Polarized Light Microscopy on the Single Frog Muscle Fibre[1)]

By

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(With 5 Text-figures and 1 Plate)

Knowledge of the submicroscopical structure of the muscle provides fundamental evidence for any modern explanation of muscular contraction. Studies on the submicroscopic structure have been attempted by the authors, applying the principle of physical optics. One of these attempts is an investigation by means of the polarizing microscope. Simple material is necessary for the experimental approach because the birefringent phenomena in the muscle are too complicated, when the whole thick muscle is used. Buchthal & Knappeis (1938) and Huxley & Niedergerke (1958) used a single muscle fibre. In the present paper, an isolated single muscle fibre or an isolated thin bundle of myofibrils in living state was adopted for the observation.

According to the general statements made in most text books, the striation pattern in the skeletal muscle fibre is due to the existence of optically different discs, the dark discs of higher refractive index are anisotropic and birefringent while the light discs of lower refractive index are isotropic or nearly so. In contradiction to these theories, the authors' findings are that the light bands in the striation pattern are uniaxial birefringent bodies and the dark bands are similar but include a light-absorbing structure. Preliminary short reports on these findings have appeared elsewhere (Washio, Kakiuchi, & Tamasige, 1960; Tamasige, 1960), and they have been discussed with various zoologists and physiologists. Gradually the findings have been accepted by them. The complete paper on the findings is given in the following lines.

Material and methods

The materials used were single living muscle fibres isolated free of injury from the hind leg muscles of the frog, *Rana temporaria temporaria* and *R. japonica*. The polarizing microscope, Nikon POH, was used with a magnification of 160 or 400 times. The preparation of the muscle fibres was placed on the microscope stage and was viewed between the polarizer and the analyser in the crossed positions, at an angle of 45° to their planes of polarization. When necessary, a gypsum test plate (retardation : 530 mμ), was interposed

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between the muscle fibre and the analyser. For the fibre this was along the fibre axis or perpendicular to it.

The change in birefringence of the muscle fibre was measured by means of a photoelectric tube (PT-17-VI) as the change in the brightness per unit area under the polarizing microscope. The tube was mounted on the ocular lens of the microscope. The cathode of the photoelectric tube was connected to a high gain direct-coupled amplifier (the amplification ; 50,000×) through a high resistor (50 Megohms) for stabilization of the input circuit. The change in brightness was read on a galvanometer scale. The first stage of the amplifier was designed to make the grid current as small as possible, in order to avoid the drop of input signal due to the high input impedance. The diagram of the experimental arrangement for studies on the change in birefringence is shown in Fig. 1.

