QUANTITATIVE STUDIES ON THE STRUCTURE OF CROSS-STRIATED MYOFIBRILS

I. INVESTIGATIONS BY INTERFERENCE MICROSCOPY

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INTRODUCTION

The cross-striated appearance of voluntary muscle fibres has its origin in the contractile material, which is contained in the myofibrils. The myofibrils, 1–2 μ in diameter, display the same pattern of bands as the intact fibre, in which they are arranged in register. The most outstanding feature of the pattern is a succession of birefringent and non-birefringent bands, known as the *A* (anisotropic) and *I* (isotropic) bands, alternating along the length of each myofibril. The *A* bands have a much higher density than the *I* bands, as may readily be seen in both the phase contrast light microscope and the electron microscope. Recently, the suggestion was put forward[1,2] that the higher density and birefringence of the *A* bands is due to the concentration there of all the myosin in the fibrils. This hypothesis was based on the observation that, when fibres or fibrils were treated with solvents known to extract myosin selectively from whole minced muscle, the material whose presence gave the *A* band its high density and birefringence was removed; a ghost fibril was left in which the original *A* band region now had the same density as the *I* bands. The density of the *I* bands, as seen in phase contrast, did not appear to change substantially during the extraction.

This concept of the localisation of myosin in the *A* bands, together with recent X-ray diffraction and electron microscope observations[3–5] has formed the basis of a model structure for muscle[1,5] in which two sets of overlapping filaments slide past each other when the muscle changes length[6,7,9]. This model can explain many of the properties of striated muscle, such as the changes in band pattern visible during contraction and stretch, and the loss of extensibility during rigor or contraction, and it seems to provide a promising basis for more detailed theories of contraction[9]. It is therefore very important that the experimental basis of the model should be established beyond all possible doubt. The existing evidence that myosin is located

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in the *A* bands, though strong, is not completely conclusive, for it needs quantitative support. The purpose of this paper, and the one that follows it, is to provide this information.

The amounts of myosin, actin and other protein fractions in the skeletal muscles of the rabbit have now been fairly well established by biochemical analysis. Myosin is known to make up about 50% of the total protein of the myofibrils. If it were possible to measure, *in situ*, the relative amounts of protein in the different bands of a myofibril, and especially the amount of "extra" material in the *A* band, then a comparison with the biochemical data would show whether this "extra" material and the myosin fraction could be identical and would give considerable information as to the general correctness or otherwise of the model. Moreover, when myosin is selectively and completely extracted from myofibrils, only a small amount (10–20%) of the other myofibrillar protein is taken out at the same time[10]. Thus, if measurements could be made of the amounts of protein removed from the different bands during myosin extraction, then a comparison with the quantities of myosin and of other proteins found in the extract would provide a most powerful test of the view that myosin is localised in the *A* bands.

The interference microscope allows such measurements to be made with a high degree of accuracy[8,11], and it has been employed here. The experiments were carried out on isolated *myofibrils*. It is undesirable to use intact fibres, for they cannot readily be obtained free from sarcoplasmic protein, and unless they are mechanically disrupted the extraction of myosin from them is not so swift and complete as it is from isolated fibrils. The accompanying paper[12] gives the results of parallel biochemical analyses of similar material.

Some interference microscope measurements of the ratios of the densities of different bands in rabbit myofibrils have already been reported, in abstract, by BENNETT[13]. The author points out that the ratios found were probably low because the length of the bands lies near the limit of resolution of the microscope, and he does not discuss their implications. This difficulty has been overcome in the present studies, in the course of which much higher and more consistent ratios have been observed.

MATERIAL AND METHODS

Glycerol-extracted myofibrils from the rabbit's psoas muscle were prepared by methods described in detail in the accompanying paper[12]. After the fibrils had been isolated they were washed to remove any materials soluble in hypotonic saline, and for the microscopic investigations they were suspended in a potassium chloride solution (either 0.1 *M* or 0.04 *M*) buffered at pH 7.0 with 0.0067 *M* phosphate buffer; 10^{-3} *M* $MgCl_2$ was also present.

The length of the fibrils was controlled by tying the bundles of psoas fibres to "Perspex" sticks before immersing them in the glycerol. Some of the fibre bundles were held at rest-length; others were stretched almost immediately after the rabbit was killed.

Two different myosin-extracting solutions were used, both giving the same results. One was a modification of the pyrophosphate-KCl solution of HASSELBACH AND SCHNEIDER[14]; it contained 0.6 *M* KCl, 0.01 *M* sodium pyrophosphate, 0.1 *M* phosphate buffer, 10^{-3} *M* $MgCl_2$. The other was 0.1 *M* pyrophosphate with 0.0067 *M* phosphate buffer and 10^{-3} *M* $MgCl_2$. The pH of both solutions was 6.4. The fibrils were irrigated under the microscope with the solution which caused the *A* substance to be rapidly and completely extracted. Before an extracted fibril was photographed, the preparation was irrigated with 0.1 *M* KCl at pH 7.0.

A Cooke-Dyson interference microscope with an objective of N.A. 1.3 was used; the light source was a 250 watt B.T.H. compact-source mercury arc lamp with a Wratten 58 filter. Photographs were taken on Kodak panchromatic microfile film at a magnification of either 350 × or

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200 ×. A fine-grain developer, D 76, was used. Care was taken to avoid irradiating the fibrils longer than necessary, for it has been observed that long exposure to light may prevent complete extraction of the *A* substance.

The photograph negatives were scanned in a microdensitometer which has been described elsewhere[15,16].

INTERFERENCE MICROSCOPY

The DYSON interference microscope has been described in detail elsewhere[17]. Essentially, it makes use of the fact that different parts of an object produce different degrees of retardation in the phase of the light they transmit, depending on their thickness and refractive index. The transmitted light is allowed to interfere with a reference beam which has followed a path parallel to the specimen beam but displaced sideways from it, so that it passes only through the fluid surrounding the specimen. The two beams are coherent. The resultant intensity depends on the phase difference between the two beams, *i.e.* the extra retardation suffered in passing through the specimen instead of through the same thickness of fluid alone. An image is thus produced whose intensity at each point is determined by the retardation along the corresponding path through the object.

DAVIES, WILKINS, CHAYEN AND LA COUR[8] and DAVIES AND DEELEY[11] have discussed how the interference microscope can be used to determine the mass of biological objects. The dry mass (m) of the object is related in a simple way to the optical path difference (Φ_w) it produces, and to the specific refractive increment (α) fo the material composing it:

$$m \text{ in grams} = \frac{\Phi_w \times \text{area in cm}^2}{100\,\alpha}$$

$$\text{and } \Phi_w = (\mu_0 - \mu_w) \times \text{thickness in cm}$$

where μ_0 and μ_w are the refractive indices of the object and of water, respectively. Φ_w is the quantity measured by the interference microscope. α has almost the same value (0.18–0.19) for a large number of proteins and is virtually independent of concentration up to 50%[18]. Thus, in a muscle fibril, consisting almost entirely of protein[19], the amount of protein present can be determined for any chosen area in the image. As the fibril is permeable to the salt solution in which it lies, no complications are introduced by the use of salt solution instead of water.

