A STUDY ON THE LOCALIZATION OF CONTRACTILE PROTEINS IN THE MUSCLE OF THE HORSESHOE CRAB (*LIMULUS POLYPHEMUS*)* **

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SUMMARY

When the glycerinated, blended and washed fibrils of the cross-striated skeletal muscle of *Limulus polyphemus* are extracted with solutions of high KCl concentrations containing pyrophosphate or ATP the A bands are removed. The protein removed from the fibrils (approximately 38% from the washed or 12% from the glycerinated unwashed fibril) consists predominately of actomyosin. This is in contrast to the results of similar treatment of rabbit fibrils in which myosin with traces of actin is extracted when the A bands are removed.

INTRODUCTION

The present revival of interest in the correlation of structure and function focuses on the molecular rather than tissue or organ level. In muscle, where considerable is known about the biochemical mechanisms of contraction, an attempt has been made to establish the precise location of the contractile proteins within the myofibrils of striated muscle (see reviews[1,2]). The methods instituted by HASSELBACH[3] and refined by HUXLEY AND HANSON[4-7] and other[8-10] have made it possible to now state, with reasonable assurance, that mammalian and bird myosin is localized in the A (anisotropic) band of skeletal, cross-striated muscle. On the basis of this localization a rather interesting concept of contraction has been developed[5,11]; that is, the myosin of the A band pulls in further, by a "slip and catch" mechanism, the smaller filaments of the I region which already extend into the A band[2,12].

In the preoccupation with the "warm-blooded" striated skeletal muscle other types of muscle, with presumably a similar contraction mechanism, have been largely overlooked. Although frog skeletal muscle exhibits much the same fine structure as its warm-blooded vertebrate counterparts[11,12] the localization of its contractile proteins is unknown. The muscle of insects and other arthropods reveals a banded pattern similar to that of vertebrate muscle[13,14,18] although other studies[15-17] seem to indicate that it lacks an I band. Attempts to localize the contractile proteins in this muscle have not been successful[15,18].

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Another arthropod, *Limulus polyphemus*, whose muscle is superficially similar to vertebrate striated muscle[19], has sufficient muscle, arranged in more or less parallel bundles, so that it can be glycerinated and treated in a manner comparable to the psoas muscle of rabbit[20,21]. We have confirmed SARKAR's findings of the contraction of glycerinated preparations of *Limulus* muscle and have further found that the A band of such preparations can be easily removed. It is thus possible to compare directly a cold-blooded, striated, invertebrate muscle with the classical warm-blooded, striated, vertebrate muscle which has been used so successfully[21].

MATERIALS AND METHODS

Limulus polyphemus were killed by cutting off all the legs with tin snips and allowing the animal to bleed. Muscle for the experiments here described was obtained from the dorsal and ventral sets of muscles joining the cephalothorax to the abdominal exoskeleton. The exoskeleton in this region was cut away and the muscles exposed. Muscles, still attached at both ends to the exoskeleton, were separated into small bundles and tied to wooden splints at rest length. Both ends, after tying, were cut and the muscle, with its splint, was placed in ice cold 50 % aqueous glycerol[20,21]. The muscles, after 24 h at 0°, were then stored for 6 to 10 days in the deep freeze at approximately —20°.

Preparation of washed fibrils was essentially the same as that used by SZENT-GYÖRGYI *et al.*[8] and VILLAFRANCA[9] for rabbit psoas. Bundles of fibers were cut from the splints, placed on absorbing paper to remove excess glycerol, weighed and cut, transversely, into small pieces. They were then blended for 1 ½ to 2 min in a Waring Blendor in 0.04 *M* KCl and 0.0067 *M* phosphate buffer, pH 7.4 (here-after to be referred to as "wash solution"). It had been noted that horseshoe crab muscle blended in this manner did not satisfactorily disperse into smaller units (either fibrils or isolated myofibrils) but, rather, tended to separate into thick sheets of material. It had also been noted[13] that arthropod muscle seemed more resistant than vertebrate muscle to breakdown and disintegrates into units about 100 μ wide, which are somewhat coarse. It was found that longer blending times usually resulted in much foam and made the preparations difficult to handle with no improvement in comminution. Glycerol (50 %), 0.155 *M* KCl, and 0.1 *M* ethylene diaminetetraacetic acid (EDTA) solutions were tested for efficacy of dispersion with blending time held to 1 ½ min. The most effective solution, however, seemed to be the "wash solution": EDTA, very interestingly, caused a total disintegration of the fibers. It was subsequently noted that larger amounts of muscle (6 g or more) rather than the 2 to 4 g employed in preparations 1 through 4 seemed to favor more homogeneous suspensions.