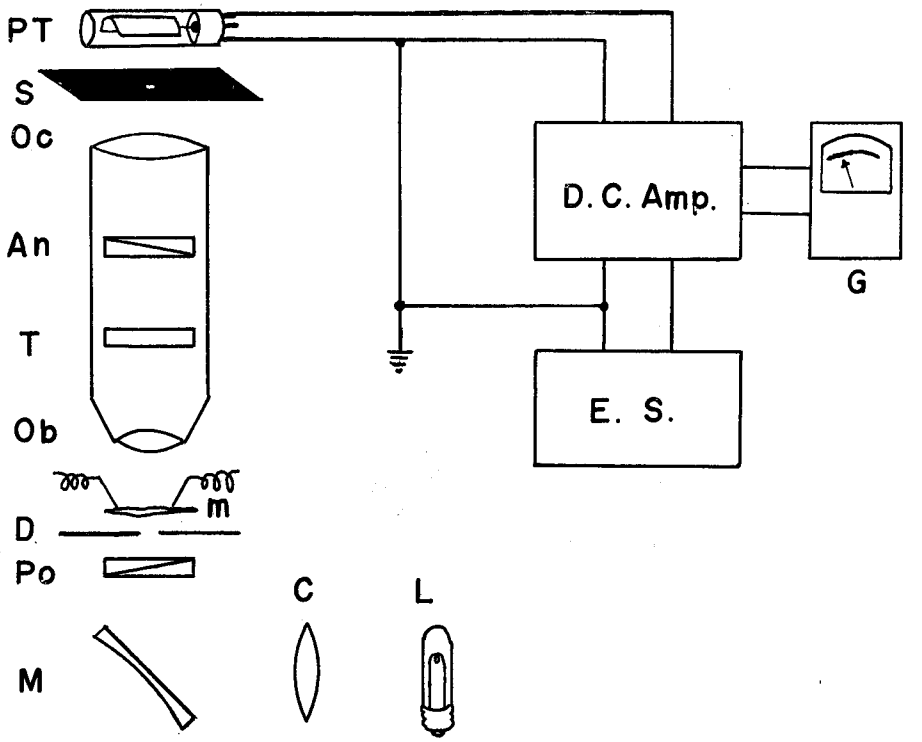

Fig. 1. Diagram of experimental arrangement for studies of birefringence in an isolated muscle fibre. An : analyser, C : condensor lens, D : iris diaphragm, D.C. Amp. : direct coupled amplifier, E.S. : electronic stimulator, G : galvanometer, L : light source, M : mirror, m : muscle fibre, Ob : objective lens, Oc : ocular lens, Po : polarizer, PT : photoelectric tube, S : slit, T : test plate.

The relation between the birefringence (n_2-n_1) and the brightness (A'^2) of the light which comes through the polarizer, a thin specimen and the analyser is as follows :

$$A'^2 = A^2 \sin 2\theta \sin^2 \frac{\pi \cdot d \cdot (n_2 - n_1)}{\lambda_0}$$

where A′, the amplitude of the polarized light which is transmitted through the polarizer, the thin specimen and finally the analyser ; A, the amplitude of the light from the polarizer (A^2, the brightness) : θ, the angle formed by the direction of vibration of the polarizer with that of vibration of the fast light resolved in the thin specimen ; d, the thickness of the thin specimen; n_1 and n_2, the refractive indicies of the polarized lights (the ordinary and extraordinary lights) resolved in the thin specimen, respectively ; λ_0, the wave length of the polarized light in air.

When A, θ and d are constant, the relation can be expressed as follows :

$$A'^2 \propto \sin^2 \frac{\pi d (n_2 - n_1)}{\lambda_0}$$

Strictly speaking, this is applicable in the case of monochromatic light. However, in order to examine a relative change in the birefringence of the thin specimen, the above relation can be applied approximately to white light also.

Results

1. Structure of muscle fibre

It has been widely accepted that the striation pattern in skeletal muscle fibre which can be seen under the light microscope is due to the alternate difference in the refractive index between the two zones and that the zone of higher refractive index (the dark band) is anisotropic or birefringent and the zone of lower index (the light band) is isotropic or nearly so.

Thus under polarizing microscope with the crossed Nicols, the zones of higher refractive index (A bands) would appear bright and the zones of lower refractive index (I bands) dark. The reversal of brightness in the striation pattern would be observed between the light and polarizing microscopes.

The muscle fibre was examined comparing the microphotographs taken with both kinds of microscopes. Charcoal granules were attached on the surface of the muscle fibre. The thin black, longitudinal streaks in the fibre seemed to be boundaries or spaces between myofibrillar bundles. The granules and the streaks served as position markers for the striation pattern. The two microphotographs taken with the two kinds of microscopes were superimposed, using the marked points so that the photographs would exactly coincide. The difference in the striation pattern between two kinds of microphotographs was inspected. The result shows that the light bands always appear bright and the dark bands always dark under both polarizing and light microscopes (Fig. 2).

There was no reversal of brightness in the striation pattern even when the muscle fibre or the analyser of the polarizing microscope was rotated by 45° in both clock- and counter clock-wise direction. Of course, the muscle fibre was entirely dark at the extinction position (where the fibre was parallel or perpendicular to the polarizer and analyser), but it was brightest (except the dark bands) when it was rotated 45° from the extinction position.

Hence, the light bands must be uniaxial birefringent bodies and the dark bands must contain a light-absorbing structure.