Experimental details

The application of this technique to the measurement of the amounts of protein in the different bands of myofibrils is simple in principle, but there are a number of important experimental considerations which must be discussed. Before doing so, however, one feature of the terminology used should be explained. These studies are mainly concerned with the measurement of the ratios between the amounts of protein per unit length of the myofibril in the different bands under consideration. It is convenient to refer to these ratios as "density ratios". It should be realised, however, that the word "density" is being used here in a special sense; it has the meaning "mass per unit length of fibril of the protein contained in the whole cross-section of the fibril". This is the quantity which is important. It is not assumed that the cross-sectional area of the fibril is the same from one band to the next, as it would have to be if "density" were used with the meaning "mass per unit volume". As

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will be seen in due course, the nature of the measurements renders such an assumption unnecessary.

Retardation measurements using the "fringe system". The Cooke-Dyson interference microscope incorporates two identical glass wedges, placed above and below the specimen. One of these wedges can be rotated in its own plane. When the axes of the two wedges (the axis being the line along which the change in thickness per unit length is maximal) are made parallel and in opposite senses (*i.e.* the thickness decreases in one wedge in a given direction along the axis, but increases in the other wedge) the wedges introduce a retardation between the direct beam and the reference beam which varies linearly across the field of view in a direction parallel to the axes of the wedges; the field is crossed by a series of dark and light fringes corresponding to loci where the path difference is an odd or an even number of half wave-lengths respectively. This retardation is superimposed on any retardation produced by the specimen. The wedges in effect introduce a comparison specimen whose thickness varies linearly across the field. The extra retardation at any point in the object can then be measured by locating the point higher up the fringe system where no object is present, but where the light intensity is the same as at the chosen point in the object (see Fig. 1). The difference in the retardations introduced by the wedges at the two points is then equal to the retardation introduced by the object; it is also proportional to the distance between the two points, which may be measured. The ratio of this distance to the distance between two bright (or two dark) fringes then gives the retardation as a fraction of one wave-length. If the object introduces a retardation of more than one wave-length, the number of whole wave-lengths of retardation can be determined by viewing the fringes in white light.

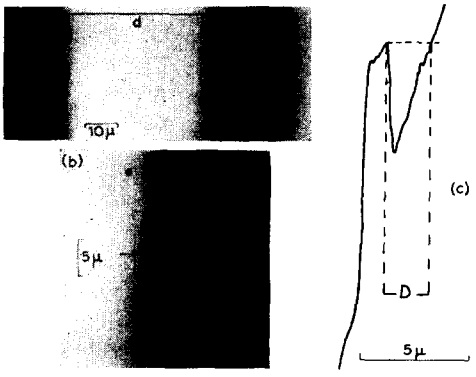

Fig. 1. Photographs of a fibril in the "fringe system" and a densitometer tracing of the negative. (a) The fibril and the fringe system. The distance "*d*" represents a change in retardation of one wave-length. (b) The fibril at a higher magnification. (c) Tracing taken across the fibril along the line marked in (b). The distance "*D*" is a measure of the retardation, Φ_w, introduced by the thickest part of this *H* zone. Φ_w (in wave-lengths) = D/d.

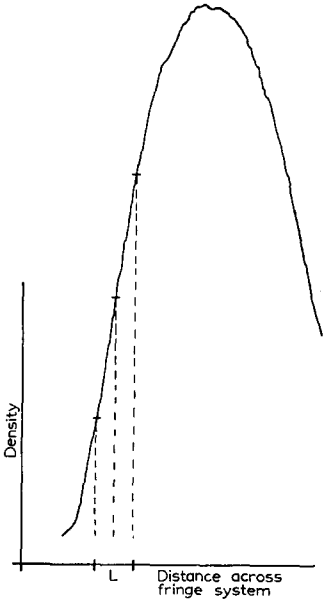

Fig. 2. Densitometer tracing across the fringe system. The relationship between retardation and photographic density is approximately linear over the region marked L.

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Retardation measurements using "even-field illumination". The wedges can also be arranged so that their axes are parallel but in the same sense; they will then introduce a retardation which is constant over the field of view, and the condition of "even field" is obtained. The field is uniformly illuminated and may be completely dark, or maximally bright, or may have an intermediate intensity, depending on the actual value of the retardation introduced, which can be varied by moving one of the wedges. Objects will appear either darker or lighter than the background, depending on whether the extra path difference they introduce brings the phase difference between the direct and reference beams nearer to an odd number of half wave-lengths, or further away. As before, the retardation at different points in the object determines the intensity at the corresponding points in the image.

It will be seen from the densitometer tracing of a photograph of the fringe system (Fig. 2) that over a certain range of retardation the film density varies almost linearly with the path difference. When an object which introduces a retardation of about one sixth of a wave-length or less (for example a myofibril) is placed at the bottom of the most nearly linear part of the fringe system, then the difference between the film density at the object and the background density at the same point is almost directly proportional to the retardation in the object. Under suitable photographic conditions, the departure from proportionality is less than about 4%.

These conditions can be duplicated with "even-field illumination" if the retardation introduced by the wedges is made equal to the retardation at the point on the fringe system where the film density/retardation curve is most nearly linear. Then provided that the photographic conditions are the same as before, the linear relation between film density and retardation will still hold. Thus a densitometer tracing along, for example, the photographic image of a myofibril (Fig. 3) will give a direct picture of the variation in the amount of protein along the fibril. The advantage of having an evenly illuminated field for this type of measurement is, of course, that the background density for all points in the object is the same and easily determined.

DAVIES AND DEELEY[11] have shown that the relation between light intensity at points in the image plane, and retardation along the corresponding path through the object is, under certain conditions, also a nearly linear one; and they have given details of the use of a photo-electric detector to measure directly the amount of material in a selected area of an object.

Comparison of "fringe" and "even field" measurements. Measurements made using the first method (the fringe method) have the advantage that they are independent of the relation between retardation and film density, for the measurement is simply one of distance between two points at which film densities are equal. However, they have the disadvantage that a densitometer tracing across the image must be made with a pinhole in order that at any given moment the measurement includes only an area of specimen over which the density is approximately constant; thus each measurement only gives the amount of protein represented by a very small area in the fibril. Provided that the cross-section of the fibril retains a constant shape at successive bands, a measurement of the ratio of the retardations at, say, the thickest part of the fibril in each band will give a correct value for the ratio of the amounts of protein present per unit length of the fibril in each band. However, if changes in shape do occur, it will be necessary to plot out the distribution of protein across

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the width of the fibril, point by point, and then perform a graphical integration. This process becomes extremely laborious when dealing with a large amount of data. Moreover, if an integration along the length of a fibril is required, then a large number of separate densitometer tracings would have to be used. Also, such pinhole tracings have a great deal of background noise due to photographic grain.

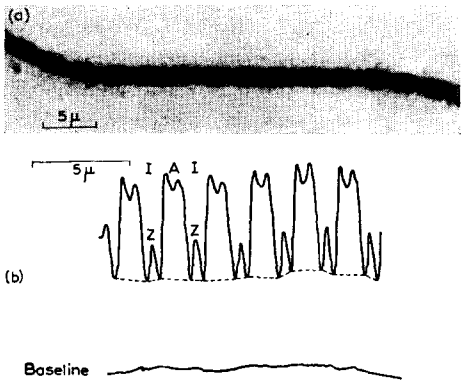

Fig. 3. Photograph of a fibril in "even field", and a densitometer tracing taken along the fibril using a narrow slit, the length of which was slightly greater than the width of the fibril. The base-line is a tracing taken through the background with the same slit. A line has been drawn connecting the level of the *I* bands, and the area enclosed between this line and the baseline gives a measure of the quantity of "*I* substance" before correction for *H* zones (see text). The areas enclosed between the line drawn at *I* band level and the outline of the *A* bands represents the quantity of "*A* substance" without correction for *H* zones. The area enclosed between the line drawn at *I* band level and the outlines of the *Z* lines represents the quantity of "*Z* substance".

