An X-ray and Electron Microscope Study of Tropomyosin

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METHODS AND RESULTS

X-rays

A technique that we have found convenient in trying to obtain oriented preparations of elongated macromolecules is to make a thin film by drying a pool of sol on a glass plate, and then to stretch narrow ribbons of the film (Astbury & Dickinson, 1940). The first step tends to leave the molecules lying parallel to the surface of the film but in all azimuths, while the second tends to draw them parallel also to the direction of stretching, so that in the end an X-ray fibre diagram should result. Even without the second step, however, an imperfect fibre diagram may be expected if the specimen is photographed with the X-ray beam parallel to the surface of the film. Further orientation and stretching effects may be produced by squeezing the film between small pieces of plate glass clamped together by screw clips.

Thin films of tropomyosin are readily prepared from the aqueous sol. The X-ray diagrams obtained when the beam is perpendicular and parallel to the film, respectively, are shown in Figs. 1*a*, *b*. They are typical wide-angle α-patterns of the keratin-myosin-epidermis-fibrinogen group. The fact that Fig. 1*b* is such a good approximation to a true fibre diagram indicates that the tropomyosin units are so elongated (and probably stiff) that the drying-contraction normal to the surface of the sol forces them to lie down almost flat. The observed spacing of the characteristic meridian arc is 5·11 A., but for the present there is some uncertainty about the side-chain spacing. It is in any case always difficult to measure exactly, but in addition we have noticed an apparent variability at room humidities that, pending further investigation, might reasonably be ascribed to inter-chain swelling by water. The higher estimated values are of the order of 10 A., which is rather greater than what is found for keratin and myosin. The photographs illustrated in Figs. 1*a* and 2*a*—they were of an earlier preparation—show also an 'extraneous' halo at about 4·2 A., which is a spacing that we have come to associate with traces of fatty substances, though it does not follow, of course, that that is necessarily the correct interpretation here.

It has not yet been found possible to produce β-tropomyosin by stretching the α-film, either air-dry or over water vapour, for it always breaks after a small percentage extension. In this respect it differs sharply from keratin and myosin. Neither has squeezing at ordinary temperatures been found effective, though it succeeds with keratin, myosin, and fibrinogen (Astbury & Sisson, 1935; Astbury & Dickinson, 1940; Bailey, Astbury & Rudall, 1943). It appears for the moment that heat is necessary: for instance, the disoriented but exceptionally well-developed β-diagram shown in Fig. 2*a* was obtained by drying a moist specimen at 105°, while Fig. 2*b* was obtained by squeezing another moist specimen between pieces of plate glass that had first been heated in steam, and then photographing with the X-ray beam parallel to the flat surface. The transformation to β-tropomyosin can take place at much lower temperatures, however, for a good β-diagram was also obtained when the plates were preheated to only 80°.

Fig. 2*b* is of the less familiar 'cross β' type, i.e. with the backbone reflexion lying on the meridian instead of on the equator; and detailed examination shows that this is due to the fact that hot-squeezing not only transforms the folded α-form into the extended β-form, but also rotates the polypeptide grids so that their side chains stand approximately normal to the plane of flattening. This effect (it indicates a building-up of ribbon-like aggregates of β-grids held together by the backbone linkage) was first observed on squeezing keratin in steam (Astbury & Sisson, 1935), and it was later observed on squeezing moist myosin even at room temperature (Astbury & Dickinson, 1940).

The backbone spacing is again 4·65 A., as found in the other members of the *k-m-e-f* group, but for the present the other two principal spacings, the side-chain spacing and the amino-acid spacing, are not so definite. As in the α-diagram, the former appears to be somewhat higher than usual (it is probably over 10 A. at room humidity), while, in the absence of the normal β-diagram, the latter can only be estimated at 3·2–3·3 A.

Preliminary X-ray examination has been made of tropomyosin crystals in the 'powder' form, air-dry and moist, but nothing has been revealed so far beyond the disoriented α-pattern, plus ammonium sulphate reflexions in the first case and a water halo in the second. It is clear that the α-pattern arises from the structure of the individual tropomyosin units; if the crystals are redissolved and a film is made, the α-pattern is found as before.

