FILAMENT LENGTHS IN STRIATED MUSCLE

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ABSTRACT

Filament lengths in resting and excited frog muscles have been measured in the electron microscope, and investigations made of the changes in length that are found under different conditions, to distinguish between those changes which arise during preparation and the actual differences in the living muscles. It is concluded that all the measured differences in filament length are caused by the preparative procedures in ways that can be simply accounted for, and that the filament lengths are the same in both resting and excited muscles at all sarcomere lengths greater than 2.1 μ, *viz.*, A filaments, 1.6 μ; I filaments, 2.05 μ. The fine periodicity visible along the I filaments also has been measured in frog, toad, and rabbit muscles and found to be 406 A.

INTRODUCTION

Hanson and H. E. Huxley (1953) studied the band pattern of striated muscle in the phase contrast microscope, and from these and other observations deduced that the band pattern arose from two overlapping sets of filaments whose lengths could therefore be measured. The same authors (H. E. Huxley and Hanson, 1954) and A. F. Huxley and Niedergerke (1954) observed the band pattern at different sarcomere lengths in the phase contrast and interference microscopes, respectively, and found that the filament lengths were constant from about 90 per cent resting length to maximum extension. On the basis of this finding, these authors proposed the sliding filament model for muscle, in which changes of muscle length result from the sliding of two arrays of filaments past each other without change in the individual filament lengths. The resolution of the light microscope (approximately 0.2 μ) limited the accuracy of these measurements, however, and it was still quite possible that slight changes in the lengths of the A and I filaments might occur according to the state of the muscle. The filament lengths can be measured more accurately in electron micrographs, providing, of course, that the necessary care is taken to ensure that the electron microscope is accurately calibrated; but there is considerable uncertainty about the extent of any length changes during preparation. In the present experiments, therefore, all possible measures were taken to determine the filament lengths in the electron microscope with the highest accuracy, and a study was made of the factors which may cause them to change during preparation, so as to find out whether or not genuine changes in filament lengths do occur under different physiological conditions.

At the same time, the fine longitudinal periodicity in the I filaments has been measured. Previous figures for this period, from electron microscope studies of both isolated fibrils and embedded material, have ranged from 250 to 450 A, in some cases varying within the same material according to sarcomere length. These earlier measurements could have been affected by possible changes in filament length during preparation, and so there has remained considerable uncertainty concerning both the actual magnitude of the periodicity and any possible functional changes in it. It has been

difficult also to know exactly how to correlate this periodicity seen in the electron microscope with the various long axial periodicities which show up in the x-ray diffraction diagram from muscle. These periodicities fall into three groups. One type, now believed to come from the actin filaments, was first measured in dried muscle by Bear (1945) who gave the axial period as possibly 350 to 420 A. Astbury and Spark (1947) and Cohen and Hanson (1956) obtained the same pattern from F actin, and, in 1953, Huxley found these "actin" reflections in living muscle. Selby and Bear (1956) were able to interpret the actin reflections in terms of either a helical arrangement or a two dimensional net, both with an axial period of either 351 A or 406 A, but still could not distinguish between these various alternatives. Worthington (1959) made very precise measurements of the layer line spacing, and supported the figure of 410 A for the actin axial periodicity, rather than 350 A.

Huxley (1953) measured other reflections from living muscle, which Elliott and Worthington (1959) have shown corresponded to an axial period of 435 A. This was significantly different from the actin period, and the reflections had other characteristics which suggested that they came from the myosin filaments.

The third type of periodicity has a value of approximately 400 A, and corresponds to reflections which Selby and Bear (1956) called "non-net" reflections, as they could not be indexed with the actin system. Worthington (1959) also found a meridional reflection corresponding to an axial spacing of about 400 A. The magnitude of this spacing varied between 360 and 410 A, depending on the previous treatment of the muscle, and thus could be distinguished from the actin spacing which disappeared after similar treatment of the muscle.

Recently, Hanson and Lowy (1962, 1963) have shown that isolated filaments of actin, examined in the electron microscope by the negative staining technique, have a helical structure with a period of 349 A. The axial spacing of subunits along the helix seen by them corresponds closely to the value obtained from x-ray diffraction by the actin filaments in intact muscle, showing that the filaments had not shortened significantly during preparation for electron microscopy by their technique.

In the present study, the periodicity seen in the electron microscope has been measured in the A bands and the I bands of muscles fixed in different physiological conditions, and corrections made for any changes produced during preparation so that these measurements could be compared with the x-ray diffraction measurements.

METHODS

Fresh frog sartorius, semitendinosus, and extensor longus digiti IV muscles, toad sartorius muscles, strips of rabbit psoas muscle, and strips of glycerinated rabbit psoas and chicken breast muscles were tied to rods at the required lengths, and fixed in a 1 per cent solution of OsO_4 buffered at pH 7.3 to 7.5 with Veronal acetate (Palade, 1952) for ½ to 1 hour at room temperature or at 0°C. The tissue was dehydrated in an alcohol series, and stained overnight with a 1 per cent solution of phosphotungstic acid (PTA) in absolute alcohol.

Frog muscles were also fixed by the method which Carlsen *et al.* (1961) have used in order to minimize possible excitation of the muscle by the fixative itself, *i.e.* the muscle, tied to a rod, was kept in Ringer's solution containing 13 mM K for 2 to 4 hours at 0°C before fixation, and the OsO_4 added stepwise in concentrations of 10^{-2} per cent, 10^{-1} per cent, and 1 per cent. These muscles were then washed in Ringer's solution for 1 hour, stained in 1 per cent PTA dissolved in 70 per cent alcohol overnight, and dehydrated in 95 per cent and 100 per cent alcohol.

In some cases the muscle was kept attached to the rod throughout dehydration and staining; in others, the muscle was removed from the rod immediately after fixation. Some frog muscles were dehydrated in acetone, not alcohol.

Frog muscles were also fixed with glutaraldehyde (Sabatini *et al.*, 1962). The fixative used contained 1 part of 25 per cent solution of glutaraldehyde to 3 parts of 0.1 M cacodylate buffer, pH 7.3. The muscles were fixed for 3 hours, dehydrated in an alcohol series, and stained overnight in a 1 per cent solution of PTA in absolute alcohol. One muscle was fixed in glutaraldehyde buffered with Veronal acetate, the fixative being added stepwise in concentrations of 0.06 per cent, 0.6 per cent and 6 per cent glutaraldehyde.

For the experiments with excited muscles, the muscle ext. long. dig. IV was used, and the tension was measured with a transducer (RCA 5734). For the potassium contractures, the muscle was mounted vertically, at a fixed length, in a narrow chamber. This was filled with a solution containing 10 mM KNO_3, 107.5 mM $NaNO_3$, and 1.8 mM $CaCl_2$ for 5 minutes, and then drained. A solution containing 50 mM KNO_3, 67.5 mM $NaNO_3$, and 1.8 mM $CaCl_2$ was then run through the chamber, and 4 seconds later a solution of 1 per cent OsO_4 in Veronal-acetate buffer containing 50 mM K was run into the

chamber. After 45 minutes the muscle was removed from the chamber, tied to a rod at its fixed length, and dehydrated in alcohol and stained as before.

For electrical stimulation, the muscle was mounted horizontally in a chamber containing Ringer's solution. In one series of experiments the muscle was held at a fixed length during stimulation. Some of the muscles were mounted in a stretched state, others were mounted at a shorter length, and the slack taken up by stimulation under zero load. The electrodes used were two parallel plates of platinum, the same length as the muscle, lying on either side of it. The muscle was stimulated with A.C. at 50 cycles per second and a voltage approximately 1½ times that which gave maximum tension. When the tension had reached its maximum a 2 per cent solution of OsO_4 was added to the solution in the chamber, giving a final concentration of 1 per cent OsO_4. After 45 minute fixation, the muscle was removed from the chamber, tied to a rod at its fixed length, and dehydrated and stained as before. In two other experiments, one end of the muscle was fixed and the other tied to a weight. The muscle was stimulated as before, and allowed to shorten against the load until it reached the length at which it just supported the load without further shortening. 2 per cent OsO_4 solution was then added to the solution in the chamber to give a final concentration of 1 per cent OsO_4. After 45 minutes the muscle was dehydrated and stained as above.

The dehydrated tissues were embedded in Araldite (50 ml Araldite, 50 ml hardener 964 B, and 1.5 ml accelerator 964 C), or in prepolymerized 15 per cent methyl methacrylate. Sections were cut on a Porter-Blum microtome with the knife-edge parallel to the fibre axis to avoid compression of the section in the direction of the filaments. (Measurements of the block face and sections confirmed that there was no length change in the section parallel to the knife edge.) If the tissue had not been stained before embedding, the sections were stained with a 1 per cent solution of PTA in absolute alcohol for ½ to ¾ hour, or with a 1 per cent aqueous solution of $KMnO_4$ for 1 hour.

The sections were viewed in a Siemens Elmiskop 1 at 60 or 80 kv, with an objective aperture of 50 μ. If the plane of the section is not exactly parallel to the fibrils, the band patterns do not correctly reflect the filament lengths (*e.g.* if the angle between the plane of the section and the fibril axis is θ, the A filament length is A cos θ, where A is the A band length). To reduce this error to less than 0.5 per cent, only fibrils which remained in the plane of the section for at least five sarcomeres were used for measurements, so that θ was less than 5°.

The magnification was determined for each grid by photographing a grating replica under the same conditions; *i.e.*, without altering the lens currents. This eliminated effects due to long term instrument variations, and to hysteresis which had been found to amount to 3 to 4 per cent even after switching the lenses on and off several times. If the position of the section within the column was different from the position of the grating replica, the focal length and hence the lens current of the objective lens had to be altered to bring the section into focus, and so the magnification of this lens was also altered. As the focal length is about 2.5 mm, a difference in object position of the order of 100 μ corresponds to a 4 to 5 per cent change in the focal length and in the magnification. Therefore any change needed in the objective lens current was recorded, and the appropriate correction applied to the magnification. The region of the grating used was calibrated in a light microscope, and a low power electron micrograph (× 1,500) of this same area used as an intermediate standard. Original magnifications were 8,000 to 15,000, and only the central area of the field, in which distortion had been found to be negligible, was used. Measurements of the fine periodicity were made on prints (enlargement × 3½), whose magnification in the direction of the fibre axis was determined directly by comparison with the plates. This eliminated the irregularities in shrinking or stretching of the print during processing, which were found to be of the order of 3 to 4 per cent, and to vary from one direction to another within one print. When the procedure outlined here was followed, the probable error in a measurement of filament length was estimated as not more than 2 per cent.