When even-field illumination is employed, with suitable photographic conditions, the film density varies almost linearly with the amount of protein present, for the reasons already discussed. Thus a densitometer tracing across a fibril will give immediately a cross-sectional view of the amounts of protein present (Fig. 4), without an intermediate stage of replotting the curve point by point. More important still, it is possible to use the even-field pictures to obtain direct optical integration of the total amount of protein present in a cross-section of a fibril.

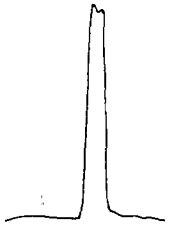

Fig. 4. A densitometer tracing across a fibril photographed in "even field".

Optical integration by densitometer. The fibrils selected for measurement were ones which appeared, visually, to have a fairly constant density across their width. Such fibrils, which are adhering to the coverslip or slide, are probably somewhat flattened. Densitometer tracings (Fig. 4) showed that, in fact, changes in density across these fibrils did not amount to more than $\pm$ 10% of the mean density, except of course at the very edge of the fibril, where the density fell to zero within a distance equal to about one quarter of the fibril width. The range of photographic density across the fibril was usually of the order of 0.4–0.5. Strictly speaking, the densitometer integrates light intensity and not film density, and so, if used with a slit system, it will give a deflection which is proportional to the average light intensity, rather than to the average film density, over the area of the slit. However, with images of the form described, it is easy to show both numerically and experimentally that the densitometer deflection obtained with a slit extending across the whole width of the fibril is proportional to the integrated film density to within about 1%; the deflection therefore varies linearly with the total amount of protein present in the whole cross-section of the fibril. If the slit is moved parallel to itself along the length of the fibril, then the densitometer will trace out the variation in the amount of protein in the different bands of the myofibril. The base-line of the tracing is of course given by

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the background density seen through the same slit. Even if the fibrils were triangular in cross-section, the error in the measurements of the ratios of the amounts of protein in the different bands would be less than 5%. It is perhaps advisable to point out, however, that this technique should not be applied indiscriminately to other microscopic objects without first finding out the magnitude of the errors involved.

Resolution of microscope. The only muscles for which reliable protein analyses are available are the skeletal muscles of the rabbit. Any comparison between the results of interference microscopy and of large-scale biochemical analyses must therefore be carried out using these muscles. In a rabbit's psoas fibril at rest-length the sarcomere is about 2.3 μ long and the distance between the dense Z line and the A band is only about 0.3 μ (Fig. 5). The length of the H zone is about 0.3 μ, and the lengths of the denser parts of the A band on either side of it 0.6 μ. These dimensions lie close to the limit of resolution of the light microscope, and it is therefore necessary to consider what inaccuracies may be introduced into the measurements made in the interference microscope and how such errors may be minimised.

The boundaries of an image formed by visible light are not completely sharp. With a conventional light microscope of N.A. 1.3, the theoretical limit of resolution is about 0.2 μ. This is the limit below which the images of two objects cannot be separately resolved; for the boundaries of the two images not to overlap, the separation of the objects must be at least twice as great. In the case of objects which have a finite thickness at their boundaries, the problem is much more complex, and

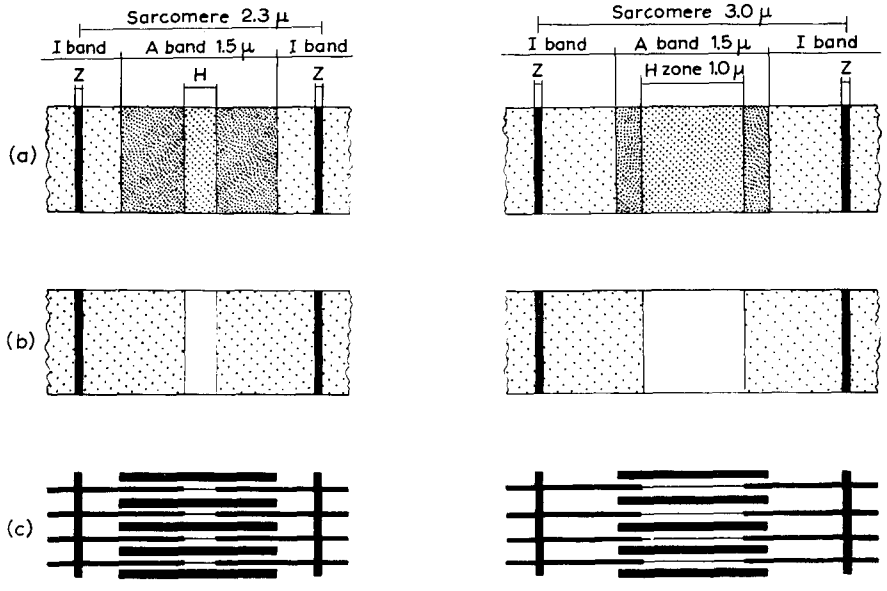

Fig. 5. Diagrams of two fibrils, one at rest-length (sarcomere length 2.3 μ) and the other stretched (sarcomere length 3.0 μ). (a) The pattern of cross-striation in the intact fibril. (b) The pattern of cross-striation in the fibril after extraction of myosin. (c) Schematic diagram of submicroscopic structure. The A band is characterised by thick filaments; these constitute the "A substance" and are taken out of the fibril by myosin-extracting procedures. The main components of the "I substance" are thin filaments extending from the Z lines to the borders of the H zone. The "S substance" is represented here by fine lines connecting the two sets of thin filaments. When the sarcomere is extended (compare the two fibrils) the lengths of I bands and H zone increase, but the length of the A band stays unchanged.

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consideration of the theory of image formation in an interference microscope introduces additional problems. It seemed, therefore, that direct experiment was the best way to determine the minimal distance from a discontinuity in the density of an object at which an accurate density measurement can be made. This was done by taking densitometer tracings across the images of suitable protein crystals photographed in the interference microscope (Fig. 6). These crystals, kindly supplied by Dr. J. C. KENDREW, were in the form of flat plates about 0.8 μ in thickness, and their edges were formed by vertical faces; such crystal faces may be assumed to be flat down to molecular dimensions and therefore provide an ideal density discontinuity.

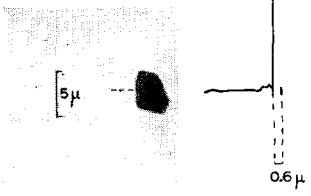

Fig. 6. A small crystal of the protein myoglobin, photographed in "even field", and a densitometer tracing across the boundary marked by an arrow. The width of the boundary zone is about 0.6 μ; hence, for objects like this crystal, which is about 0.8 μ thick, an accurate measurement of optical retardation cannot be made less than 0.3 μ away from a density discontinuity.