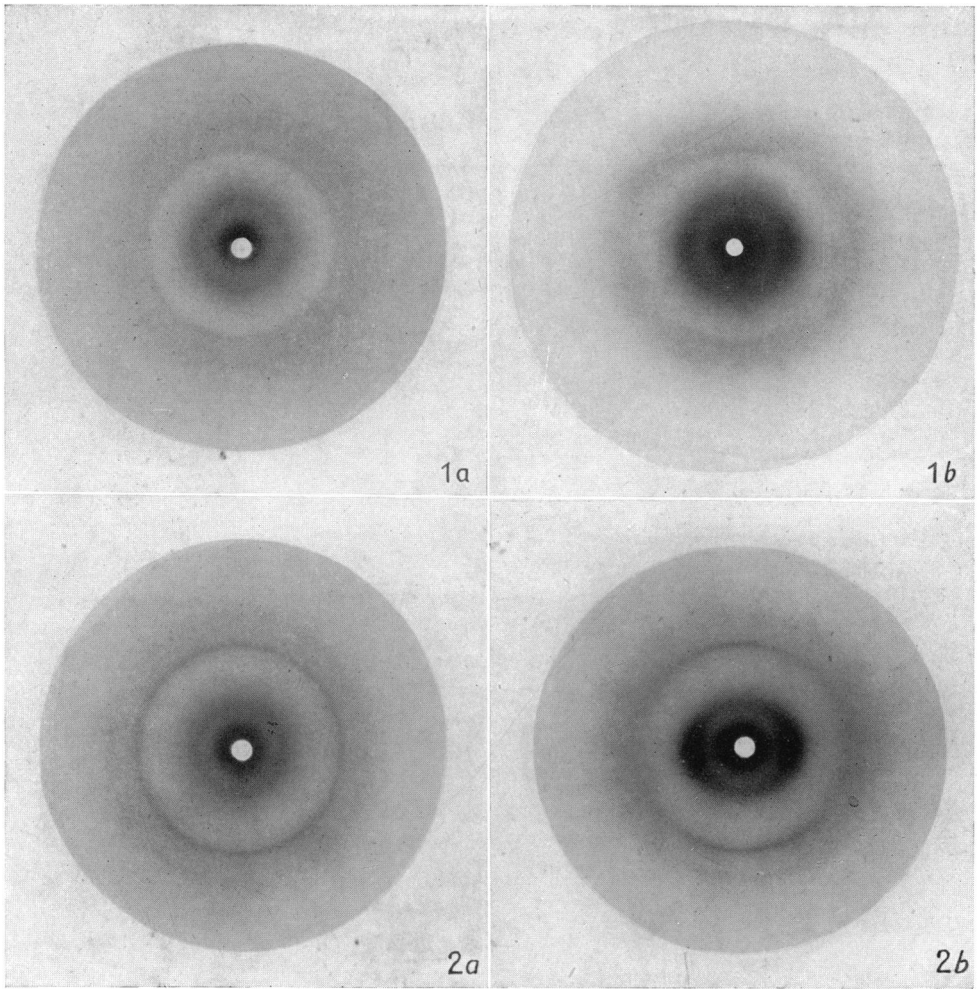


Fig. 1. X-ray photograph of tropomyosin film at ordinary humidity. (*a*) Beam perpendicular to the surface. (Disoriented α.) (*b*) Beam parallel to the surface. (Partially oriented α.)

Fig. 2. (*a*) Disoriented β-diagram obtained by drying moist tropomyosin at 105°. (*b*) Cross β-diagram obtained by squeezing moist tropomyosin between glass plates that had been heated in steam. Beam parallel to the surface.

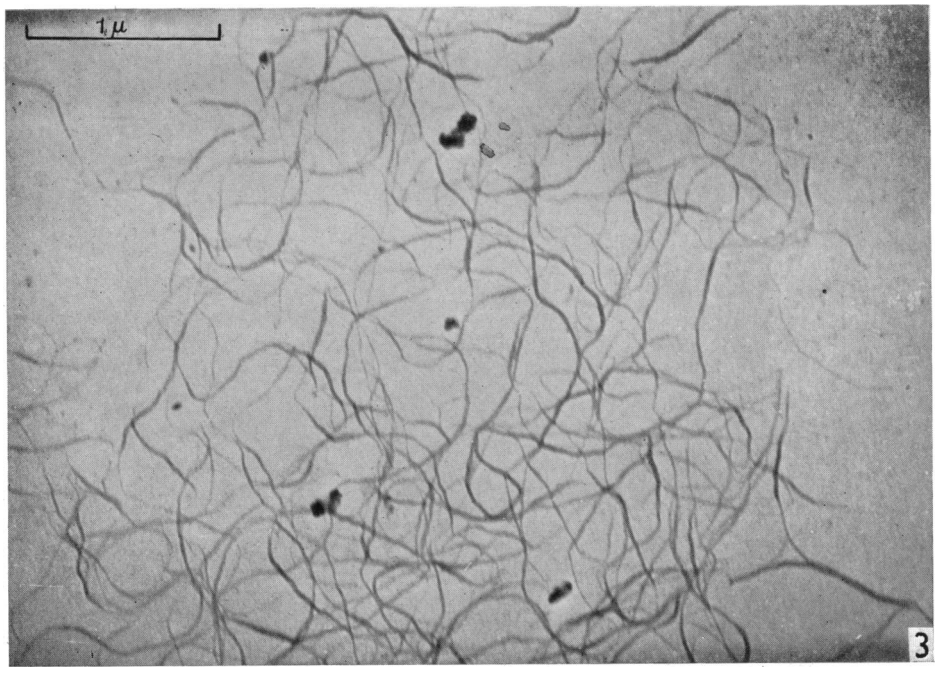


Fig. 3. Tropomyosin fibrils deposited from aqueous solution.

