X-ray Diffraction Studies of Muscle: Observations on the anterior byssus retractor muscle of *Mytilus edulis* and the insect flight muscle from *Sarcophaga bullata*.

by

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Abstract

The interfilament spacing of the anterior byssus retractor muscle from *Mytilus edulis* was studied as the muscle was extended. It was found that variations in this spacing were very small and consistent with the hypothesis that the interfilament spacing was independent of the extension of the muscle.

It was observed that the interfilament spacing was dependent on the osmolarity of the bathing medium. In concentrated solutions of the artificial seawater, the interfilament spacing decreased; while in dilute solutions of artificial seawater, it was observed that the interfilament spacing was increasing.

X-ray diffraction patterns were obtained from fresh, and glutaraldehyde fixed, specimens of insect flight muscle from *Sarcophaga bullata*. There patterns were in general agreement with previous X-ray diffraction studies of insect flight muscle. A reflexion at 93Å was observed and interpreted as arising from diffraction in the mitochondria. Specimens of dried insect flight muscle produced a diffraction pattern consisting of arc and ring reflexions. This was interpreted as suggesting an ordered arrangement of cristae, in the mitochondria from these muscles.

General Introduction

Animals have a basic characteristic in their ability to move purposefully. This ability has arisen from the development of muscles (or some primitive version of them).

Muscles are usually classified as striated or smooth, depending on their appearance under the ordinary light microscope. Functionally, striated muscles are usually muscles which act quickly and are under voluntary control, for instance, limb muscles in vertebrates; while smooth muscles act slowly and involuntarily, for instance, muscles of the gut or uterus.

As most of the research on muscles was performed with striated muscle, then the knowledge of these is in a more advanced state; however, much of this knowledge is believed to be directly applicable to smooth muscle.

Early theories to explain muscle contraction on the molecular level usually involved the folding or coiling of the protein chains in the muscle.

The theories have differed in the type of folding assumed and in the nature of the forces that were supposed to produce the folding, but they have been remarkably uniform in assuming that shortening takes place in each filament at a number of points in series, so that the decrease in length of the whole filament is the sum of the individual amount of shortening (A.F. Huxley, Muscle Structure and Theories of Contraction, Progress in Biophysics 7, 1957, p 279).

These theories were strongly encouraged in 1940, when Astbury and Dickinson found that changes in structure occur when a myosin film was stretched or made to shorten. However, W.T. Astbury (1947) reported that there did not seem to be any important changes in the wide angle X-ray diffraction pattern between a contracted muscle and a resting muscle. The theories based on the alterations of the configurations of the

polypeptide chains would require large changes in the diffraction pattern. It therefore appeared that units, larger than the individual polypeptide chains, were involved in the contraction process.

In 1951, Huxley, using a specially designed low angle camera and a fine focus X-ray tube designed by Ehrenberg and Spear (1951), recorded an equatorial X-ray diffraction pattern which showed that there was present, in living muscle, a hexagonal array of long filaments spaced about 400Å apart. Huxley (1953) also found that the spacing between filaments varied with the muscle length, such that the volume of the array remains approximately constant. The live muscle also showed a pattern of meridional reflexions indicating that the protein molecules are arranged in a regular way. The spacing of these meridional reflexions did not change when the muscle was stretched.

The early X-ray results also indicated that there was a second set of longitudinal filaments present and that these gave rise to prominent regions of electron density at the trigonal positions of the hexagonal lattice in muscles in rigor. The evidence for this was that the X-ray patterns from muscle in rigor showed a very strong (1,1) reflexion; whereas, in living muscles this reflexion was weaker than the (1,0) reflexion. This indicated that, at the trigonal points of the hexagonal lattice, there has been an increase in density when the muscle goes into the rigor state (Huxley, 1952).

The electron microscopy techniques were being improved and they were soon at the necessary level for the two types of filaments to be seen. The expected double hexagonal arrays were seen, with crossbridges between the filaments, but also it was noted that the double arrays were not seen everywhere, only in the lateral parts of the A bands of the muscle (Huxley, 1953b).

Hanson and Huxley (1953) were able to propose the overlapping filament model, which stated that the myofibrils are made up of a succession of partially overlapping, interpenetrating arrays of protein filaments, and the filaments in these arrays are in a regular hexagonal lattice.

Hanson and Huxley then set out to determine the constancy of the A band and the I band filaments using light microscopy in muscles under various conditions. The constancy in length of the A band was found by H. E. Huxley and Hanson (1954) and, independently, by A. F. Huxley and Niedergerke (1954). This enabled both groups simultaneously to propose the sliding filament model.

In the sliding filament model the arrays of filaments slide past each other as the muscle length is changed. The individual filaments do not change their length, and the contractile force is generated by a process which actively translates one type of filament past the neighbouring filament of the other type.

More recently, other evidence to support this model has been collected. The meridional reflexions of the diffraction patterns have been identified to reveal two sets of reflexions. By comparing one set with patterns from oriented preparations of purified actin (Astbury and Spark, 1947), it was possible to identify this set as originating from the actin filaments.

The second set is from a helical structure and this is believed to arise from the myosin filaments, the pattern being dominated by the projecting crossbridges (Worthington, 1959; Elliott and Worthington, 1959; and Elliott, 1964).

The constancy of the spacing of these reflexions in actively contracting

muscle was also demonstrated (Huxley, Brown and Holmes, 1965; Elliott, Lowy and Millman, 1965; Elliott, Lowy and Millman, 1967; Huxley and Brown, 1967).

Elliott, Lowy and Worthington (1963), demonstrated that not only the lattice spacing, but also the relative intensity of the equatorial reflexions, changed with the sarcomere length. They noted that the (1,1) reflexion became progressively weaker relative to the (1,0) as the sarcomere length was increased. This meant that there was a progressive decrease in the amount of material at the trigonal positions of the hexagonal lattice, and this observation agreed with the sliding filament model which required the extent of overlap of the thick and thin filaments to be dependent on the sarcomere length.

Electron micrograph evidence also supported the sliding filament model. Transverse sections through the muscle showed the regions of thin filaments, regions of overlap of the thick and thin filaments in a hexagonal lattice, and regions of thick filaments alone in a hexagonal lattice (Huxley, 1957).

Changes in the extent of overlap of the thick and thin filaments were seen in many electron micrographs. The filament lengths were closely measured in a series of experiments by Page and Huxley (1963), and Page (1964), and it was found that the filament lengths did not vary with the sarcomere length. The amount of filament overlap had increased, or decreased, to compensate for the change in sarcomere length.

New techniques in electron microscopy allowed the myosin and actin molecules to be studied. Hall (1955) had developed a negative staining technique in which the specimen under examination is embedded in a thin film of a very dense material. The regions of the specimen which are not penetrated by the dense material allow the electron beam to pass through with less absorption and therefore appear by negative

contrast. Also, Rice (1961) was adapting the technique of shadow casting to myosin molecules. This technique involves spraying the specimens at an angle with a vapourized heavy metal. The metal particles build up on one side of the specimen and a shadow is formed on the far side.

Huxley (1961, 1962) developed these techniques further for muscle. Unfortunately the techniques can only be applied successfully to small specimens but by placing the myofilaments in a relaxing medium and then placing them in a Waring blender, the thick and thin filaments could be separated to form specimens small enough for these techniques.

Huxley (1963) obtained electronmicrographs of myosin filaments prepared from the homogenized muscle. They are characterized by having a central zone, which is free of projections, and projections clustered along the rest of the filament. However, the remarkable observation was the synthetic filaments prepared from a precipitate of pure myosin also had this same form. Huxley (1963) found that the length of the synthetic filaments depended on the conditions of the precipitation, but the central projection free area always had the same length.

The shadow-casting technique had shown that the individual myosin molecules had a globular head and a straight tail (Rice, 1961; Huxley, 1963). Therefore, Huxley (1963) was able to propose that the thick filament is an aggregation of myosin molecules. These would arrange themselves so that the molecules are oriented in one of two directions, depending on which end of the filament the myosin molecule is attached. The central, projection-free, area of length 0.15 to 0.2 μ would be formed by the overlapping of the tails of the molecules. The internal structure of the myosin filaments has now been observed from cross-sections of striated muscle in the electron microscope (Pepe and Drucker, 1972).

Hanson and Lowy (1963) were applying the same techniques to the actin filaments. They found that the actin filaments had a beaded structure and appeared to consist of two chains of gobular subunits which were twisted around each other. Hanson and Lowy (1964) also observed the actin filaments from a large variety of species and found no difference in their appearance.

Huxley (1963) had added heavy meromyosin (HMM) to the actin preparation and discovered that the actin chains had a definite structural polarity. The HMM attached to the actin filaments in a specific way to give an arrowhead appearance. Furthermore, the polarity reversed across the Z line so that the actin filaments were in the same orientation to the bi-polar myosin filaments. The actin filaments to which HMM has been added are known as decorated thin filaments. Moore, Huxley, and De Rosier (1970) have performed a detailed study of these, and of the thin filaments, by applying Fourier analysis to the electron micrographs.

Recently, the structure of the myofilaments has been demonstrated using the technique of freeze-fracturing and etching (Rayns, 1972). Clearly visible in the electron micrographs were the crossbridges between the thick and thin filaments, and the arrays of filaments.

The protein biochemists were also looking at proteins of muscle. It was known that the myosin molecule could be broken down with enzymes and Gergely (1950) found that the ATP-ase activity was not linked to the structural integrity of the whole molecule.

Mihelyi and Szent-Györgyi (1953) were are to establish that the myosin molecule was split by the enzyme trypsin into two discrete components; one of these components possessed ATP-ase activity and also a capacity for binding to actin. Szent-Györgyi (1953) introduced the terminology of meromyosin for these components. The component which possessed the

ATP-ase activity and the capacity for binding to actin was termed heavy meromyosin (HMM), and the other component was termed light meromyosin (LMM), in respect to their behaviour in the ultracentrifuge. Szent-Györgyi also found that the LMM had the same aggregation properties as the myosin molecules. The LMM was also determined to be a helical molecule by Szent-Györgyi, Cohen, and Philpott (1960), using optical rotatory dispersion.

Lowey and Cohen (1962) performed further studies on the products of enzymic breakdown of myosin. They were able to estimate, using standard biophysical techniques, the molecular weights of the meromyosins. Huxley (1963) was able to demonstrate the general appearance of LMM and HMM using electron microscopy and shadow casting techniques. The light meromyosin appeared as a rod-shaped particle and the heavy meromyosin appeared as a rod with a large globular region at the end.

Koming, Mitchell, Nihei and Kay (1965) used the enzyme papain to break-down the myosin. They discovered that there was another peak present in their ultracentrifugation data. Therefore, they concluded that as well as LMM and HMM there was also another fragment present.

Lowey, Goldstein and Luck (1966) suggested that this new fragment was part of the HMM fragment. Lowey, Goldstein, Cohen and Luck (1967) were able to verify this. They found that HMM could be split to form two fragments, HMM subunit - 1 and HMM subunit - 2. The HMM S-1 was found to bind to actin while the HMM S-2 was found to be water soluble with little tendency to aggregate.

Lowey, Slayter, Weeds and Baker (1969) determined the size and shape of fragments from biophysical methods and from measurements on electron micrograph shadow casts. From this, they were able to propose a model of the substructure of a myosin molecule.

Summarizing, the myosin molecule is composed of three major fragments, LMM, HMM S-1 and HMM S-2. The light meromyosin is rod shaped and readily formed aggregates; the HMM S-1 is globular, has ATP-ase activity, and a strong tendency to combine with actin; and the HMM S-2 is rod shaped but soluble; i.e., it has no tendency to aggregate.

Huxley and Hanson (1960) had suggested that the bridges were formed by the HMM and the thick filaments were formed by the light meromyosins. The previous results have given very strong support to this hypothesis.

Current views in biochemistry suggest that if one protein molecule forms a specific complex with another then the complex will have a precisely defined configuration. However, as previously stated, the lattice of thick and thin filaments expands or decreases as the muscle is contracted or stretched, to give a constant volume effect. Therefore, the crossbridges would have to operate over a wide range of angles.

Lowey (1967) suggested that the reason the myosin molecule was susceptable to enzymic attack at the two specific locations was that the two specific locations were areas of flexibility in the molecule. Therefore, with two or more flexible areas, the heads of the myosin molecules could have attached at the correct angle to actin over a wide range of interfilament spacings.

As well as myosin and actin, striated muscle also contained other proteins. α-actinin (Ebashi and Ebashi, 1965; Masaki, Endo and Ebashi, 1967; Goll, Mommaerts, Reedy and Seraydarian, 1969; Goll, Suzuki, Temple and Holmes, 1972) and M-protein (Masaki, Takaiti and Ebashi, 1968) appeared to be necessary to aid the formation of the arrays of filaments. The α-actinin organized the thin filaments, and the M-protein organized the thick filaments at the M-line. Also tropomyosin and troponin Ebashi and Kodama, 1965, 1966) were present. These proteins regulated

the contractile process.

The contractile process involved the release of calcium ions from the sarcoplasmic reticulum. The calcium ions caused the inhibitory effect of the troponin-tropomyosin regulatory proteins to cease. The interaction between actin and myosin proceeded, with the utilization of ATP.

The binding capacity for calcium ions by the sarcoplasmic reticulum was greater than that of the contractile system; therefore, when the sarcoplasmic reticulum reassumed its calcium-accumulating activity, it recaptured the calcium ions from the contractile system. The inhibitory effect of the troponin-tropomyosin regulatory proteins re-appeared and this resulted in the cessation of the interaction of the filaments, and consequently relaxation, a passive process, followed.

The concentration of ATP also affected the contractile process. In the presence of calcium, high or low concentrations of ATP caused relaxation in the muscle. However, in normally contracting muscle the concentration of ATP was kept constant and the physiological regulating factor was the concentration of the calcium ion (see reviews by Ebashi and Endo, 1968; Ebashi, Endo and Ohtsuki, 1969).

In the presence of the regulatory proteins the contractile activities required calcium concentrations of around 10^{-5} M. When the calcium concentration was below 10^{-7} M the contractile activities were inhibited and the myosin and actin were unable to interact (Ebashi, 1961; Weber and Winicur, 1961; Weber and Herz, 1963). Ebashi and Ebashi (1964) found that if the regulatory proteins were removed then contraction could occur in the absence of calcium. As previously stated, Ebashi and Kodama (1965, 1966) had identified the regulatory proteins as tropomyosin and troponin. The troponin component has

been resolved into two components, troponin A and troponin B by Hartshorne et. al. (1969). They proposed that troponin A is responsible for the calcium sensitivity. Schaub and Perry (1969) confirm the resolution of troponin into two components; one component, in conjunction with tropomyosin, having an inhibitory effect on ATP-ase activity, and the other component carrying the calcium ion sensitizing factor. They also found that the troponin required tropomyosin to inhibit ATP-ase activity.

Ohtsuki, Masaki, Nonomura and Ebashi (1967), using ferritin-labelled antibodies, have shown that the tropomyosin combined with the thin filaments. The troponin also appears to bind periodically, around every 400Å to the thin filaments. Highashi and Ooi (1968, 1969); Nonomura, Drabikowski and Ebashi (1968) have demonstrated the binding of troponin to crystals of tropomyosin.

In view of the large separation of the troponin molecules, Ebashi and Endo (1968) and Ebashi, Kodama and Ebashi (1968) suggested that there might be a conformational change on troponin which would have an effect on the actin structure. Tonomure, Watanabe and Morales (1969) investigated this proposal using a spin labelling technique. They found that a spin label attached to tropomyosin responds to calcium ion fluctuations only when troponin is present, and that a spin label attached to actin responds to the calcium ion fluctuations when both troponin and tropomyosin are present. They, therefore, concluded there was a structural change communicated to actin, via tropomyosin, from the troponin.

It therefore appeared that muscle contraction, in vertebrate striated muscle, takes place by the sliding filament system. Crossbridges are formed by projections from the thick filaments attaching to the thin filaments. The process was regulated by the concentration of the calcium ions which interact with the regulatory proteins attached to the thin filaments.