It was found that the total width of the boundary of the image was about 0.6 μ; thus, taking the true boundary to be half way across the observed boundary zone, the conclusion was reached that accurate density measurements may be made at distances not less than 0.3 μ from a density discontinuity within an object. Thus, if an accurate density measurement is to be made of one band in a fibril, the minimal requirements are that the band should be of uniform density and at least 0.6 μ long. This condition is not satisfied in the I bands of rest-length myofibrils from rabbits; they are bisected by Z lines, and the distance from Z to A is only about 0.35 μ. To satisfy the resolution requirements, the distance from the Z line to the end of the A band must be at least 0.6 μ. Thus the total I band length, including the Z line, must be at least 1.3–1.4 μ; this condition obtains when the sarcomere length is 2.8–2.9 μ, the A band having a constant length of 1.5 μ (Fig. 5). The resting length of the sarcomere is about 2.3 μ, and so fibrils which have been stretched by 20–30% will be satisfactory. Fibrils shorter than this will give artificially high values for I band density and artificially low A/I ratios.

Measurement of the density of the A band is complicated by the presence of the H zone which has a lower density than the rest of the A band. At rest-length the H zone is about 0.3 μ long, and the denser zones on either side of it (referred to here as A^* zones) are each 0.6 μ long; in a stretched fibril with a sarcomere length of 2.9 μ, the H zone is 0.9 μ long and each A^* zone 0.3 μ (Fig. 5). Thus it is never possible to obtain an accurate value for the ratio A^* density: I density, because at rest-length the I band is too short, and in stretched fibrils with I bands sufficiently long for measurement, the A^* zone is too short. However, the H/I ratio in a stretched fibril can be determined with an accuracy that is not seriously affected by the limited resolution of the microscope; thus, for example, at 125% rest-length, the H zone is 0.9 μ long and the I band (Z to A) 0.6 μ. It will be shown later how this H/I ratio can be used in determining the amount of A substance.

Integration methods. The difficulties which the limited resolving power of the microscope imposes can be avoided to some extent in a rather different way. It has

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already been explained that a value for the total material present in a fibril can be obtained by measuring the area under a densitometer tracing taken along a fibril photographed under even field conditions, provided that the tracing is made using a slit placed at right angles to the long axis of the fibril, with the slit length greater than the width of the fibril. Effectively, this technique measures the volume underneath the density envelope of the photographic image recorded in the interference microscope. The accuracy of this measurement is virtually independent of the scale of the microscopic detail within the object relative to the resolving power of the microscope. Provided that the numerical aperture is large, it is the distribution of light intensity in the image, rather than its total amount, which is affected by the finite resolving power.

Thus it is possible to compare the total material present in a fibril before and after myosin extraction without having to take into account the detailed characteristics of the image. Another quantity that can be measured in a similar way is the "extra" material of the *A* band. This can be done simply by measuring the area under the tracing above the *I* band level (Fig. 3) in a stretched fibril, and adding to that quantity a correction term to allow for the fact that after extraction of the *A* substance the *H* zone has a lower density than the *I* band (Figs. 8b and 9, pp. 239 and 240 respectively); only the correction term depends on the measurement of the relative density and length of a particular region within the *A* band.

The integration method is also used to estimate the fraction of the total myofibrillar material present as "extra" material (*i.e.* excess over the *I* band level, Fig. 3) in the *Z* lines. The width of a *Z* line is only about 0.1 μ, as seen in the electron microscope, so that its true peak density cannot be measured.

RESULTS

The following terminology, illustrated in Fig. 9 will be used. *Z* substance will mean the "extra" material which raises the density of the *Z* lines above the general level of density of the *I* bands. *I* substance will mean the material which extends from the *Z* line, through the *I* band, into the *A* band, and terminates at edge of the *H* zone. *S* substance will mean the material which bridges the gap in the middle of the *A* band between adjacent regions of *I* substance, and which remains when the *A* substance is removed. *A* substance will mean the "extra" material of the *A* band which gives it a density excess over the levels of density of the *I* substance and *S* substance. Density ratios will be quoted in the form "*H*/*I* ratio", "*S*/*I* ratio", etc.

Measurements of density ratios

H/I *ratio in intact stretched fibrils.* The *H*/*I* ratios measured by the fringe method on stretched fibrils are listed in Table I. A typical set of measurements from an individual fibril is given in Table Ia, and Fig. 7 shows the photographic image and typical densitometer tracings across the *H* and *I* bands of the same fibril.

The *H*/*I* ratios measured by the even field method on stretched fibrils are listed in Table II. A typical set of results from an individual fibril is given in Table IIa, and Fig. 8a shows such a fibril and the densitometer tracing along its length.

The overall average for the *H*/*I* ratio, using the results obtained by both methods, is 2.17 $\pm$ 0.05.

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TABLE I

H/I RATIOS OF INTACT STRETCHED FIBRILS, FRINGE METHOD

Expt. No.	*Number of fibrils*	*Number of ratios measured*	*Average ratio*
1	3	52	2.28 ± 0.1
2	4	50	2.10 ± 0.1
3	2	12	2.05 ± 0.2
4	1	6	2.25 ± 0.2
		120	
	Weighted average		2.20 ± 0.1

TABLE Ia

TYPICAL SET OF H/I RATIOS FROM A SINGLE FIBRIL (Fibril number 120)

*H density**	*I density**	*Ratio*
10.5	5 4	2.3
9.5	5 4	2.1
10.0	4 4	2.5
10.0	4 5	2.2
10.5	5.5 4	2.2
	Average	2.26 ± 0.05

* In each sarcomere there are two half I bands (Z to A) and one H zone.

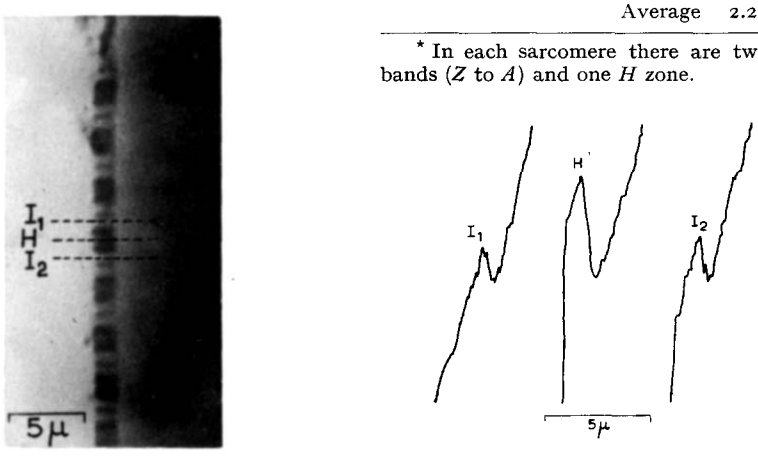

Fig. 7. Photograph of fibril number 120 in the "fringe system", and densitometer tracings taken across the fibril through the H zone and the two adjacent I bands marked on the photograph. Table I a lists the measurements made from a series of such tracings through bands in the same fibril, and the H/I density ratios calculated from these measurements.

S/I *ratio after myosin extraction.* This ratio and, in fact, all further ratios reported in this paper, was measured by the even-field method only, for by this stage in the investigation the advantages of this method had been fully appreciated, and its validity confirmed by the measurements already described. The results, obtained on stretched fibrils, are given in Table III, and Table IIIa gives a typical set of values from a single fibril whose photograph and densitometer tracing are given in Fig. 8b.

The average value for the S/I ratio after myosin extraction is 0.24 ± 0.02.

Ratio of density of I *band after and before myosin extraction,* I *after*/I *before.* This ratio is required for two reasons, in the first place in order to place the density of the H zone after myosin extraction (the S density) on the same scale as its density in the intact fibril, and, secondly, to enable a calculation to be made of the amount of I substance removed during myosin extraction.