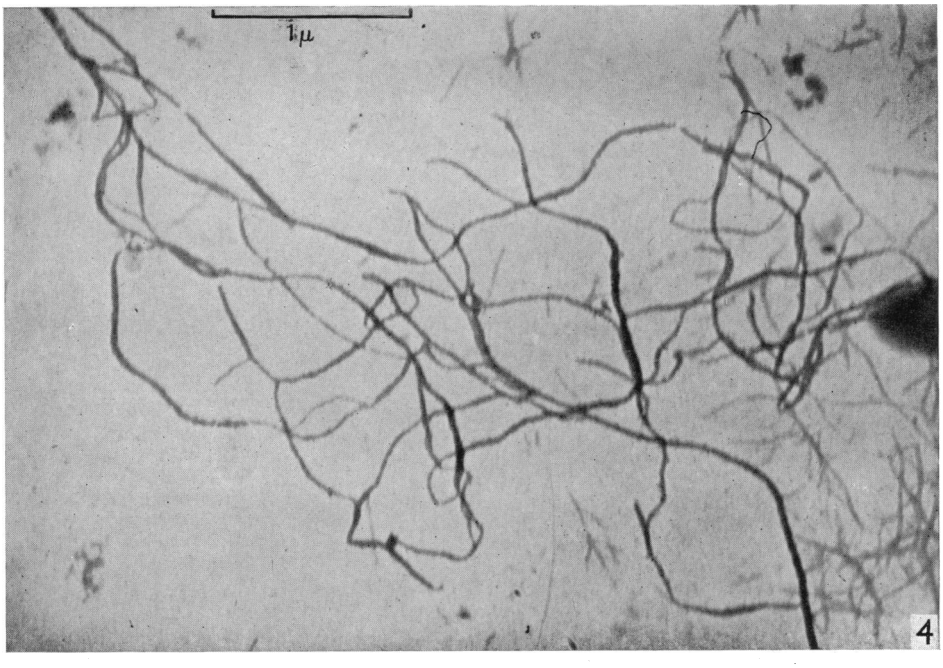


Fig. 4. Tropomyosin fibrils deposited from aqueous solution and stained with osmic acid vapour.

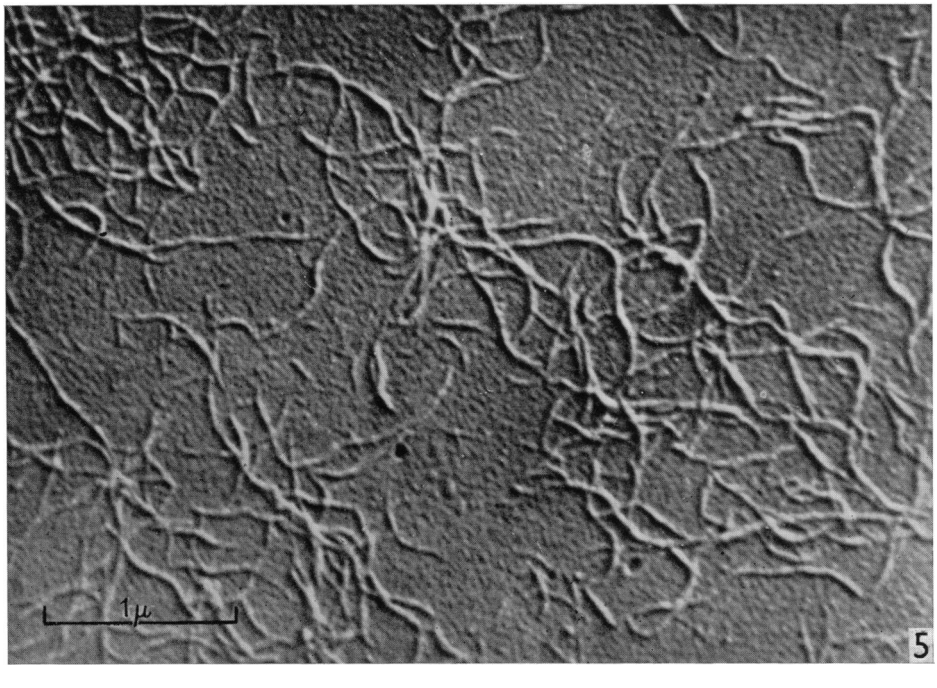


Fig. 5. Tropomyosin fibrils deposited from aqueous solution and shadowed with gold.