The regulation of contraction in molluscan muscle appeared to differ from that of the vertebrate striated muscle. The calcium dependent factors which regulate contraction are associated with myosin. The actin-containing thin filaments did not bind calcium. They will activate the ATP-ase activity of rabbit myosin; however, this activity was not calcium dependent (Szent-Györgyi, Cohen, and Kendrick-Jones, 1970). The molluscan thin filaments will combine with tropomyosin and troponin proteins of rabbit and will then behave as rabbit thin filament preparations.

The components responsible for the calcium binding and the calcium dependence are not removed from the molluscan myosin by normal procedures, and the protein troponin did not appear to be present in the molluscan muscles (Kendrick-Jones, Lehman and Szent-Györgyi, 1970).

Introduction to the Anterior Byssus Retractor Muscle (ABRM)

Molluscan muscle fibers may be classified into three distinct structural types (Hanson and Lowy, 1960, pp. 265-335 in Bournes Vol. 1). These types are:

(i) Cross-striated
(ii) Obliquely-striated
(iii) Smooth

In common, they have thick filaments which vary in length and in size according to the fiber type but which contain the proteins myosin and tropomyosin A (often referred to as paramyosin). The proportion of these two proteins vary with the muscle type - the smooth muscles containing the greatest proportion of the tropomyosin A. Also in common are the thin filaments which have a thickness of about 80 Å. These filaments consist primarily of the protein actin.

The physical properties of the molluscan muscles appear to be related to the structural type (Hanson and Lowy , 1960).

The cross-striated muscles, eg. Pecten cross-striated adductor, contract and relax rapidly, and behave similarly to vertebrate cross-striated muscle. The thick filament lengths are about 1.6μ and the thick filament diameters are around 300 Å. The overlapping of the thick and thin filaments gives rise to the striations (Millman, 1967).

The obliquely-striated muscle fibers may be further distinguished into (a) regular obliquely-striated fibers, in which the contractile part is arranged around a central core of mitochondria and (b) irregular, obliquely-striated fibers where there is no mitochondrial core. In these fibers the centre as well as the outside contains contractile filaments but the order in the central region breaks down.

The regular, obliquely-striated muscles, eg. the mantle and funnel retractor muscles of octopus and cuttlefish (Sepia) contract and relax like the cross-striated muscles, but have slower speed, whereas the irregular, obliquely-striated muscles, eg. the translucent section of the adductor muscle of the oyster (Crassostrea) contract rapidly but relaxation may be relatively slow, the rate of relaxation being dependent on the period of stimulation (Millman, 1967).

The regular, obliquely-striated muscles have a thick filament length similar to the cross-striated muscles, and an average width in the central region of the filament of 130 Å, whereas the irregular, obliquely-striated muscle has a thick filament length of between 5-15μ and a filament width of around 400 Å (Millman, 1967).

The anterior byssus retractor muscle (ABRM) is an example of smooth molluscan muscle. The smooth muscles have an extremely interesting physiological behaviour, in that they are able to maintain tension for long periods of time. Smooth adductor muscles can hold the shells of bi-valve molluscs closed for many days against the force of the hinge-ligament and, because of this ability, the smooth muscles are also known as 'catch' muscles.

Smooth muscles contract slowly and can exhibit two distinct types of relaxation, slow (phasic) and extremely slow (tonic). Phasic relaxation has an expontential time constant of about 5 seconds, whereas the exponential time constant of tonic relaxation ranges from 5 to 100 minutes (Lowy and Millman, 1963; Millman, 1967).

The rate of relaxation appears to be controlled by the amount of neuromuscular transmitter, 5-hydroxytryptamine, which is released by 'relaxing nerves' (Twarog, 1966). Tonic relaxation can be changed to phasic relaxation in the ABRM (Mytilus) by direct application of 5-hydroxytryptamine or by repetitive electrical stimulation. Twarog, 1967, found that the ABRM muscle appeared to be doubly innervated, one set of nerves releasing acetylcholine (ACH) to produce tonic contraction. The second set of nerves released 5-hydroxytryptamine (5-HT) to give rapid relaxation;

and simultaneous stimulation of both sets of nerves produced phasic contractions.

The thick filament length is longest for smooth muscle, being up to 100 μ, (oyster opaque adductor), and 10 μ to 30 μ for ABRM (Mytilus). The thick filaments are tapered, the width in the central area being around 700 Å (oyster), and 500 to 1,000 Å for the ABRM (Mytilus); Millman, 1967.

The ABRM contains the proteins actin, myosin and tropomyosin B. These proteins are normally found in other types of muscle (eg. vertebrate muscle). The ABRM in common with most other molluscan muscles, also contains large quantities of the protein tropomyosin A (often referred to as paramyosin) (Millman, 1967).

Szent-Györgyi, Cohen and Kendrick-Jones (1971) have found that the protein troponin, which is a regulating protein found in vertebrate muscles is not present in the molluscan muscles. Kendrick-Jones et.al. (1970) observed that the molluscan thin filaments (actin containing filaments) do not have the calcium binding properties associated with vertebrate thin filaments. However, when these thin filaments from molluscs were combined with the relaxing proteins from rabbit muscles, an association readily took place and the thin filaments then behaved like rabbit thin-filament preparations. Further they found that it was the paramyosin filaments that contained the calcium binding properties.

X-ray diffraction of the ABRM produces a very characteristic pattern. Two series of reflexions are observed on or near the meridian. One of the series arises from the paramyosin filaments (Bear and Selby, 1956). This series consists of sharp reflexions which can be indexed on a rectangular, non-primative, net measuring 720 Å x 340 Å (Bear and Selby, 1956; see also Elliott, 1967). The second series which is more diffuse has been attributed to the thin, actin containing filaments (Selby and Bear, 1956).

The equatorial patterns show a diffuse reflexion at about 120 Å (Elliott and Lowy, 1961). Elliott and Lowy, 1961, and Elliott, 1964, had at first believed this to arise from layered structures within the paramyosin filament; Lowy and Vibert, 1967, however, had found that under certain conditions they could achieve a sharp sampling along the 59 Å actin layer line at a position corresponding to the equatorial reflexion. They, therefore, concluded that partial-ordering of actin filaments was the structure that was giving rise to the equatorial reflexion.

X-ray diffraction from concentrated sols of actin (Spencer, 1969) and electron microscope observations of the ABRM (Hanson and Lowy, unpublished results; H. E. Huxley, unpublished results; Twarog, 1967; Heumann and Zebe, 1967) were in agreement with this conclusion. The electron microscope observations indicated that there are regions of the ABRM containing only thin filaments, arranged at a regular average spacing but without any long range order of the filaments.

The protein tropomyosin A (paramyosin) was observed using X-ray diffraction by Bear and Selby (1956), as noted before, and indexed on a rectangular net. Cohen and Holmes (1963) obtained X-ray diffraction results from the ABRM which agreed with this indexing, and further indicated that the paramyosin diffraction pattern was originating from a coiled-coil configuration of α-helical molecules.

Lowey, Kucera and Holtzer (1963) performed light scattering, viscosity and sedimentation experiments on solutions of tropomyosin A. Their data indicated that the molecule was a rod approximately 1,330 Å long and 20 Å in diameter. This data, together with a further examination of the data of Cohen and Holmes by Fraser, MacRae and Miller (1965) indicated a two chain coiled-coil configuration.

Elliott, Lowy, Parry and Vibert (1968) investigated the evidence for the coiled-coil model. They concluded that the diffraction patterns were not produced by a simple coiled-coil model and that the diffraction patterns were modified by side-chain interactions.

The coarse structure of the paramyosin was studied by Elliott and Lowy (1970). Using electron microscopy, X-ray diffraction and optical diffraction, they observed that the paramyosin molecules (tropomyosin A) could be formed into thin sheets which could be indexed according to the Bear and Selby hexagonal net. Under normal circumstances the thin sheets would form cylindrical filaments. The paramyosin molecules would be in a spiral cylindrical lattice. The X-ray diffraction patterns and electron micrographs agree with this proposed model. Elliott and Lowy (1970) further suggest that the arrangement of the paramyosin molecules on the surface of the filament would be quite compatible with an attachment of myosin molecules onto this surface.

Kendrick-Jones et.al. (1969) and Szent-Györgyi et.al. (1970) referred to the similarity of the aggregation properties of myosin and tropomyosin A. This would be consistent with the Elliott and Lowy proposal.

It would seem that the X-ray diffraction patterns from paramyosin filaments are due to the tropomyosin A but there is a possibility that myosin as well as tropomyosin A might contribute to the diffraction pattern at low angles (Cohen and Holmes, 1963).

Hanson and Lowy (1959) proposed that contraction in the "catch" muscles is produced by a mechanism between the thick and thin filaments. Two hypotheses have been proposed to explain a tension maintenance, with a small energy expenditure. In one hypothesis, the tension is maintained by the slow breaking of actin-myosin cross linkages (Lowy and Millman, 1959, 1963; Lowy, Millman, and Hanson, 1964) and the difference between phasic and tonic contraction is controlled externally by the concentration of 5-hydroxytryptamine. In the second hypothesis the tension developed by the actomyosin system is conserved by a formation of crosslinks between paramyosin molecules (Rüegg 1958, 1961; Johnson, Kahn and Szent-Györgyi, 1959; Johnson, 1962).

The second hypothesis has been supported by the solubility properties of tropomyosin A (RÜegg 1961, 1964), however, no evidence of any "crystallization" or configurational change has been observed in X-ray diffraction studies (Millman and Elliott, 1972).

Method Section

The experiments which were performed on the ABRM, are as follows:

Firstly, to investigate the change of the equatorial spacing as the muscle was lengthened. Electrical stimulation was used, and in a second series of experiments 5-HT was used to relax the muscle.

From the large distribution of equatorial spacings recorded in the length change experiments, it was decided to investigate whether the equatorial spacing was influenced by the concentration of the bathing solution.

Experiments were also performed to see whether the contractile properties of the muscle were directly linked to the equatorial spacing.

Optical diffraction was performed, on masks, to simulate the diffraction patterns of possible thick and thin filament arrangements in the muscle. This was used to verify that the equatorial reflexion could arise from an actin lattice, and to gain experience with this technique.

Dissection of the ABRM:

The anterior byssus retractor muscle, ABRM was dissected from Mytilus edulis. The dissection procedure was as follows:-

The shell of the Mytilus edulis was broken near the posterior adductor muscle, using bone cutters, to allow for the insertion of a narrow bladed scalpel. The posterior adductor muscle was cut, allowing the shell to open. Access to the anterior adductor muscle was then provided and this muscle was also cut to allow the shell to be opened more fully.

At this stage of the dissection, the length of the ABRM was measured using a pair of dividers so that the length of the muscle inside the shell was

determined (accuracy±1mm) before the muscle had been disturbed.

The foot was then removed by cutting with a pair of scissors. The Mytilus is essentially symmetrical and it was found that cutting the Mytilus into two halves at the byssus organ, with a scalpel, facilitated an easier dissection. Using a pair of forceps, the gill and all other tissues were removed leaving only the ABRM and the posterior byssus retractor muscle attached to the half-shell.

A platinum connector was tied to the muscle with soft cotton thread at the former position of the byssus. The anterior end of the ABRM is connected to the shell. This shell was carefully cut with bone cutters, in order that the muscle remained attached. This provided an anchorage position to which another platinum connector was tied. The ABRM was then severed from the posterior byssus retractor muscle and the portion of the shell holding the anterior end of the ABRM was also detached from the rest of the shell. At this stage, the ABRM, with a platinum connector at either end, was free of any attachments to the mollusc. During this procedure, the muscle had been frequently bathed with sea water.

The dissection of the ABRM normally resulted in contraction and therefore, the muscle was hung (with a two to five gram weight) in an aerated small container of seawater for approximately one hour. This resulted in the muscle relaxing to its normal length.

The muscle was then hung in the X-ray beam. The lower connector on the muscle was held by a platinum hook, while the other connector was attached to a Palmer stand. Increases in the height of the Palmer stand carriage were transmitted as increases in tension until such time as the muscle has relaxed to the new length.

The muscle was irrigated with aerated seawater, which was dripped slowly onto the muscle (approximately 1 drop/sec.) For the length change experiments, the seawater was at room temperature (approximately 20^{o}C); however, for the experiments with varying concentrations of seawater and the experiments with penicillin and glucose added to the seawater, the seawater was chilled (approximately 10^{o}C at the muscle position).

When the muscle length was changed by adjusting the carriage, the muscle was given an electrical stimulus through the platinum connectors. The stimulus produced a phasic contraction in the muscle and then relaxation of the muscle to the new length occurred. The stimulus given was an eight second duration of square wave pulses, each pulse was one millisecond long, 35 volts in height, and the pulses were delivered at a frequency of 100 Hertz.

After the muscle was placed on the set and given a stimulus, it was allowed to relax. Then the length of the muscle was measured with a pair of dividers and an exposure was taken (each exposure required a minimum of six hours). The adjustable carriage was then moved (normally 2mm). The muscle was given a stimulus and allowed to relax, the length again measured with a pair of dividers, and another exposure taken. This was repeated for increasing muscle lengths and also for decreasing lengths until the muscle was back to its starting length. For each exposure, the specimen to film distance was also measured.

The experiment was stopped if the muscle failed to respond or gave only a small contraction. A streaking or loss of the diffraction pattern also indicated muscle deterioration. This is in agreement with observations by Elliott and Lowy (1961).

A series of these experiments, in which the muscle length was changed, was performed. A second series was also performed, using the same method but with the addition of 5-hydroxytryptamine added to the seawater. The 5-hydroxytryptamine concentration was 10^{-3} g/litre of seawater.

The seawater which was used in these experiments was artificial seawater. The seawater was made by dissolving a package of 'Instant Ocean' (Aquarium Systems Inc.) according to the package instructions into the requisite quantity of tap water.

The X-rays were produced by a Hilger Watts type Y33 microfocus X-ray generator. The generator was set for 100 micron line focus operation. The 100 micron line filament was set in the vertical position. As the camera is actually seeing the beam at a low angle of incidence (approximately 3-5^{o}), then the effective width of the beam is considerably smaller than 100 microns because of this foreshortening.

The camera used was a Franks camera (Franks, 1955) which had been adapted (Elliott and Worthington, 1963) specifically for low angle diffraction. The camera contains mirrors which focus the X-rays by reflecting them through very small angles.

The beam is made essentially monochromatic (selecting the CuKα radiation) by the focussing procedures.

Four X-ray beams are produced by the camera. Two of the beams have been reflected by one of two mirrors in the camera, another beam has been reflected by both, and the fourth beam has missed both mirrors.

For an X-ray diffraction pattern which required both meridional and equatorial information, the doubly reflected beam would be used (in conjunction with a point focus source). However, in this application, where only equatorial information was required, it was possible to increase the intensity of the line focus by overlapping the doubly reflected beam with the appropriate singly reflected beam. The unwanted beams are removed, after focussing, using adjustable lead collimators.

The non-scattered beam was collected on a platinum backstop which was positioned just in front of the film. The backstop was required because the diffracted beams are extremely weak compared to the main beam (approximately by four orders of magnitude). If the main beam was allowed to strike the film, the area of interest would have been completely masked by secondary electron scattering from the film.

The X-ray film used was Ilford Ilfex and this was developed in an FR X-ray developer (FR Corporation) using normal techniques (5 min. at 68^{o}F).

The X-ray beam was focussed into a line in the vertical direction, such that the beam passed through the muscle parallel to the axis of the muscle. This resulted in the equatorial reflexions, on the film, being elongated into lines in the vertical direction. The line focus prevented any observation of the meridional pattern.

The diffraction patterns were measured using an optical comparator (Scherr Tumico, Inc.). The interfilament spacings in the ABRM were then calculated by applying Bragg's formula,

$$d = \frac{n \lambda}{2 \sin \theta}$$

where d is the separation of the filaments

n is the diffraction order (n=1 for these experiments)

λ is the wavelength of the X-rays (λ = 1.540 Å for CuK_{α})

2θ is the angle between the main X-ray beam; in these experiments, θ was very small, hence the approximation $\sin\theta = \theta$ radians was used, and the expression reduced to

$$d = \frac{3.08F}{E} \text{ Å}$$

where F is the specimen to film distance and E is the separation of the equatorial reflexions.

