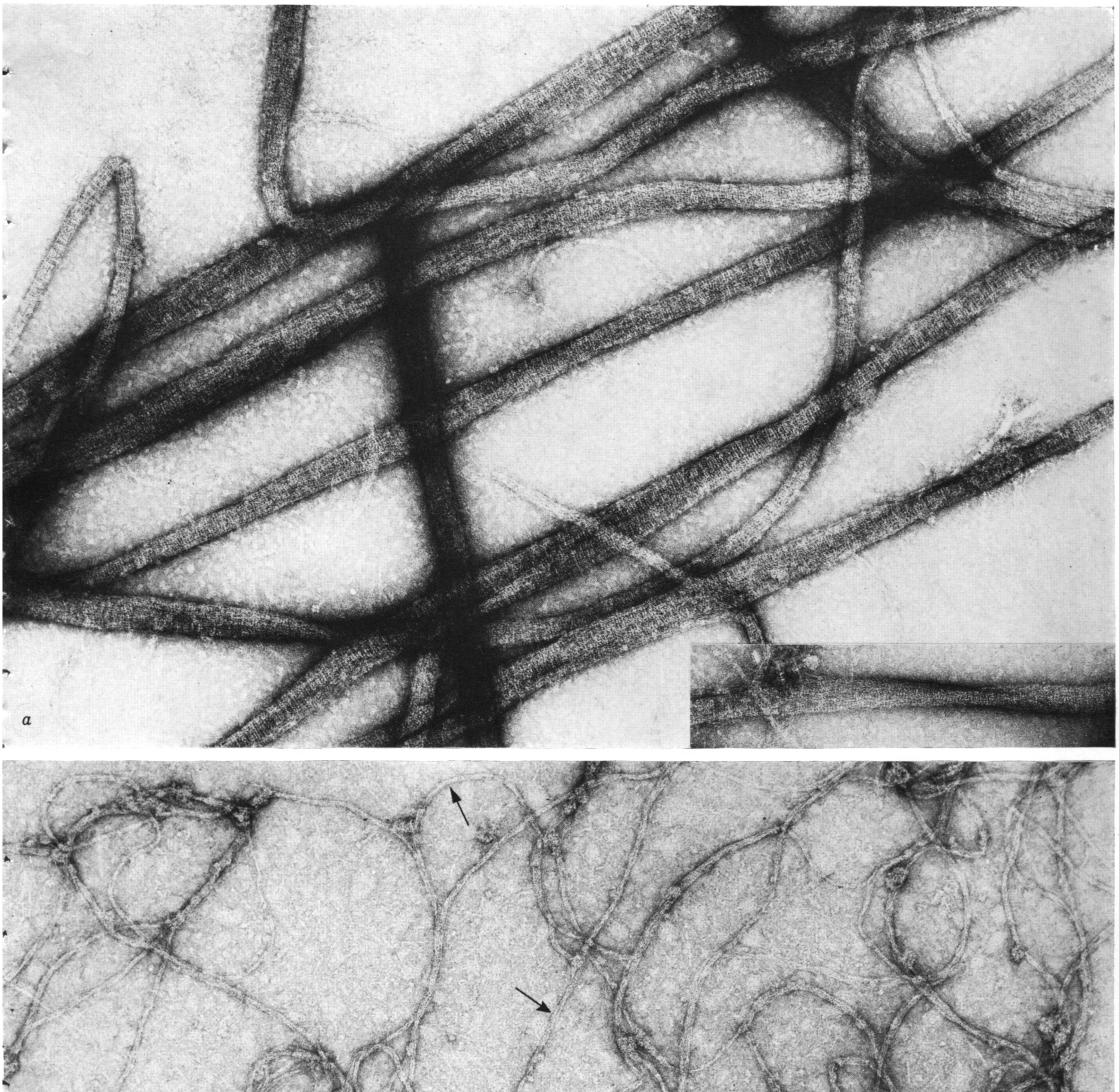

FIGURE 1. (*a*) Partially purified ribbons obtained from chicken gizzard (see Methods). Inset: micrograph illustrating the ribbon-like shape of these structures. (Magnifications of both, × 90000.) (*b*) Preparation obtained by dialysing ribbons such as those shown in (*a*) against distilled water. The ribbons have dissociated into fine filaments (see text). At the arrows subfilaments can be seen. (Magn. × 110000.)