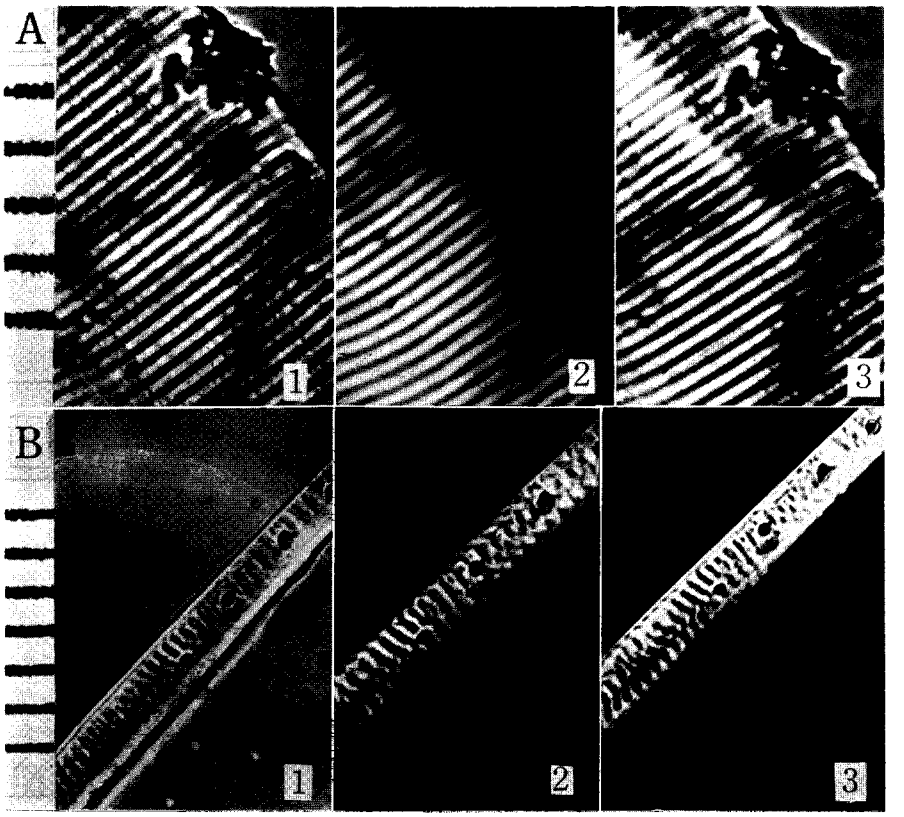

Fig. 2. Comparison of the striated pattern of muscle fibre under polarizing and ordinary light microscopes. The photomicrographs taken with the polarizing microscope (2) and the light microscope (1) are superimposed (3). The back ground of the fibre in the microphotograph is dark under the polarizing microscope (2). A, a muscle fibre in the Ringer's solution ; B, a bundle of myofibrils separated in oil. Each division in the scale means 10μ.

2. Myofibril

Myofibrils can be separated longitudinally from a muscle fibre, placed in transparent oil, for example, the cod liver oil or the liquid paraffin, according to Natori's method (1949).

Thus striation pattern of myofibrils was observed clearly as well as that of a single muscle fibre ; and the myofibrils also exhibited the strong birefringence

(Pl. XII. C). Then the difference in striation pattern between the light and polarizing microphotographs was examined also in the isolated small bundles of myofibrils (of less than 10μ in thickness).

The results were exactly similar to that obtained in an intact single muscle fibre. The application of a small amount of sodium chloride crystals to the isolated myofibrils in oil induced a local contraction around the NaCl-application site, and also the strength of the birefringence was reduced at the contracted areas of myofibrils.

3. Muscle fibre contraction

A) *Contracture induced by a chemical* : Chemicals induce many types of contraction in a living muscle fibre. In this experiment, the effect of chloroform on muscle fibres was investigated. Upon the removal of the chemicals in the early stage of contraction, muscle fibres were restored to their normal condition.

The striation pattern of a contracted single muscle fibre, immersed in a bath of saturated chloroform-Ringer's solution, disappeared completely, but did not appear completely in the diluted (0.0025 or 0.001 per cent) chloroform-Ringer's solution. In the latter case, the light bands shorten by about 40 per cent while the dark bands remained unchanged (Pl. XII, A,B).

The change in brightness of the fibre in polarized light microscopy is shown in Fig. 3A. The result shows that the brightness is reduced by 20 per cent of the initial value, and the strength of the birefringence is equally reduced by contraction of the muscle fibre.

B) *Continued contraction produced by repetitive electrical stimulation* : A living muscle fibre was fixed at one end with a hook in the Ringer's solution bath. The electrical square pulses at frequency of 50 c/sec. were applied through a pair of stimulating electrodes of Ag-AgCl type immersed in the Ringer's solution.

In this case, under isotonic contractions the light bands were shortened to 40% of the resting length but the dark bands remained unchanged, similar to that observed in the chemically induced contracture. The decrease in brightness in the polarized light microscopy during contraction of the fibre was as much as 10 per cent of the initial value (Fig. 3B).

This value was less than that in the case of chemical contracture. The explanation may be that the contraction height in the case of continued contraction produced electrically is smaller than that of the contracture by chemicals.