Effect of Seawater Concentration

In this series of experiments the muscle was hung from a fixed bar with a two gram weight suspended from the muscle to keep it under a small tension. The muscle was irrigated with aerated cooled seawater (approximately $10^{o}C$), the concentration of which was changed after exposure. The experimental procedure was similar to the previous series except that the length of the muscle was not varied, instead the concentration of the seawater was altered.

After each exposure, the seawater concentration was changed and then the muscle was given at least twenty minutes to equilibrate before the next exposure was taken. This was then repeated.

The exposures required a minimum of six hours for a measurable diffraction pattern.

The physiology of the muscle was also observed at various concentrations of seawater, in a separate series of experiments.

The ABRM was hung in a seawater bath (at approximately $20^{o}C$). The ABRM had been prepared according to the dissection procedure previously described. The platinum connectors were attached at the lower end to a fixed hook and at the upper end to a pointer.

The pointer was an extension of a torsion lever, so that a change of tension in the muscle resulted in a displacement of the pointer. The muscle could be stimulated both electrically and chemically, and the reaction of the muscle was recorded, by the pointer, on the smoked drum of a kymograph. The bathing solution was removed before stimulation was applied.

The muscle was stimulated at five minute intervals with an eight second

duration of pulses; the pulses were one millisecond long, at a frequency of 10 hertz, and at a height of approximately six times the threshold voltage of the muscle. The muscle was also stimulated with acetylcholine (Ach) at a concentration of 10^{-5} g/ml, in place of an electrical stimulation. Following the application of Ach, the muscle was rinsed well to remove all the excess Ach. After the muscle had recovered from the Ach contraction, the seawater was changed and the series continued in seawater of a different concentration.

Results Section

Lengthened ABRM Experiments

Determination of the actual length of the muscle on the X-ray set was difficult, in that although the attachment to the shell defined one end of the muscle, the position of the posterior end could not be precisely determined. In practice, the point at which the lower platinum connector was tied to the muscle was used as this reference point. The length of the muscle at each stage was measured with a pair of dividers (accuracy approximately ±1mm).

Since the muscle length could only be measured with limited accuracy, it was assumed that a change in the Palmer stand carriage position would produce a constant relative change in the muscle length.

It was found that a change in carriage height of 0.20cm produced an average change in muscle length of 0.16cm. The series in which 5-HT was included in the bathing solution, where very little tension was produced by the stretch, gave an average change in muscle length of 0.20cm for a change in carriage position of 0.20cm.

For each muscle, the average initial length and the incremental lengths were calculated by applying these corrections to the averaged measurement of the muscle length.

Also, the interfilament spacing was found to vary substantially from muscle to muscle (range of 25Å). Therefore the data from each muscle was converted into percentage changes, for both changes in interfilament separation and of changes in muscle length.

The results without 5-HT are shown in Figure 1 (p 34) and the results from the series of experiments with 5-HT are shown in Figure 2 (p 35).

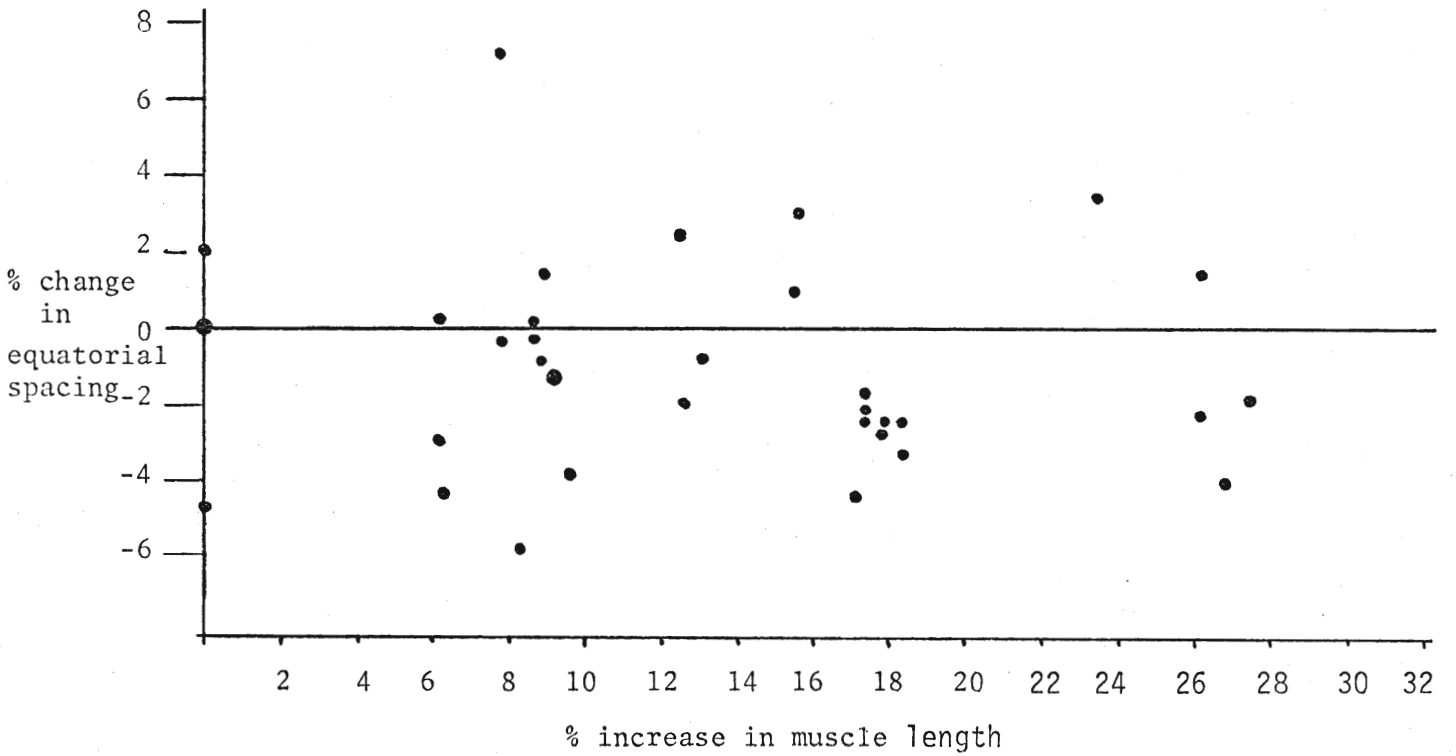

Figure 1.

Percentage change in equatorial spacing against percentage increase in muscle extension, for ABRM muscles (without 5-HT)

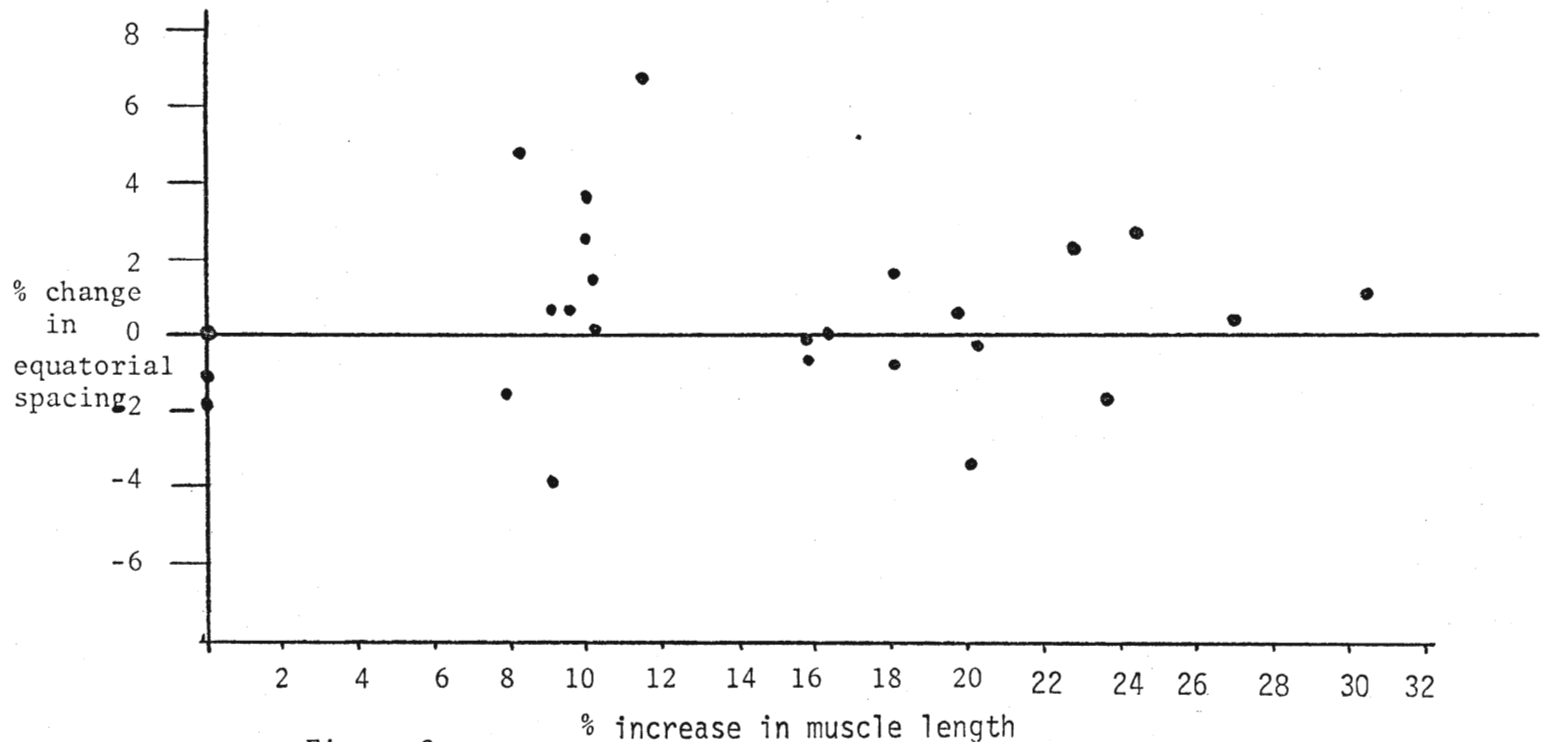

Figure 2

Percentage change in equatorial spacing against percentage increase in muscle extension, for ABRM muscles in the presence of 5-HT

The complete data from these graphs is tabulated in appendix p 101, 102. It was observed that the results lie on both sides of the zero spacing change axis. The hypothesis that the change in the equatorial spacing was independent of the change in the extension of the muscle was considered.

As the data was expressed in percentage changes, a non-parametric statistical test[1] was chosen (Probability and Statistics, Alder and Roessler, Freeman Press).

The Null hypothesis, consistent with the hypothesis that the equatorial spacing was independent of the extension of the muscle, was that there would be no significant difference between the sample at zero extension and the sample when the muscle was extended.

The results from the Wilcoxon rank test supported this hypothesis at the five percent level of significance; both for the data without 5-HT and for the data with 5-HT (see appendix A-3, A-4, p 103, 106).

Therefore, the hypothesis that the equatorial spacing was independent of the extension of the muscle was valid.

The hypothesis, that there was no significant difference between the data without 5-HT and the data with 5-HT, was also tested. The Wilcoxon rank test was applied and the hypothesis was rejected at the five percent level of significance, but accepted at the one percent level of significance (see appendix A-5 , p 108).

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1. At first it was thought that the percentage data could be transformed into normalized data using the transform of percentage data into the arcsin of the square root of the percentage data. However, certain conditions for the application of this transformation were not fulfilled. (Statistics for Experimentalists, B. E. Cooper, Pergamon Press).

This indicated that the two sets of data were not highly consistent with each other at the 5% level. However, both sets of data were consistent with the hypothesis that the equatorial spacing was independent of the extension of the muscle.

The graphs were again examined. It appeared that the data from experiments without 5-HT had a slight negative trend with respect to the data from experiments with 5-HT. For each series of experiments, the data were separated into three groups and averaged (see appendix A-6 , p 110). This provided an average of the change in equatorial spacing at :

(i) the initial muscle length
(ii) a medium value of muscle extension
(iii) a high value of muscle extension

In practice, the average values for the data of zero extension, zero to +15% extension (exclusive of zero), and from +15% to +31% extension, were calculated. These three groups included approximately 99% of the recorded results (see Figure 3, p 38).

A straight line was drawn to indicate the trend of the data. For the experiments without 5-HT, a negative trend was observed, the slope of the line was -0.05 % Δ spacing/% Δ length. For the experiments with 5-HT, a positive trend was observed, with a slope of +0.04 % Δ spacing/ % Δ length.

Therefore, even at a muscle extension of +30%, the change in the interfilament spacing was less than two percent.

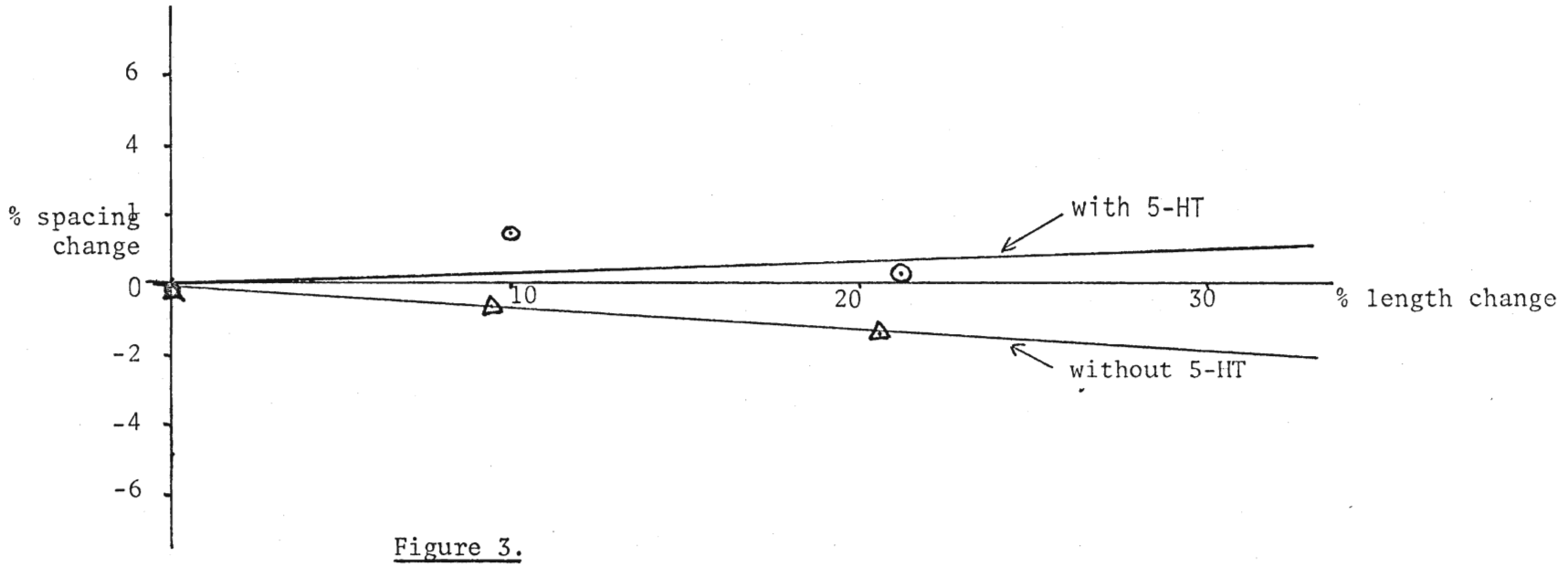


Figure 3.

Graph of grouped data from ABRM extension experiments:

△ Denotes averages for muscles without 5-HT

○ Denotes averages for muscles with 5-HT

The data from experiments without 5-HT was fitted by a line with slope - 0.046

The data from experiments with 5-HT was fitted by a line with slope +0.035

Mean Equatorial Spacing

The spacing of the equatorial reflexion from these two series of muscles was found to vary considerably, over a range of approximately 25Å. The mean initial equatorial spacing of the muscles and the standard deviation were calculated for each series of experiments.