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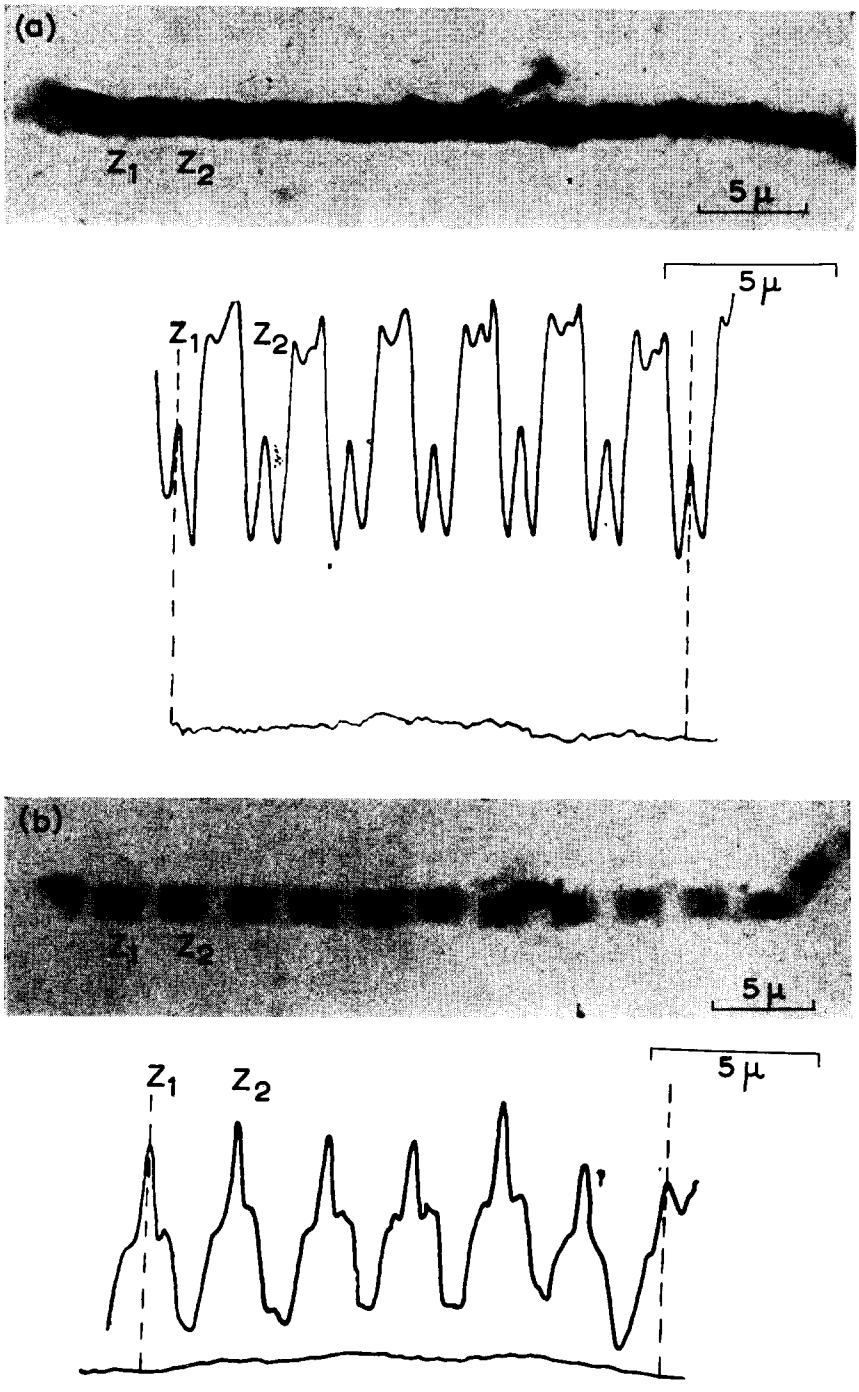

Fig. 8. Photographs and densitometer tracings of a fibril, number 163, (a) before and (b) after myosin extraction. The photographs were taken under even-field conditions. The tracings were obtained using a slit to integrate across the width of the fibril. Measurements made from these tracings are listed in Tables IIIa and IVa.

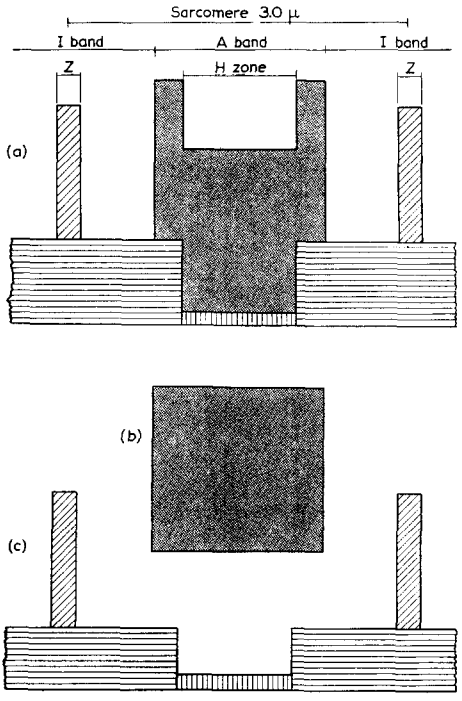

Fig. 9. Histograms representing quantitatively the distribution of protein in a sarcomere 3.0 μ long. Vertical dimensions represent the proportion of each component in the total dry mass of the sarcomere. Horizontal dimensions represent the length of each band of the sarcomere. The "*A* substance" is shown in grey. The "*S* substance" is shaded with vertical lines. The "*I* substance" is shaded with horizontal lines. The "*Z* substance" is shaded with slanting lines. (a) Intact fibril. Compare with Fig. 3 and Fig. 8 (a) which show densitometer tracings, made with a slit, along the length of similar fibrils in "even field"; like the histogram, these represent the distribution of protein along the length of the fibril, and in Fig. 3 the "*I* substance" is indicated. (b) The *A* substance. (c) The fibril after removal of myosin. Compare with Fig. 8 (b) which shows a densitometer tracing along the length of a similar extracted fibril in "even field".

TABLE II

H/I RATIOS OF INTACT STRETCHED FIBRILS, EVEN-FIELD METHOD

Expt. No.	*Number of fibrils*	*Number of ratios measured*	*Average ratio*
1	6	68	2.12 ± 0.05
2	8	118	2.14 ± 0.05
3	7	88	2.21 ± 0.05
		274	
		Weighted average	2.16 ± 0.03

TABLE IIa

TYPICAL SET OF *H/I* RATIOS FROM A SINGLE FIBRIL (Fibril number 3)

H density	*I density*	*Ratio*
3.75	1.55	2.24
	1.8	
4.0	1.8	2.19
	1.85	
4.25	1.9	2.21
	1.95	
4.2	1.95	2.00
	2.05	
4.1	2.15	2.00
	1.95	
	Average	2.13 ± 0.05

The *H* zone itself often becomes partly obscured during extraction, either through slight shortening of one part of the cross-section of the fibril as it tends to detach itself from the coverslip when the *A* substance is dissolved out, or sometimes through incomplete extraction of the *A* substance in the *H* zone. Fibrils with perfectly good *H* zones can always be found in a preparation after extraction of myosin, but very often the fibril which was photographed before extraction is not amongst them. It is much more economical of time, therefore, to measure the *S/I* ratio on good examples of extracted fibrils and to correct for changes in the *I* density afterwards.