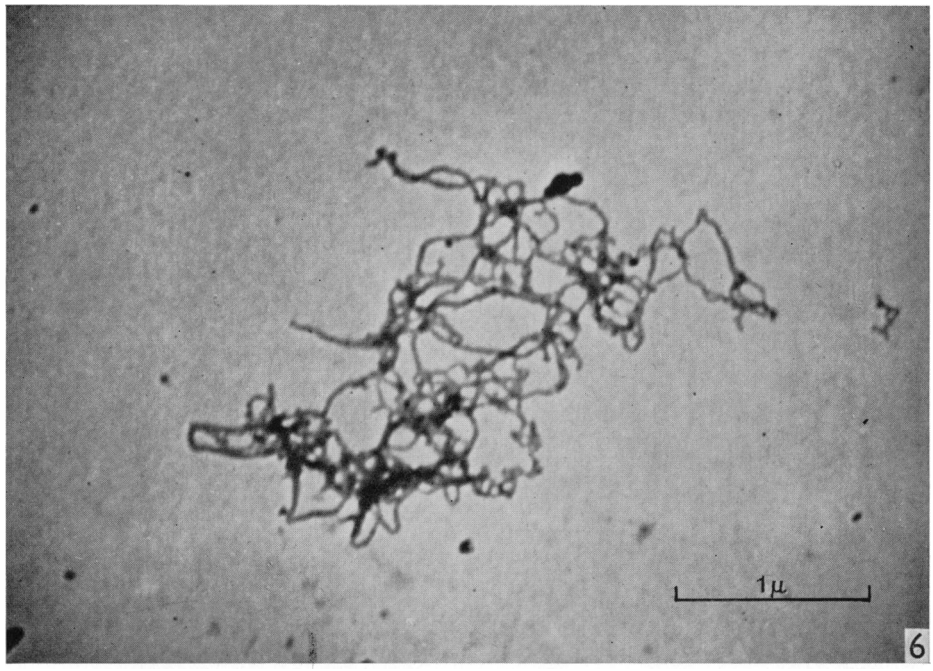


Fig. 6. Tropomyosin fibrils deposited from an aqueous solution that had been heated to 80° for 5 min.

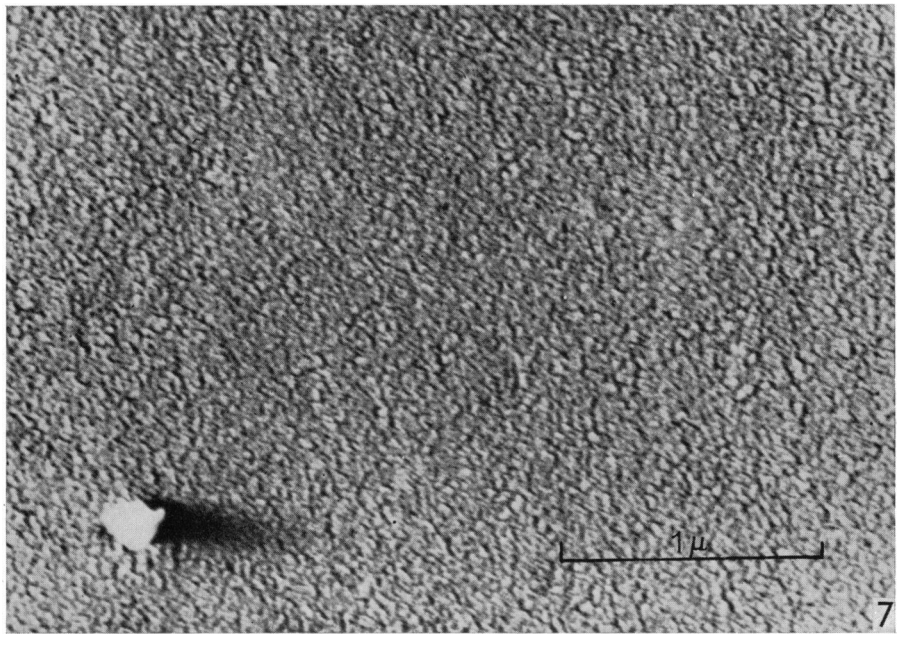


Fig. 7. Deposit from a solution of tropomyosin in 0·1 M-KCl. Gold shadowed.

A few preliminary observations have also been made on the property of 'supercontraction' that tropomyosin might be expected to possess in common with the other members of the *k-m-e-f* group. It was found to be no exception. For this test, short lengths (about 1 cm.) of narrow ribbon cut from a thin film were exposed to steam, and in every case a rapid contraction was recorded, amounting in the mean to some 30% of the initial length. These were admittedly only rough trial experiments, but they leave no doubt that tropomyosin, too, has the power of supercontracting strongly. It may be recalled that similar experiments with myosin films in which the molecules lay approximately flat, but in all azimuths, gave supercontractions of the order of 20% of the initial length (Astbury & Dickinson, 1940).

Electron microscope

The photographs were taken with an RCA instrument, type B. The preparations studied were formed from drops of solution placed on filmed specimen grids and dried by one or other of the following means: air drying at room temperature; accelerated air drying at about 50°; desiccator drying; rapid vacuum drying. The aqueous solutions, at pH 6–7, were made by dissolving in thrice-filtered distilled water the horny solid obtained on drying a concentrated solution, and then diluting to about 1 in 50,000. Two salt solutions were examined, one in 0·1 M-KCl and the other in 0·033 M-KCl, at a concentration of 0·002% protein. For these a drop of solution was placed on the filmed grid and then immediately drained off with filter paper so as to leave a thin layer. The results described here were obtained with tropomyosin prepared from rabbit skeletal muscle, but towards the end of the investigation similar photographs were obtained with fish tropomyosin.