Fresh frog fibrils and glycerinated chicken fibrils were prepared by blending the shredded muscles in a medium containing 10^{-2} M Mg^{++}, 10^{-3} M EDTA (ethylenediaminetetraacetate), and 10^{-1} M KCl in 6×10^{-3} M phosphate buffer, pH 7 in a Measuring and Scientific Equipment, Ltd. homogeniser. Isolated I segments (bundles of actin filaments on either side of a Z line) were prepared from these by blending in the Mg/EDTA/salt solution containing in addition 0.16 gm ATP/100 ml of solution. These were collected on grids and either negatively stained or shadowed, sometimes after fixation in OsO_4 vapour for 15 seconds, or in 5 per cent formalin, or after freeze-drying. The solutions used for the negative staining were 1 per cent aqueous uranyl acetate and 1 per cent aqueous PTA adjusted to pH 7 with dilute NaOH. The metal for the shadowing was a 3:1 mixture of Pt and Pd.

In the light diffraction experiments, frog sartorius muscles, or fibre bundles from glycerinated rabbit psoas muscles, were laid flat on a glass slide, and clamped at either end. This slide was held vertically

in a glass chamber with flat parallel sides. Light from an ultraviolet lamp with a green filter and a narrow slit in front of it was focused on the muscle, and the diffraction pattern recorded on a photographic plate mounted 10 cm from the muscle. The muscle was then fixed with 1 per cent buffered OsO_4 and dehydrated, and the diffraction pattern from the same area again recorded. The accuracy of the measurements of the spacings was about 2 per cent.

DESIGN OF EXPERIMENTS

The problem of looking for changes in filament lengths during preparation is one of considerable complexity. Such changes could possibly occur during any of the stages of preparation, and if they are reflected in the over-all length of the muscle, their presence is easy to detect. However, it would not be easy to determine whether in such circumstances both sets of filaments were changing, or only one; and hence one would not know the actual amount of change that is taking place in each set of filaments. On the other hand, the filaments may possibly slide past each other as they change in length; or one set of filaments may shorten and the other set stretch. Both these types of change could leave the sarcomere length unchanged, and would be very much more difficult to detect and measure.

To overcome some of these difficulties, various experiments can be devised. In the first place, it must be ascertained whether over-all length changes do take place in a muscle free to shorten or stretch, during any one stage of preparation. Then, if length changes are found, the effect of holding a muscle at a fixed length during preparation can be studied and filament lengths in the held and unrestrained muscles compared at different muscle lengths.

If changes occur during fixation with OsO_4, the effect of other fixatives can be studied and compared with the OsO_4-fixed muscles. This may throw some light on the nature and extent of possible changes caused by the OsO_4. Similarly, the muscles may be dehydrated with acetone instead of alcohol, in the hope that differences between the two will lead to more information about possible changes. Further, to avoid treatment with fixatives and dehydrating agents altogether, isolated bundles of filaments can be prepared, at any rate in the case of I filaments, and negative staining and shadowing techniques used for viewing them in the electron microscope.

There is one other method available for gaining further information about length changes in the I filaments. If it is assumed that the fine periodicity is the same along the whole length of the living filaments, then measurements of this in different parts of the I filaments (*e.g.* in the A band and in the I band) will show whether length changes are uniform along the whole filament.

RESULTS

INVESTIGATION OF POSSIBLE LENGTH CHANGES DURING FIXATION, DEHYDRATION, AND EMBEDDING: Since the contractile structure runs continuously from one end of the fibre to the other, the simplest way to investigate whether or not length changes take place during preparation would be to measure the length of fibre bundles before and after each stage; and indeed this can be readily done in the case of dehydration and embedding. However, this is not possible for the fixation stage, because OsO_4 causes excitation and consequent contraction of fresh muscle. The next best approximation is to prefix the muscle in formalin for 10 minutes, after which time the muscle is inexcitable, and then to investigate the effect of OsO_4 on the length of fibre bundles of this material, bearing in mind that the effect of OsO_4 on the formalin-fixed muscle may not be the same as on fresh tissue. The results of such measurements are given in Table I. These show that, even after prefixation in formalin, the OsO_4 solution caused about 4 per cent shortening of the fibre bundles, and further shortening

TABLE I

Longitudinal Shortening of Unrestrained Fibre Bundles

	Fixation*	Dehydration‡	Embedding in Araldite
No. of measurements.	14	14	19
	per cent	*per cent*	*per cent*
Mean per cent shortening	3.5	9	0.5
Minimum and maximum shortening	1 to 7	4 to 13	0 to 2

* Fibre bundles from muscles prefixed in 5 per cent neutral formalin for 10 minutes.

‡ Fibre bundles from muscles fixed in 1 per cent OsO_4 solution or by stepwise addition of OsO_4 solutions.

(~9 per cent) occurred during dehydration. The shortening observed during embedding in Araldite (½ per cent) is within the experimental error of the measurements and not significant. Thus, one or both sets of filaments tend to shorten during both OsO_4 fixation and dehydration.

In these experiments, the muscle fibres were unrestrained and free to shorten. In the next series, the muscles were held at a fixed length. It was then still possible that some parts would shorten while others lengthened. The diffraction pattern from a muscle clamped at each end was recorded before and after fixation, and again after dehydration. Care was taken to ensure that the same area of the muscle was examined each time, so that variations in sarcomere length along the fibre should not confuse the results. The pattern from the fresh muscle was not so sharp as that from glycerinated tissue, but within the experimental error (2 per cent) no change in the spacing of the pattern, and hence of the sarcomere length, was detected during either fixation or dehydration of both the fresh and the glycerinated tissue (Table II). These results show that holding the muscle at a fixed length during these procedures does prevent shortening of sarcomeres, but they give no information about the two sets of filaments; both sets may shorten (slipping past each other) while the sarcomere length stays constant, or one set of filaments may shorten while the other is stretched.

TABLE II

*Changes in sarcomere lengths measured by diffraction**

	Fixation	Dehydration	Fixation + dehydration
Glycerinated muscle‡			
No. of measurements	9	3	1
Mean shortening, *per cent*	0.5	0.5	0
Minimum and maximum shortening, *per cent*	0–2	0–1	
Fresh muscle§			
No. of measurements	4	1	2
Mean shortening, *per cent*	1	0	1.5
Minimum and maximum shortening, *per cent*	0–3		0–3

* Muscles clamped at both ends

‡ Accuracy of measurements: 1.5 per cent.

§§Accuracy of measurements: 2 per cent.

CHANGES IN FILAMENT LENGTH DURING DEHYDRATION: Filament lengths from muscle that had been held at a constant length during fixation and dehydration were compared with those from muscle that was unrestrained during dehydration (Table III). It was found that the I filaments were shorter in the muscle that had been free to shorten during dehydration, but that there was no great difference in the A filament lengths. For example, in a frog sartorius muscle fixed in the usual 1 per cent OsO_4 solution, the I filaments were 1.85 μ, whereas in the unrestrained muscle they were only 1.68 μ. (Throughout this text, the "I filament length" refers to the length from the boundary of one H zone to the next. Knappeis and Carlsen, 1962, have shown that one I filament is not continuous through the Z line, so that the length of an isolated I filament is in fact one half the value given here.) The A filament length in both these muscles was 1.44 μ. Further, in the held muscle the I filament lengths were constant at different sarcomere lengths, but in the unrestrained muscle, the longer the sarcomere, the shorter were the I filaments, *i.e.* the longer the I band, the more shortening of the I filaments took place. For example, in the frog semitendinosus fixed by the method used by Carlsen *et al.* (1961) on resting muscles, the I filament length in the held muscle was 1.8 μ over a range of sarcomere lengths from 2.05 to 2.8 μ; in the unrestrained muscles, similarly fixed, the I filaments varied from 1.8 to 1.7 μ over the same sarcomere range, and had dropped to 1.6 μ at a sarcomere length of 3 μ, when the corresponding value in the held muscle was 1.7 μ. (The I filament

TABLE III

Filament Lengths in Embedded Material

Muscle	Fixation	Dehydration	Sarcomere	I filament			A filament			No. of periods in I band
					N	SD		N	SD	
			μ	μ		μ	μ		μ	
Sartorius	Normal OsO_4	Held*	2.7	1.85	12	0.02	1.44	12	0.01	15
"	" "	"	2.5	1.80	6	0.03	1.50	6	0.01	13
"	" "	Held* NPTA‡	2.6	1.87	15	0.05				
"	" "	Not held§	2.4	1.68	11	0.02	1.44	15	0.01	13–14
"	" "	" "	2.1	1.75			1.49			7–8
"	" "	Acetone, held¶	2.4	1.88	24	0.03	1.51	20	0.03	
"	" "	" not held**	2.1	1.90	14	0.02				
"	Depolarized + OsO_4‡‡	Held*	3.0	1.91	14	0.02	1.54	14	0.005	6
"	Depolarized + OsO_4‡‡	"	2.1	1.85	10	0.005	1.49	10	0.005	13–14
Semitendinosus	Normal OsO_4	"	2.05	1.87	8	0.02	1.53	8	0.01	6
"	" "	"	2.9	1.92	7	0.01				16–17
"	" "	" NPTA‡	2.2	1.97						6
"	" "	" "	3.3	1.93	9	0.03				21
"	" "	" "	3.7	1.92	10	0.02				24
Sartorius	Carlsen‖	Held*	2.0	1.80	5	0.01	1.49	6	0.02	4–5
"	"	Not held§	2.15	1.67	5	0.03	1.44	5	0.02	8
Semitendinosus	Carlsen‖	Held*	2.05	1.80			1.47			6–7
"	"	"	2.2	1.80	20	0.02				10
"	"	"	2.4	1.77						12
"	"	"	2.75	1.78	12	0.04	1.52	12	0.03	15–16
"	"	"	2.95	1.68	28	0.02				19–20
"	"	"	3.55	1.91	10	0.02				24
"	"	"	3.8	1.88	21	0.01				24
"	"	Not held§	1.9	1.79	13	0.03	1.54	6	0.02	3–4
"	"	" "	2.3	1.76	16	0.03	1.51	13	0.02	10–11
"	"	" "	2.55	1.70	28	0.03	1.50	28	0.02	13
"	"	" "	2.65	1.72	19	0.03	1.51	6	0.01	15
"	"	" "	2.75	1.71	12	0.02	1.51	11	0.02	17–18
"	"	" "	2.9	1.62	22	0.02	1.57	6	0.03	19–20
"	"	" "	3.0	1.59	22	0.02				21
Sartorius	Glutaraldehyde	Held*	2.1	1.96	12	0.05	1.60	18	0.01	
"	"	Not held§	2.05	1.94	8	0.02	1.60	8	0.01	
"	" in steps§§	Held*	2.8	1.98	18	0.03	1.57	18	0.01	
Semitendinosus	Glutaraldehyde after 13 mM K‖‖	Held	3.25	1.99	17	0.03	1.59	5	0.02	

* Muscle held at constant length during dehydration.