C) *Local contraction* : When a slightly fatigued single muscle fibre was stimulated electrically through an intracellular micro-electrode, a local contraction could be produced (*Pl.* XII. *E*). The tip diameter of the micro-electrode used was less than 1 μ. A remarkable change in birefringence occurred at the very point of stimulation. The birefringence changed in proportion to the degree of contraction.

D) *Propagated contraction* : An attempt was made to observe the change

in birefringence during propagation of the contraction wave along the muscle fibre. Fig. 4 shows the polarizing microphotographs in which a slight decrease in the birefringence at the head of the contraction wave propagated along the fibre.

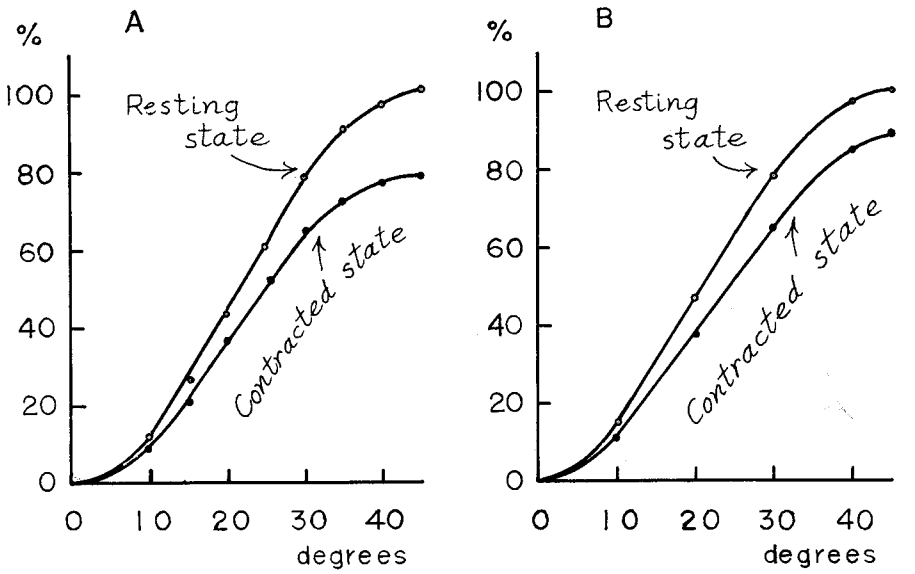

Fig. 3. The change in brightness of the living single muscle fibre in polarized light during contraction produced by chemical (chloroform-Ringer) (3. A) and electrical (3. B) stimulation. Abscissa : an angle of rotation of the fibre from the extinction position. Ordinate : the change in brightness of the fibre.

4. Passive elongation

To examine the effects of elongation on the birefringence of the muscle fibre, the fibre was stretched. The length of the light bands was obviously increased by the passive stretching while the dark bands were only slightly elongated under both polarizing and light microscopes. The result agrees with that obtained by the previous authors, e.g. Huxley and Niedergerke, 1958. The change in birefringence of the living single muscle fibre by passive stretch could not be clearly detected. It may be explained as follows : the birefringence of the muscle fibre is very strong already at the normal length and further increase in the birefringence by passive stretching is very slight.

5. Effects of hypertonic or hypotonic solutions

The volume of a single muscle fibre was changed by immersion in hypertonic or hypotonic solutions to investigate the effects of volume changes on the striation