The blended material was washed 7 times, or until very little protein appeared in the supernatant after centrifugation. The final residue was then extracted with 0.48 *M* KCl, 0.01 *M* pyrophosphate, 0.1 *M* phosphate buffer pH 6.5, 0.001 *M* $MgCl_2$ (HASSELBACH-SCHNEIDER solution as employed by HANSON AND HUXLEY[4]) or, in one case, preparation 4, with 0.3 *M* KCl, 0.15 *M* phosphate buffer pH 6.5 and $2 \cdot 10^{-3}$ *M* ATP (GUBA-STRAUB solution also as used by HANSON AND HUXLEY[4]).

Phase contrast observations were made with either a Leitz Ortholux or an A/O microscope and the photographs were taken on Kodak M plates with a Makam camera. Material for the electron microscope observations was fixed with 1 % osmic

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acid in "wash solution". Fixed material was dehydrated with isopropyl alcohol, counterstained with 2% phosphotungstic acid in isopropyl alcohol and embedded in methacrylate. Sections of this material were examined under an RCA model EMU-2B Electron microscope.

Protein was estimated as 6.2 times the amount of nitrogen determined by the semi-micro Kjeldahl procedure. Crystalline disodium ATP was purchased from Pabst Co.

RESULTS

Washing the blended fibers in the buffered "wash solution" removed approximately 70% of the protein originally present in the glycerinated fibers (Table I). This was calculated rather than directly determined and is, undoubtedly, higher than the true value due to some loss of smaller fibrils when the washings were decanted. This suggests a higher concentration of "soluble" sarcoplasmic protein in *Limulus* muscle than in rabbit psoas muscle. The latter yields 42%[8], 50%[9], or 28%[7] under comparable conditions.

When the washed residue was extracted with the HASSELBACH-SCHNEIDER or the

TABLE I

AMOUNT OF MATERIAL REMOVED FROM GLYCERINATED *Limulus* MUSCLE PREPARATIONS BY WASHING 7 TIMES WITH 0.04 *M* KCl AND 0.0067 *M* PHOSPHATE BUFFER, pH 7.4

Prep.	*Protein in homogenate before wash (mg)*	*Protein in final wash (mg)*	*Protein left in fibrils (mg)*	*Percent left in fibrils*
1	555.7	9.24	142.0	25.7
2	516.8	4.0	148.7	28.7
3	534.2	0.0	154.4	28.9
4	220.2	5.44	60.8	28.9
6	924.0	—	257.0	27.8
8	567.0	—	201.5	35.5

TABLE II

ANALYSIS OF MATERIAL EXTRACTED, WITH HIGH CONCENTRATIONS OF KCl, FROM THE WASHED FIBRILS

Prep.	*Extract. time (min)*	*Protein in fibrils (mg)*	*Protein in extract (mg)*	*Percent fibril extract*	*mg prot. per ml*	*Rel. visc.*	*Rel. visc. ATP*	*ATP sens.*
1a	15	50.4	13.4	26.6	1.68	1.68	1.34	74.7
1b	30	50.4	14.0	27.8	1.75	1.73	1.39	72.8
2a	5	40.6	14.5	35.7	1.53	1.46	1.24	67.3
2b	10	40.6	16.4	40.5	1.74	1.48	1.27	67.9
2c	15	40.6	18.1	44.7	1.92	1.46	1.26	65.1
3	15	126.4	48.3	38.2	2.46	2.32	1.57	87.2
4*	15	41.7	17.8	42.7	1.99	—	1.58	—
5	2	319.0	128.2	40.2	3.21	5.10	1.85	165.0
6	15	199.0	93.3	46.9	1.30	2.45	1.65	78.2
8	15	104.5	40.1	38.3	1.34	3.60	1.89	101.0

* Extracted with 0.3 *M* KCl, 0.15 *M* phosphate buffer pH 6.5, and $2 \cdot 10^{-3}$ *M* ATP.