‡ Muscle not stained with PTA before embedding.

§ Muscle not held during dehydration.

‖ Muscle fixed by the method used by Carlsen *et al.* (1961) to produce minimal excitation by the fixative.

¶ Muscle dehydrated in acetone while being held at constant length.

** Muscle dehydrated in acetone, not being held during this stage.

‡‡ Muscle depolarized in isotonic KCH_3SO_4 before fixation in 1 per cent OsO_4 solution.

§§ Glutaraldehyde fixative added stepwise in concentrations 0.06 per cent, 0.6 per cent, and 6 per cent.

‖‖ 6 per cent glutaraldehyde solution added to muscle previously soaked in Ringer's containing 13 mM K.

lengths in the held muscles were found to be greater than 1.8 μ when the sarcomere length was 3.5 μ or more; this will be discussed later.) These results show either that any shortening of the A filaments that takes place during dehydration is not prevented by holding the muscle at a constant length during this step, or alternatively that very little shortening of the A filaments occurs during dehydration. On the other hand, some shortening of the I filaments is prevented by holding the muscle, and when shortening of the muscle is allowed, the I filaments apparently show a greater tendency to shorten within the I band than in the A band.

The 400 A period was measured in the muscles of the last experiments in both the A and the I bands. As can be seen from Table IV, the periodicity in the held muscles was found to be the same

(355 to 360 A) in both bands, indicating that any shortening that has occurred during fixation and dehydration is uniform along the whole filament (assuming that the periodicity is the same along the whole filament in the living muscle), and that the part of the I filament within the I band has not been stretched by any possible shrinking of the A filaments. In the muscle free to shorten during dehydration, the periodicity in the A band remained the same (355 to 360 A), but that in the I band was less (315 to 340 A). The amount of shortening was not precisely the same in each

TABLE IV

Periodicities in A and I Bands of Semitendinosus Muscle

Preparation	Sarcomere	I filament	Periodicity in I band	A band	Periodicity in A band
	μ	μ	A	μ	A
held*	2.05	1.80	356	1.47	352
"	2.75	1.78	354	1.52	352
"	3.55	1.81	365		
not held‡	2.3	1.76	340	1.51	360
" "	2.55	1.70	331	1.50	353
" "	2.75	1.71	338	1.51	356
" "	2.9	1.62	324	1.57	363
" "	2.95	1.57	313		

All the muscles were fixed by the method used by Carlsen *et al.* (1961) for minimal excitation by the fixative.

* Muscle held at fixed length during dehydration.

‡ Muscle not held at fixed length during dehydration.

preparation, but the period was always significantly less in the I than in the A band. These measurements therefore confirm that, in a muscle allowed to shorten during dehydration, the I filaments shorten to a greater extent in the I band than in the region of overlap. The fact that the A band period is the same, independent of whether the muscle is held during dehydration, suggests that if any shortening of the A filaments occurs it does so during fixation only, and not during dehydration.

In one experiment, the tissue was dehydrated with acetone instead of alcohol. In this case, the I filament length in the held muscle (1.88 μ) was the same as in the unrestrained muscle (1.9 μ).

EFFECT OF DIFFERENT FIXATION PROCEDURES: Table III shows that the filament lengths in the embedded muscle were found to vary also with the method of fixation. Muscles fixed by the method used by Carlsen *et al.* (1961) on resting muscles (*i.e.* stepwise addition of the OsO_4 fixative following depolarization in 13 mM K) had an I filament length of 1.8 μ; those fixed in the normal 1 per cent OsO_4 solution had I filaments 1.85 to 1.95 μ long. The A filaments were about 1.5 μ in each case. To determine whether this difference could be ascribed to changes produced by the fixative, some muscles were fixed with glutaraldehyde (Table III). This fixative, at a concentration of 6 per cent and buffered with cacodylate, causes contraction of the muscle, like OsO_4; but when it is buffered with Veronal acetate and added stepwise in concentrations of 0.06 per cent, 0.6 per cent, and 6 per cent, it produces no visible contraction nor extension in a muscle lying freely. When the full strength fixative was used, the I filaments were found to be 1.95 to 2.0 μ long. When the stepwise addition was used, the I filaments still measured 1.98 μ. By comparing these values, using glutaraldehyde, with those mentioned previously using OsO_4, it would appear first of all that the OsO_4 causes some shortening of the I filaments. Secondly, the question arises whether the observations on the muscles fixed in OsO_4 by the two different methods could be explained by the filaments being shorter in the living resting muscle than in the muscle stimulated by the fixative. Since no differences between the stimulated and unstimulated muscles are observed using glutaraldehyde fixative, it seems unlikely that it is the stimulation *per se* that is the responsible factor. The explanation for the differences in I filament length between the stimulated and unstimulated muscles fixed in OsO_4 must therefore be sought in a different direction. The most likely is that some shortening of the I filaments is produced by OsO_4 in each case, but that for some reason this OsO_4 induced shortening is greater with the unstimulated muscle.

The measurements on the glutaraldehyde fixed muscles show that the A filaments also are longer in these muscles (1.6 μ) than in those fixed in OsO_4 (1.45 to 1.55 μ). This probably indicates that the OsO_4 causes shortening of the A filaments as well as of the I filaments, rather than that the A filaments are stretched in the glutaraldehyde fixed muscles.

ISOLATED I SEGMENTS: As a further comparison for the I filaments, isolated I segments (bundles of I filaments attached to a Z line, Fig. 1) were prepared, and their lengths measured by methods that did not involve any fixative or treatment with alcohol. Considerable difficulty was experienced in obtaining I segments from fresh frog muscle, and so glycerinated chicken breast muscle was used for most of the experiments. For reasons discussed later, these chicken I filaments are believed to have the same length as the fresh frog filaments. Table V shows that the length of the I segments after negative staining with uranyl acetate was 2.0 μ. With PTA negative staining, the segments were found to be 2.06 μ. However, it seemed possible that staining with uranyl acetate or PTA caused some shortening of the filaments. Measurements were made therefore on shadowed preparations, in some of which the I segments were frozen-dried before shadowing. The length of these shadowed segments was found to be 2.05 μ, the same as the PTA stained material. If the segments were treated with OSO_4 vapour, or with formalin, after they were collected on the carbon film of the grid, their length was unchanged. When the I segment is lying on the carbon film it is sufficiently firmly attached to resist being washed off by the washing fluid, and shortening may be inhibited in this condition. In some experiments, therefore, OsO_4 solution was added to the suspension of segments in the blending medium, in the hope of demonstrating an OsO_4-induced shortening directly. In these preparations, however, the filaments were disorganized, not lying straight on either side of the Z line, so that, although the length of these segments was only 1.8 μ (after uranyl acetate negative staining), it was not possible to say how much of this shortening was due to the OsO_4 and how much to the disarray of the filaments. However, the measurements do tend to confirm the view that the I filament length is 2.0 to 2.05 μ, and that it is well preserved by glutaraldehyde, but reduced by OsO_4.

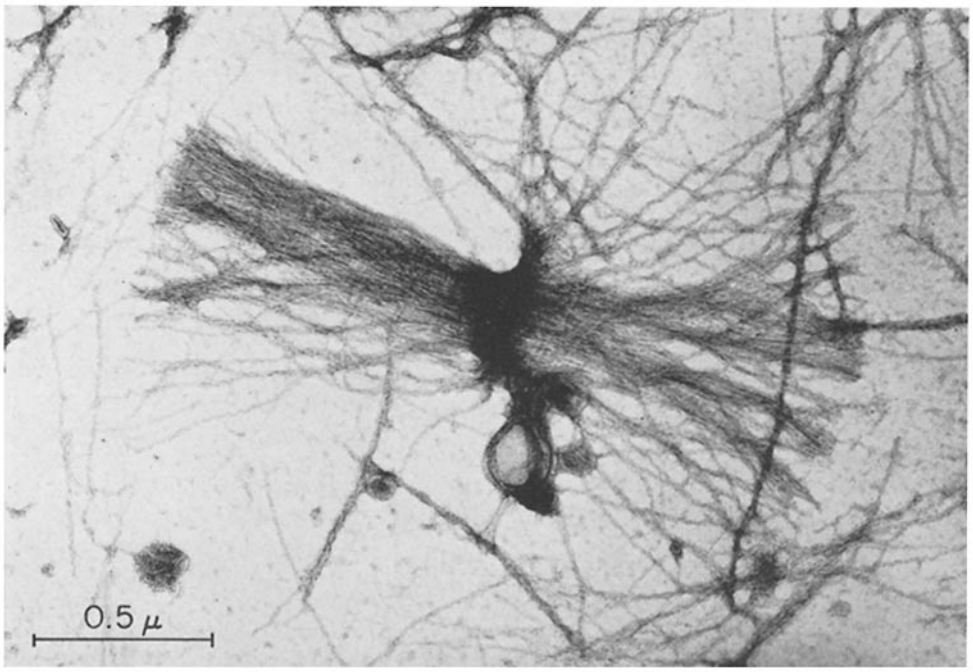


FIGURE 1 Isolated I segment from glycerinated chicken breast muscle, negatively stained with uranyl acetate. × 47,500.