The mean initial equatorial spacing (i.e., before the muscles had been extended) for the series of experiments without 5-HT was 134Å (standard deviation, σ =7Å, 10 muscles).

For the series with 5-HT added, the mean initial equatorial spacing was 139Å (σ =6Å, 8 muscles).

The Wilcoxon test was applied (see appendix A-5 , p 108), and it was found that the difference between the two means was significant at the 5% level. The effect of 5-HT on the initial equatorial spacing was, therefore, investigated under closely controlled conditions and found to produce no significant change (see p 40).

Effect of 5-HT on the Equatorial Spacing

In the previous section, the mean of the average initial spacing from experiments without 5-HT was compared to the mean of the average initial spacing from experiments with 5-HT. However, in this comparison different muscles were used in each series. To eliminate any variations due to differences in individual muscles, spacings were measured from the same muscles both with and without 5-HT.

Twelve experiments were performed and it was found that the average increase in equatorial spacings, with the addition of 5-HT (at 10^{-6} g/ml) was 0.6Å (σ=5.4Å). The average spacing of the equatorial reflexion, without 5-HT, in this series of experiments was 126Å (σ=7Å) (see appendix A-7 , p 111).

The hypothesis, that 5-HT has no effect on the initial equatorial spacing, was considered. Using the Wilcoxon test for paired cases (Probability and Statistics, Alder and Roessler, Freeman Press) on the results, it was found that this hypothesis was strongly supported (see appendix A-7, p 111).

Hence, it was concluded that the addition of 5-HT (at 10^{-6}g/ml) to the seawater produced no significant change in the initial spacing of the equatorial reflexion.

Effect of Variations of the Seawater Concentration on the Equatorial Spacing

The seawater used in these experiments was artificial seawater which had been prepared by dissolving packages of 'Instant Ocean', according to the directions, in tap water.

1.0S is used to refer to the ionic concentration of the artificial seawater when it was prepared according to the directions for normal or standard seawater. Hence 2.0S would refer to a more concentrated seawater, in which only one-half of the regular amount of tap water was used (hence it would have twice the ionic concentration of the standard seawater). Similarly, 0.5S seawater would have one-half of the ionic concentration of the standard seawater.

In this series of experiments, 2.0S seawater was first prepared and this was diluted to produce 1.5S, 1.0S and 0.5S concentrations.

At 0.5S ionic concentration of the seawater, the equatorial reflexion had become very diffuse and measurement of the separation of the reflexions was not possible. However, it was clear that the diffuse reflexions had less separation and hence the interfilament spacings had increased. An accurate measurement of the diffuseness of the reflexions was not possible, as they were merging with the background radiation near the backstop.

The mean equatorial spacing at 1.0S ionic concentration (normal seawater concentration) was 147Å (σ=8Å, 5 muscles) in this first series of experiments (December 1970, January 1971) (see Table 8 in appendix).

At a seawater concentration of 1.5S, the mean equatorial spacing had decreased to 118Å (σ =7Å, 5 muscles), hence the spacing had decreased by 20%.

In a further series of experiments, performed in September 1972, the effect of 2.0S seawater on the equatorial spacing was also investigated. At this concentration the average spacing was 105Å (σ=3Å, 3 muscles). Hence in 2.0S seawater, the equatorial spacing had also decreased by about 20%. It should be noted that in these experiments the average 1.0S spacing was 131Å (σ=3Å, 3 muscles), as compared to 147Å in the first series (December 1970, January 1971); and therefore, it was possible that the percentage decrease of the 2.0S spacings was being limited by the size of the thin filaments themselves.

The experiment of 17/12/70 showed that the effect of the variation of the seawater concentration was largely reversible. Figure 4 (p 43) shows a series of diffraction patterns from this muscle in various concentrations of seawater. The specimen to film distance was constant; and therefore, the films may be compared to each other. A slight overall decrease in the spacings appeared to have occured throughout the experiment. However, more experiments would be required to establish whether the decrease was significant.

Figure 4

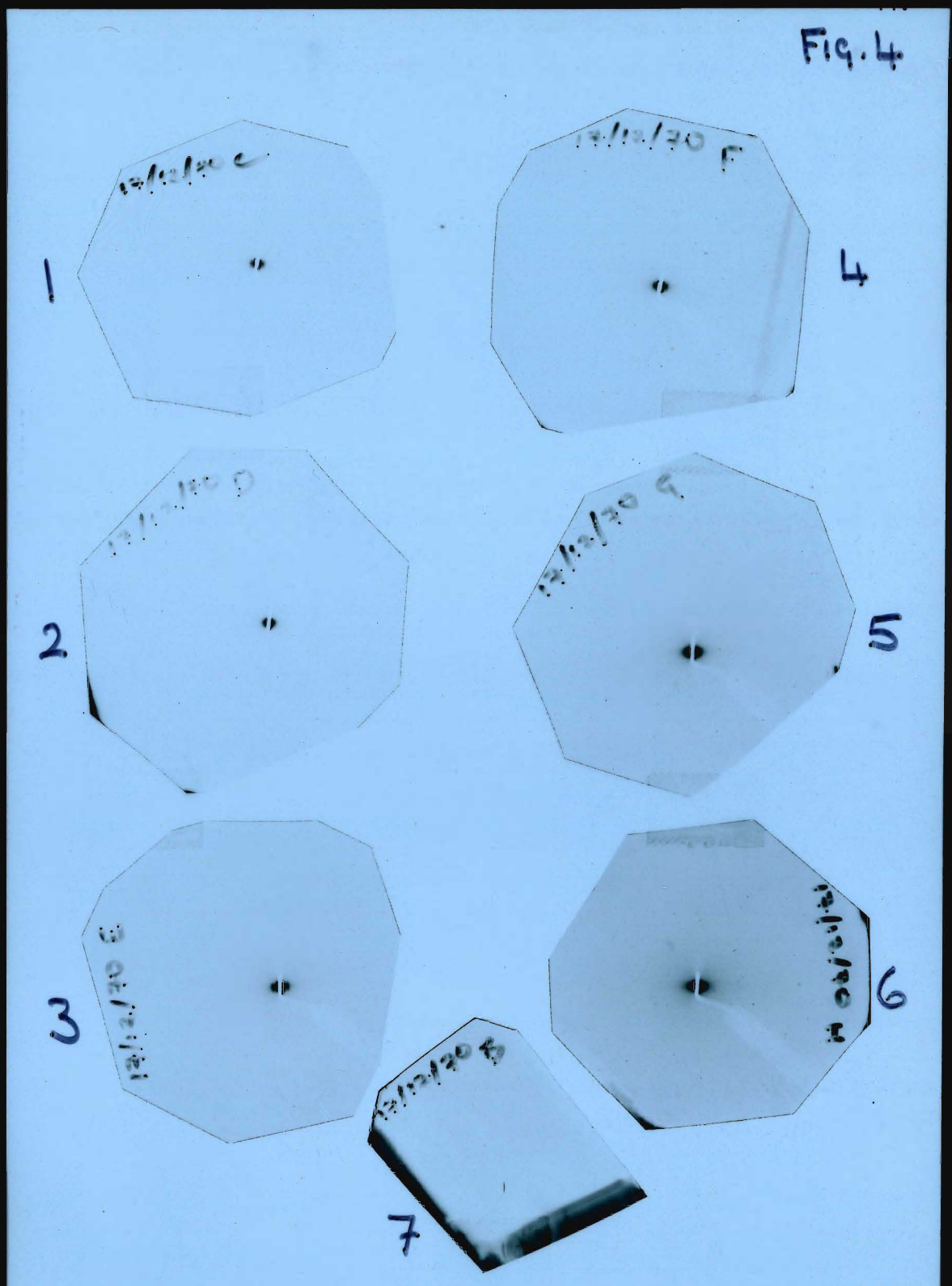
ABRM diffraction patterns from muscles in various concentrations of seawater (contact print).

#	Muscle Index	Seawater Conc.	S.F.D. (cm)	Averaged Measured Separation of Reflexion (mm)	Equatorial Spacing Å
1	17/12/70C	1.0S	9.38	1.92	151 Å
2	17/12/70D	0.5S	9.38	diffuse	
3	17/12/70E	1.5S	9.38	2.46	118 Å
4	17/12/70F	1.0S	9.38	2.04	142 Å
5	17/12/70G	0.5S	9.38	diffuse	
6	17/12/70H	1.5S	9.38	2.56	113 Å

where S.F.D. refers to the Specimen to Film Distance.
The above diffraction patterns were from the same muscle, taken in sequence.

7 17/12/70B was an image of the X-ray beam showing that the diffuseness of the X-ray reflexions was a function of the specimen and not of the X-ray source.

Fig. 4



Effect of Penicillin and Glucose

Before it was realized that the equatorial spacing could vary between muscles over a range of approximately 25Å, the means of the extension series, both without and with 5-HT, were compared to the results of Elliott and Lowy (1961). They had briefly looked at the equatorial spacing and found that it did not appear to change on extension. However, the mean of their equatorial spacings was around 123Å. They had used natural seawater, to which they had added glucose (10 mg/ml) and penicillin G (250 units/ml), in their experiments. Therefore, the possibility that these additions had caused the low value of the equatorial spacing was considered.

Three experiments were performed in which glucose and penicillin G, to the above concentrations, were added to the seawater.

Experimental Index	Averaged Spacing in Seawater	Averaged Spacing in Seawater and Glucose and Penicillin G
16/8/71	134.0 Å	127.7 Å
18/8/71	135.6 Å	131.6 Å
11/9/71	135.5 Å	132.6 Å
Average of 3 muscles	135.0 Å	130.6 Å

The decrease in spacing due to the addition of glucose (10 mg/ml) and penicillin G (250 units/ml) was approximately 3%.

The addition of the glucose to the seawater would increase the molarity by approximately 4.4%. The experiments on variations of seawater concentration (p 41) have indicated that a 50% increase in molarity of the seawater produces a 20% decrease in the equatorial spacing. Hence, assuming a linear response, the expected decrease due to the glucose would be approximately 2% which is in general agreement with the observation.

It would therefore appear that other factors were responsible for the difference between the average equatorial spacings obtained by Elliott and Lowy (1961) and those obtained by the experiments described in this thesis.

Contraction of the Muscle in Various Concentrations of Seawater

This experiment was performed to investigate whether there might be a direct relationship between the equatorial spacing and the physiological properties of the muscle.

The results of the experiment indicate that the force generated by the muscle, both by electrical stimulation and also by acetylcholine (Ach) stimulation, was greatest when the muscle was in artificial seawater of standard concentration.

The relaxation of the muscle, as measured by the half-peak decay time (i.e., the time taken for the tension of fall to one-half of its peak value; Lowy and Millman, 1963) was slower when the ionic concentration of the seawater was increased. However, when the muscle was in seawater at 0.5 times standard ionic concentration, the recovery of the muscle was the same as when it was in seawater at the standard ionic concentration.

After stimulation of the muscle by Acetylcholine (Ach), the muscle was observed to be in a tonic contraction state at all of the ionic concentrations used in this experiment. The relaxation time, of the tonic contraction state, was not measured.

In Figure 5 (p 48), the results from one experiment are shown. These results were typical of the other experiments (see appendix A-9 ,p 114).

The effects of the concentration of the seawater appear to be approximately reversible both for the tension generating properties and for the relaxation of the muscle.

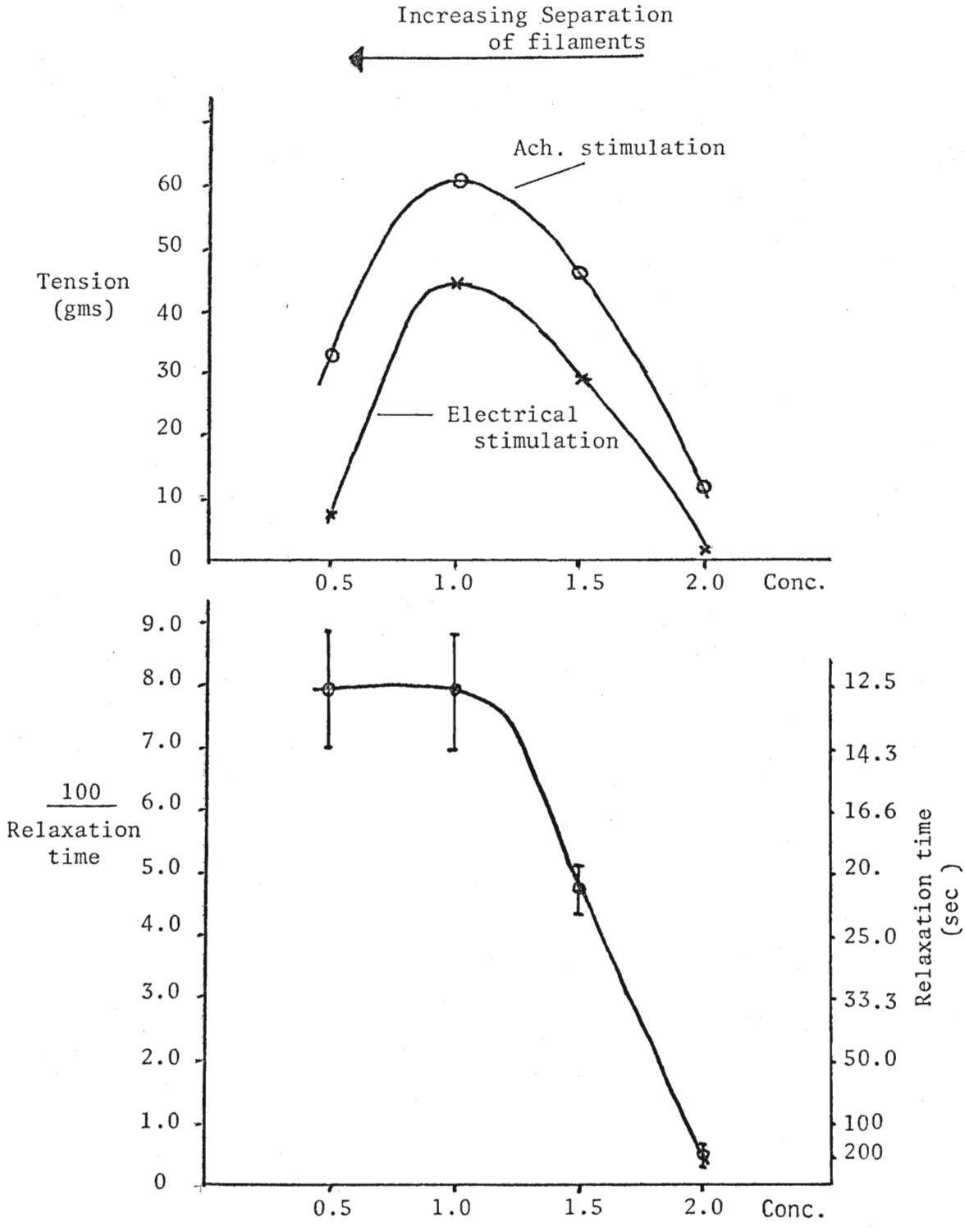


Figure 5. Tension and relaxation times for the ABRM in various concentrations of seawater

Variations in the Means of the Equatorial Spacing

The means of the initial equatorial spacing from different experiments varied by up to 21Å in normal seawater.

Page	Experimental Series	Experimental Period	Average Spacing
39	Extension experiment	Feb.-April 1970	134Å (σ=6, 10 muscles)
39	Extension experiment (with 5-HT)	March-May 1970	140Å (σ=6, 8 muscles)
41	Varied seawater concentration	Dec. 1970-Jan. 1971	147Å (σ=8, 5 muscles)
45	Addition of Glucose & Penicillin	Aug.-Sept. 1971	135Å (σ=2, 3 muscles)
40	Effect of 5-HT	Aug.-Sept. 1972	126Å (σ=7, 9 muscles)
42	Varied seawater concentration	Sept. 1972	131Å (σ=3, 3 muscles)

There were large variations in the equatorial spacing between muscles in the same series. It was observed that the average spacing from the experimental series performed in winter and spring tended to have larger equatorial spacings than those performed during the summer. These results would be consistent with the possibility of a seasonal change in the physiology of the mollusc.