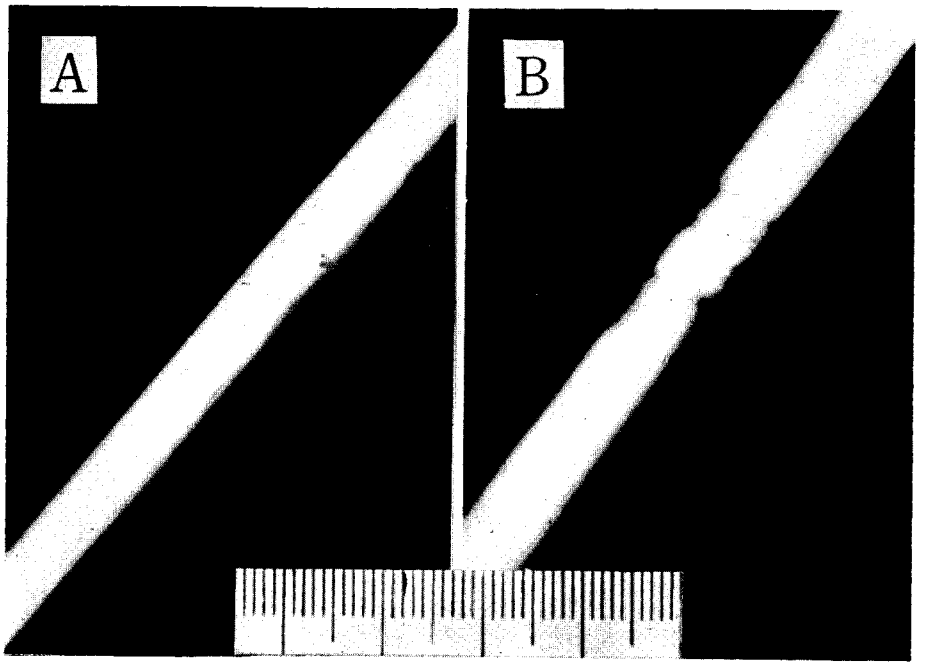

Fig. 4. The change in birefringence at the front of contraction wave which spreads along the muscle fibre under the polarizing microscope. A : in the resting state. B : in the active state. X-ray films were used, and the electronic stimulator was synchronized with the Xenon lamp flash through a suitable delay circuit. Scale division, 10μ.

pattern and birefringence of the fibre. Hypertonic solutions were made by dilution of the stock solution of 10 times the concentration of the standard Ringer's solution in all ionic constituents. Hypotonic solutions were made by dilution of the standard Ringer's solution with distilled water. The concentrations thus are expressed relatively to that of the standard Ringer's solution, as 10× Ringer, 2× Ringer, 1/2 Ringer, and 1/10 Ringer.

A) *Changes in hypertonic solutions*: When a single muscle fibre was immersed in hypertonic solutions, it shrank and the boundary between light and dark bands became obscure. But the striation pattern still remained. A change in birefringence was not evident.

B) *Changes in hypotonic solutions*: In the hypotonic solutions the muscle fibre swelled and in the more hypotonic solutions, less than 1/5 Ringer the striation pattern gradually became obscure. In the more hypotonic solutions than 1/20 Ringer, it disappeared rapidly. Thus, a swollen and continuously light fibre over the entire length was obtained. It was remarkable that muscle fibre,

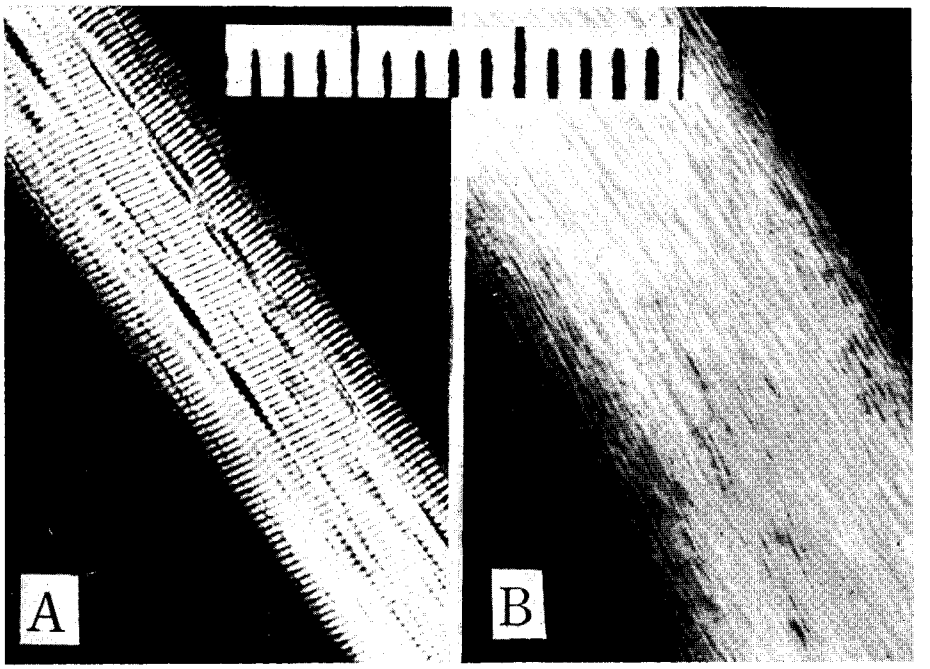

Fig. 5. Effect of a hypotonic solution upon the striation pattern of muscle fibre. A, a fibre in the normal Ringer's solution ; B, the same fibre in 1/20 Ringer's solution. Note the fibre is swollen, the dark bands of the striation pattern disappear and only a long bright (birefringent) fiber over the entire length remains. Scale division, 10μ.

in which the striation pattern had disappeared, still exhibited the strong birefringence (Fig. 5) and also maintained contractility. A slow and local contraction was induced by both strong electrical and chemical stimulations. Consequently it is probable that the dark bands are not very important for the fundamental contractile mechanism, and the light absorbing structure is easily broken down by the swelling of the myofibrils (only the long continuous myofibrils remained) when the fibre is immersed in the hypotonic solutions.