EXTENT OF EXCITATION OF FIXED MUSCLES: OsO_4 itself causes excitation of muscle, and a muscle lying freely will contract when OsO_4 is poured onto it until the H zone just disappears and the I filaments meet in the centre of the A band. To obtain information on the state of the muscle excited by OsO_4, and how it differs from

the fully excited muscle, the tension developed during fixation of a toe muscle was compared with the tension record of the same muscle during a potassium contracture at the same length (Fig. 2 *a* and *b*). The maximum tension in the potassium contracture was reached in 9 seconds; the tension caused by the 1 per cent OsO_4 solution, added in the same way, was 12 per cent of that in the potassium contracture, being reached in 4 seconds, and then maintained. The muscle fixed in this way therefore is not in a maximally excited state when fixed, and, as shown later, can be distinguished by differences in filament lengths from a muscle fixed while being stimulated. It could be potassium contracture is given in Fig. 2 *d*, together with the record of a previous potassium contracture of the same muscle (Fig. 2 *c*). The drop of tension on the addition of the OsO_4 solution, amounting to 30 to 40 per cent, is greater than the drop due to "fatigue" over the same time interval. The paired muscle from the opposite foot was used to measure this fatigue, and, for three or four successive contractures, with 30 minutes' rest between each, as was used for the fixed muscle, there was no appreciable difference in the tension records. A tension drop with a time course similar to that shown in Fig. 2 *d* was observed on the addition of OsO_4 to an electrically stimulated muscle, and the

TABLE V

Isolated I Segment Lengths

	Uranyl acetate negative stain				PTA negative stain	Shadowed segments		
	No fixation	3 per cent formalin	OsO_4 vapour	OsO_4* solution	OsO_4 vapour	No fixation	OsO_4 vapour	Freeze-dried
Fresh frog								
Mean value, μ	1.95							
No. of measurements	15							
Standard deviation, μ	0.03							
Glycerinated chicken								
Mean value, μ	1.99	2.0	1.98	1.8	2.06	2.05	2.04	2.05
No. of measurements	24	16	30		10	23	6	7
Standard deviation, μ	0.05	0.03	0.01		0.03	0.03	0.01	0.03

* Segments treated with OsO_4 in solution, not on grid.

that only some of the fibres or fibrils are excited, and so only part of the full tension of which the muscle is capable is developed. However, all parts of the normally fixed muscle are different from the excited muscle, and it is considered unlikely that some of the muscle is excited by the fixative, and that some of it is at rest.

(A muscle that is depolarized in isotonic KCh_3SO_4 and allowed to relax from the resultant potassium contracture still will shorten when OsO_4 is poured onto it, and it cannot be distinguished from a normally fixed muscle in terms of its filament lengths. This suggests that the stimulating effect of the OsO_4 is not simply due to depolarization of the surface membrane.)

The tension record of a muscle fixed during a maintained tension was again about 60 to 70 per cent of the maximum tetanic tension.

FILAMENT LENGTHS IN EXCITED MUSCLE: Measurements of filament lengths in excited muscle (*i.e.* fixed during a potassium contracture, or while being tetanically stimulated) are given in Table VI. In the isometrically contracting muscles the A and I filament lengths were found to be virtually constant over the range of sarcomere lengths studied (2.2 to 3.7 μ). The I filaments were 2.0 to 2.05 μ long, *i.e.* longer than in the other OsO_4-fixed muscles (1.7 to 1.95 μ) but very little more than in the glutaraldehyde-fixed muscles (2.0 μ), and the same as the isolated I segments. The A filament lengths in the excited muscle (1.6 μ) were also found to be longer than those in

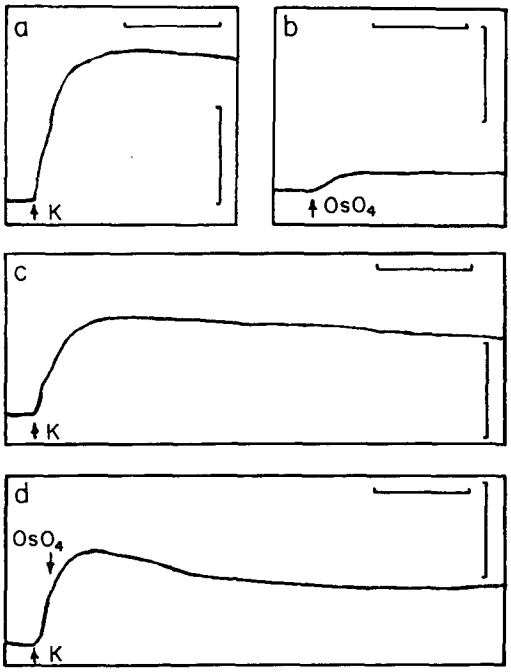


FIGURE 2 (Horizontal bar: 10 seconds; vertical bar: 0.5 gm)

Fig. 2 *a*. Record of tension developed in a potassium contracture of a frog toe muscle.

Fig. 2*b*. Record of tension developed during OsO_4 fixation of the same muscle as in Fig. 2*a*.

Fig. 2*c*. Record of tension developed in a frog toe muscle on addition of a 50 mM solution of potassium.

Fig. 2*d*. Record of tension developed in the same muscle as in Fig. 2*c* on addition of a 50 mM solution of potassium (first arrow) followed by a 1 per cent solution of OsO_4 containing 50 mM potassium (second arrow).

the other OsO_4-fixed muscles (1.45 to 1.55 μ) but the same as in the glutaraldehyde-fixed tissue. In the case of the muscle with a gap between the two sets of filaments, the I filaments were a little shorter (1.98 μ), although the A filament length was still 1.6 μ.

The fine periodicity was measured in these muscles in both the A and the I bands (Table VII). With one exception, the periodicity in the A band was about 400 A; and in the I band, apart from one exception, it was also found to be about 400 A; *i.e.*, the same in both bands. In the first exception, in a muscle where the A filament length was shorter (1.54 μ) than in the other muscles, the periodicity in the A band was only 380 A; and in one other muscle, the periodicity in the I band was higher (429 A) and the I filament was longer (2.09 μ) than in the rest of the muscles.

Both the A and the I filament lengths (1.6 μ and 2.0 to 2.05 μ) in muscles which had shortened against a load were found to be the same as in those muscles which had either not shortened or shortened against zero load before the contraction became isometric at the length in question.

MEASUREMENT OF THE FINE PERIODICITY: Measurements of the fine periodicity in frog, toad, and rabbit muscles are given in Table VIII. Because of the additional and uneven shortening of the I filaments which has been shown to occur if the muscle is unrestrained during dehydration, measurements were made only on muscles that were held during dehydration. It can be seen that, in frog muscle, the periodicity varies with the I filament length from 350 to 405 A. As the previous experiments have indicated that the I fila-

TABLE VI

Filament lengths in Excited Frog Toe Muscles

Sarcomere	I filaments			A filaments			Method of excitation
		N	SD		N	SD	
μ	μ		μ	μ		μ	
3.3	1.99–2.06			1.56–1.63			Shortened under load
2.2	2.04	23	0.02	1.62	11	0.02	Potassium contracture (isometric)
2.3–2.6	2.04	23	0.02	1.56	17	0.02	Electrical stimulation "
2.6–2.8	2.01	8	0.02				Potassium contracture "
2.7–3.1	2.03	21	0.02	1.56	12	0.02	Potassium contracture "
3.3	2.05	15	0.02	1.61	10	0.01	Electrical stimulation "
3.7	1.98	17	0.02	1.61	4	0.01	Electrical stimulation "

TABLE VII

Periodicity in Excited Muscles

Sarcomere	I filament	400 A in I band	A filament	400 A in A band	Method of excitation
μ	μ	A	μ	A	
2.3	2.0	407	1.6	400	K contracture
2.5–2.6	2.09	429	1.6	395	stimulation
2.7	2.01	410	1.54	381	K contracture
3.35	2.05	403	1.6	399	stimulation
3.8	1.98	397			stimulation

TABLE VIII

Measured Periodicities

Muscle	Preparation	Sarcomere	I filament	Periodicity	n	SD	Corrected periodicity
		μ	μ	A		A	A
Frog sartorius	1 per cent OsO_4	2.7	1.85	365	43	10	403
" "	KCH_2SO_4, 1 per cent OsO_4*	3.0	1.92	380	31	10	406
" "	Glutaraldehyde‡	2.8	1.98	389	15	12	403
Frog semitendinosus	1 per cent OsO_4	2.05	1.92	387	12	18	414
" "	" NPTA§	3.7	1.93	379	9	11	403
" "	Carlsen‖	2.05	1.80	354	23	9	402
" "	"	2.75	1.78	353	28	15	407
" "	"	3.55	1.81	365	10	13	413
Frog ext. dig. long. IV	Excited¶	2.3	2.00	407	9	15	417
" "	"	3.35	2.05	403	23	6	403
" "	"	3.8	1.98	397	14	12	411
			Mean value for frog		217		406
Toad sartorius	1 per cent OsO_4	2.3	1.84	354	9	15	395
" "	"	2.5	1.70	337	46	11	407
" "	"	3.0	1.71	338	44	9	408
			Mean value for toad		99		406
Rabbit psoas	1 per cent OsO_4	2.4	2.11	384	14	8	408
" "	"	2.8	2.12	380	21	6	402
			Mean value for rabbit		35		404
		Mean value for all animals			351		406

* Muscle depolarized in isotonic KCH_3SO_4 before fixation in 1 per cent OsO_4.

‡ Muscle fixed in glutaraldehyde, added stepwise in concentrations 0.06 per cent, 0.6 per cent, and 6 per cent.

§ Muscle not stained with PTA before embedding.

‖ Muscle fixed by the method used by Carlsen *et al.* (1961) on resting muscles.

¶ Muscles in K contracture or electrically stimulated when fixed.

ment length in the living frog muscle is 2.05 μ, and that the lower values in OsO_4-fixed tissue are most likely due to OsO_4 and alcohol induced shortening only, (these conclusions are argued in more detail later), a correction factor of (2.05 ÷ I filament length in the section) has been applied to these measurements. The same factor has been applied to the measurements on the toad muscle, as its I filament length is believed to be the same as that in the frog (see below); for the rabbit muscle where the I filament length is 2.24 μ (H. E. Huxley, personal observation) the correction factor used was (2.24 ÷ I filament length in the section). The corrected values, which are listed in the final column of Table VIII, show that the periodicity is the same in the frog (406 A), the toad (406 A) and the rabbit (404 A). The mean value for all three animals is 406 A (351 measurements, standard deviation of the mean, 12 A).