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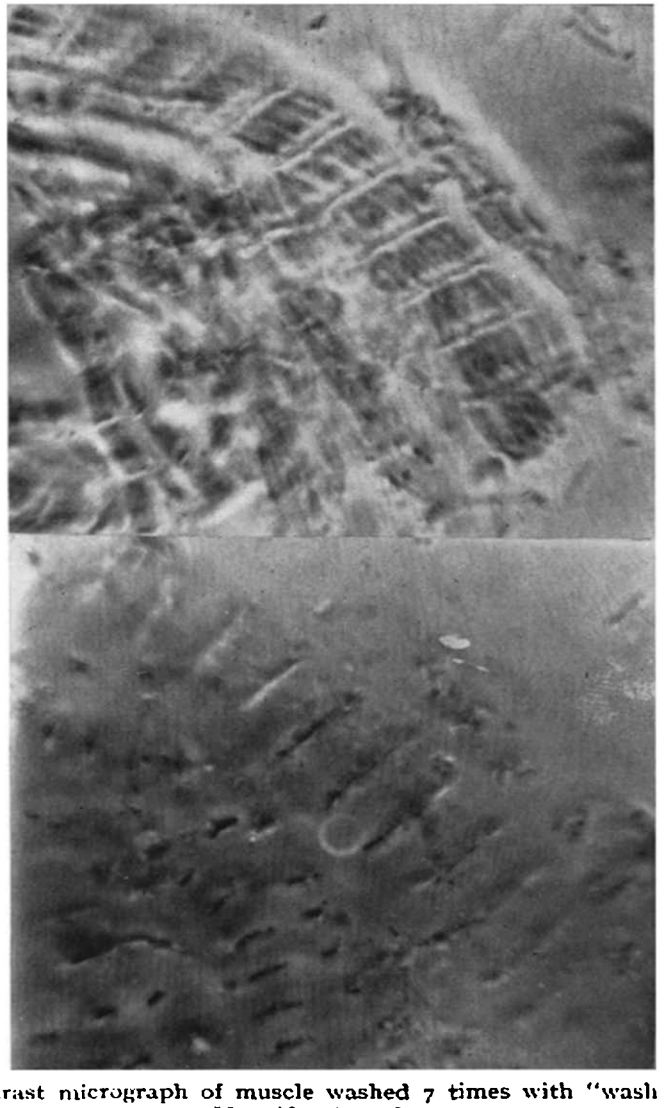

Fig. 1. Phase contrast micrograph of muscle washed 7 times with "wash solution". Prep. 5. Magnification 682 ×.

Fig. 2. Phase contrast micrograph of the same fibrils as in Fig. 1 but after extraction with HASSELBACH-SCHNEIDER solution. Photograph taken 2 min after flooding the slide with the extracting solution. Magnification 682 ×.

GUBA-STRAUB solutions an average of 38.2 % of the fibril protein was removed (Table II). The amount extracted is increased slightly with increased time of extraction as can be seen in the table (preps. 1a, b or 2a, b, c). Re-extraction of the fibrils, in the one case attempted (prep. 8), netted only 4.4 % additonal material; *i.e.* a total of 42.7 % of the washed fibril. From the viscosity data (ATP sensitivity[22]) it is readily apparent that the extracted material represents a protein solution comparable to actomyosin from rabbit: that is, when ATP was added to the extract there followed a pronounced drop in viscosity. It is, furthermore, evident, since the increased

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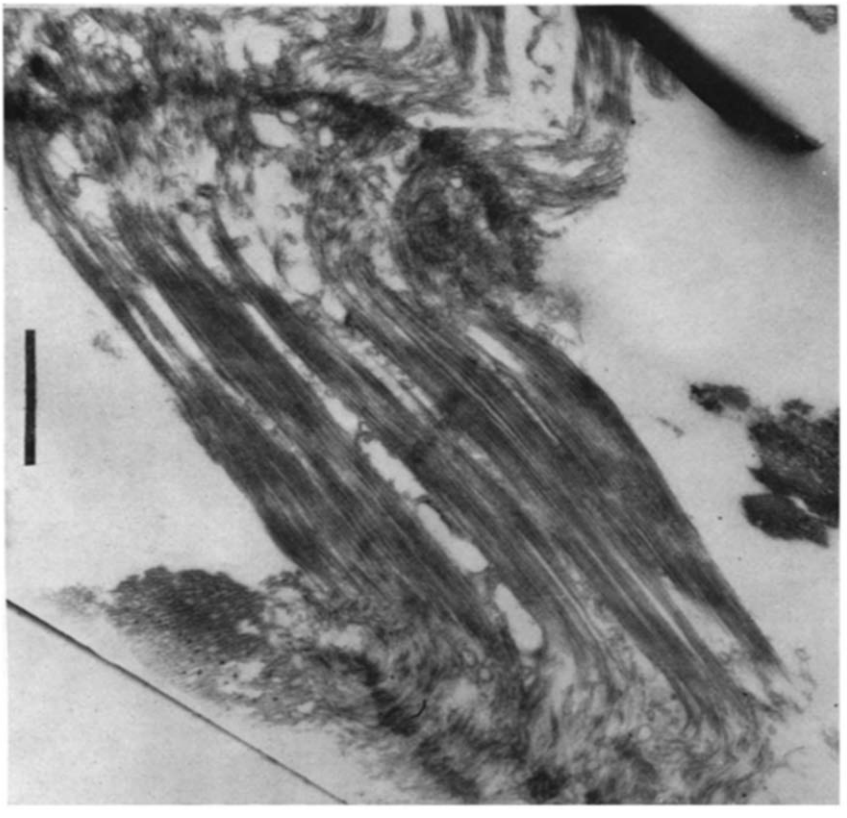

Fig. 3. Electron micrograph of muscle washed 7 times with "wash solution". Material the same as in Fig. 1 (prep. 5). Black line is 1 micron: magnification 18,500 ×.

extraction time does not greatly change the ATP sensitivity, that actomyosin is extracted as such, rather than first an extraction of myosin which is then followed by a disruption of the fibrils allowing more and more actin to go into solution. When prep. 4 was dialyzed for 20 h against 0.04 *M* KCl the precipitate exhibited an ATP sensitivity of 43 in 0.6 *M* KCl.