Aqueous solutions were thus shown to give a mesh of very long, fine, and remarkably uniform fibrils, as illustrated in Fig. 3, which is a straightforward micrograph without either staining or metal-shadowing.

Similar dry preparations deposited from aqueous solution were also exposed to the vapour of 1% osmic acid solution, at room temperature, for periods of 1–30 min., and afterwards washed with distilled water. This treatment made the fibrils much bolder in the viewing screen, and in addition it revealed in places a transverse banded, and somewhat saw-tooth, appearance. Fig. 4 illustrates a micrograph of this sort.

The most striking photographs (e.g. Fig. 5) were obtained after shadowing the dry preparations with gold, by the now well-known technique developed by Williams & Wyckoff. The fibrils are seen to be of the order of 200–300 A. thick, and though there were sometimes indications of the banded or beaded appearance suggested by the osmic acid photographs, it was not found possible to confirm this finding outright because of suspicions that the gold may have aggregated. However, it is hoped to repeat these experiments later using chromium.

The action of heat on aqueous solutions of tropomyosin is illustrated by Fig. 6. The solution was heated at 80° for 5 min. and a small drop was quickly transferred to a filmed specimen grid that had been warmed to 50° in a dry tube, after which the preparation was dried as quickly as possible. It will be seen that there is a well-marked change in the character of the fibrils: the network has shrunk and the fibrils have less sharp outlines and have become curled up and often twisted together. Similar experiments at 100° or for longer times produced still more marked effects of this kind.

The action of KCl is illustrated in Fig. 7. The effect is to disperse the fibrils more or less completely; in fact, the 0·1 M solution gave hardly anything on which to focus. After staining with osmic acid vapour a few small fibrils and particles were revealed in the deposit from the 0·03 M solution; but from the 0·1 M solution there were fewer such particles and only poorly resolved micro-fibrils, perhaps about 100 A. thick and a few hundred A. long.

DISCUSSION

The following discussion is based partly on the X-ray and electron microscope results just described and partly on data given in the preceding papers by Bailey (1948) and Bailey, Gutfreund & Ogston (1948).

Tropomyosin is distinguished from other members of the *k-m-e-f* group (except perhaps fibrinogen) in that it consists of relatively short identical chains, or combinations of chains, from which can be prepared both fibres and visible orthodox crystals. It is a 'monomer' of the group (Astbury, 1947*a*, *b*), so to speak, the other members having proceeded presumably to the further stage of end-to-end addition to form much longer chains. It may be a breakdown product of some larger complex already built up in this way, or it may represent 'monomeric' units isolated before 'polymerization', but in either case there can be no doubt of its great importance for the study of the fibrous proteins. It is a key discovery that properly exploited might very well make the decisive contribution towards elucidating the *k-m-e-f* group completely.

Taking the probable value of the molecular weight to be about 90,000, this is equivalent to 90,000/116·4 = 773 residues; and if these residues were incorporated into a single polypeptide chain in the fully extended configuration, their total length would amount to $773 \times 3{\cdot}33 = 2574$ A., and the average side dimensions of the chain would be approximately 10 A. by 4·5 A. The mean axial ratio would thus be well over 300. We have, of course, to consider all possibilities in relation not only to X-ray

data but also to those provided by other techniques, but there is no really convincing argument in favour of this interpretation. Such diffusion and viscosity evidence as is available for the present, though admittedly imperfect, at least points to a considerably smaller axial ratio, and it is hard to believe that such long, flexible chains would build large crystals of the commonplace habit observed. The X-ray photograph given by tropomyosin in its normal state is always an α-diagram, and quite apart from any question of the true nature of the α-configuration, no doubt now remains as to what kind of diagram is given by the fully extended or β-configuration: there is always a strong backbone reflexion of spacing $4{\cdot}6_5$ A., not to mention other characteristic reflexions of weaker intensity. There is nothing in the normal tropomyosin diffraction pattern to suggest either extended chains of the full length quoted above or combinations of similar shorter chains: the diffraction pattern of aggregates of extended chains appears only after treatment to that end.