BAND PATTERNS IN SHORTENED MUSCLE: In most of the muscles studied, the fibrils within a given region of a fibre had fairly uniform sarcomere lengths, within 0.1 or 0.2 μ of each other, although they varied more than this from one part of the fibre to another. In the muscles which shortened from a stretched length, however, some areas were found which showed a large variation in sarcomere length and in band pattern from one sarcomere to the next (Fig. 4). Some sarcomeres had shortened to such an extent that there was no I band, others had wide I bands with very little amount of overlap with the A filaments. In some cases, one half of the sarcomere had no I band, whereas the other end had one, and there was sometimes the same asymmetry about the Z line; *i.e.*, on one side of it the A filaments reached the Z line, and on the other side there was a wide I band. In the sarcomeres in which the I band was absent, the region of greater density within the A band, reflecting the length of overlap of the A and I filaments, could be seen ending beyond the M line. But in all cases the I filaments were about 2.05 μ long; *i.e.*, the same after active shortening as after passive shortening or stretch and subsequent isometric stimulation.

The figures chosen to accompany this text have been selected because they illustrate details concerning the filaments, and the sarcoplasmic reticulum is poorly preserved in them. However, in material where it has been well fixed, the organization of this system has been compared in excited and resting msucles. No differences have been found.

DISCUSSION

The experiments reported here confirm the deductions of A. F. Huxley and Peachey (1961) that the preparative procedures do produce changes in the lengths of the filaments, and further show that the extent of these changes varies considerably with the method of preparation. The results of these experiments are summarized below:—

1. *Changes During Fixation*

Both sets of filaments shorten during OsO_4 fixation if free to do so. The extent of this shortening, which is uniform along the length of the I filament, depends on the method of fixation, and the state of the muscle at the time of fixation, and can be as much as 10 per cent in the I filaments and 6 to 7 per cent in the A filaments.

Glutaraldehyde probably causes some shortening (2 to 3 per cent) of the I filaments, but very little, if any, shortening of the A filaments.

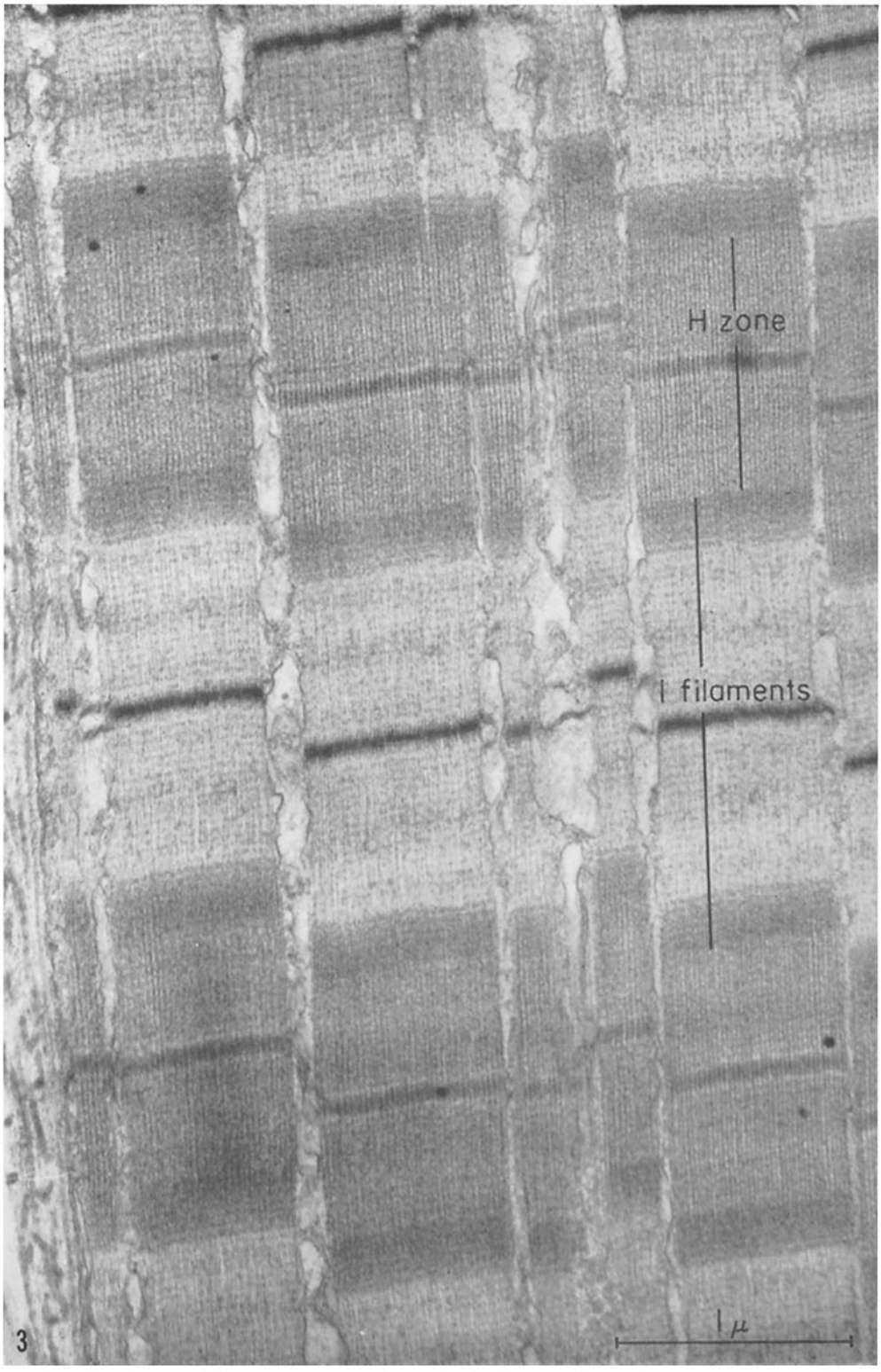
2. *Changes During Dehydration*

Alcohol causes shortening of the I filaments if they are free to shorten (up to 10 per cent), whereas acetone causes little, if any.[1] The length of the I filaments within the I band has a greater tendency to shorten than the part within the A band.

The A filaments probably do not shorten appreciably during dehydration.

[1] The greater shortening produced by the alcohol compared with the acetone is of interest in view of Selby and Bear's finding (1956) that acetone did not destroy the x-ray reflections from actin, although alcohol did.

FIGURE 3 Frog sartorius muscle, depolarized in isotonic KCH_3SO_4 solution before fixation in 1 per cent OsO_4 solution, and held at a constant length during dehydration, stained with PTA before embedding. The fine periodicity along the length of the I filaments, ending at the H zone, is clearly visible. The I filament length in this muscle is 1.91 μ, which is believed to be shorter than the real value in the living muscle, because of shortening during fixation (see page 386). × 35,000.


H zone
I filaments
1 μ
3

Some of the shortening of the I filaments during dehydration is prevented by holding the muscle at a constant length during this step.

3. *Changes During Embedding in Araldite*

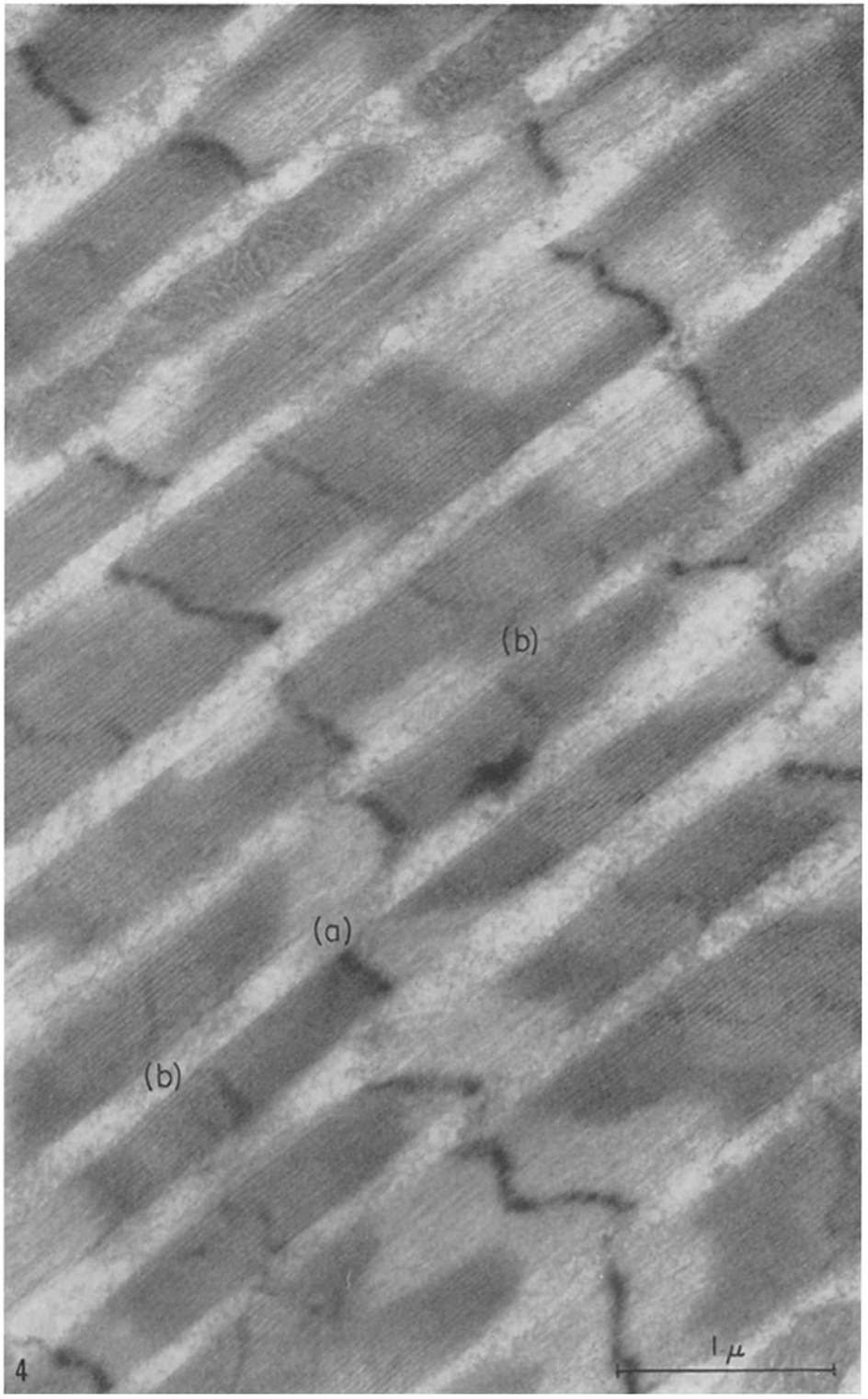
Little change occurs during this stage. A simple general explanation, based on the observations made here, can be proposed for the variations in the filament lengths with the state of the muscle when fixed. If there are links of any kind between the two sets of filaments, and if the total length of the muscle is held constant, then these links will tend to reduce shortening of the filaments (or to produce stretching of one set of filaments while the other shortens). The actual amount of shortening that takes place would be governed by the nature and number of these links (and the magnitude of the resistance they could offer to slipping of the filaments), and by the time at which they were formed.