In an attempt to assay the amount of actomyosin present and to ascertain whether any other protein was extracted from the fibrils, the extract of preparations 6 and 8 were diluted with 10 vol. of ion-free cold water and the resulting precipitate collected by centrifugation. The precipitate (67.5 and 74.3 % of the extract), when dissolved in 0.6 *M* KCl, exhibited the typical actomyosin viscosimetric response upon the addition of ATP (ATP sensitivities of 100 and 118 respectively). In 0.15 *M* KCl it exhibited the typical superprecipitation reaction when ATP was added: at 0.3 *M* KCl the precipitate which was present appeared to go into solution in the presence of ATP. The ATPase activity was 1200 and 2400 gamma P/mg protein/h at pH 9.0, 0.005 *M* $CaCl_2$ and 0.06 *M* KCl. Although the ATPase activity and solubility properties (that is, soluble in high salt and insoluble in low salt) cannot, at this time, distinguish between actomyosin and myosin, they do, however, lend support to the view that myosin, or actomyosin, has been extracted from the fibrils. The viscosity response and

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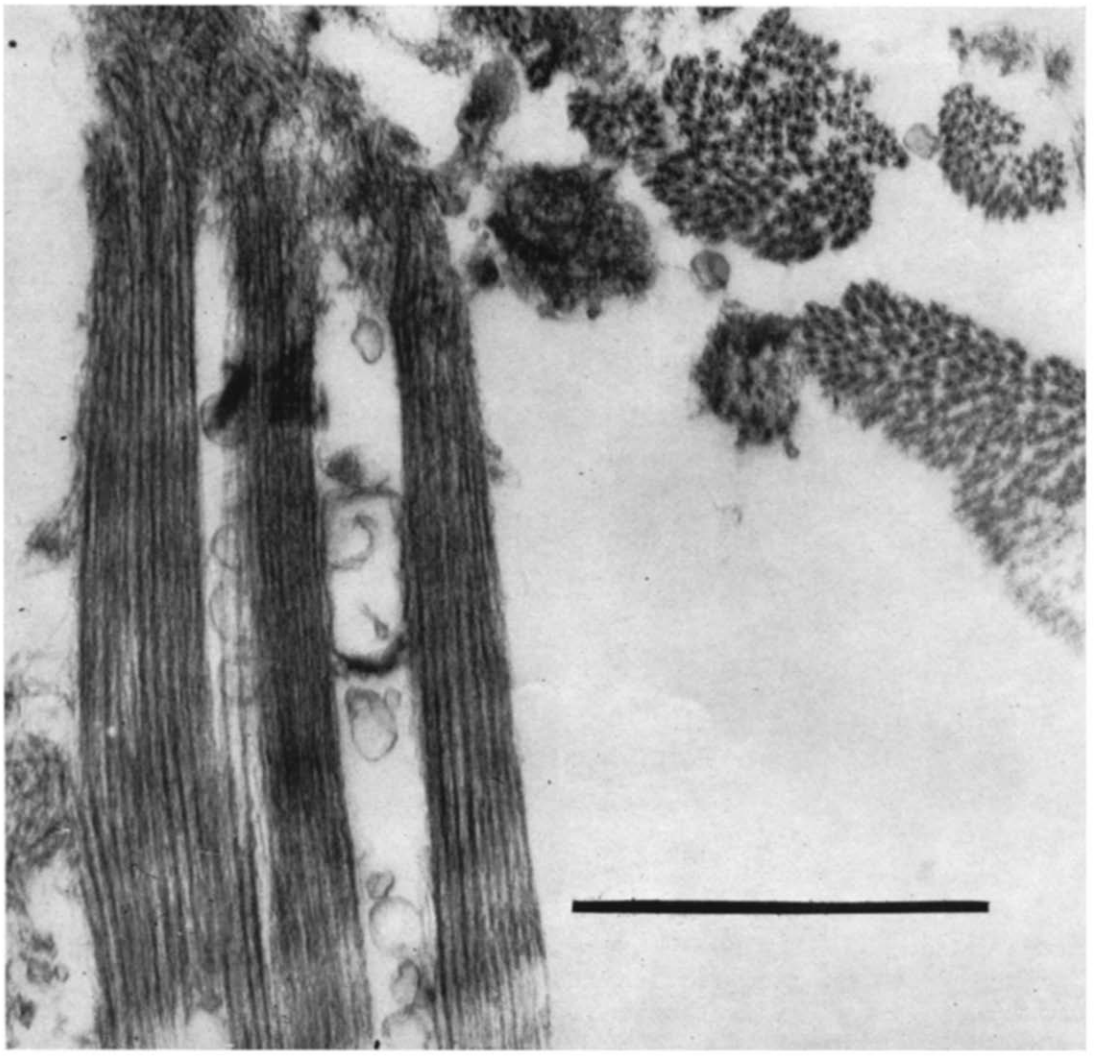

Fig. 4. As in Fig. 3. Cross sections through the A band region in the upper right hand corner of the photograph. Line is 1 micron: magnification 48,000 ×.

superprecipitation reaction definitely indicate that it is actomyosin rather than myosin.