6. Injured muscle fibre

A local injury to a single muscle fibre spread along the fibre with the lapse of time. At first, the injured part contracted, then the striation pattern disappeared and also the birefringence decreased. In Plate XII Figure F, a polarizing microphotograph of the injured part, the Ca-coagulum of sarcoplasm is shown. In such a state, the normal part of the fibre exhibited a strong birefringence whereas the birefringence of the injured part vanished entirely as is seen in the figure.

7. Glycerinized muscle fibre

A single muscle fibre isolated from a glycerinized rabbit psoas muscle was investigated under the polarizing microscope. The muscle fibre gives no response to electrical stimulation (Umezawa, 1956). But under the polarizing microscope there was no difference between the living and glycerinized muscle fibres. The application of adenosinetriphosphate (ATP) to the glycerinized muscle fibre induced a remarkable contraction, but after about 10 sec. the contraction reached a stationary state. The striated pattern and then the birefringence of the fibre vanished completely.

Discussion

The facts about the structure of a striated muscle fibre, that the zones of higher refractive index are birefringent, while the zones of lower refractive index are optically isotropic or nearly so, have been established by a number of microscopists since the nineteenth century (e.g. Brücke, 1858). A bands (anisotropic) have been called dark bands and I bands (isotropic) light bands. But the interpretation of the striation pattern is quite difficult in a thick specimen such as a whole sartorius muscle of the frog. According to A.F. Huxley (1957) the striated appearance depends on the focussing condition of the microscope. But in the present observations, no light-dark reversal of the striation pattern due to focussing of the light microscope could be observed. Furthermore, the appearance of both bands never changed even under the polarizing microscope. The same phenomena were observed on small bundles of myofibrils of less than 10μ in thickness which were separated longitudinally from a muscle fibre in oil. The results are at variance with Huxely's description, but observation on the isolated thin bundle of myofibrils is more accurate and has the advantage of reliance on more minute details than Huxley's observation of the muscle fibre.

From the result 1 stated above, the light bands are uniaxial birefringent bodies while the dark bands contain a light-absorbing structure. The fact is further confirmed by the result 2, from the observation of isolated thin bundles of myofibrils.

According to results 3 and 4, the lengths of the light bands are remarkably changed by either the active contraction or the passive stretching of the fibre whereas those of the dark bands are not. This may be explained by the fact that the light absorbing structure of the dark bands is a transversely arranged net-work of inter-myofibrillar connective fine-filaments. The long parallel myofibrils are transversely bound together at spaced intervals along the length of the fibre. The transversely bound areas of the myofibrils can scarcely be contracted or elongated at all by stimulation or by passive stretching. But the existence of such an interfibrillar transverse connection has much advantage for the maintenance of the long parallel arrangement of the contractile elements and for the rapid develop-

ment of strong tensions in a definite direction along the length of the fibre. The situation is quite different from that in smooth muscle which contracts rather slowly in two or three directions.

Furthermore, a bundle of long myofibrils without the light absorbing structure (the dark band) can be prepared by immersion of the muscle fibre in the hypotonic solutions (1/5–1/20 Ringer in the result 5), but it is different from the myosin-free muscle prepared by treatment of the muscle with such a solution as the one devised by Hasselbach & Schneider (1951). The former is still strongly birefringent and contractile in response to electrical or chemical stimuli. The structure of dark appearance in the striation pattern is not so important in the fundamental mechanism of contraction. It may have some other functions in the arrangement of the contractile structure.

The relation between the change in length and the birefringence of the muscle fibre, in the above results, in general agrees with that obtained by the previous authors (Muralt, 1932 ; Fischer, 1936 ; Bozler & Cottrell, 1937 ; Buchthal & Knappeis, 1938). The birefringence decreased with the height of the contraction of the fibre and with the degree of disorderness of the submicroscopical arrangement of the contractile elements. Thus the injured sarcoplasm or the Ca-coagulum of the sarcoplasm has no birefringence. The contractility of the sarcoplasm is kept remained as long as the birefringence can be detected (the results 5 and 6).