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TABLE III

S/I RATIOS AFTER MYOSIN EXTRACTION

Expt. No.	*Number of fibrils*	*Number of ratios measured*	*Average ratio*
1	1	7	0.21
2	1	7	0.26
3	1	5	0.35
4	1	6	0.30
5	1	9	0.16
6	1	5	0.19
7	1	18	0.24
8	1	9	0.31
9	1	9	0.20
		75	
		Weighted average	0.24 ± 0.02

TABLE IIIa

TYPICAL SET OF S/I RATIOS FOR A SINGLE EXTRACTED FIBRIL (Fibril number 163)

S density	*I density*	*Ratio*
3	16	0.19
	16	
8	17	0.42
	21	
7	21	0.35
	19	
6	19	0.315
	19	
5	17.5	0.260
	21.5	
5	17	0.285
	18	
	Average	0.31 ± 0.03

Table IV contains the results of measurements of I after/I before in stretched fibrils; a typical set of values for an individual fibril before and after myosin extraction is given in Table IVa, and photographs of the fibril itself and its densitometer tracings are given in Fig. 8.

The average value for I after/I before is 0.725 ± 0.03.

TABLE IV

RATIO OF I BAND DENSITY AFTER EXTRACTION TO I BAND DENSITY IN INTACT FIBRIL, STRETCHED FIBRILS

Fibril number	*Number of ratios measured*	*Average ratio*
82	34	0.72
83	8	0.73
86	10	0.73
93	8	0.70
104	8	0.86
108	12	0.65
111	8	0.62
160	16	0.77
162	24	0.70
164	12	0.75
166	22	0.70
168	20	0.75
170	20	0.75
	202	
	Weighted average	0.725 ± 0.03

TABLE IVa

TYPICAL SET OF VALUES FOR I BAND DENSITY BEFORE AND AFTER EXTRACTION, FOR A SINGLE FIBRIL

Fibril number 163

I before	*I after*	*Ratio**
2.3	1.6	0.68
2.4	1.6	
2.6	1.7	0.73
2.6	2.1	
2.4	2.1	0.835
2.4	1.9	
2.4	1.9	0.775
2.5	1.9	
2.3	1.75	0.81
2.4	2.05	
2.45	1.7	0.72
2.4	1.8	
	Average	0.76 ± 0.02

* Average for two half I bands in each sarcomere.

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When a correction term for the change in I band density is included, the ratio [density of H zone after extraction] *i.e.* density of S substance, to [density of I band in the intact fibril] is found to be $0.17_4 \pm 0.02$.

$$\frac{H \text{ after}}{I \text{ before}} = \frac{S}{I} = 0.17_4 \pm 0.02.$$

Amount of Z substance. The amount of Z substance was measured by the even-field integration method in the manner already described. The results, which show some scatter, are given in Table V. The average was 6% ± 0.5% of the total myofibrillar material.

Calculation of amount of A *substance, etc. from density ratio measurements.* The relevant ratios are:

H/I ratio in intact fibril = 2.17 ± 0.05,
S/I ratio in extracted fibril = 0.24 ± 0.02,
I after/I before (after and before extraction of myosin) = 0.725 ± 0.03.

TABLE V

PERCENTAGE OF TOTAL MATERIAL PRESENT AS Z SUBSTANCE

Fibril number	*Number of measurements*	*Average % of Z substance*
3	6	5·5
4	8	4·7
5	11	3·45
7	8	6·55
8	7	8.6
15	9	8.0
16	7	6.1
18	3	6.45
	59	
	Weighted average	6.02 ± 0.5%

Hence the ratio S after/I before = 0.17 ± 0.02.
Now, for sarcomere lengths 2.8–3.2 μ:

the total length of I substance = 2.0 μ (unaffected by sarcomere length),
the length of A substance = 1.5 μ (unaffected by sarcomere length),
the length of S substance = 0.8–1.2 μ (according to sarcomere length).

Hence:

the amount of A substance = $1.5 \times (2.17 - 0.17)$ = 3.0 units,
the amount of I substance = 2.0×1.0 = 2.0 units,
the amount of S substance = 1.0×0.17 = 0.17 units,
the amount of Z substance = 6% of the total material.

Expressed as percentages of the total myofibrillar protein, and taking into account errors from all sources, the amounts of these substances are:

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A substance	54.5% ± 2%,
I substance	36.4% ± 2%,
S substance	3.1% ± 0.3%,
Z substance	6.0% ± 1%.

During extraction of myosin, all of the *A* substance is removed, together with 27.5% of the *I* substance (ratio *I* after/*I* before = 0.725), *i.e.* in addition to *A* substance, another 10% of the total myofibrillar protein is removed. Thus one would anticipate from the ratio measurements that a total of 64.5% by weight of the myofibrillar protein would be removed during myosin extraction.

Measurements carried out by integration of even-field tracings

Amount of "extra" material in the A *band.* This quantity has to be found in three stages. In the first stage the total excess density of the *A* band above the level of the *I* bands is measured by integrating the region shown on the densitometer tracing in Fig. 3 (see also Fig. 9). The quantity obtained is *not* the amount of *A* substance, for no allowance has been made for the fact that the *I* substance does not bridge the *H* zone, *i.e.* that the *S* substance has a lower density than the *I* substance. The most elementary form of correction is then applied, taking the density of the *S* substance to be zero. A second correction can then be applied by making use of the ratio *S* after/*I* before. Thus the final value obtained for the amount of *A* substance depends on a ratio measurement only in its second correction term, which is small; otherwise, it is obtained purely by integration.

The values obtained for "*A* substance before correction" for a number of fibrils are given in Table VI, and the average is found to be 40.5% of the total myofibrillar material.

TABLE VI

A SUBSTANCE BEFORE CORRECTION FOR *H* ZONE

Fibril number	*Number of sarcomeres*	*Uncorrected A substance as % of total fibrillar material*
3	6	42.0
4	8	39.6
5	11	41.0
7	8	40.7
8	7	38.4
15	9	43.5
16	7	38.6
18	3	41.5
	Average	40.5% ± 0.4%

The corrected value for the *A* substance can now be calculated:

A substance minus additional *A* substance in *H* zone = 40.5%,

Z substance = 6%.

Hence: additional *A* substance in *H* zone + *I* substance + *S* substance = 53.5%.

If density of *S* substance is zero, then in an *H* zone 1.0 μ long the quantity of additional *A* substance = 17.8%.

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Hence the second value for the total *A* substance = 58.3%.

If density of *S* substance is 0.17 (p. 240), then the total amount of *S* substance = 3.0%.

Hence the final corrected value for the total *A* substance = 55.3%.

Total amount of material removed during myosin extraction. The results of measurements under even-field conditions of the total material removed during myosin extraction from both rest-length and stretched fibrils are given in Table VII. A typical pair of photographs and tracings are shown in Fig. 8. The rest-length fibrils appear to lose slightly less material than the stretched ones, but it is not considered that this difference is significant. It is found that the fibrils lose on the average 61.5% of their total material.

The difference between this figure of 61.5% and the theoretical figure of 64.5% (54.5% *A* substance, 10% of the *I* substance) obtained from the ratio measurements is probably accounted for by the tendency for the extraction of the *A* substance in the *H* zone to be slightly less than complete. Fibrils which after extraction had very well defined *H* zones gave values around 65% for the amount of material removed as measured by integration.