Whether the material (about 30 %) which does not precipitate when the ionic strength of the extract is lowered with 10 vol. of water is EP[8,9] cannot be definitely stated at this time. Ultracentrifugal analysis of the extract itself (prep. 8) shows at least three distinct peaks with approximate sedimentation constants (S_{20}) of 22.02, 11.33, and 6.24.

The striations of *Limulus* muscle fibrils washed 7 times were completely preserved through the washings (Figs. 1 and 3), and the fibrils also retained their ability to contract upon the addition of ATP in 0.04 *M* KCl and 0.4 *M* $MgCl_2$. No lengthy

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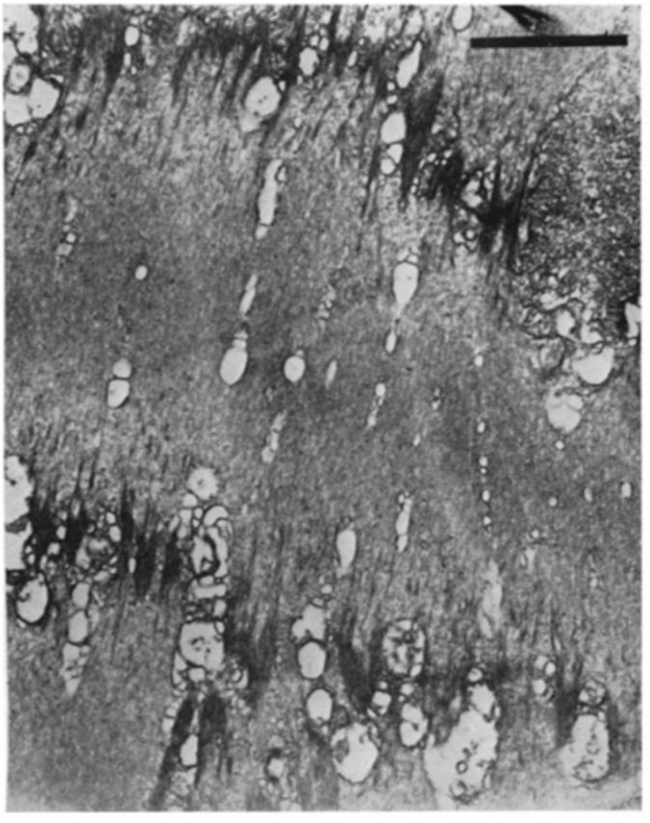

Fig. 5. Electron micrograph of washed muscle (prep. 5) but after extraction with HASSELBACH-SCHNEIDER solution: that is, the material is comparable to that in Fig. 2. Line is 1 micron: magnification 18,000 ×.

discussion of the fine structure of this muscle will be included here*: it is sufficient to point out that the A, I, and Z bands characteristic of vertebrate muscle are also present in *Limulus* muscle (Figs. 1 and 3). As in the Coleoptera[15] no M line has yet been observed. This is in contrast to other arthropod muscle[15–18]. It will be noted that the A band consists of large filaments about 150 Å in diameter. The A band also contains material of smaller diameter (60 Å) which either may exist in the form of a ring around the larger filaments (as in Fig. 4, cross-section), or as cross-bridging "filaments" between the large filaments, or as the secondary filaments of HUXLEY[12]. The precise arrangement cannot be definitely stated at this time. Similarly, the Z band appears to be filamentous in structure (Figs. 3 and 5).

When the washed fibrils were extracted with the "myosin" extractives the A band disappeared completely leaving behind the Z bands (Figs. 2, 5, 6, and 7). Fig. 2 shows the same fibrils as in Fig. 1 but after the extracting solution had been drawn under the cover slip of the phase contrast microscope. Fig. 8 shows a similar, but longer (30 min as opposed to 2 min) time sequence of extraction of a single fibril.

The electron micrographs were made from the residual plug after the extraction mixture was centrifuged. The small "filaments" (Fig. 5) are apparently lined up, which

* Manuscript on the fine structure of *Limulus* muscle is in preparation.