ABRM

Discussion

The experiments described in this thesis have indicated the following:

(i) When the ABRM is lengthened there is no significant change (> 2%) in the spacing of the equatorial reflexion.

(ii) The interfilament spacing varied from muscle to muscle over a wide range. The averages from the different experimental series range from 126Å to 147Å.

(iii) The spacing of the filaments was not changed by the addition of 5-HT (at a concentration of 10^{-6}g/ml).

(iv) The spacing of the filaments was dependent on the osmolarity of the artificial seawater. As the concentration was increased, the equatorial spacing decreased. As the seawater was diluted, then the reflexion became very diffuse and the movement of the intensity of this reflexion towards the backstop indicated that the filament separation was increasing but poorly ordered. These effects were largely reversible.

(v) The tension generating properties of the muscle were greatest at the normal concentration of seawater. As the seawater concentration was increased or decreased, then the tension generated decreased. The relaxation of the muscle was slower when the concentration was increased; however, it remained constant when the concentration decreased. Again the effect was largely reversible.

The behaviour of the equatorial reflexions from the ABRM was very different to the behaviour of the equatorial reflexions from a vertebrate striated muscle. In the former, the filament spacing as measured from the equatorial reflexion does not change as the muscle length is changed.

In a vertebrate muscle system, the lattice spacing changes as the muscle is lengthed or shortened. These changes are such that the product of the sarcomere length and the square of the lattice spacing remain constant; i.e., a constant volume effect (Huxley, 1953; Elliott, Lowy and Worthington, 1963; Rome, 1967, 1972). The spacing of the vertebrate filament lattice can also be changed experimentally, without affecting the sarcomere length by altering the medium around the filaments (Rome, 1968).

The equatorial diffraction from the ABRM arises from arrays of actin filaments; whereas, the equatorial diffraction from the vertebrate muscles arises essentially from a lattice of myosin filaments. In the vertebrate striated muscle, the intensity of diffracted x-rays arising from areas of actin filaments (i.e., the I-band) is largely masked by the diffraction from the ordered lattice of myosin filaments. Rome (1972) was able to observe a faint reflexion at around 146Å with glycerol-extracted vertebrate striated muscles stretched to very long extensions. This was attributed to actin filaments in the I-band region. However, the effect of a muscle length change on the actin filament spacing in the I-band is not known.

The interfilament spacing of the ABRM did not change significantly as the muscle was lengthened; however, the interfilament spacing was found to vary with the concentration of the seawater.

Elliott (1968) had suggested that the behaviour of the filament lattice was similar to a classic colloidal system, the separation of the filaments being governed by long-range forces. Elliott (1968) had shown, in principle, that a stable balance would be achieved between the van der Waals attractive forces and the Coulombs repulsive forces for hexagonally-packed, charged cylinders in an ionic solution. The position of this balance depended critically on the value of:

(i) the van der Waals constant;

(ii) the Debye-Huckel parameter;
(iii) the surface potential of the polyelectrolyte; and
(iv) the radius of the polyelectrolyte cylinder.

Elliott and Lowy (1968) studied the organization of the actin in mammalian smooth muscle and were able to relate equatorial reflexions to areas of actin filaments in the muscle. Vibert (1969) found that this spacing varied with the tonicity of the medium.

Spencer (1969) found that concentrated solutions of oriented F-actin gave a strong equatorial reflexion. The position of this reflexion varied with the concentration in the manner expected for an array of regularly packed, mutually repulsive cylinders. This indicated the existence of long-range forces between filaments and supported the Elliott model.

The ABRM, after being placed in concentrated solutions of seawater, experienced decreases in the interfilament separation. This was consistent with an increase in the ionic strength of the medium between the filaments and hence, a change in the dielectric constant of the medium between the filaments. In dilute solutions of seawater, the ionic strength of the medium between the filaments was decreased and this was consistent with an increase in interfilament separations. Therefore, the results in this thesis were also in accordance with the Elliott (1968) proposal.

As the initial spacing of the filaments was not changed by the addition of 5-HT to the solution (at a concentration of 10^{-6}g/ml), it appeared that the actin filaments do not experience a change in surface potential by the addition of 5-HT to the bathing medium.[2] A measureable change

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2. The equatorial reflexion was originating from areas of actin filaments; hence, any change occurring on the paramyosin filaments would not be observed.

in spacing was not expected to occur solely from this small change in the osmolarity of the seawater.

The interfilament spacings varied over a wide range of approximately 25Å between muscles. Elliott and Lowy (1961) had recorded an average spacing of 122Å and Millman and Elliott (1972) had recorded an average spacing of 134Å, for the ABRM. The cause for this was not known. There was an indication that there may be a seasonal variation in the equatorial spacing; however, other factors such as the age of the mollusc, etc., may also be contributing to this range.

The tension generating properties of the ABRM were greatest when the muscle was in seawater of normal concentration, and decreased in both hyper-osmolar and hypo-osmolar seawater. No direct correlation between the interfilament separation and the tension generating properties was observed. The relaxation of the muscle was slower in hyper-osmolar seawater; however, it remained constant in hypo-osmolar seawater. Again, there was no direct correlation between the interfilament separation and the relaxation of the muscles.

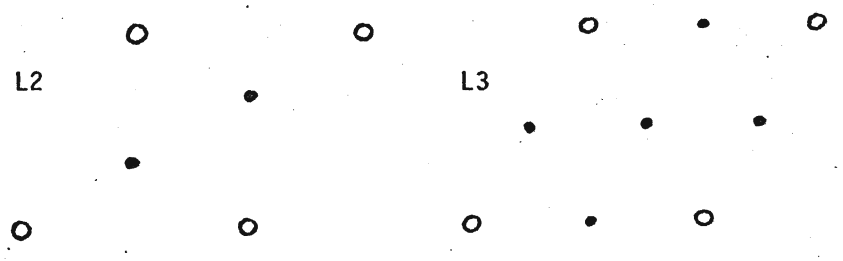
Neither hypothesis for the 'catch' mechanism in the ABRM was favoured by the results of this thesis. In general terms, it appeared that the equatorial reflexion, arising from groups of actin filaments, was influenced by long-range forces. The equatorial reflexion responded to changes in the osmolarity of the bathing solution. The addition of 5-hydroxytryptamine, a chemical which produces relaxation (Twarog, 1966), did not influence the interfilament separation of the actin filament groups.

Insect Flight Muscle

Insect flight muscles may be divided into two groups, synchronous, in which each muscle contraction is triggered by one or a short burst of motor nerve impulses, and asynchronous, in which repetative muscle contractions are maintained by a self-oscillatory mechanism, and only the initiation and termination is controlled by the motor nerve (Roeder, 1951). Synchronous flight muscles are found in grasshoppers, moths and dragonflies; and asynchronous flight muscles (commonly called insect fibrillar muscles) are found in bees, wasps and beetles (Roeder, 1951). The oscillatory activity was not intrinsic in the muscles and it depended on the presence of a suitable mechanical load (see review by J. W. S. Pringle, 1967).

Insect fibrillar muscle is also characterized in that the overlap between the myosin and the actin containing filaments of the myofibrillar array is initially almost complete and the muscles are not able to support an extension of more than about 10% without tearing (see review J. W. S. Pringle, 1967; also electronmicrographs of insect flight muscle in M. K. Reedy, 1968, 1969, and D. S. Smith, 1972). Auber and Couteaux (1963) and Garamvölgyi (1965) have found that the tapered ends of the A band filament, in blowfly and bee flight muscle, are continued as a filament of lower density into the Z band. These connecting filaments have been termed C filaments.

The transverse packing of the filaments differed between the insect muscle and the vertebrate muscle. Both types of muscle conformed to a hexagonal packing; however, in vertebrate muscle there were only two actin filaments per unit cell whereas the insect muscle lattice contained three actin filaments per unit cell (Worthington, 1961). These lattices have been termed L2 and L3 respectively.



The L3 lattices from a variety of different insect flight muscles maintained approximately the same dimensions, with a side of 560 Å (Worthington, 1961). Miller and Tregear (1971) gave the side of the hexagonal net as 530 Å (glycerinated water bug flight muscle).

Reedy, Holmes and Tregear (1965) studied glycerinated insect flight muscle from the giant tropical water bug (Lethocerus maximus). They observed this muscle in both the rigor and the relaxed state using both X-ray diffraction and electron microscopy, and found that the transition between the two states is accompanied by a re-orientation of the cross-bridges between the actin and myosin filaments. The electron micrographs of muscles in rigor showed an axial periodicity of 380 Å which arises from the crossbridges joined to the actin in symmetrical pairs. These form actin-centered chevrons which point away from the Z band (see diagram 6). The electron micrographs of the relaxed muscle showed a period of 143 Å which arises from crossbridges extending perpendicularly from the myosin filaments (see diagram 7).

This agrees with the X-ray diffraction results. Figure (10) shows the diffraction pattern from the muscle in rigor. Apparent are the layer lines which arise from a 388 Å repeating unit. Figure (10) also shows the diffraction pattern from the relaxed muscle. There is a strong 146 Å meridional spot. In both patterns the 59 Å layer line, which arises from the actin filaments is clearly visible. The small differences in spacings between the X-ray and electron microscope measurements are shrinkage artifacts caused by the processing of the specimen which is required for electron microscopy (Reedy et al, 1965).

Further studies on the ultrastructure of Lethocerus flight muscle have been performed by Reedy (1967, 1968). As well as the electron microscopy and X-ray diffraction techniques, optical transforms of the electron micrographs were used to yield additional information. His results indicated that the crossbridges originate in symmetrical double pairs. These occurred at an axial separation of 146 Å and with a 67.5° change in azimuth. The four bridges from one of the pairs on the myosin filament formed the arms of a "flared X" or chiasmatic configuration.

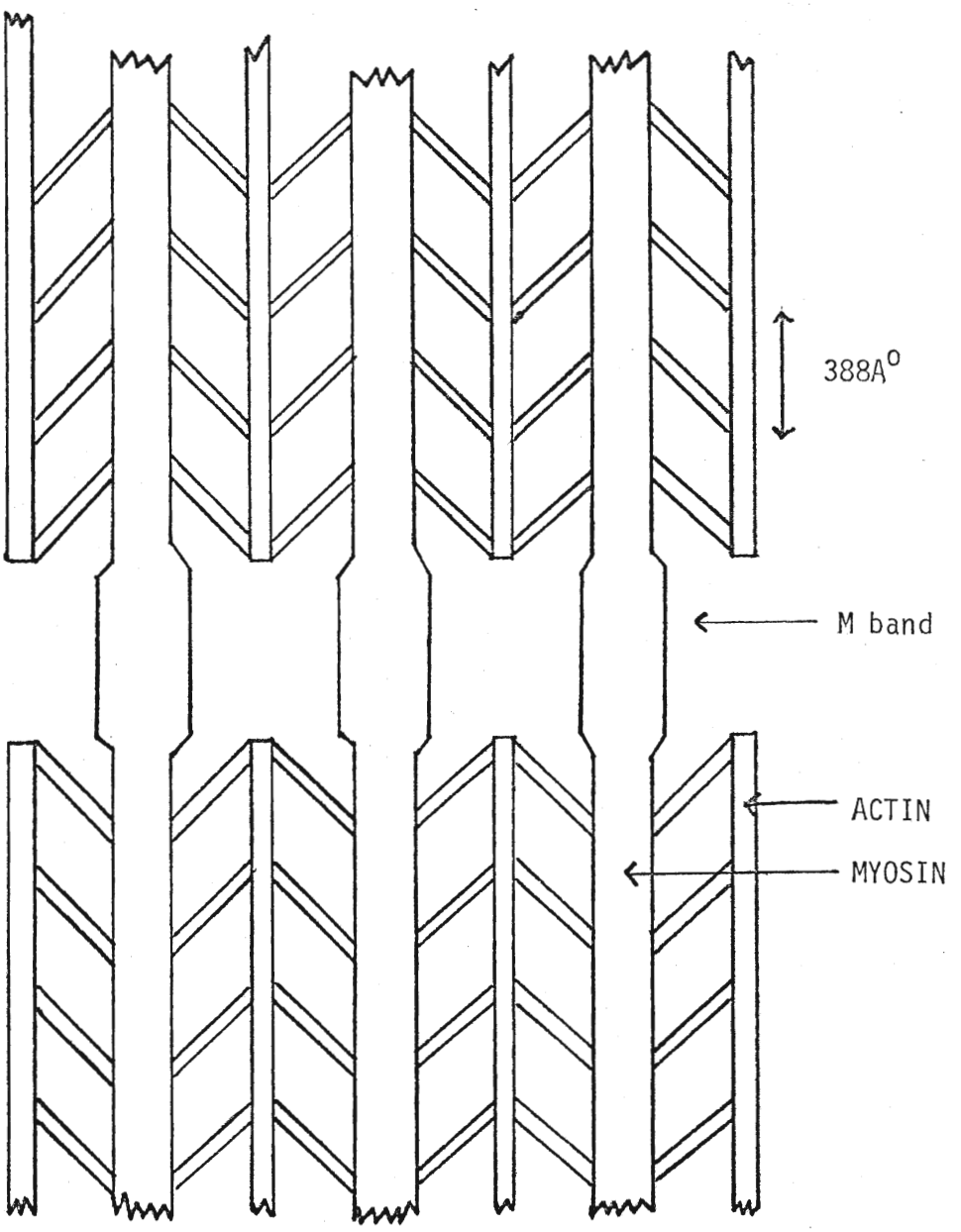


Fig. 6

Diagram of single layers of actin and myosin filaments. Crossbridge positions characteristic of the rigor state.
(from Reedy, Holmes, and Tregear; 1965)

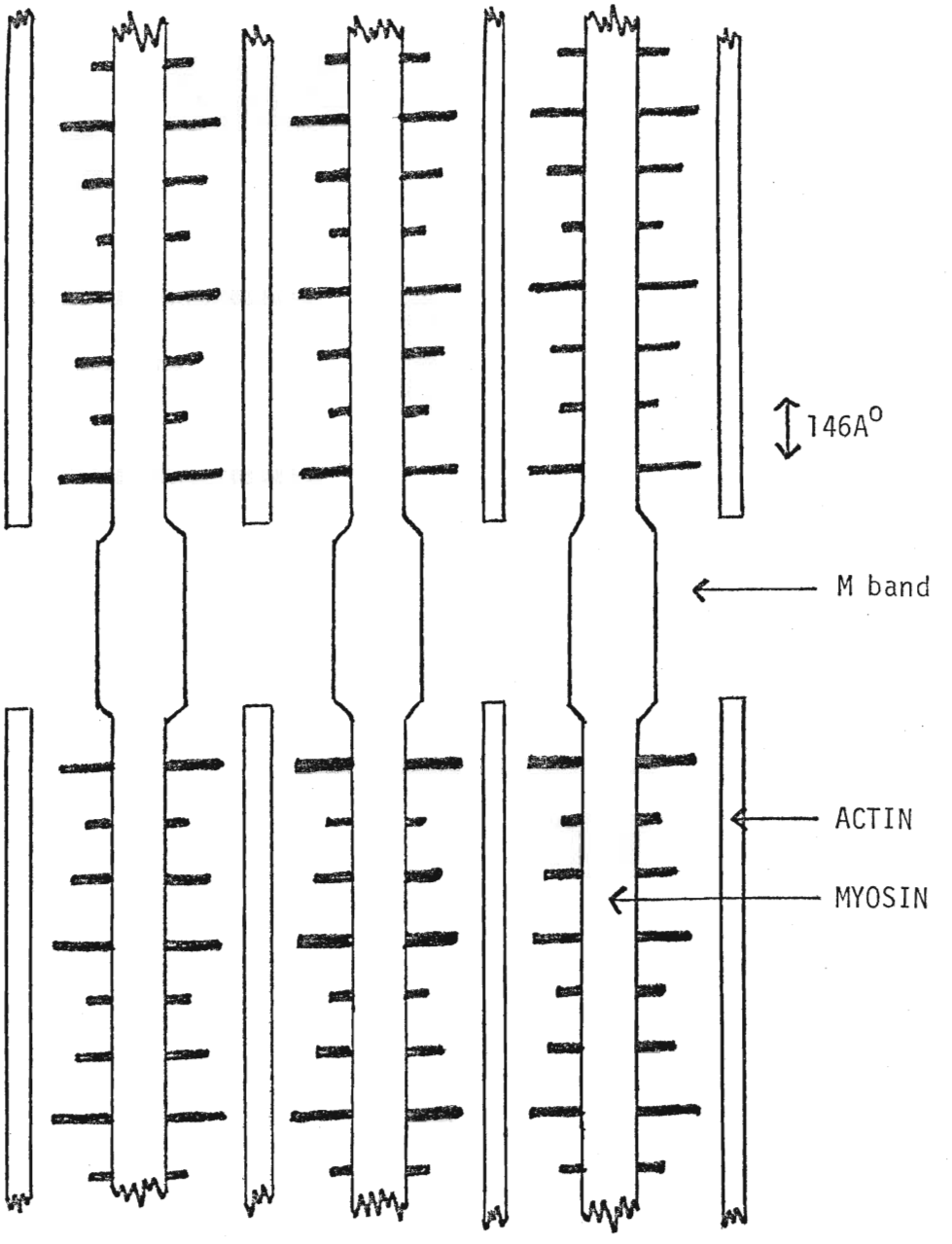


Fig. 7

Diagram of single layers of actin and myosin filaments. Crossbridge positions characteristic of the released state
(from Reedy, Holmes, and Tregear; 1965)

A right-handed helical structure is indicated for the actin filaments from observations of successive crossbridge pairs in serial sections (Reedy, 1968). This agrees with the screw sense found by Depue and Rice (1965) on the shadowcasts of purified rabbit F-actin filaments. The actin filaments were double-helical with a half-turn interval of 388 Å (X-ray measurements) and the crossbridge origins on the myosin filaments were also in a double helical arrangement such that the half turn interval is 388 Å (Reedy, 1968).