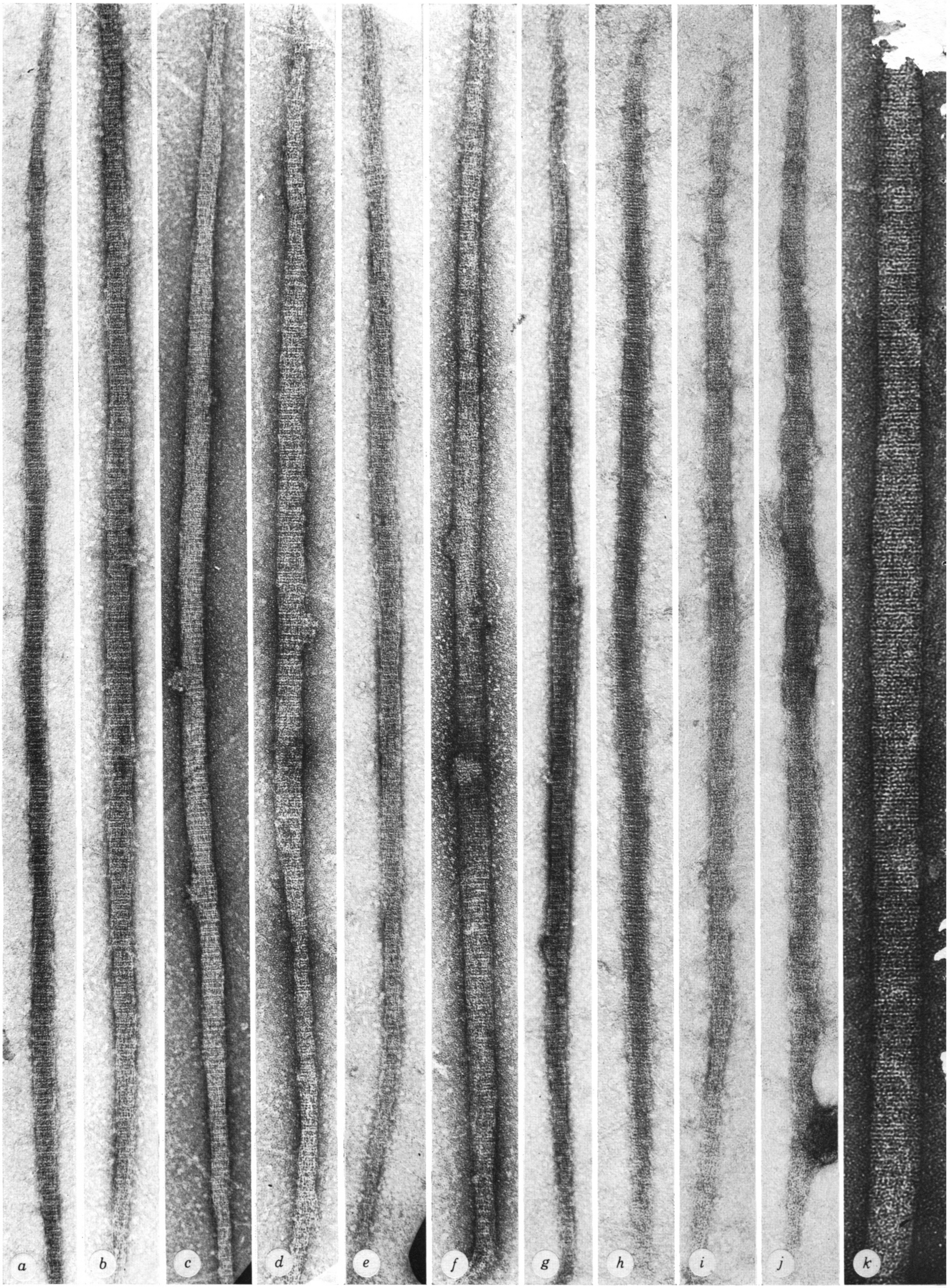

FIGURE 2. Long ribbon-shaped structures observed in preparations obtained from different muscles. (*a*) to (*f*), chicken gizzard, c.m.f. with added 50 mmol/l $CaCl_2$; (*g*) to (*i*), vas deferens, c.m.f.; (*j*), (*k*), taenia coli, c.m.f. (Magnification range from $\times 80000$ to $\times 150000$; finest period 5.6 nm.)