According to views most favoured at the moment (Astbury & Bell, 1941; Astbury, 1942), the α-form is produced by a regular folding of the backbone (in a plane transverse to the side chains) which reduces the length by very nearly one half. On this interpretation we have, therefore, to consider chains of length about 1287 A., or combinations of submultiple chains which are either distinct or are only loops in the longer chain. Table 1 gives the approximate dimensional characteristics of some of the more

Table 1. *Some possible dimensions of the tropomyosin molecule*

Effective no. of α-chains	Length (A.)	Side dimensions (A.)	Mean axial ratio
1	1287	10 × 9·5	132
2	644	20 × 9·5 or 10 × 19	44
3	429	30 × 9·5 or 10 × 28·5	22

plausible possibilities. These dimensions refer, of course, to the anhydrous molecule, and they are intended to be no more than rough guides for comparison with results that may be obtained by other methods. The alternative side dimensions correspond to the two principal modes of linking parallel polypeptide chains, either by their side chains to form a 'grid' of two or three chains, or by their backbones, in which case they lie on top of one another like centipedes. The first possibility seems the more reasonable, but arguments can be adduced in support of the second.

Table 1, tentative as it is, serves at least to bring out the marked dimensional differences to be expected, and it should not be too difficult to distinguish between the various possibilities by means of critical measurements of diffusion, viscosity, and light scattering. Such measurements are for the present incomplete, but it is informative even now

Table 2. *Calculation of D and ν from possible axial ratios of the tropomyosin molecule*

(See text for explanation of columns.)

(1) Mean axial ratio	(2) D_0/D	(3) D	(4) ν
132	~4·7	$\sim 1{\cdot}54 \times 10^{-7}$	~996
44	2·78	$2{\cdot}60 \times 10^{-7}$	143
22	2·07	$3{\cdot}49 \times 10^{-7}$	45

to make a few comparisons. Table 2 gives estimates of quantities related to diffusion and viscosity. The explanation of the four columns is as follows:

(1) These axial ratios are taken from Table 1. For the purpose of subsequent calculations the assumption is that the molecule approximates to an unhydrated elongated ellipsoid.

(2) D_0 is the diffusion constant of a sphere of the same mass and volume as the ellipsoid. Its radius is given by

$$4/3\pi r^3 \times 1{\cdot}28 = 90{,}000 \times 1{\cdot}65 \times 10^{-24},$$

whence $$r = 30{\cdot}3 \text{ A.}$$

Also $$D_0 = \frac{kT}{6\pi\eta r},$$

where k is Boltzmann's constant ($1{\cdot}380 \times 10^{-16}$ erg/degree), T is absolute temperature, and η is the viscosity of the solvent (in this case a buffer at pH 6·5, consisting of 0·2 M-KCl, 0·0133 M-Na_2HPO_4, 0·0267 M-NaH_2PO_4, for which Bailey found a viscosity of approximately 0·0098 poise). Thus D_0 at 20° is found to be $7{\cdot}23 \times 10^{-7}$ cm.²/sec. The ratio D_0/D is equivalent to f/f_0, the 'frictional ratio' of Svedberg & Pedersen (1940), and it is obtained from the axial ratio by aid of a table (Cohn & Edsall, 1943), based on F. Perrin's (1936) equation.

(3) D follows from the ratio D_0/D and the calculated value of D_0.

(4) The quantity ν is the so-called 'viscosity increment' of the solute, and is given by

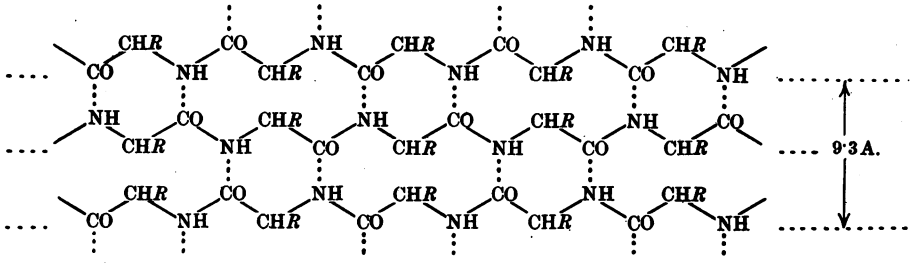
$$\frac{1}{\phi}(\eta/\eta_0 - 1) \equiv \nu,$$

where η is the viscosity of the solution, η_0 that of the solvent, and ϕ is the volume fraction of the system occupied by the solute molecules. It is obtained from the axial ratio by aid of a table based on Simha's equation (Mehl, Oncley & Simha, 1940; Simha, 1940; Cohn & Edsall, 1943).

The available experimental values of D are 2·22 and $2{\cdot}26 \times 10^{-7}$ found by Ogston, and $2{\cdot}43 \times 10^{-7}$ found by Gutfreund on extrapolating to a protein concentration of 0·6 % the values obtained at concentrations of 1·2 and 0·7 %. Bailey (personal communication) has found values of ν equal to 79 and 65 at ionic concentrations of 0·27 and 1·07, respectively. If we assume that axial ratios intermediate between those quoted are ruled out—an