The myosin filaments in one sarcomere were all in helical register and the actin filaments were also in a helical register such that the six actin filaments around each myosin filament differ in helical register by 60^{o} but such that this arrangement repeats transversely through the myofibril.

Tregear and Miller (1969) collected evidence of crossbridge movement during the contraction of insect flight muscle. They used glycerol-extracted flight muscle from the water bug, Lethocerus cordofanus, which they placed in a solution containing MgATP and Ca^{2+} ions, and then subjected to a forced longitudinal oscillation. This caused the muscles to work in a similar fashion to their performance during flight. They measured the intensity of the 145 Å meridional reflexion (N.B. this reflexion has been previously described as 146 Å) with a proportional counter which was scanned synchronously with the oscillation. The 145 Å reflexion is believed to originate from the regular axial repeat of the crossbridge origins on the thick filaments (Reedy, Holmes and Tregear, 1965; Huxley and Brown, 1967). A variation of the intensity of this reflexion is interpreted as a movement of the crossbridges (Tregear and Miller, 1969). Tregear and Miller found that there was a large intensity fluctuation when the muscle is repetatively activated by the forced oscillation and that the fluctuation in the intensity of the 145 Å reflexion varied in antiphase with the tension (ie. when the tension of the muscle was greatest then the intensity was lowest).

Again, using glycerol-extracted Lethocerus flight muscle in various solutions, Miller and Tregear (1970) measured the intensities of the (1, 0, $\bar{1}$, 0) and the (2, 0, $\bar{2}$, 0) equatorial reflexions of the muscle during relaxed conditions, during rigor conditions, during peak tension of oscillation, and during static Ca^{2+}-activated conditions. From these intensities they calculated the electron density distribution for the unit cells of the muscle in each condition. They found that, although there was a slight shift of electron density to the position of the actin filaments for the muscle in both a static Ca^{2+}-activated state and also at the peak of tension state, compared to the relaxed state, this re - distribution was small. However, as expected from the electron micrographs of rigor muscle, it was found that electron density was transferred away from the region between the filaments, and towards the actin filaments, when compared to the relaxed muscle.

Miller and Tregear interpret these results as an indication that in the activated preparations only a small fraction of the crossbridges appear to be attached to the actin filaments at any one time. This was also consistent with vertebrate (rabbit) muscle. Eisenberg and Moos (1968) compared the ATP-ase activity of rabbit muscle with that of a completely dissociated acto-heavy meromyosin complex. Their conclusion was that crossbridges in active rabbit muscle are dissociated from the actin filaments.

The X-ray diffraction patterns of insect flight muscle have been reviewed by Miller and Tregear (1971) and a summary of their findings is given below. The results which are reviewed are mostly from glycerol-extracted preparations of water bug flight muscle.

Essentially, there were two very distinct patterns, one arising from relaxed muscles, and the other from rigor muscle.

The diffraction pattern from relaxed muscle has a strong meridional reflexion

of spacing 145.1 ± 0.3 Å. Reedy, Holmes and Tregear (1965) have also reported a strong second order of this reflexion; however, this is not referred to by Miller and Tregear (1971). In the relaxed muscle, there was a layer line of 388 Å (Miller and Tregear also mention this layer line as 385 Å), but the intensity on this layer line was weak. There was another weak layer line of spacing 235 Å. Off-meridional reflexions also occurred on layer lines of spacings 59 Å and 51 Å. These, and weak meridional reflexions with spacings 128 Å and 27.1 Å, are believed to originate from the actin filaments.

This diffraction pattern is readily interpreted by comparison with the distribution of crossbridge origins on the surface of the myosin filament as suggested by Reedy (1968); see Figure p 61. The spacing of the layer lines at 145, 235 and 388 Å suggested a double helix of pitch = 770 Å, axial displacement = 145.1 Å and average radius around 80-130 Å. One strand of the double helix is related to the other by an azimuthal rotation of 180°. Miller and Tregear suggested the crossbridges were perpendicular to the filament axis, although not normal to the cylindrical surface. Their calculations indicate that the crossbridges are confined to horizontal planes with an axial displacement of 145 Å from each other. This wrapping of the crossbridges close to the thick filaments resulted in the strong intensity of the 145 Å reflexion, but the 388 Å and the 235 Å reflexions are weakened.

The diffraction pattern from rigor muscle showed a series of layer lines. The 388 Å layer line (N.B. also recorded as 385 Å) was very strong and there was a strong second order layer line at 193 Å (N.B. also mentioned as 192.5 Å). The actin layer lines at 51, 59, 70, 85.5, and 110 Å spacing are all intensified. The weak layer line of spacing 235 Å of the relaxed muscle is not observed in the rigor state. In rigor muscles, the 145 Å and the 127 Å meridional reflexions were weak. This is believed to be caused by the crossbridges no longer being perpendicular to the filament axis.

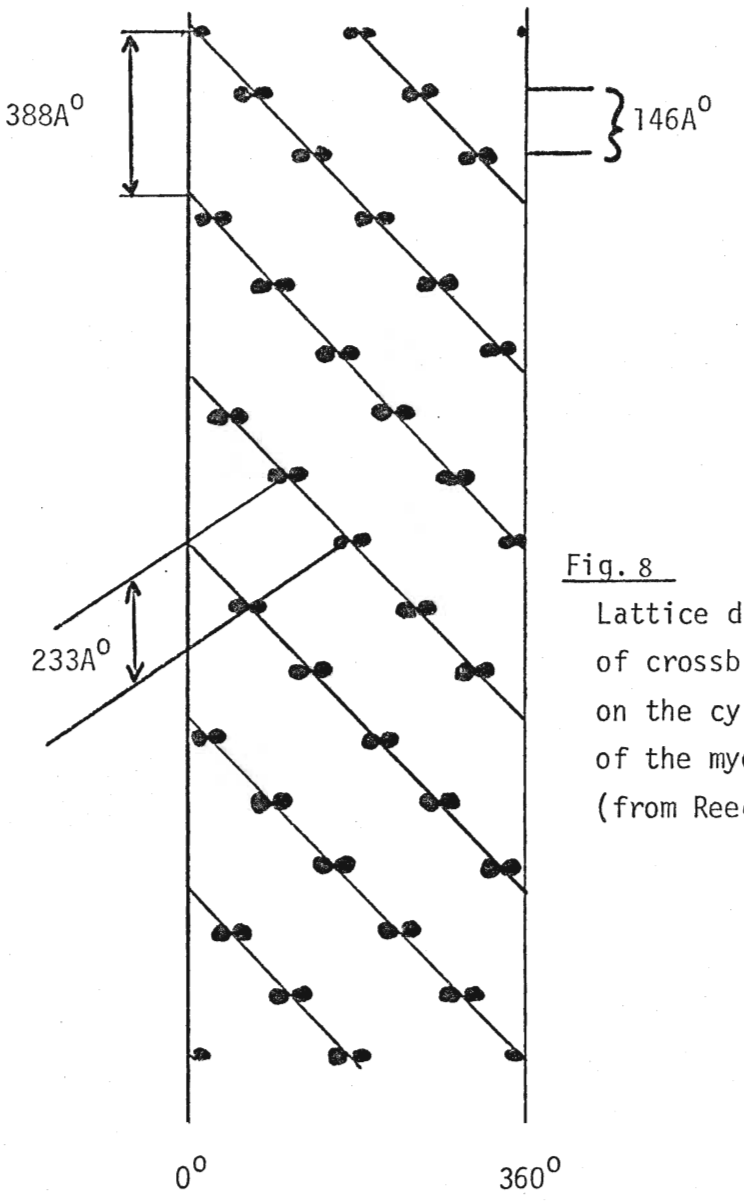


Fig. 8
Lattice distribution of crossbridge origins on the cylindrical surface of the myosin filament (from Reedy, 1968)

The actin helix is described as a double helix of pitch around 760 Å, axial displacement = 54.2 Å, and radius around 24 Å. The helix was a double helix in which the strands were related by an axial displacement of 27.1 Å and an azimuthal rotation of just under 180°.

There are two anomalies which remain in the diffraction patterns. These are the occurrence of the 127-128 Å meridional reflexion and the observation that the maximum intensity on the 388 Å layer line is too close to the meridian to be explained by crossbridges at a radius of 80-130 Å. If the crossbridges were at a larger radius, this would be expected to produce intensity close to the meridian of the 235 Å layer line.

These two anomalies are explained if the thin filaments around any one thick filament are in helical register, with pitch - 770 Å and axial displacement - 128 Å. This helical register appeared to be present in both the rigor and relaxed state, and Reedy (1968) had proposed this helical register from electron micrograph observations.

The relaxed and rigor states both gave strong equatorial reflexions corresponding to the (1, 0, $\bar{1}$, 0) and the (2, 0, $\bar{2}$, 0) reflexions from a hexagonal unit cell of side 530 Å (glycerol-extracted muscle). Also other weaker reflexions are observed (Worthington, 1961), but the (1, 1, $\bar{2}$, 0) is not observed in either state because of the positions of the thin filaments in the unit cell (cf. Equatorial Reflexions from Vertebrate Muscle, General Introduction, p 10).

In actively contracting flight muscle, the diffraction pattern appeared the same as in relaxed muscle, except that there was a slight variation of the intensities of the 145 Å and the equatorial reflexions. The other reflexions were too weak to be studied. As previously mentioned, the intensity of the 145 Å reflexion varies in antiphase with the tension.

Miller and Tregear (1971) also gave the intensities of some of the X-ray

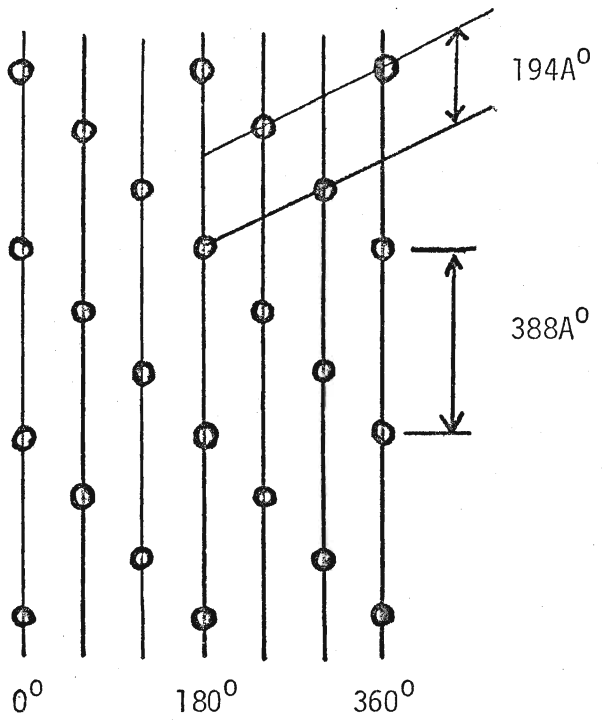


Fig. 9

Circles represent the optimum sites for crossbridge attachments along the actin filaments. These crossbridge attachments are helically distributed around each myosin filament.
(from Reedy, 1968)

reflexions from the muscle in different physiological states. These are tabulated below:-

Muscle Condition	Diffracted Intensity (as % of relaxed $(1,0,\bar{1},0)$ reflexions):- 145 Å	$(1,0,\bar{1},0)$	$(2,0,\bar{2},0)$
Relaxed (ATP, no Ca^{2+})	17	100	52
Rigor (no ATP)	2	71	84
Activated (ATP, 10^{-7}M Ca^{2+})			
1. Static	17	103	56
2. Vibrated	13	103	57

The intensity change of $(1, 0, \bar{1}, 0)$ and $(2, 0, \bar{2}, 0)$ reflexions between rigor and relaxed is expected. Electron density is transferred from the region between the filaments towards the actin filaments when the muscle is in the rigor state.

Worthington (1960) reported a diffraction pattern from air-dried insect flight muscle (the specimens were from the blowfly, Calliphora, and the water beetle, Dytiscus). The pattern consisted of a series of meridional arcs which were orders of the period d=300±5 Å. Worthington has attributed this pattern to diffraction from air-dried mitochondria in the muscle. By treating the muscle with various solvents before air-drying, the period could be increased by up to almost 25% for a muscle placed in 30% acetone in water for one day before air-drying. Frequently, as the intensity of the meridional diffraction decreased, a moderate angle X-ray diffraction pattern would appear, usually in the form of a ring at 47 Å. Worthington suggested that this arises from lipid crystallizing out as a separate phase.

Insect Flight Muscle

Method Section

The species of insect used in these experiments was Sarcophaga bullata Park which has a common North American Name of Flesh Fly. A colony of these flies was reared at Brock Univeristy by Miss Dianne Richardson. The Flesh Fly is a very large fly which makes handling and dissecting easier.

Dissection of the Fly

The dissections began by anesthetizing several flies with carbon dioxide gas. This was simply accomplished by passing CO_2 gas from a cylinder into one of the jars from the colony. The anesthetic effect would last for around two minutes during which time suitable specimens would be selected and decapitated. The legs and the wings were then dissected from the thorax using a pair of fine scissors. To facilitate further dissection the fly would be affixed to a piece of discarded X-ray film. A hole, approximately 3mm in diameter, was first punched in the film and then adhesive was placed around three quarters of the hole. A carboxylate cement 'Durelon' was initially used but discontinued in favour of 'Devcon 5-Minute Epoxy' (Devcon Corp., Danver, Mass., U.S.A.) which was a fast setting, low heat producing epoxy resin. The fly was positioned, ventral side uppermost, onto the epoxy resin such that the middle of the thorax was over the centre of the hole, and the abdomen was in the section without the epoxy resin.

When the epoxy resin was set, the epidermis between the thorax and the abdomen was cut with a scalpel and the abdomen gripped, with a pair of forceps. By carefully pulling with the forceps, the abdomen was separated from the thorax and also the digestive tract was removed from the thorax.