Noll and Weber (1934) showed that the birefringence of the A bands could be accounted for by the birefringence of myosin threads. In the present experiments, evidence is presented that the myosin threads are not confined to the dark bands, but spread over the entire length of the muscle fibre.

The light-dark reversal in the striation pattern was demonstrated with the interference microscope or the phase-contrast microscope (Hanson & Huxley, 1955 ; A.F. Huxley & Taylor, 1958). This was very natural on the basis of physical optics. However, no polarizing microphotograph has been published which shows the light bands changed into dark ones in appearance or the dark bands(so called 'anisotropic') into bright ones when observed under the polarizing microscope.

Summary

1. The striated pattern of living skeletal muscle fibre isolated from the hind legs of the frog was investigated under both the polarizing and light microscopes. In any position of the focus of the microscopes, no reversal of the pattern of dark and light bands did occur.

2. The change in birefringence of the muscle fibre was recorded as the change in brightness under the polarizing microscope measured by means of a photoelectric tube. The decrease in the birefringence during isotonic contraction produced by electrical and chemical stimulation was confirmed by this method,

while the change in birefringence caused by the passive stretch of the fibre was very slight.

3. The length of the dark bands was unchanged while the length of the light bands was changed during contraction and stretch under the light and polarizing microscopes.

4. The light bands were uniaxial, strong birefringent bodies, and the dark bands seemed to be of the same nature but included light absorbing structures. The light absorbing structure is considered to be a transverse connective net-work of inter-myofibrillar fine-filaments.

5. The dark bands of muscle fibre disappeared in hypotonic solutions and only the long light fibres remained, but they still had strong birefringence and contractility in response to electrical and chemical stimuli.

6. The submicroscopic structure of muscle fibre and the relation between contraction and birefringence is discussed.

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References

Bozler, E. and Cottrell, C.L. 1937. The birefringence of muscle and its variation during contraction. J. Cell. and Comp. Physiol. 10 : 165–182.

Brücke, E. 1858. Untersuchungen über den Bau der Muskelfasern mit Hülfe des Polarisierten Lichtes. Denkschr. Akad. Wiss. Wien. 15 : 69–84.

Buchthal, F. und Knappeis, G.G. 1938. Untersuchungen über die Dopplebrechung der einzelnen, lebenden, quergestreiften Muskelfaser. Skand. Arch. Physiol. 78 : 97–116.

Fischer, E. 1936a. The changes in birefringence during contraction and stretch of a smooth muscle (retractor of phascolosoma). J. Cell. and Comp. Physiol. 8 : 503–514.

Fischer, E. 1936*b*. The submicroscopical structure of muscle and its changes during contraction and stretch. Cold Spring Harbor Symposia 4 : 214–223.

Hansen, J. and Huxley, H.E. 1955. Fibrous proteins and their biological significance. Symposia Soc. Exptl. Biol. 9 : 228–264.

Hasselbach, W. und Schneider, G. 1951. Der L-Myosin und Aktingehalt des Kaninchenmuskels. Biochem. Zeit. 321 : 461–475.

Huxely, A.F. 1957. Muscle structure and theories of contraction. Progr. Biophys. 7 : 255–318.

Huxley, A.F. and Niedergerke, R. 1958. Measurement of the striation of isolated muscle fibres with the interference microscope. J. Physiol 144 : 403–425.

Huxley, A.F. and Taylor, R.E. 1958. Local activation of striated muscle fibres. J. Physiol. 144 : 426–441.

von Muralt, A. 1932. Ueber das Verhalten der Doppelbrechung des quergestreiften

Muskels während der Kontraktion. Pflüg. Arch. ges. Physiol. 230 : 299–326.
Natori, R. 1949. Studies on the mechanism of muscle contraction. J. Physiol. Soc. Jap. 11 : 14–15 (Report of 26th General Meet, in Japanese).
Noll, D. und Weber, H.H. 1934. Polarisationsoptik und molekularer Feinbau der Q-Abschnitte des Froschmuskels. Pflüg. Arch. ges. Physiol. 235 : 234–246.
Tamasige, M. 1960. Polarized light studies on contraction of the frog single muscle fibre. J. Physiol. Soc. Japan. 22 : 724 (in Japanese).
Umezawa, S. 1956. Electrical stimulation of single muscle fibre with the electrodes placed out or inside of cell membrane. Zool. Mag., Tokyo. 65 : 427–434 (in Japanese).
Washio, H., Kakiuchi, Y. and Tamasige, M. 1960, Polarizing microscopy on contraction of an isolated muscle cell. Zool. Mag., Tokyo. 69 : 45 (in Japanese).