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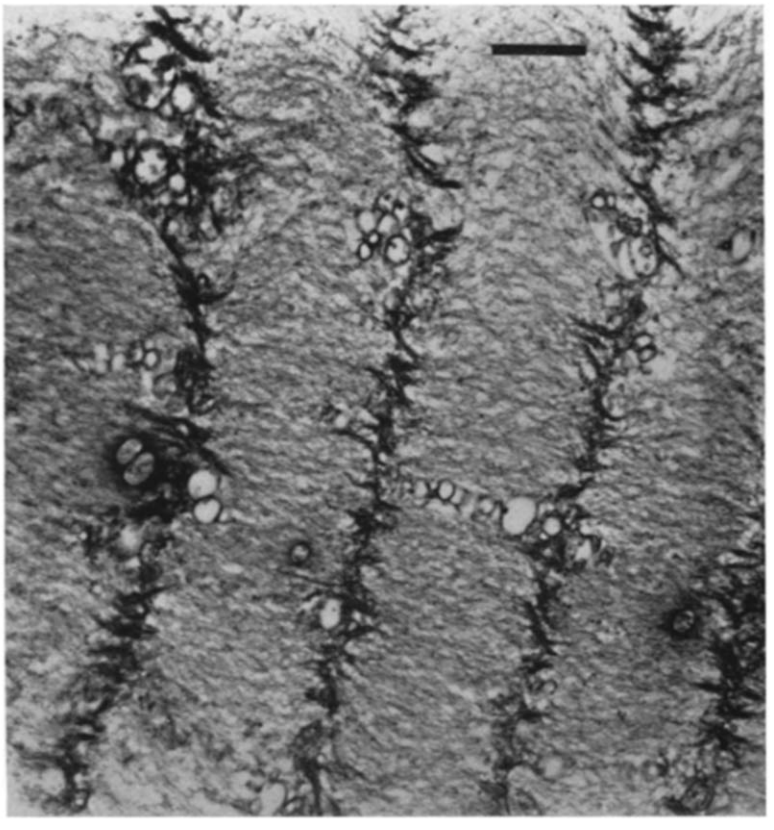

Fig. 6. As in Fig. 5. Fibrils were probably contracted before the HASSELBACH-SCHNEIDER extraction. Line is 1 micron; magnification 6,000 ×.

may be due either to the fact that the fibers are more stretched than in Figs. 6 and 7 (sarcomere length 5.0 μ as opposed to 2.7 μ) or, possibly, that only part of the large filaments have been extracted. The Z bands (Fig. 5) can be seen as a parallel array of intermediate sized filaments. In Figs. 6 and 7 the filaments of the Z band, or perhaps of contraction bands, run more or less transversely to the major axis. The residual material here is more disorganized than in Fig. 5: this is apparently more typical. They are probably not actin filaments since extraction of such fibrils (prep. 8) with a 0.6 *M* KI – 0.0005 *M* ATP mixture yielded a low viscosity solution after prolonged dialysis against several changes of 0.0005 *M* ATP. The viscosity did not increase when $KCl–MgCl_2$ were added but, rather, decreased slightly.

Whether or not the vesicular material present in almost all of the electron micrographs represents the endoplasmic reticulum[23] is unknown: it is, however, interesting to note that they aggregate around the Z bands and between myofibrils. They may represent a primitive conducting system similar to the arrangement postulated for vertebrate muscle[24]. A few of them may represent mitochondrial material with inner structure (see Fig. 6).

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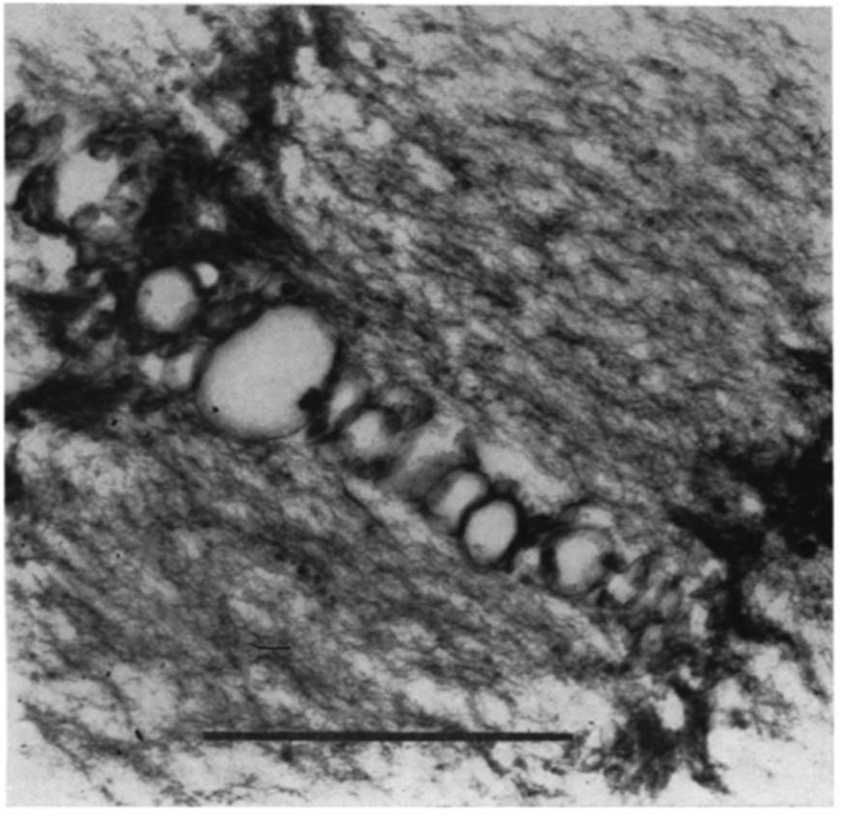