The thorax was now cut along both sides using surgical eye scissors and the ventral surface removed by carefully lifting with a pair of fine forceps. The flight muscles werenow exposed lying longitudinally in the thorax with the remnants of the leg muscles on either side.

In view of the fragile nature of the flight muscles they were normally left in the thorax. The dorsal section of the thoracic exoskeleton which was covering the hole in the film was also removed and the film trimmed to an appropriate size for mounting in a specimen chamber.

The specimen chamber was a Plexiglass container with two windows of thin mica. The film holding the specimen would be placed in position, such that the X-ray beam would pass through the hole in the film and into the flight muscle, and held in position with plasticene (modelling clay). A small amount of water would be placed in the bottom of the specimen chamber and the chamber would then be made airtight to prevent drying during exposure.

Fixation

Fixation was performed on some flies before insertion into the specimen chamber. Fixation was accomplished using one of two methods. The first method (Dr. M.K. Reedy, personal communication) was to add buffered glutaraldehyde to distilled water to give an appropriate concentration (between 2-5%). The dissected fly was placed in this solution for an appropriate time (between 1-3½ hours) and then washed and placed in position in the specimen chamber. The second method was similar to the first except that insect ringer was used in place of distilled water. The composition of the insect ringers used for these experiments was as follows:

146.5mM NaCl
6.5mM KCl
2.0mM $CaCl_2$
2.0mM $MgCl_2$
10.0mM Sodium Phosphate buffer pH 7.0.

For both methods, variations on the times and the concentrations of glutaraldehyde were tried.

Osmium Tetroxide Stain

Some of the glutaraldehyde fixed specimens were placed in a solution containing 2% osmium tetroxide for approximately 2 hours and then washed and inserted in the X-ray beam. I wish to acknowledge assistance by Kwai Yui Lee in the preparation of the osmium stained specimens.

Development and Measurement of the Diffraction Patterns

The methods of the development and measurement of the diffraction patterns were the same as those applied in the section referring to ABRM diffraction patterns.

Insect Flight Muscle Results

Equatorial Spacing

The equatorial spacings of two muscles were measured to give a value for the side of the hexagonal net. The reflexions that could clearly be identified were the (2, 0, $\bar{2}$, 0) reflexion, the (2, 2, $\bar{4}$, 0) and (3, 1, $\bar{4}$, 0) doublet and the (2, 3, $\bar{5}$, 0) reflexion. The (1, 0, $\bar{1}$, 0) reflexion was not resolved and was being partially collected on the backstop.

The (2, 2, $\bar{4}$, 0) and the (3, 1, $\bar{4}$, 0) reflexions are very close together (see Worthington, 1961) and were not resolved. The theoretical ratios of these spacings are 0.289 and 0.277, respectively, relative to the (1, 0, $\bar{1}$, 0) reflexion. Worthington (1961) obtained an average ratio of 0.282 for these two reflexions, by comparing numerous patterns.

The (1, 0, $\bar{1}$, 0) reflexion will occur at a spacing of $\frac{\sqrt{3}\,a}{2}$, where (a) is the side of the hexagonal net. The (2, 0, $\bar{2}$, 0) reflexion is, therefore, found at a spacing of $\frac{\sqrt{3}\,a}{4}$ and the (2, 3, $\bar{5}$, 0) reflexion occurs at $\frac{\sqrt{3}}{2} \cdot \frac{a}{\sqrt{19}}$.

Worthington (1961) gave the side of the hexagonal net to be 560Å (living specimens) and Miller and Tregear (1971) obtained a value of 530Å (glycerol-extracted specimens). The results for the freshly dissected, unfixed flight muscle from Sarcophaga bullata are tabulated below:

TABLE 1

Muscle	Reflexion Index	Average Spacing Å	Side (a) Å	Average (a) Å
24/6/70	(2, 0, $\bar{2}$, 0)	233.3	538.7	
	(2,2,$\bar{4}$,0)&(3,1,$\bar{4}$,0)	131.2	537.2	538.2
	(2, 3, $\bar{5}$, 0)	107.0	538.6	
26/3/71A	(2, 0, $\bar{2}$, 0)	231.7	535.0	
	(2,2,$\bar{4}$,0)&(3,1,$\bar{4}$,0)	126.2	515.9	523.8
	(2, 3, $\bar{5}$, 0)	103.4	520.4	

A line focus was used for muscle 24/6/70 and a point focus was used for 26/3/71(A). The line focus facilitated more accurate measurement of the relexions.

The fly (index 19/10/70, fresh, unfixed) showed strong equatorial reflexions and also sampling on the 1st layer line. Unfortunately, the fly was positioned for recording the axial reflexions and therefore only part of one side of the low angle equatorial region was seen because of the position of the backstop.

Six reflexions were seen, the first two strong reflexions are indexed as the $(2,2,\bar{4},0)$ $(3,1,\bar{4},0)$ doublet, and the $(2,3,\bar{5},0)$ reflexion. The position of the main beam (i.e. (0,0,0,0) reflexion) was calculated from the separation of the two reflexions and with this information, the spacing of all the reflexions was determined.

The $(2,2,\bar{4},0)$ $(3,1,\bar{4},0)$ reflexion and the $(2,3,\bar{4},0)$ reflexion are clearly seen on the 1st layer line (i.e. the $(2,2,\bar{4},1)$ $(3,1,\bar{4},1)$ and the $(2,3,\bar{4},1$ reflexions are seen), the other reflexions are only seen on the equator.

The spacings are as follows:

$(2,2,\bar{4},0)$ $(3,1,\bar{4},0)$	133 Å ± 3Å	These spacings gave (a) as 544 Å
$(2,3,\bar{5},0)$	108 Å ± 3Å	
	93.5 Å ± 2Å	
very weak	76.0 Å ± 2Å	
weak	69.6 Å ± 2Å	
weak	61.4 Å ± 2Å	

The reflexions may be indexed as one, or a combination of diffraction planes.

Spacing Å	Possible Indexes
93.5	$(5,0,\bar{5},0)$
76.0	$(6,0,\bar{6},0)$, $(4,3,\bar{7},0)$, $(5,2,\bar{7},0)$
69.6	$(6,1,\bar{7},0)$, $(4,4,\bar{8},0)$, $(7,0,\bar{7},0)(5,3,\bar{8},0)$
61.4	$(7,1,\bar{8},0)$, $(5,4,\bar{9},0)$, $(6,3,\bar{9},0)(8,0,\bar{8},0)$

The average value for the side of the hexagonal net, from three experiments, was 535 ±5Å.

Insect Flight Muscle

Results Section

Axial Spacings

Miller and Tregear (1971) have summarized the values of the spacing of reflexions from previous work on insect flight muscle. As the diffraction patterns obtained from Sarcophaga bullata flight muscles are in general agreement with the patterns previously described, then the Miller and Tregear spacings will be used to identify the reflexions in the diffraction patterns.

The diffraction patterns that were obtained were:-

(i) relaxed type patterns - from fresh flight muscle
(ii) rigor type patterns - from fresh flight muscle
(iii) rigor type patterns - from fixed flight muscle
(iv) dried pattern (mitochondria pattern) - from dried specimens
(v) other reflexions from deteriorating specimens

In both the rigor and the relaxed muscle, the 59 Å actin reflexion can usually be accurately measured and, therefore, the results are also given in terms of the ratio of the reflexions compared to the 59 Å reflexion. This procedure aids the elimination of errors in the averaged spacing of the reflexions which could arise from small errors in the specimen to film distance, and it is particularly useful with respect to the fixed specimens in which the possibility of a systematic error from fixation shrinkage also arises (Page and Huxley, 1963).

Often reflexions could be resolved by eye on the films, but could not be accurately measured due to low intensity of the reflexion or high background intensity in the region of the reflexion.

Figure 10

Diffraction Patterns from the Insect Flight Muscle of *Sarcophaga bullata*

(contact print)

#	Muscle Index	Specimen to Film Distance (cm)	
1	8/3/71	9.24	Relaxed type pattern from fresh muscle
2	9/3/71	9.49	Rigor type pattern from fresh muscle
3	11/3/71	9.52	Relaxed type pattern from fresh muscle
4	19/10/70	8.65	Rigor type pattern from fresh muscle
5	10/8/70A	8.83	Rigor type pattern from fresh muscle
6	17/3/71	9.38	Rigor type pattern from fixed muscle
7	24/3/71	11.32	Arc and ring reflexions from air dried fresh muscle
8	24/3/71	11.32	

Fig. 10

(i) Relaxed Type Patterns From Fresh Flight Muscle

The relaxed type diffraction patterns can readily be identified as they show a strong 59 Å spacing together with a strong meridional 72 Å spacing, and have the absence of the series of layer lines which are clearly seen in the rigor pattern (see Figure 10).

The table below records the values of the reflexions. Within each experiment, the ratio of the spacings, relative to the actual measurement of the '59 Å' spacing, was found. By combining these ratios from the individual experiments, it was possible to calculate an average ratio for each identified spacing. Hence, after averaging the measured spacing of the 59 Å reflexions, the spacings of the reflexions from Sarcophaga bullata may be generated.

TABLE 2

Miller and Tregear Spacing	Exp. # 8/3/71	Exp. # 11/2/71	Exp. # 14/3/71	Exp. # 15/3/71	$\bar{X}$ of Spacing Ratios	$\bar{X}$ Spacing ref. 59.0 Å
27.1 (weak)						
51	51.1		51.0		0.873	51.5±
59	58.8	58.8	58.3	60.1	1.00	59.0±0.3
72.5	71.7	71.8	70.5	72.1	1.21	71.5±0.7
	91.9		92.2	96.2	1.58	93.3±1.2
128 (weak)						
145	142.0		144.0	146.0	2.44	144.0±1.0
235 (weak)	232.0				3.96	234.0±
385 (weak)	404.0				6.87	405.0±

A reflexion which has not previously been reported is appearing at 93 Å. It is a meridional reflexion, rather than a layer line, and its possible origin will be discussed later.

No splitting of the 72 Å or the 145 Å reflexions across the meridian was observed in the diffraction patterns (see Elliott, 1965). However, the 40 micron spot focus of the X-ray generator was not used; and therefore, any splitting would not necessarily be observable.

(ii) Rigor Type Patterns From Fresh Flight Muscle

The specimens which produced rigor type were prepared in the same manner as those which produced relaxed type patterns. It is believed that the physical dissection of the fly and/or the activity of the fly prior to the dissection resulted in changes to the state of the muscle.

The rigor pattern is identified by the presence of a series of layer lines at around 51, 59, 70, and 85 Å, and also by a strong first order layer line near 385 Å (see Figure 10).

The recorded values of the reflexions and the ratio of the reflexions to the 59 Å reflexion are tabulated below. As before, some reflexions could be visualized, but were too faint to be accurately measured.

TABLE 3

Miller and Tregear Spacing	Exp. # 10/8/70	Exp. # 19/8/70	Exp. # 21/8/70	Exp. # 19/10/70	Exp. # 30/6/71	$\bar{X}$ of Spacing Ratios	$\bar{X}$ Spacing of 59.46 ± 0.17 Å
27.1 (weak)							
51	51.9	50.2	50.8	51.1		0.858	51.0±0.6
59	59.3	58.9	59.7	59.8	59.6	1.00	59.5±0.2
70	70.6	69.3	70.7	71.1		1.19	70.5±0.4
85.5	86.2	87.4				1.47	87.3±
110							
128 (weak)				127		2.12	126 ±
145 (weak)				147		2.45	146 ±
193				194		3.25	193 ±
385		375		374	391	6.39	380 ±

(iii) Rigor Type Patterns From Fixed Flight Muscle

The diffraction patterns obtained from flight muscle which had been fixed with glutaraldehyde resembled in appearance the rigor patterns from unfixed flight muscle. No patterns from the fixed flight muscle which resemble the relaxed type pattern were obtained. The recorded reflexions are tabulated below:-

TABLE 4

Miller& Tregear Spacing	Exp. # 9/3/71	Exp. # 15/3/71	Exp. # 17/3/71	Exp. # 22/3/71	Exp. # 7/7/71	$\overline{X}$ of Spacing Ratios	$\overline{X}$ Spacing ref. 58.2± 0.6	$\overline{X}$ Spacing Using $_{o}$ 59.5 A for 59 Spacing
26.1 (weak)								
51	49.2		49.6	50.8		0.862	50.2±0.9	51.3
59	57.7	59.3	56.9	58.9		1.00	58.2±0.6	59.5
70	67.8	70.9	66.7	69.9		1.18	68.8±1.2	70.3
				72.4		1.23	71.6±	73.1
85.5	81.9					1.42	82.6±	84.4
110	104			107		1.81	105	107
128 (weak)								
	135					2.33	136	139
145 (weak)			147			2.58	150	153
193	190		192	202	200	3.37	196 ±5	200
385	359		376	392	404	6.50	378 ±13	387

Exp. # 9/3/71 Glutaraldehyde fixation - 3% for 3 hours
Exp. #15/3/71 Glutaraldehyde fixation - 2% for 3 hours
Exp. #17/3/71 Glutaraldehyde fixation - 3% for 3½ hours
Exp. #22/3/71 Glutaraldehyde fixation - 3% for 3½ hours
Exp. # 7/7/71 Glutaraldehyde fixation - 3% for 3½ hours

As in previous tables, the average spacing of the 59Å actin reflexion from the fixed rigor specimens was used to calculate the values of the other reflexions. The average value of the 59Å actin reflexion from the unfixed rigor results (59.5Å) was also used to generate the values of the reflexions, as a compensation for fixation shrinkage (we assume that the myosin and actin components shrink approximately equally). Page and Huxley (1963) found that with vertebrate muscle, glutaraldehyde probably caused some shortening of the I filaments (2 to 3%) but very little shortening of A filaments.

The average values for the 59Å reflexion were:

(i)	for the relaxed patterns	59.0Å±0.3Å (standard error on 4 results)
(ii)	for the unfixed rigor patterns	59.5Å±0.2Å (standard error on 5 results)
(iii)	for the fixed rigor patterns	58.2Å±0.6Å (standard error on 4 results)

In view of the limited number of results, the difference between the average 59Å reflexion of the fixed and unfixed rigor patterns cannot be considered statistically significant. However, Reedy (1965) gives a value of approximately 2% for the shrinkage of the specimen after fixation for electron microscopy, which was consistent with the variation of the 59Å spacing observed.

Although no patterns from the fixed flight muscle which resembled the relaxed type patterns were obtained, the pattern from one muscle (index 22/3/71) showed a meridional spot at corrected spacing of 71.6Å, as well as the actin layer line at 68.8Å. This was thought to arise from part of the muscle being in the relaxed state when fixed. Scattered radiation, in the meridional direction has masked the observation of the expected first order, at around 145Å.

The corrected spacing of the 136Å reflexion seen in film 9/3/71 is

believed to be an average value of the weak 128A and 145A reflexions.

The rigor type patterns from both the fixed and the unfixed flight muscles were very similar in appearance. The average ratios of the spacing between the fixed rigor spacings and the fresh rigor spacings did not differ substantially. Any differences between the spacing observed in the fixed and unfixed rigor muscle were consistent with the 2% shrinkage artifact reported by Reedy (1965). It would, therefore, appear that any changes in the muscle structure between fresh rigor and fixed rigor were small.

Insect Flight Muscle

Results Section

(iv) X-ray Diffraction Patterns from Dried Muscle

The X-ray diffraction pattern from dried muscle appeared as a series of arcs and rings (see Figure 10). Meridional (or near meridional) reflexions were also seen in film (24/3/71). These were, with the exception of the 26.5Å reflexion, very weak. Under magnification the reflexions were difficult to see and therefore, the positions of the reflexions were marked onto paper and the measurements made from the paper. Although this does introduce additional errors, this technique is permissible for moderate-angle reflexions because of the inverse relationship between the separation of the reflexions and their spacings; i.e., an error in measurement of a reflexion with a spacing of 200Å might change the spacing by many Angstroms; whereas, the same error in measurement of a reflexion at 10Å might only change the spacing by a fraction of an Angstrom.