TABLE VII

TOTAL AMOUNT OF MATERIAL REMOVED FROM FIBRILS DURING MYOSIN EXTRACTION, INTEGRATION METHOD

Expt. No.	*Number of fibrils*	*Number of sarcomeres measured*	*Average amount removed as % of total fibrillar material*
1 (rest-length fibrils)	9	73	57.7 ± 2
2 (stretched fibrils)	9	88	63.5 ± 2
3 (stretched fibrils)	6	28	61.3 ± 1
		Weighted average	61.5 ± 4%

A possible source of error in the calculation of amount of A *substance*

Only one assumption has been made so far that does not have rigid experimental support. It concerns the behaviour of the *S* substance during extraction of myosin. It has been assumed, effectively, that the density of the *A* substance is given by the density of the *H* zone in an intact fibril minus the density of that *H* zone after extraction—*i.e.* minus the density of the *S* substance after extraction. If, in fact, some *S* substance was removed during extraction, then the real density of the *A* substance would be slightly lower than has been estimated.

It is possible to set a limit to the size of the error which could be involved. This can be done in two ways. In the first place, one can attempt to measure the ratio of the density of the denser terminal zones of the *A* band to the density of the *H* zone, *i.e.* the A^*/H ratio. This gives a measure of the drop in density at the *H* zone and can be used to calculate the density of the *S* substance before extraction. As the *A* band is only 1.5 μ long, the best conditions for measuring the A^*/H ratio occur when the *H* zone is 0.5 μ long and the A^* zones are also each 0.5 μ long. Such a length, however, is still too small to permit of accurate density measurements. The *H* zone will appear to have a higher density and the A^* zones a lower density than they possess in fact. Thus the density of the *S* substance will be overestimated and the value for the amount of *A* substance will be too low. This type of calculation, therefore, enables a lower limit to be set, and the actual amount of *A* substance must exceed this limit.

Measurements of the A^*/H ratio on fibrils with a sarcomere length of about 2.6 μ gave an average value of 1.28 ± 0.02 (54 measurements, six different fibrils).

The density of the *S* substance calculated from this ratio depends slightly on the particular assumptions made about the behaviour of the *S* substance during stretch from a sarcomere length

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of about 2.6 μ at which A^*/H is measured to a sarcomere length of about 3.0 μ at which H/I is measured. The following three models will cover the whole possible range of variation:

1. Where any change in the density of the S substance during stretch is neglected.
2. Where the density of the S substance varies inversely with the length of the sarcomere.
3. Where the density of the S substance varies inversely with the length of the H zone.

Table VIII lists the results for these three models.

TABLE VIII

LOWER LIMITS FOR AMOUNT OF A SUBSTANCE CALCULATED FROM A^*/H RATIOS

Model	A^*/H	S/I *(Calculated)*	*Amounts expressed as % of total fibrillar protein*			
			A substance	*I substance*	*S substance*	*Z substance*
1	1.28	0.39	49.6	37.2	7.2	6
2	1.28	0.37	50.0	37.1	6.9	6
3	1.28	0.19	54.0	36.5	3.5	6

Thus these measurements set a lower limit (49.6–54 %, depending on which assumption is made) which is only slightly below the value of 54.5 % for the amount of A substance calculated in the straightforward way.

A lower limit can be set in another way, by attempting to measure A^*/I ratios on fibrils at intermediate lengths. If fibrils with a sarcomere length of about 2.6 μ are used, then the length of the "clear" region of the I band (Z to A) is 0.5 μ and the length of the A^* zone is 0.5 μ. Again, these lengths are on the borderline at which accurate density measurements can be made; the ratios obtained are likely to be low, and the values for the amount of A substance will represent lower limits.

Measurements of the A^*/I ratio, on eight fibrils, representing 80 separate measurements, gave an average value of 2.85. Table IX lists the corresponding values for S substance and A substance etc. calculated for the three different models.

Again the lower limits calculated from these measurements (51.2–53.1 %, depending on which assumption is made) are not very far below the original figure of 54.5 % for the amount of A substance.

TABLE IX

LOWER LIMITS FOR AMOUNT OF A SUBSTANCE CALCULATED FROM A^*/I RATIOS

Model	A^*/I	S/I *(calculated)*	*Amounts expressed as % of total fibrillar protein*			
			A substance	*I substance*	*S substance*	*Z substance*
1	2.85	0.32	51.2	37.9	5.9	6
2	2.85	0.22	53.1	36.5	4.4	6
3	2.85	0.32	51.2	37.9	5.9	6

Summary of results

The results may now be summarized. Table X shows the values obtained by the two principal methods, all expressed as percentages of total fibrillar protein. Fig. 9 gives the results in histogram form.

These values are calculated on the assumption that no S substance is removed during myosin extraction. If this is not the case, then other measurements can be used to fix a value which the amount of A substance must *exceed*; this value lies somewhere between 49% and 54%, depending on how the calculation is carried out.

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TABLE X

SUMMARY OF RESULTS

	Quantity expressed as % of total myofibrillar protein	
	Ratio method	*Integration methods*
Amount of *A* substance	54.5 ± 2.0	55.3 ± 2.0
Amount of *I* substance	36.4 ± 2.0	35.7 ± 2.0
Amount of *S* substance	3.1 ± 0.3	(3.1 ± 0.3)
Amount of *Z* substance	(6 ± 1)	6 ± 1
Amount of *A* substance extracted	54.5 ± 2.0	—
Amount of *I* substance extracted	10.0 ± 1.0	—
Total amount extracted	64.5 ± 3	61.5 ± 4

DISCUSSION

For the purposes of discussion the interference microscope results may be summarised as follows. The "extra" material of the *A* band accounts for 50–55% of the total myofibrillar protein. When myosin is extracted all of this extra material is removed, together with a further 10% which comes from the *I* substance. In all, 60–65% of the total myofibrillar protein is removed.

By large-scale chemical analysis, several different estimations have been made of the amounts of myosin and other protein fractions in whole fresh rabbit muscle. The most recent and probably the most reliable data are due to HASSELBACH AND SCHNEIDER[14] whose results are given in Table XI where, in order to facilitate comparison with the interference microscope results—which apply to washed fibrils—they are also expressed as percentages of the total protein excluding the sarcoplasmic fraction.

TABLE XI

COMPARISON OF BIOCHEMICAL MEASUREMENTS BY THE PRESENT AUTHORS (H–H)[12] WITH THOSE BY HASSELBACH AND SCHNEIDER[14] (H–S) AND THOSE BY SZENT-GYORGYI *et al.*[10] (S–G)

Figures in parentheses have been calculated by the present authors.

Protein fraction	*As % of total protein in fresh muscle*			*As % of total protein in glycerol-extracted muscle*			*As % of total protein in washed fibrils*		
	H–S	*S–G*	*H–H*	*H–S*	*S–G*	*H–H*	*H–S*	*S–G*	*H–H*
Soluble	28	(48)	34	—	42	28	—	—	—
Myosin	38	(26)	34	—	29	37	(53)	50	51
X-Protein	—	(9)	7	—	10	8	—	18	11
Myosin + *X*-protein	—	(35)	41	—	39	45	—	68	62
Actin + residue	34	(17)	25	—	19	27	(47)	32	38

The values obtained by HASSELBACH AND SCHNEIDER for the stroma and possibly also for the urea-soluble fraction no doubt include a contribution from extra-fibrillar structures—mainly connective tissue—so that the values for other proteins would be a little higher if they were expressed as percentages of myofibrillar protein. Thus, if 20% of the residue left after the extraction of myosin and actin is of extra-fibrillar origin, the figure for myosin would be 55%.