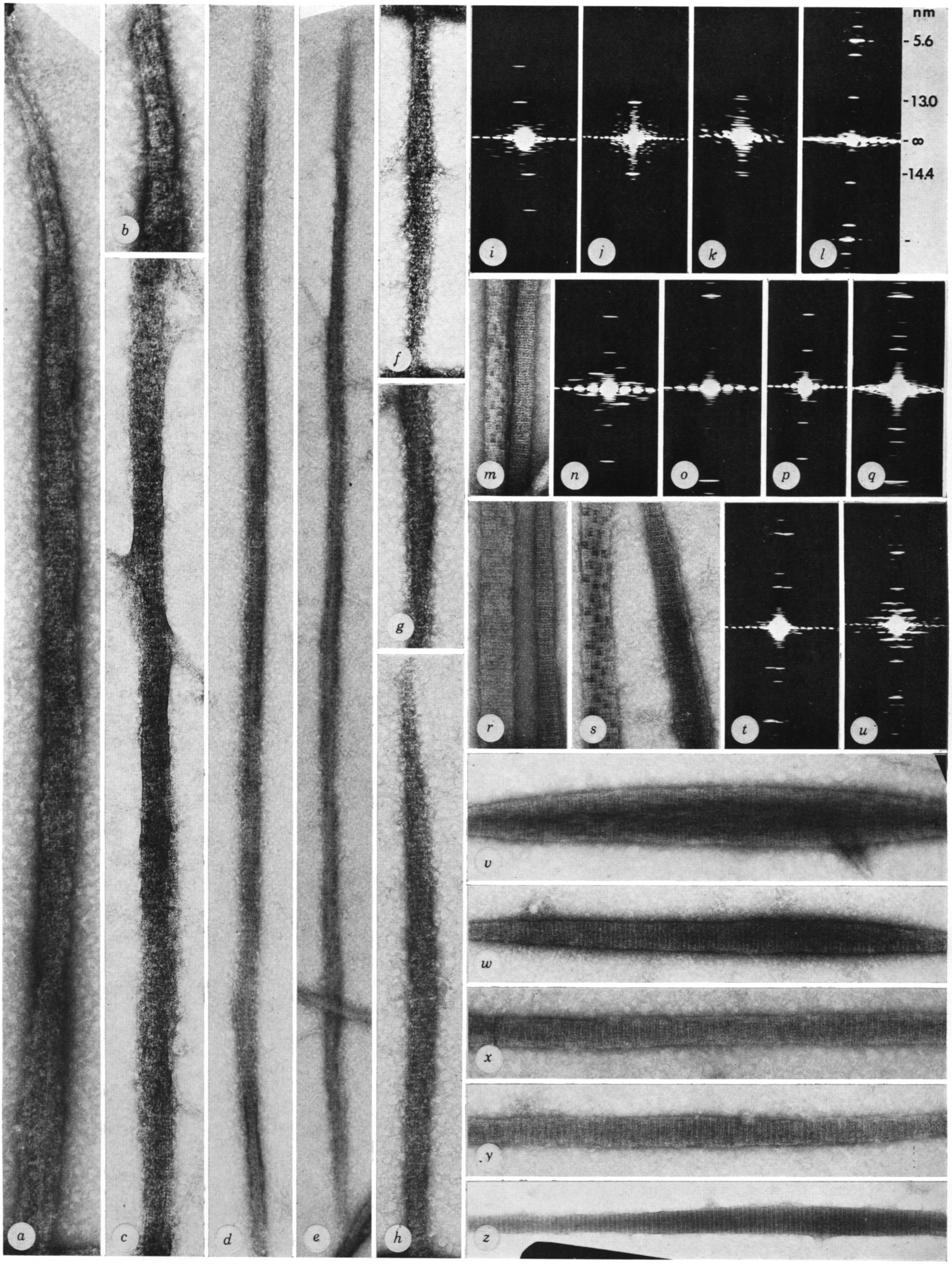

FIGURE 3. For legend see facing page.