Fig. 7. As in Fig. 6 but at a higher magnification to show the fine filaments (about 60 Å) left after actomyosin extraction. Line is 1 micron: magnification 42,500 ×.

DISCUSSION

It is of interest to note that the striations of horseshoe crab muscle are quite similar to those found in vertebrate skeletal muscle. As previously reported by JORDAN[19], we also found a distinct I band, as well as A and Z bands. This pattern is similar to that reported for glycerinated preparations of blowfly muscle[18] and for the crab (*Paragrapsus*)[13] although the horseshoe crab differs from the blowfly in that it apparently lacks an M line. The *Limulus* muscle pattern differs from some insect muscle preparations[15–17] which do not show a distinct I band but do exhibit M lines. Even within the class Insecta there are differences since the firefly may well be lacking an M line[15]. It is possible that different methods of fixation bring out differences in the cross-striated patterns and that the differences mentioned here might not be real. If, however, such real differences do exist one must proceed with caution, as in the past, in formulating theories of contraction on purely morphological grounds alone or on the morphology of one specialized muscle.

When treated in precisely the same manner as rabbit psoas[8,9] *Limulus* muscle yields substantially different results. When extracted with mammalian myosin extractants the A band and its large filaments disappear as they do in the mammalian

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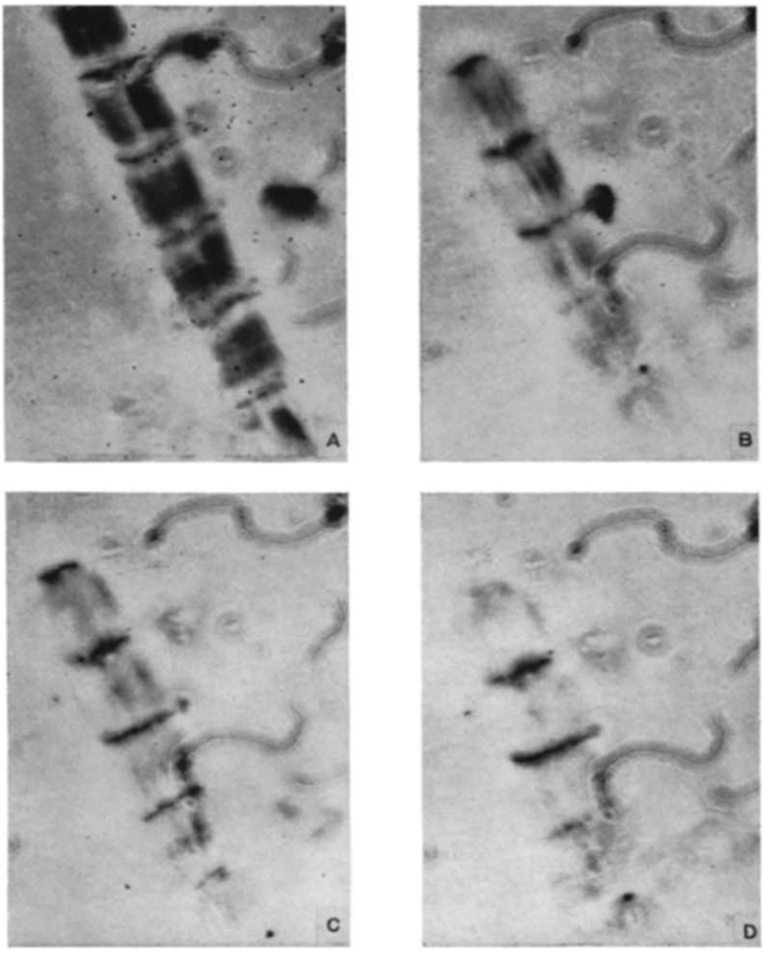

Fig. 8. Series of phase contrast micrographs of the same fibril during the course of extraction with HASSELBACH-SCHNEIDER solution under the microscope. Time between A and D was approximately 30 min. Magnification 845 ×.

muscle but instead of myosin, with traces of actin, actomyosin as such is extracted from the horseshoe crab. Apparently actomyosin exists as a discreet entity in the *Limulus* muscle rather than in a partially dissociated form, actin and myosin, or myosin layered over actin as may be the case in rabbit muscle[12].