The results of HASSELBACH AND SCHNEIDER show that about 53%, possibly

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slightly more, of the fibrillar protein is myosin. Thus there is very close correspondence with the interference microscope value of 50–55% (54.5% being the preferred value) for the amount of *A* substance. The interference microscope results show that only about 10% of the total myofibrillar protein, apart from the *A* substance, is removed during myosin extraction. Thus the hypothesis that myosin is the *A* substance is in perfect agreement with the comparison between the results of interference microscopy and these biochemical measurements, and it also follows that at least four-fifths of the myosin must *necessarily* be present as the *A* substance.

More recently, SZENT-GYÖRGYI, MAZIA AND SZENT-GYÖRGYI[10] have reported biochemical analyses on glycerol-extracted rabbit psoas fibrils. These are of great interest, for the material is identical with that used in the interference microscope studies. Their results, which are summarized in Table XI, show rather a wide spread. However, taking average values, they found that 68% of the total fibrillar protein is removed during myosin extraction, that about 50% is myosin, and that the unknown fraction extracted together with myosin is about 18%. This unknown fraction is for convenience here called the *X*-protein.

SZENT-GYÖRGYI *et al.* suggested that the *X*-protein might be the *A* substance and that myosin might be distributed continuously along the length of the fibril. Such a model is incompatible with the interference microscope result that of the 60–65% total fibrillar protein which is removed during myosin extraction, 50–55% is the *A* substance and only about 10% comes from elsewhere in the sarcomere. If their model were correct the figures should be about 10% for myosin and 50% for the unknown fraction, *i.e.* practically the reverse of the ones found.

The amount of myosin (about 50%) found by SZENT-GYÖRGYI *et al.* is in quite good agreement with the amount of *A* substance measured by interference microscopy, and our own data from chemical analysis also show that the agreement is close.

The results obtained by chemical analysis and described in the accompanying paper[12] are given in Table XI and may be summarized as follows: The amount of protein removed from washed, glycerol-extracted fibrils by the same myosin-extracting solutions as were used in the interference microscope studies is, on the average, 62% of the total fibrillar protein. Of this 62%, about 51% precipitates when the extract is diluted with water to reduce its ionic strength to 0.04; thus about 51% of the total protein of washed fibrils is considered to be myosin. This figure is in remarkably good agreement with the interference microscope value of 50–55% for the amount of *A* substance; it should also be pointed out that the interference microscope studies show that all the *A* substance is removed when myosin extraction is performed on the fibrils.

If the extract is dialysed to reduce its ionic strength to 0.04, then the quantity of precipitate is lower. Thus the value obtained by the dilution method for the percentage of myosin in the extract (83%) is higher than that obtained by SZENT-GYÖRGYI *et al.* (73%) who used the dialysis method; and although the present experiments give a figure for total protein extracted somewhat lower than that of SZENT-GYÖRGYI *et al.*, the amount of myosin found is very similar. The essential difference is that SZENT-GYÖRGYI *et al.* found about 18% of *X*-protein (expressed as % of total fibrillar protein), whereas the present experiments give a value of about 11%. This latter value may be compared with the interference microscope value of 10% for the amount of *I* substance removed when the *A* substance is extracted.

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The identity of the *X*-protein is somewhat uncertain. At first sight it would not appear to be actin, for the viscosity of the whole extract does not appreciably decrease when adenosine triphosphate is added (SZENT-GYÖRGYI *et al.*[10]). However, some recent work of PERRY[20,21] suggests that part of the *X*-protein might very well be an inactive form of actin. He found that about 13% of the total protein of washed fibrils, isolated from fresh muscle, slowly leached out of them when they were stored in 0.078*M* borate buffer. About one-third of this extract was tropomyosin, but the remainder, called "pseudoglobulin", was apparently identical with a protein obtained by dialysing *F*-actin against 0.078*M* borate buffer. PERRY considers that the pseudoglobulin is an inactive form of actin, and it appears likely that the *X*-protein may contain this pseudoglobulin as, indeed, PERRY suggested with reference to the results of SZENT-GYÖRGYI *et al.* This might explain why myosin-extracting procedures applied to *fresh* fibrils remove actomyosin[20], whereas the same procedures applied to glycerol-extracted fibrils apparently do not. It is not necessary to suppose that glycerol extraction or the development of *rigor mortis*[2] renders actin insoluble; a more plausible hypothesis is that the same actin fraction is removed from both fresh and glycerol-extracted fibrils, but that as a result of glycerol extraction this fraction has become modified and will not form actomyosin. This hypothesis would explain why the total soluble protein found by HASSELBACH AND SCHNEIDER is lower than the [soluble protein + *X*-protein] found by SZENT-GYÖRGYI *et al.* and by ourselves, and why the quantity of material remaining after myosin extraction (about 47% expressed as percentage of myofibrillar protein) found by HASSELBACH AND SCHNEIDER[14] in fresh muscle *fibres* is higher than the quantity found by SZENT-GYÖRGYI *et al.* (32%) and by ourselves (38%) in glycerol-extracted muscle *fibrils*. HASSELBACH AND SCHNEIDER believe that the fact that their material is not broken down into fibrils prevents the highly polymerized fibrous actin from being extracted; thus their residue would contain all the actin originally present, whereas the residue from fibrils would have lost some actin.

To summarize, then, all the biochemical analyses agree that the percentage of myosin in myofibrils is about 50–55%; the interference microscope value for the amount of *A* substance in myofibrils is also 50–55%. When myosin extraction is performed on myofibrils, interference microscope measurements show that all the *A* substance is removed together with a further 10% of total fibrillar protein derived from the *I* substance. Biochemical analysis of the extract shows that it contains, in addition to all the myosin of the fibrils, other protein components amounting to about 11% of the total fibrillar protein. There is some reason for believing that modified actin makes up a substantial part of this protein fraction.

The most straightforward conclusion is clearly that the whole of the myosin in muscle is located in the *A* bands; that actin is present as part of the *I* substance; and that when myosin is extracted from glycerinated fibrils, some of the actin is also removed, in a partially denatured form. The results cannot exclude, however, the possibility that a small part of the myosin is present in the *I* substance, and that some or all of the *X*-protein is present in the *A* substance; the quantities involved have a maximum value of 10% of the total fibrillar protein, and so at least four fifths of the myosin must necessarily be present as the *A* substance. But the simpler hypothesis, that all the myosin is present in the *A* substance, is in such excellent agreement with the facts that it seems much the more probable one.

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SUMMARY

The quantity of *A* substance in glycerol-extracted fibrils of rabbit psoas muscle has been determined by interference microscopy and found to be 50–55 %, most probably 54.5 %, of the total protein of the fibril. When myosin-extracting procedures are applied to these fibrils, all of the *A* substance is removed, together with another 10 % of the total protein, making a total of 60–65 % extracted.

In the accompanying paper[12] it has been shown by chemical analysis of similar fibrils that myosin constitutes about 51 % of the total protein of the fibril, and that the myosin-extracting procedures remove about 62 % of the total protein.

These comparative measurements by interference microscopy and chemical analysis prove that at least four-fifths of the myosin in these fibrils is present as the *A* substance, and the results are in excellent agreement with the hypothesis that *all* the myosin is concentrated in the *A* bands.

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