assumption which seems reasonable in the light of all that we know now about the *k-m-e-f* group—then it is fair to say that the diffusion data support tolerably well the double α-chain structure of mean axial ratio about 44. We should expect the observed diffusion constant to be appreciably less than the calculated, principally because we have made no allowance for hydration, which can hardly be neglected with such a polar molecule as tropomyosin, and because, strictly speaking, we need to know the diffusion constant either at infinite dilution or at at least for a region of concentration where it does actually remain constant. These omissions represent only the more obvious difficulties: there are, besides, the experimental difficulties associated with the exact measurement of diffusion constants, and in any case the theoretical formulae used are still to be considered as no more than hopeful approximations, pending thoroughgoing test on suitable molecules whose characteristics have been definitely established by independent methods. There would be little justification at this stage in trying to estimate, for example, the degree of hydration required to bridge the gap between prediction and observation, but on the whole it does appear that agreement with a double α-chain is not unsatisfactory. As regards the viscosity data, however, the situation is less encouraging, for the present available values of ν point to something between the axial ratios of 44 and 22; that is, the observed viscosity is *less* than might be expected for a double α-chain. The explanation of this discrepancy may lie in Simha's formula, which is meant to apply only if orientation effects produced by the shearing forces are so small that all molecular orientations may be taken as equally probable. With greatly elongated structures such as we have reason to believe tropomyosin molecules are, the velocity gradients required to meet this condition must be very small indeed, otherwise the recorded viscosity will always be low. At present the experimental data are insufficient to guarantee that Simha's equation would be valid.

The suggestion that a fundamental unit of the *k-m-e-f* group comprises at least two parallel chains (either distinct or forming a loop in a single longer chain) is the more readily acceptable because there are also certain X-ray indications to that effect (Astbury & Woods, 1933). The crystallographic interpretation of the β-diagram requires a unit of pattern which repeats in one of the lateral directions at a distance of *twice* the backbone spacing, and our original explanation of this, which appears to be generally approved, was that it arises from two chains running lengthways but in opposite directions and linked by a succession of CO···NH bridges (now known as the 'backbone linkage') thus:



(The side chains are perpendicular to the plane of the paper and are pointing alternately up and down)

Fibre axis

The electron microscope studies show that the very high viscosity and the easy flow birefringence of aqueous solutions of tropomyosin are consequences of the units aggregating into long, fine fibrils. The latter are observed visually only after evaporation, but there is no doubt that they exist as such in solution so long as salt is absent: when potassium chloride is added the fibrils are broken down again into their units, but the change is reversible on removing the salt. As already mentioned, it is remarkable how uniform the fibrils often are, as though they tend to an almost crystallographic regularity, but this is perhaps not surprising in view of the well-formed and comparatively large crystals that can be grown by other procedures. The micrographs taken after treatment with osmic acid strengthen this impression by their occasional banded appearance, and it may be that some of the micrographs taken after gold-shadowing, though at present open to suspicion, are to be interpreted similarly. By analogy with the yarn-like structure of so many other fibres it would be natural to assume that the long tropomyosin units lie lengthways in the fibrils, overlapping one another irregularly in many regions, but lying strictly abreast or at least overlapping with geometrical regularity in those places where transverse bands are found, but it is not yet possible to verify this assumption directly

by means of the X-ray diagrams because of the difficulty of stretching strips of film without rupture. All that photographs of unstretched film show is that elongated units lie approximately parallel to the surface of the film, but whether parallel or transverse to the fibrils is uncertain; but if a suitable stretching technique could be devised, then it might be possible to orient the fibrils approximately parallel to a single direction and thus reveal which way the units lie *within* the fibrils. It seems very probable that they lie lengthways, otherwise we might expect the flow birefringence to be negative with respect to the direction of flow, whereas it is observed to be positive. Again, the observed usual fibril thickness is of the order of 200–300 A. (and finer fibrils are found too), which is less than the presumed length of even a three-chain unit, let alone the two-chain unit suggested by the diffusion data.

Tropomyosin fibrils present an important example of a phenomenon which has come to the fore only recently, but which promises at last to throw light on the mechanism of formation of protein fibres *in vivo*. It is now well established (Astbury, Dickinson & Bailey, 1935), and indeed has led to successful industrial development (cf. Astbury, 1945), that artificial protein fibres can be made by unfolding and subsequently drawing out the polypeptide chains from the specific configurations of many corpuscular proteins, but whether this process bears any relation to what happens in nature is another question. Actually, the evidence is beginning to point in a direction that is rather an extension of the familiar mechanism of chain formation by polymerization and polycondensation that has been exploited so strikingly in the manufacture of the other great group of man-made fibres, such as nylon and the like, which are truly synthetic. It is for this reason that we have considered tropomyosin as a kind of 'monomer' of the *k-m-e-f* group (Astbury, 1947*a*, *b*): it has the capacity of building up fibres, but as so far observed *in vitro* these are only of an impermanent character; the constituent units are held together by no more than secondary linkages and they can be dispersed again simply by the addition of salt. We can, however, conceive of a further and more permanent step whereby long fibres are built up by the incorporation of covalent linkages between the 'monomeric' units, and it is a 'polymerization' step of this kind that now seems one of the likely final stages in the formation of keratin, myosin and fibrin, and other natural protein fibres. Two other recently recognized examples of the reversible aggregation of corpuscular units to form long, uniform fibrils are fibrous insulin (Waugh, 1946; Hall, 1947) and *F*-actin (Jakus & Hall, 1947; Astbury, Perry, Reed & Spark, 1947). The latter, discovered by Straub (1942, 1943), has been shown by Szent-Györgyi (1942, 1947) and his school to play a critical part in the mechanism of muscular contraction, but its significance in the deeper family relationships of the *k-m-e-f* group may turn out to be more fundamental still (Astbury, 1947*b*, *c*).