Before the amount of shortening in a particular set of circumstances can be discussed, the length of the living filaments must be established. In the case of the I filaments, a value for this can be obtained from the measurements on the unfixed isolated I segments. Of the various methods used to prepare these, that in which the segments were frozen-dried and then shadowed is considered least likely to produce any shortening. There is no treatment with any chemical in this procedure, and no drying of a liquid phase when surface tension effects may interfere. The I segment length of glycerinated chicken muscle is 2.05 μ after this method of preparation, which is longer than when the segments are negatively stained with uranyl acetate, but the same as with the PTA negative stain and the other shadowed preparations. This length is therefore taken as the length of the I filaments in the glycerinated chicken muscle before further treatment. The true length may be longer, as some shortening may have taken place, but is probably not shorter since the same result is obtained by two different methods, in neither of which stretching would be expected.

On the basis of the following evidence, this value of 2.05 μ is considered to be the length of the I filaments in the living frog muscle, also. The chicken and frog muscles studied have the same number of fine periodicities along the length of the I filaments; *i.e.*, 24 on either side of the Z line. Since the value of this period is probably the same in both animals, (all the muscles that have been studied by x-ray diffraction methods have the same actin period), it follows that the lengths of the actin filaments are the same in both the living muscles. Now the measurements on the isolated I segments were made on glycerinated muscle, not living tissue. However, Worthington (1959) has shown that the x-ray reflections from actin, as well as the 410 A reflection, are unchanged after glycerol extraction of rabbit muscle; the actin filament lengths in the glycerinated chicken muscle therefore are taken to be unchanged by this treatment. This value of 2.05 μ is greater than the length of the few fresh frog I segments that were obtained (which were 1.95 μ long), but these were negatively stained with uranyl acetate which has been shown to cause shortening of the glycerinated segments, and so the longer length is considered more reliable.

A value for the I filament length can be derived in a different way. If the periodicity observed is considered to correspond to one of the axial periods shown up in the x-ray diffraction patterns, it must have a value of either 350 or 410 A. (The 435 A period is not included as it is believed to arise from the myosin filaments.) The measured value of this period is greater than 350 A in sections of muscles in which little shortening has taken place (Table VIII), so the figure of 410 A is taken. Multiplying this by 48 (Fig. 3) the number of periods seen along the length of the I filaments, and adding the width of the Z line as measured from sections, we arrive at a value of 2.05 μ for the I filament length. (The same calculation, but based on the value of

FIGURE 4 Frog toe muscle, shortened against a load, and then fixed in OsO_4 while being stimulated. Stained with PTA before embedding. There is considerable variation in band pattern and in the amount of overlap between the two sets of filaments, even within a given sarcomere. There is also asymmetry about the Z line in some cases (*a*). When there is no I band, the I filaments can be seen to end beyond the M line (*b*). Despite the very great changes in band pattern, the A and I filament lengths can be seen to be constant. × 32,000.


(b)
(a)
(b)
1 μ
4

350 A, gives an I filament length of 1.75 μ, a value that is less than the I filament lengths measured in sections of muscles which have been clamped at a fixed length during dehydration, Table III.)

There is no "reference" preparation such as the I segments in which the A filament length could be measured before fixation or dehydration. When isolated A filaments were prepared from fresh frog muscle, they were found to break up into shorter lengths. A few bundles of whole A filaments (lengths 1.6 μ), attached at the M line, were prepared from glycerinated chicken muscle, but there is no evidence to show that the frog and chicken A filaments have the same length. (Their lengths are much the same in embedded muscles, but, because of the changes known to be produced during preparation, this is not sufficient evidence.) The A filaments in glutaraldehyde-fixed muscle (1.6 μ) are longer than in the OsO_4-fixed tissue. It has been shown that glutaraldehyde causes very little shortening of the I filaments, so it may also preserve the length of the A filaments well. They are not likely to have stretched, and so the length of the A filaments in the living muscle is probably not less than 1.6 μ, although it may be a little greater.

The sum of the A and I filament lengths deduced here (1.6 μ and 2.05 μ) is 3.65 μ, a little greater than the value of 3.52 μ given by A. F. Huxley and Peachey (1961). However, the correction factor applied by these authors to compensate for filament shortening during preparation did not include any allowance for shortening during the fixation stage, which may account for the difference between the two figures.

Given, then, the figures of 2.05 μ for the I filament length and 1.6 μ for the A filament length in the living resting frog muscles, it is apparent that the I filaments in both the OsO_4- and the glutaraldehyde-fixed muscles have shortened (ignoring for the moment the excited muscles), and that the A filaments have shortened in the OsO_4-fixed tissue. The question then arises whether this shortening takes place during fixation, dehydration, or both. Since very much more shortening occurs in the OsO_4-fixed muscle than in the glutaraldehyde-fixed muscle, even though both have the common dehydration step, this suggests that much of the shortening of the OsO_4-fixed tissue occurs during the fixation. It might be argued that the OsO_4-fixed filaments shorten more than do those in the glutaraldehyde-fixed material

FIGURE 5 All the muscles were stained with PTA before embedding.

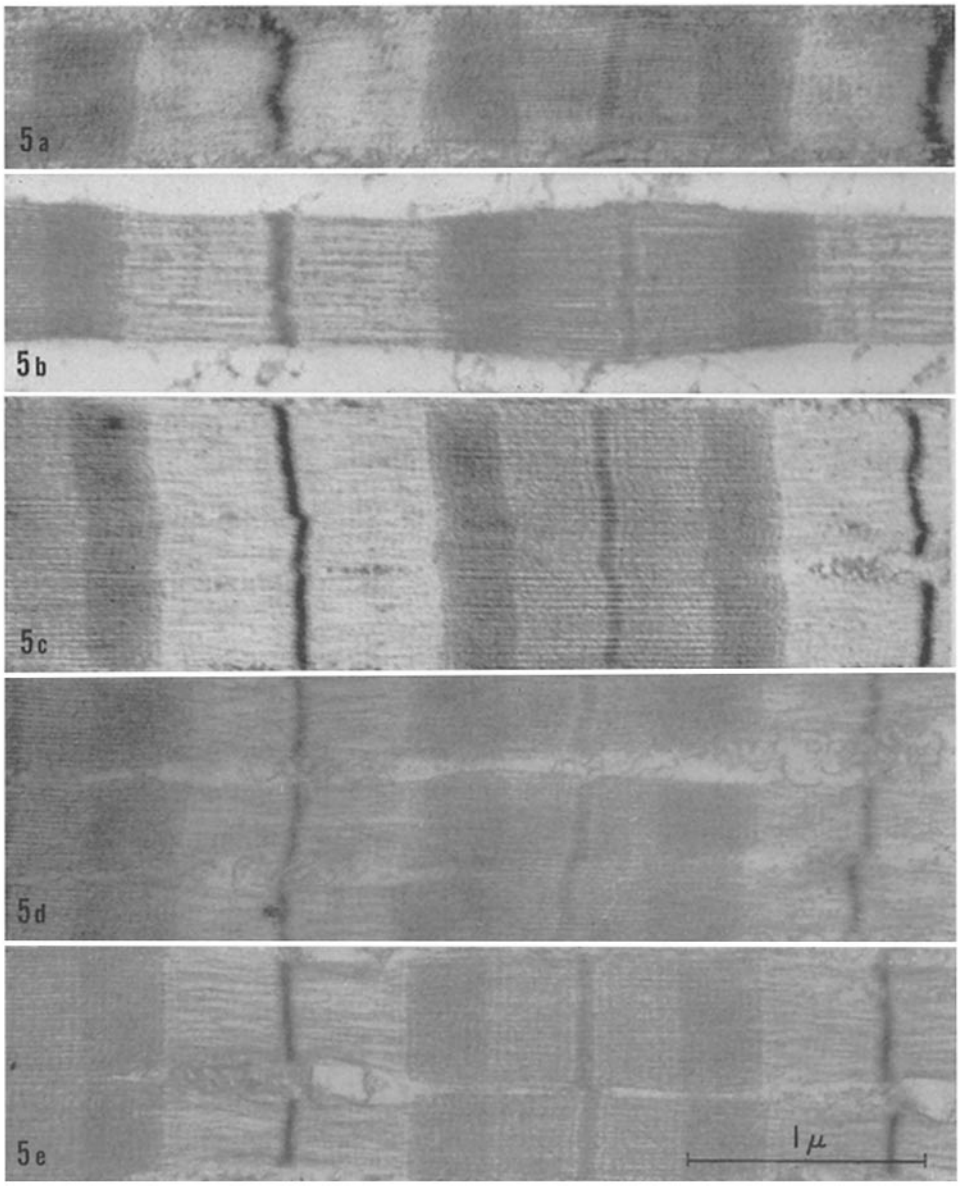
Fig. 5*a*. Frog toe muscle, fixed with OsO_4 during a potassium contracture, and held at a constant length during dehydration. The A filament length is 1.56 μ and the I filament 2.03 μ; these values approximate to what are believed to be the real values in living muscle. This is considered to be because the filaments are prevented from shortening, during fixation, by cross-links between the A and I filaments developed as a result of stimulation.

Fig. 5 *b*. Frog sartorius, fixed with glutaraldehyde added stepwise in concentrations of 0.06 per cent, 0.6 per cent and 6 per cent. Held at constant length during dehydration. Again, the values of the A and I filament lengths (1.50 μ and 2.00 μ, respectively) approximate to what are believed to be the true values in living muscle. It is thought that the glutaraldehyde, unlike OsO_4, causes little shortening of the filaments during fixation (see page 371).