Explanation of Plate XII

All of the microphotographs were taken by the interposition of a gypsum test plate (retardation : 530 mμ) between the fibre and the analyser. Scale division, 10μ.

A. A living single muscle fibre isolated from the frog in the resting state under a polarizing microscope.

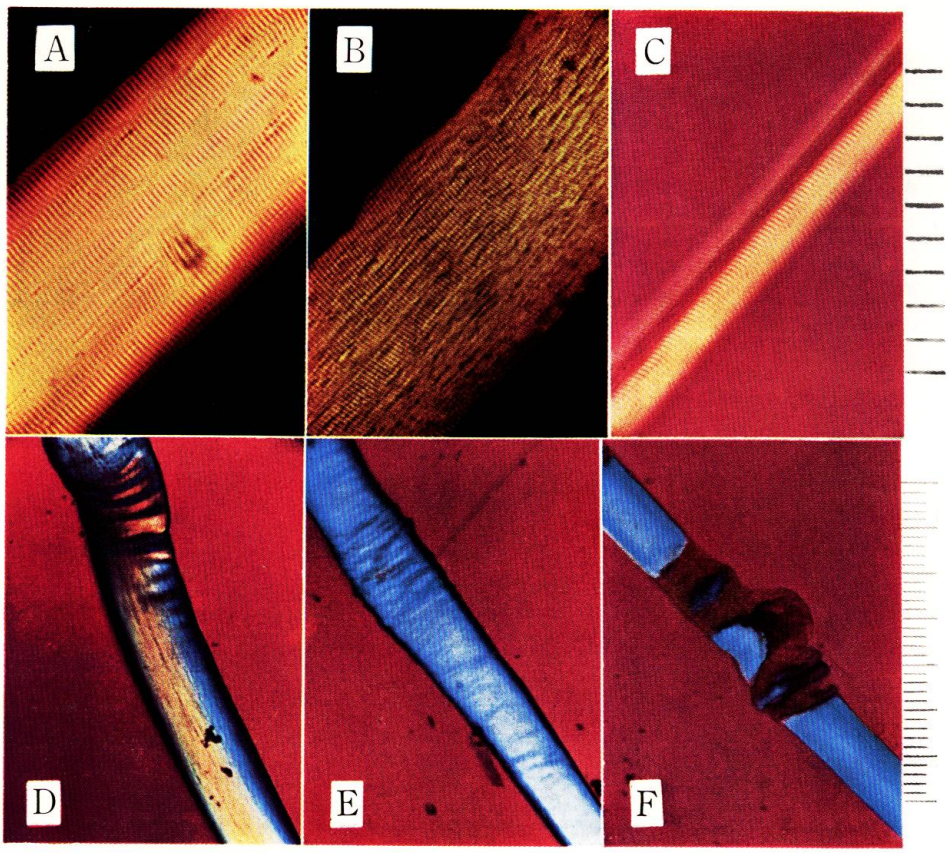
B. The same fibre as in A in the contracted state induced by chloroform-Ringer's solution. The light bands are shortened by about 40% of the initial length while the dark bands remain unchanged.

C. Two bundles of myofibrils isolated and separated longitudinally from a muscle fibre in cod liver oil. They also exhibit a strong birefringence. The striation pattern is still seen clearly.

D. Microphotograph of local contraction induced by saturated chloroform-Ringer's solution. The strongest contracted parts exhibit the same interference color as that of the back ground. The birefringence was completely lost.

E. Microphotograph of local contraction. A slightly fatigued single muscle fibre was stimulated electrically through an intracellular micro-electrode. A decrease in birefringence is seen around the tip of the micro-electrode.

F. Microphotograph of an injured muscle fibre. In the injured area, the sarcoplasm combined with Ca-ions in the surrounding medium, and the Ca-coagulum of sarcoplasm resulting, has no birefringence and the interference color in the microphotograph is the same as that of the back ground, whereas that of the normal sarcoplasm shows the blue color and strong birefringence.



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