The macroscopic crystals of tropomyosin that Bailey has succeeded in growing arouse the greatest interest. Above all they offer an opportunity of single-crystal study by X-ray methods, with the object of discovering ultimately at least the detailed shape if not the actual structure of the units. For the present what has been ascertained is that in the powder form the crystals give the same type of large-angle diffraction pattern as is given by films and by the other members of the *k-m-e-f* group, but it is hoped soon to be able to operate with single crystals. The thoroughly orthodox habit alone is something of a surprise, for it is the sort of thing one associates not with greatly elongated rods but rather with molecules much nearer the spherical; but even more astonishing is the very high water content, amounting to almost 90%. Bailey's figures are: water 89·6, ammonium sulphate 1·74, and protein 8·76%; from which it follows that there are on the average about sixty-six water molecules per amino-acid residue. Another way of describing the situation is to say that on the basis of parallel two-chain units of lateral dimensions approximately 20 × 10 A., each encased in a uniform thickness of water molecules, the mean distance between the surface of one unit and the next comes to about 40 A.! Of course, the longer side chains will project beyond the average, but in the case of the longest (arginine) this will only reduce the distance between the ends of side chains by perhaps 10 A., and we are still left with the impression of a structure that is hardly more than an organized gel, an impression that is confirmed by the great difficulty in even moving the crystals without distorting them.

SUMMARY

1. The large-angle X-ray diffraction diagram of tropomyosin is the characteristic α-diagram of the keratin-myosin-epidermis-fibrinogen group. A similar diagram is obtained whether the specimen is thin film prepared from aqueous solution or a crystal 'powder': presumably, therefore, it arises from the intramolecular pattern of the tropomyosin units themselves.

2. It has not yet been found possible to produce the β-diagram by stretching or squeezing at room temperature, but a well-defined β-diagram appears on heating to above about 80°, and hot-squeezing orients the side chains perpendicular to the plane of flattening, as in other members of the *k-m-e-f* group.

3. Preliminary tests indicate that tropomyosin film shows the property of 'supercontraction' found in other members of the *k-m-e-f* group.

4. In the electron microscope, a deposit from aqueous solution is seen to consist of remarkably uniform fibrils mostly about 200–300 A. thick. These fibrils are completely dispersed into their constituent units by the action of potassium chloride.

5. When stained with osmic acid, and possibly also on shadowing with gold atoms, the fibrils show indications of cross striations.

6. Fibrils deposited from preheated aqueous solution have a shrunken, curled-up and twisted appearance.

7. The structures of the tropomyosin units, fibrils and crystals are discussed in the light of the above findings and data reported in the accompanying communications. It is suggested that tropomyosin is a typical 'monomer' of the *k-m-e-f* group, the long fibres of which are built up by a process of 'polymerization' involving linkages of a more permanent character than those which operate in the formation of the fibrils found in aqueous solution. The latter, however, would appear to be of the nature of impermanent prototypes of the fibres formed *in vivo*.

8. Available diffusion and viscosity data lend reasonable support to the hypothesis that the tropomyosin unit consists of a pair of chains, or a looped single chain, in the α-configuration.

It is clear that the investigation described in this paper could not have been carried out without Dr K. Bailey's close co-operation, and we wish gratefully to acknowledge this and the help of many discussions.

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A Comparison of the Decomposition of Hydrogen Peroxide by Catalase, Ferrous and Ferric Ions, Haemin and Ferrous Phthalocyanine

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The catalytic decomposition of hydrogen peroxide into water and molecular oxygen has been shown to be a very complicated reaction. In the earlier work up to 1923 it was established that the reaction with many different catalysts is quasi-unimolecular: for instance, there are the typical experiments of Bredig & Berneck (1899), Duclaux (1922) and Bertelan (1920) on the decomposition catalyzed by colloidal metals and ferrous and ferric salts. From 1920 to 1932 more detailed kinetic analyses were carried out on the bromine-bromide ion and iodine-iodide ion systems, and the first general kinetic mechanism for the decomposition was devised by Abel (1920) and Bray & Livingston (1923). This is the principle of compensating reactions in which both the oxidized and reduced forms of the catalyst react with the peroxide, giving a dynamic equilibrium between these two states and a resultant catalytic decomposition of the peroxide. For the heavy metal catalysis the chemical mechanism was represented by more or less cumbrous stoicheiometric equations.