Fig. 5 *c*. Frog sartorius, fixed in 1 per cent OsO_4, and held at a constant length during dehydration. The A and I filament lengths here (1.44 μ and 1.82 μ, respectively) are believed to be shorter than the true lengths because of shortening of the filaments caused by OsO_4. This is a relatively relaxed muscle, and so this shortening will not be inhibited completely by cross-links.

Fig. 5 *d*. Frog semitendinosus, soaked in Ringer's solution containing 13 mM K, and then fixed with OsO_4 added stepwise in concentrations of 0.01 per cent, 0.1 per cent and 1 per cent. The I filaments are even shorter in this muscle (1.75 μ; A filament length 1.46 μ). This is probably due to complete freedom of the filaments to slide past each other during the early stages of fixation in this relaxed muscle.

Fig. 5 *e*. Frog semitendinosus, fixed as for 5 *d*, but muscle free to shorten during dehydration. The A and I filament lengths are 1.47 μ and 1.71 μ, showing that there is additional shortening of the I filaments during dehydration. × 31,500.


5a
5b
5c
5d
5e
1 μ

in the alcohol, but this is not thought to be the case, for the following reasons. The A band does not shorten further in the unrestrained than in the clamped muscle in the OsO_4-fixed tissue, and this suggests that the A filaments do not shorten in either case during dehydration. Alternatively, it may be that they shorten to the same extent in both the clamped and unrestrained muscles. However, as the I filament period within the overlap region of the A band is the same in both cases, it is likely that by the end of fixation there is some sort of linking between the A and I filaments that prevents shortening of the I filaments in this region of overlap. If this is so, and indeed by the end of fixation the muscle is so rigid that it cannot be stretched without tearing, then further shortening of the A filaments during dehydration can only occur if the I filaments are stretched within the I band, and the period within the A band would be less than that within the I band. This does not appear to be the case, and hence, the A filaments probably do not shorten in the clamped muscle in OsO_4-fixed material during dehydration, nor in the unrestrained muscle either. The shortening of the A filaments therefore must occur during fixation, and, by the same argument, the I filaments also do not shorten during dehydration of the clamped muscle.

Further evidence that, in the case of the muscles fixed in 1 per cent OsO_4 solution, little shortening occurs during dehydration of the held muscle, comes from a comparison of the muscles dehydrated in alcohol with those dehydrated in acetone. After acetone treatment, the I filaments in the unrestrained muscle are no shorter than those in the held muscle, even though the filaments are free to shorten in the unrestrained muscle and not in the held muscle, (or less so). This indicates therefore that there is no tendency for the I filaments to shorten during acetone dehydration, and that all the shortening in these muscles must have taken place during fixation. Now, the lengths of both the A and the I filaments in the muscle dehydrated in acetone are the same as those in the alcohol dehydrated, held muscles, so that in these muscles, also, the shortening of the filaments most probably occurs during fixation, not dehydration.

It has been found also that the more relaxed the muscle is in the early stages of OsO_4 fixation, the greater is the shortening of the I filaments below the *in vivo* length. We have just argued that this shortening occurs mainly in the OsO_4, not during dehydration, so we can say that the amount of shortening produced by the OsO_4 is greatest under conditions in which, according to the sliding filament model, links between the two sets of filaments are absent, so that they are able to slide past each other. Hence shortening would be able to proceed freely even though the muscle is held at a fixed length; and the short I filaments (1.8 μ) in the muscles fixed by the method of minimal excitation can be accounted for in this way. However, this does not explain why those muscles, fixed in this manner, which have a very long sarcomere (more than 3.5 μ), also have longer I filaments (1.9 μ). It may be that, in these muscles, shortening of the I filaments by OsO_4 is inhibited, not by links between the two sets of filaments, but by some sort of elastic connections between the I filaments across the H zone, the S filaments of Hanson and H. E. Huxley (1955), which it was suggested were responsible for some of the considerable resting tensions at these lengths.

Muscles that are fixed in the normal 1 per cent OsO_4 solution are weakly excited by the fixative, so that, according to the sliding filament model, one would expect that some links would be formed between the A and I filaments and that these would inhibit some of the OsO_4-induced shortening. (The tension developed by these muscles is smaller, probably because only a few links are formed between each pair of filaments, and these might be too few, or not strong enough, to prevent all the shortening.) The I filament lengths in these muscles (1.85 to 1.95 μ) are in fact found to be greater than those in the more completely relaxed muscles discussed in the previous paragraph (1.8 μ) and yet shorter than in unfixed I segments (2.05 μ); and so again the observations can be accounted for quite well. (See Fig. 5.)

In the muscles fixed while in potassium contractures or during tetanic stimulation, one would expect that there would be many links between the A and I filaments before fixation, and hence the shortening of both sets of filaments during fixation should be more strongly inhibited or even fully prevented, unless one set of filaments shortens and stretches the other. In practise we find that the I filament length is 2.05 μ, and the A filament length 1.6 μ. The same values are found also in the muscles which have previously shortened against a load and where contraction had become isometric before fixation. Much the simplest explanation of these values is that they are the same as in the

living excited muscle and that no shortening or stretching of either has occurred during preparation. Since the periodicity is the same in both the A and the I bands, if any shortening or stretching of the I filaments has occurred it has been uniform along their whole length. This argues against shortening of one set of filaments while the other stretches, since that part of the I filaments within the A band would be expected to change as the A band does, not as the I band. It could be that the periodicity is different in the region of overlap in the living muscle, but, if so, it would have been a remarkable coincidence that this region should have been stretched or shortened, during fixation and dehydration, just enough to match the rest of the I filament. Again, it could be that the whole length of the I filaments shortens and that only the H zone region of the A filaments is stretched; but the constancy of the A filament lengths at different sarcomere lengths, and the fact that they are not stretched by comparison with the glutaraldehyde-fixed muscles, make this an unlikely explanation.

In two cases, some shortening or stretching of the filaments does appear to have occurred. In one, the I filament length was found to be 2.09 μ and in this muscle the periodicity in the I band was correspondingly high, 429 A. In the other, the A band filaments are shorter than 1.6 μ, and the periodicity within the A band is correspondingly low, 380 A. Further, in the muscles with sarcomere lengths of 3.7 μ, there is no overlap between the two sets of filaments. The shorter I filaments in these (less than 2.0 μ) can thus be accounted for by the lack of links which may inhibit OsO_4 induced shortening. Presumably there is some restraint from the S filaments, but not so much as from links between the filaments.

The question arises whether these muscles were fully active when actually fixed, as only 60 to 70 per cent of the maximum tension is maintained during fixation. No variation in filament lengths was found between different fibres or between different parts of the same fibre, so presumably all the fibres were in the same condition at the time of fixation, and the tension drop was not due to the inner fibres fatiguing before the fixative reached them. Although this drop in tension occurs during the early stages of fixation and is caused by some action of the fixative, it does not follow that it is due to relaxation of some kind initiated by the OsO_4; there is little reason to expect that a fixed muscle should generate as much tension as an actively contracting one, particularly once the series elastic element is also fixed. On the other hand, Carlsen *et al.* (1961), working with single fibres, found that the tension dropped less than 2 per cent on addition of the fixative; this suggests that the muscles in the present experiments were not fully active when fixed. However, they were at least 60 to 70 per cent active, and could clearly be distinguished from the muscles fixed while resting.

Taking, then, the measured values of the A and I filament lengths in the embedded excited muscles (1.6 μ and 2.05 μ, respectively) as being those in the living excited muscles, whether after active shortening or not, and comparing these with the values of 1.6 μ and 2.05 μ deduced for the lengths in the living, resting muscles, we find that they are the same in both physiological states of the muscle. Further, these values for both sets of filaments do not vary with sarcomere length in either the resting or the excited muscle (Fig. 6). These two findings provide therefore very strong support for the sliding filament theory of muscular contraction. It is true that, because of the changes produced by the preparative procedures, the constancy of the filament lengths has not been demonstrated directly, and indeed this would seem to be impossible until a method of preparation known to produce no length changes is available. But as far as we have been able to investigate the length changes in the different muscles, it does appear that these changes are all produced during preparation, and that the filament lengths are constant over the whole range of functional states of the living muscle which have been examined. These results do not exclude, of course, the possibility of small local length changes in either filaments, associated with different physiological states of the muscle, provided that the over-all length change so produced is less than about 2 per cent.

This conclusion is rather different from that reached by Carlsen *et al.* (1961). Although these authors found values much the same as those in this present study for the filament lengths in the "resting" muscles and in those muscles which had actively shortened and whose sarcomere lengths were more than 2.1 μ (*i.e.* where there was an H zone so that the I filament length could be measured), their interpretation of these results was different in one important aspect. They recognized that changes in filament length during preparation could affect the results, but, because of their finding that the sarcomere length was un-