All attempts made to extract myosin, directly and without actin, from arthropod muscle have failed[25-27]. Either actomyosin is extracted or else no contractile material is removed. This appears to be true of the amphibians[28,29] and may be true of cold-blooded animals in general. The work reported here thus strongly supports a difference in protein binding in the cold-blooded *vs.* the warm-blooded animal.

It is now generally believed that myosin makes up the major portion of the large (110 Å) filaments lying in the A band of vertebrate skeletal muscle, while actin filaments run from Z band to H zone[12]. Where EP[8,9] actually resides is, at the moment, unsettled. If one, therefore, argues by analogy the case of *Limulus* muscle, the data presented here strongly support the hypothesis that in the muscle of this animal the

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large filaments of the A band are formed of actomyosin: the actin and myosin moieties probably being more firmly united. If this is true then it becomes more difficult to visualize a "slip and catch" mechanism of contraction in which the myosin pulls the actin into the A band region[2,5,11]. It is true that smaller "filaments" remain after actomyosin extraction but what they are, morphologically or chemically, has not been determined. The one experiment reported here suggests that they are not actin but may be tropomyosin. It seems, therefore, more probable that in *Limulus* the actomyosin complex functions as a unit. We can neither support nor disprove a "slip and catch" mechanism but, rather, suggest, if this hypothesis be followed, that another element (other than actin) might serve as the "passive" filament of the I band.

ACKNOWLEDGEMENTS

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REFERENCES

1 S. V. PERRY, *Physiol. Revs.*, 36 (1956) 1.
2 J. HANSEN AND H. E. HUXLEY, *Proc. Soc. Exptl. Biol. Med.*, 9 (1955) 228.
3 W. H. HASSELBACH, *Z. Naturforsch.*, 8b (1953) 449.
4 J. HANSON AND H. E. HUXLEY, *Nature*, 172 (1953) 530.
5 H. E. HUXLEY AND J. HANSON, *Nature*, 173 (1954) 973.
6 H. E. HUXLEY AND J. HANSON, *Biochim. Biophys. Acta*, 23 (1957) 229.
7 J. HANSON AND H. E. HUXLEY, *Biochim. Biophys. Acta*, 23 (1957) 250.
8 A. G. SZENT-GYÖRGYI, D. MAZIA AND A. SZENT-GYÖRGYI, *Biochim. Biophys. Acta*, 16 (1955) 339.
9 G. W. DE VILLAFRANCA, *Arch. Biochem. Biophys.*, 61 (1956) 378.
10 H. HOLTZER, J. M. MARSHALL AND H. FINCK, *J. Biophys. Biochem. Cytol.*, 3 (1957) 705.
11 A. F. HUXLEY AND R. NIEDERGERKE, *Nature*, 173 (1954) 971.
12 H. E. HUXLEY, *J. Biophys. Biochem. Cytol.*, 3 (1957) 631.
13 J. L. FARRANT AND E. H. MERCER, *Exptl. Cell Research*, 3 (1952) 553.
14 H. E. JORDAN, *J. Morphol.*, 96 (1955) 513.
15 G. B. CHAPMAN, *J. Morphol.*, 95 (1954) 237.
16 D. E. PHILPOTT, *Biol. Bull.*, 107 (1954) 302.
17 D. E. PHILPOTT AND A. SZENT-GYÖRGYI, *Biochim. Biophys. Acta*, 18 (195) 177.
18 A. J. HODGE, *J. Biophys. Biochem. Cytol.*, 1 (1955) 361.
19 H. E. JORDAN, *Carnegie Inst. Wash. Publ.*, 251 (1917) 273.
20 N. K. SARKAR, *Enzymologia*, 14 (1951) 288.
21 A. SZENT-GYÖRGYI, *Chemistry of Muscular Contraction*, 2nd Ed., Academic Press Inc., New York, 1951.
22 H. H. WEBER AND H. PORTZEHL, *Advances in Protein Chem.*, 7 (1952) 161.
23 K. R. PORTER AND G. E. PALADE, *J. Biophys. Biochem. Cytol.*, 3 (1957) 269.
24 A. F. HUXLEY AND R. E. TAYLOR, *Nature*, 176 (1955) 1068.
25 D. GILMOUR AND J. H. CALABY, *Enzymologia*, 16 (1953) 23.
26 G. W. DE VILLAFRANCA, unpublished.
27 E. M. SZENT-KIRALYI, personal communication.
28 G. W. DE VILLAFRANCA, *Biol. Bull.*, 111 (1956) 320.
29 J. GODEAUX, *Bull. classe sci., Acad. roy. Belg.*, 38 (1952) 1103.