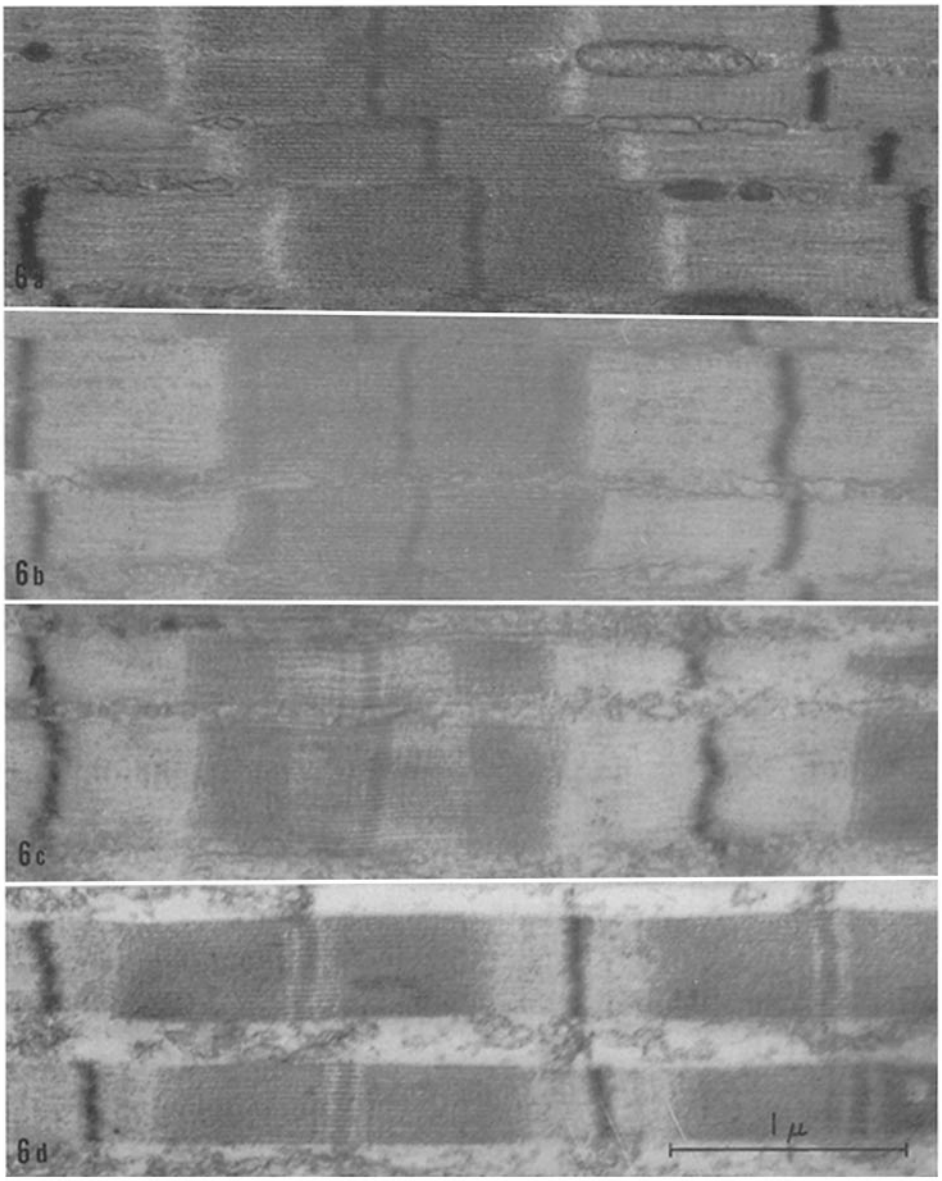


FIGURE 6 Frog toe muscles, fixed with 1 per cent OsO_4, held at a constant length during dehydration, and stained with PTA before embedding.

Figs. 6 *a* and 6 *b*, fixed while being isometrically stimulated.

Figs. 6*c* and 6*d*, fixed during a potassium contracture.

The A and I filaments are approximately 1.6 and 2.0 μ in each muscle; *i.e.*, over the range of sarcomere lengths from 3.7 to 2.15 μ. These lengths approximate to what are believed to be the true lengths in living muscle, because shortening of the filaments during fixation is considered to be prevented by cross-links between the two sets of filaments, developed as a result of stimulation. In the very stretched muscle, where there is no overlap between the filaments, the fine periodicity clearly stops at the end of the I filament. (See I filaments at the right of Fig. 6 *a*.) × 31,500.

changed by their preparative procedures, they applied no correction for such shortening and took the measured values as being very probably the same as those in the living fibre. The present studies, however, have shown that shortening of the filaments can and does take place while the sarcomere length remains constant, so that the filament lengths in the embedded muscle may be very different from those in the living muscle. In the case of the isometrically contracting muscles, the values of the filament lengths reported here (1.6 μ and 2.05 μ) are much larger than those of Carlsen *et al.* (1961) (1.4 μ and 1.7 to 1.8 μ). This difference is at the moment inexplicable.

Since the I filament length in all states of the living frog muscle is believed to be 2.05 μ, and since all deviations from this value in the embedded material are considered to be entirely due to shortening during preparation, the measurements of the fine periodicity in the frog muscles were corrected by the factor (2.05 ÷ I filament length in section). The toad muscles have the same number of periods along the length of the I filaments as the frog muscles (24 on either side of the Z line), and, as has already been discussed, this indicates that the I filaments are the same length in both animals; the same correction factor therefore was used for the toad. The rabbit psoas muscle, on the other hand, has 26 periods on either side of the Z line, and, since H. E. Huxley (personal observation) has shown that its unfixed I filament length is 2.24 μ, the correction factor used for this muscle was (2.24 ÷ I filament length in section). The corrected mean value of these measurements (406 A) then can be compared with the values for the axial periods obtained from the x-ray diffraction data. As already discussed, it has not been possible from the x-ray evidence to decide with certainty between the values of 350 A and 410 A for the actin axial period. We can say now that 410 A is the probable actin period in living muscle, since this is the periodicity visible along the length of the actin filaments in the electron microscope.

Recently, Hanson and Lowy (1962, 1963) have studied isolated actin filaments from fresh and glycerinated muscles by negative staining techniques, and have found that the filaments have a two-stranded helical structure (not a planar one) with a repeat distance between the helical cross-over points of 350 A. There are two lines of evidence, in addition to the one already mentioned in the introduction, which indicate that the difference between this figure and that measured in whole fixed muscle is unlikely to be due to shortening of the isolated filaments during preparation. First, the isolated I segments measured in the present study were prepared by similar methods, including negative staining. Their lengths, however, were 2.05 μ, which has been shown to correspond to a 410 A repeat, there being 48 periods along the filament; whereas if the filaments had shortened until the periodicity was 350 A, the lengths of the I segments would have been approximately 1.75 μ (48 × 350 A + Z line). Moreover, H. E. Huxley (personal observation) has found that the methods of isolation and negative staining do not produce length changes in rabbit I segments. If, however, the pitch of the helix is altered when the filaments are isolated from muscle, or when they are negatively stained, so that the distance between cross-over points becomes 350 A, not 410 A, (*i.e.* 13 residues per turn instead of 15 per turn), the length change brought about by this would be roughly not more than 1 per cent (Hanson and Lowy, personal communication).

The simple helical structure found by Hanson and Lowy (1962, 1963) in actin filaments isolated from muscle and in filaments from F actin preparations would not give rise to the periodic variation in density seen along the filaments in sections. The regions of greater density, therefore, must be due to something attached to the actin helix, which is stained both by OsO_4 and by other electron stains such as PTA, and which is not extracted by glycerol, since the periodicity is sometimes seen in glycerinated muscle. Perry and Corsi (1958) and Corsi and Perry (1958) found that tropomyosin and actin were extracted in constant relative proportions when muscle fibrils were treated with certain solvents, even though the absolute amounts might vary. When such fibrils were examined in the phase contrast microscope, it was found that the I band was removed. They suggested therefore that the two proteins were associated in some way in the muscle. It may be that it is the tropomyosin, attached to the actin helix, which produces the fine periodicity of the I filaments. Whatever it is, it is probably this material which in the x-ray diffraction diagram of muscle gives rise to the "non-net" reflections observed by Selby and Bear (1956) and the non-actin 410 A spacing seen by Worthington (1959). This reflection is on the meridian, and so does not come from a helical structure. It has the same magnitude as that ob-

served in the electron microscope; and further, it is shortened to as little as 360 A by treatment with formalin and PTA in the same way that the periodicity seen in the electron microscope shortens when treated with OsO_4 and with alcohol.

As has been shown, because of the various length changes which take place during preparation, it is not easy to determine natural filament lengths in the electron microscope. However, now that the magnitude of the fine periodicity is known, a simple method of determining the I filament length in other muscles is available, assuming that this periodicity is the same in all animals. The number of fine periods along the length of the I filaments can be counted, and this, multiplied by 410 A, together with the width of the Z line measured from sections, gives a value of the I filament length. Thus in rat leg muscle, which has 27 periods on either side of the Z line, a value of 2.3 μ for the I filament length may be deduced, as the Z line is about 800 A wide. Similarly, human muscle has at least 30 periods on either side of the Z line, so that its I filament length is at least 2.55 μ. Such differences in I filament lengths in different muscles may have measurable physiological consequences.

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REFERENCES

ASTBURY, W. T., and SPARK, L. C., 1947, *Biochim. et Biophysica Acta*, **1**, 388.

BEAR, R. S., 1945, *J. Am. Chem. Soc.*, **67**, 1625.

CARLSEN, F., KNAPPEIS, G. G., and BUCHTHAL, F., 1961, *J. Biophysic. and Biochem. Cytol.*, **11**, 95.

COHEN, C., and HANSON, J., 1956 *Biochim. et Biophysica Acta*, **21**, 177.

CORSI, A., and PERRY, S. V., 1958 *Biochem. J.*, **68**, 12.

ELLIOTT, G. F., and WORTHINGTON C. R., 1959, *J. Physiol.*, **149**, 32P.

HANSON, J., and HUXLEY, H E., 1953 *Nature*. **172** 530.

HANSON, J., and HUXLEY, H. E., 1955, *Symp. Soc. Exp. Biol. and Med.*, **9**, 228.

HANSON, J., and LOWY, J., 1962, *in* 5th International Congress for Electron Microscopy, Philadelphia, 1962, (S. S. Breese, editor), New York, Academic Press, Inc., **2**, 09.

HANSON, J., and LOWY, J., 1963, *J. Mol. Biol.*, **6**, 46.

HUXLEY, A. F., and NIEDERGERKE, R., 1954, *Nature*, **173**, 971.

HUXLEY, A. F., and PEACHEY, L. D., 1961, *J. Physiol.*, **156**, 150.

HUXLEY, H. E., 1953, *Proc. Roy. Soc. London, Series B*, **141**, 59.

HUXLEY, H. E., and HANSON, J., 1954, *Nature*, **173**, 973.

KNAPPEIS, G. G., and CARLSEN, F., 1962, *J. Cell Biol.*, **13**, 323.

PALADE, G. E., 1952, *J. Exp. Med.*, **95**, 285.

PERRY, S. V., and CORSI, A., 1958, *Biochem. J.*, **68**, 5.

SABATINI, D. D., BENSCH, K. G., and BARRNETT, R. J., 1962, *Anat. Rec.*, **142**, 274.

SELBY, C. C., and BEAR, R. S., 1956, *J. Biophysic. and Biochem. Cytol.*, **2**, 71.

WORTHINGTON, C. R., 1959, *J. Mol. Biol.*, **1**, 398.