The meridional (or near meridional) reflexions from film (24/3/71) were 8.75Å, 10.7Å, 12.9Å, 17.7Å and 26.5Å respectively. These were attributed to actin since they corresponded closely to the 9.05Å, 10.8Å, 13.4Å, 18.0Å and 26.5Å actin reflexions recorded from dried invertebrate muscle by Worthington (1956).

The arc reflexions were the second, third and fourth orders of an average spacing of 280Å±4Å (meridional direction). The first order reflexion was lost in the scatter near the backstop. The major part of the intensity of these reflexions was seen in the axial direction. The third order, which was the strongest order, also was seen to be continuous to the equator. The value of the spacing in the equatorial direction

Insect Flight Muscle

TABLE 5

X-ray Diffraction Pattern from Dried Insect Flight Muscle of Sarcophaga bullata

Muscle Index	Treatment	Ring Spacing	Arc Spacing: M denotes meridional measurement, E denotes equatorial measurement
29/3/71	dried	37.8Å 47.3Å	70.2Å(M) 93.9Å(M), 84.5Å(E) 140 Å(M)
1/3/71	dried	37.2Å NB: 2nd ring visible but too weak for measurement.	69.9Å(M) 95.2Å(M), 82.7Å(E) 138 Å(M)
24/3/71	dried	36.4Å 46.6Å 62.6Å	69.6Å(M) 92.0Å(M), 80.7Å(E) 139 Å(M)
	NB: Also meridional or near meridional reflexions at 26.5Å, 17.7Å, 12.9Å, 10.7Å and 8.75Å.		
30/9/70	glutaraldehyde fixed and dried	40.9Å	98.7Å(M) 148 Å(M) - diffuse arc

was smaller than the value in the meridional region, average values being 82.5Å (equatorial) and 93.7Å (meridional) for one reflexion (see Table 5, p 79). It was possible that the equatorial reflexion was a distinct reflexion, and separate in origin from the meridional arc; however, the general appearance of all the arc reflexions suggested that the arc reflexions were continuous.

The third order, at an average spacing of 93.7Å±1.2Å (mean ± standard error), was the strongest, and the ratio of the spacings of the second and fourth orders relative to the third order was calculated for each muscle. The average values of the second and fourth orders were thus determined as 139Å and 70.0Å respectively. The average value of the equatorial reflexion was calculated as 82.6Å±2.2Å.

The average value for the number of layers in each diffracting unit with the 280Å repeat was also calculated. The 'elliptical' nature of the reflexion indicated that the period within the muscle varied according to the orientation of that layer. The calculation of the average number of reflecting planes (see appendix A-10 , p 115) did however, give a minimum value. Applying the calculation to the 280Å reflexion, the average number of reflecting planes was around 7. Deviations of the repeating distance would increase the angular width of the reflexion and hence, could give a false lower value for the number of reflecting planes.

The arcs (Figure 10 , p 71) appeared to closely resemble the observations made by Worthington (1960). Worthington attributed the series of meridional arcs to diffraction arising from regular packing of cristae in mitochondria which are found between the muscle fibres. Using specimens of the blowfly, Calliphora and of the water beetle, Dytiscus, he obtained a spacing of 300Å±5Å for the cristae in these muscles. He also observed that the period could be altered by various treatments.

Smith (1963) found that the cristae in mitochondria of Calliphora flight muscles were well organized, compared to mitochondria from other sources, and that there were around 30 - 35 cristae per micron (e.m. measurements). This supports Worthington's assumption that the arced reflexions arose from the crista to crista spacing.

Worthington (1960) observed the first, second, third and fourth orders of diffraction to be very strong, weak, medium and very weak. This was in agreement with the arc reflexions from Sarcophaga bullata in which the second, third and fourth orders were respectively weak, medium and very weak. The first order was presumably masked by the central scatter near the backstop. Although Worthington (1960) reported that up to eight orders have been recorded, no more than four orders were observed in the present work.

The ring reflexions were indexed as the third, fourth and fifth orders of a spacing of 187Å±4Å. The fifth order of this spacing was the strongest, with an average spacing of 37.1Å±.6Å (standard error of 3 readings). The ratios of the other spacings relative to the fifth order spacing were calculated and averaged to give the fourth order spacing as 46.9Å. The fourth order reflexion was weak and the third order reflexion was very weak.

The ring appearance of the reflexions indicated that the reflecting planes are randomly oriented. A spacing of around 187Å may be expected from a double membrane structure. It was, therefore, reasonable to suggest that the 187Å spacing arose from arrays of double membranes randomly oriented. If we assume a constant lattice spacing of 187Å for the layers of double membranes then the average number of layers in each group can be calculated from the angular width of the reflexion (see appendix A-10 , p 115). This calculation gave an average value of around 10 for the number of layers in each diffracting unit.

Worthington (1960) did not observe these ring reflexions; however, as the eigth order of the 300Å arc reflexions would occur at 37.5Å, it is possible that a faint 37.1Å reflexion might have been identified as the eigth order of 300Å rather than the fifth order of a 187Å ring reflexion. No illustrations containing orders greater than the fourth order are shown in Worthington (1960).

Millman (unpublished results) has obtained rings of about this spacing in several different species of dried invertebrate muscle.

The 30/9/71 specimen which had been placed in a glutaraldehyde fixative before drying showed a 40.9Å ring and arcs at 98.7Å and 148.2Å. The reflexions were more diffuse than in unfixed dried specimens, and the values of the spacings were greater. It appeared that the action of the fixative either produced an increase in the thickness of the mitochondria membranes, or prevented the shrinkage of mitochondria membranes during the drying process.

(v) Other Reflexions

If the wet, unfixed specimens did not give the rigor, relaxed or dried patterns, then normally only diffuse scatter, such as you might expect from an amorphous substance, would appear in the region of the backstop. On two occasions, a ring at 45.7Å was seen.

Wet, fixed specimens which did not give the rigor or dried patterns frequently showed a ring at 47.1Å±0.8Å (S.E. of 8 readings); otherwise, the diffuse scatter appeared. Worthington (1960) also reported a ring at 47Å. He found that this frequently occurred after treatments with solvents and suggested that it originated from lipid crystallizing out as a separate phase from the lipoprotein complex.

It is noted that the 47.1Å ring and the fourth order ring reflexion, at 46.9Å, observed in dried muscle have the same spacing. However, in dried muscle the fifth order at 37.1Å was much stronger than the fourth order.

The muscle specimens that were fixed in glutaraldehyde and stained with osmium tetroxide gave no diffraction pattern apart from diffuse scatter. It appeared that the ultrastructure was lost during the glutaraldehyde fixation, as other specimens which were fixed with glutaraldehyde at the same time, but not stained, also gave no diffraction pattern.

Insect Flight Muscle

Discussion

The X-ray diffraction patterns obtained from the Sarcophaga bullata were consistent with the results reviewed by Miller and Tregear (1971) from previous examinations of insect flight muscle.

Equatorial Spacing

The side of the hexagonal net from freshly dissected Sarcophaga bullata flight muscle was found to be 535Å ±5Å. This dimension was in general agreement with the results from Worthington (1961) and the summary by Miller and Tregear (1971). Their findings were, respectively, 560Å for living specimens of various insects and 530Å for glycerol-extracted Lethocerus flight muscle.

Relaxed Type Pattern from Fresh Sarcophaga bullata Flight Muscle

The relaxed type diffraction patterns were readily identified by the strong 59Å and the strong 72Å meridional reflexions and the absence of the series of layer line reflexions which were clearly seen in the rigor pattern.

The weak 27.1Å reflexion and the weak 128Å reflexion were not observed, and in general, the 235Å and the 385Å reflexions were too weak to be measured.

A reflexion which had not previously been reported, was occurring at 93Å. This was a meridional reflexion rather than a layer line reflexion. Layer line reflexions from the actin filaments would be expected at spacings of 51, 59, 70, 85.5 and 110Å; and therefore, it appeared that this reflexion was not attributable to the actin filaments.

X-ray diffraction patterns from dried insect flight muscle have been attributed to diffraction from the mitochondria (Worthington, 1961). In the results obtained from dried Sarcophaga bullata flight muscle there was an arc reflexion of medium intensity at 93.7Å which was believed to arise from the mitochondria. Therefore, it is suggested that the 93Å reflexion, observed in the relaxed type pattern, was originating from mitochondria in the insect flight muscle.

Miller and Tregear (1971) did not observe the 93Å reflexion; however, their observations were on glycerol-extracted Lethocerus muscle. As glycerol-extraction 'dissolves' the muscle membranes then glycerol-extracted, relaxed flight muscle would not be expected to produce this reflexion. An attempt to study glycerol-extracted Sarcophaga bullata was frustrated by a refrigeration failure; and therefore, the possible elimination of the 93Å reflexion with glycerol-extraction has not been investigated. The 93Å reflexion was not observed with rigor type patterns; however, it was possible that the layer line reflexions were masking the observations of this meridional reflexion.

Rigor Type Patterns

The rigor type patterns were identified by the series of layer line reflexions at around 51, 59, 70 and 85Å and also by the strong first order layer lines. The weak 27.1Å reflexion was not observed.

The reflexions were all consistent with the interpretation by Reedy (1968) that the rigor pattern was produced by crossbridges attached to the actin filaments; the actin filaments also being in helical register about the myosin filaments.

Rigor type patterns were also obtained from glutaraldehyde fixed Sarcophaga bullata flight muscle. Again, the recorded reflexions were all consistent with the interpretation by Reedy (1968).

The 59Å reflexions were compared between fixed rigor and unfixed rigor patterns. The results were consistent with a 2% fixation shrinkage observed by Reedy (1965).

The X-ray Diffraction Patterns From Dried Flight Muscle

The X-ray diffraction patterns from dried muscle appeared as a series of arcs and rings. Worthington (1961) has attributed the arced diffraction pattern to mitochondria in the air-dried muscle.

The ring reflexions were indexed as orders of 187Å ±4Å. The angular width of the reflexion indicated that the average diffracting unit had approximately ten layers, and the ring appearance indicated that these arrays were randomly oriented.

The cristae, in the mitochondria, have a thickness of between 150 - 200Å (Malhortra, 1966), and they have been identified with a double membrane structure (Robertson, 1959). It is therefore suggested that the origin of these reflexions was from layers of double membranes packed together in small groups and at various orientations. This type of order might possibly be the result of a partial breakdown of the mitochondrial structures. A concentric layering of the double membrane structures (similar to the layering of an onion) could also produce a ring type reflexion. There does not appear to be any electron microscopy studies conducted on dried flight muscle which would suggest a preferred arrangement.

The 'elliptical' arc reflexions were indexed as orders of a 280Å ±4Å spacing (meridional direction). The spacing in the equatorial direction was less at approximately 248Å. The large period was in the meridional direction which was the direction of the muscle filaments.

The meridional spacing was similar to the 300Å arc reflexion recorded by Worthington (1960) from Calliphora and Dytiscus flight muscle, and identified as the crista to crista distance. Measurement of the angular width of the reflexions indicated the average number of reflecting planes to be approximately seven.

The 'elliptical' reflexion was best fitted by a relationship in which the difference between the meridional period and the equatorial period varied as the square of the cosine of the angle as measured from the equatorial line. The reflexion was interpreted as the crista to crista spacing in which the drying process had produced a lateral force causing the crista to crista separation to be less in this direction. The shrinkage of the muscle during drying was observed to occur in the equatorial direction, the length of the muscle being fixed by physical constraints.

If the 187Å spacing was the thickness of a crista, then the arrangement of the cristae in the meridional direction would be such that the centre to centre distance would be 280Å, producing a separation of 93Å between adjacent cristae (see Figure 11).

The 93A reflexion was observed to be strong relative to the second and fourth orders. Unfortunately, the first order reflexion was masked by scatter radiation near the backstop. Assigning the values of 16, 2, 6, 1 respectively to the intensity of the first, second, third and fourth orders; an approximate Patterson function for the structure was calculated. This was shown in Figure 12. The overall shape of the Patterson was in general agreement with the concept of crista (double membrane structures) separated by a distance of one-half of the crista thickness.

The ring reflexions were also assigned approximate values for the

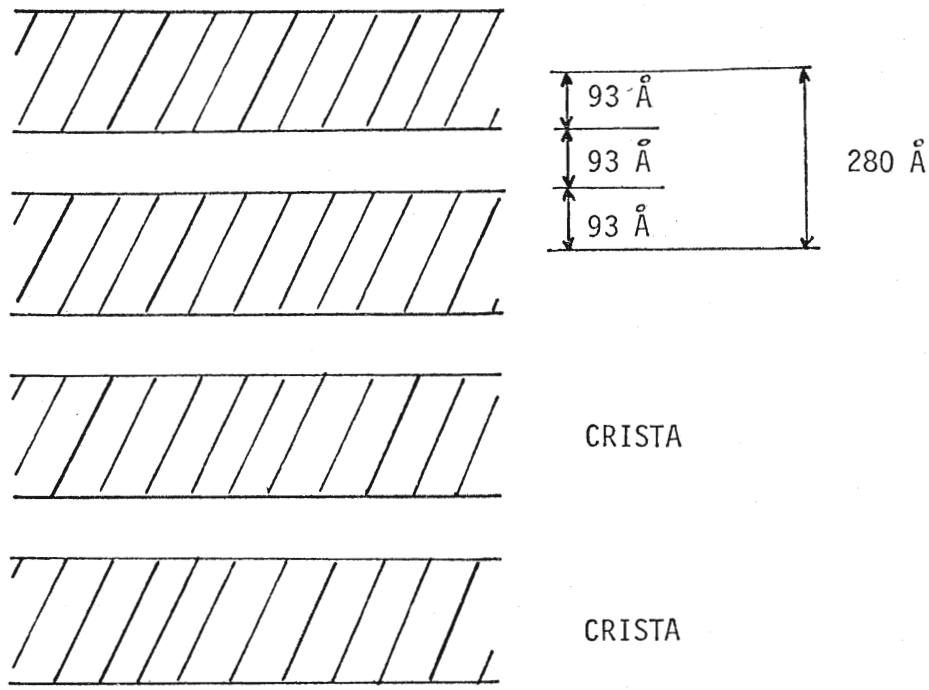


Figure 11.
Diagram indicating the separation of cristae in the mitochondria of Sarcophaga bullata insect flight muscle.

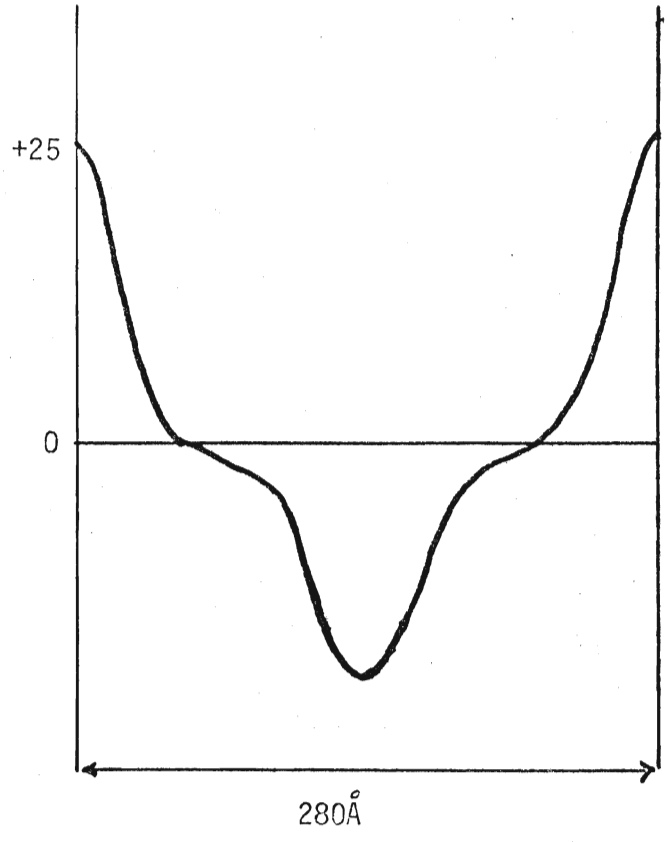


Figure 12-1

Patterson Function applied to the arc reflexions

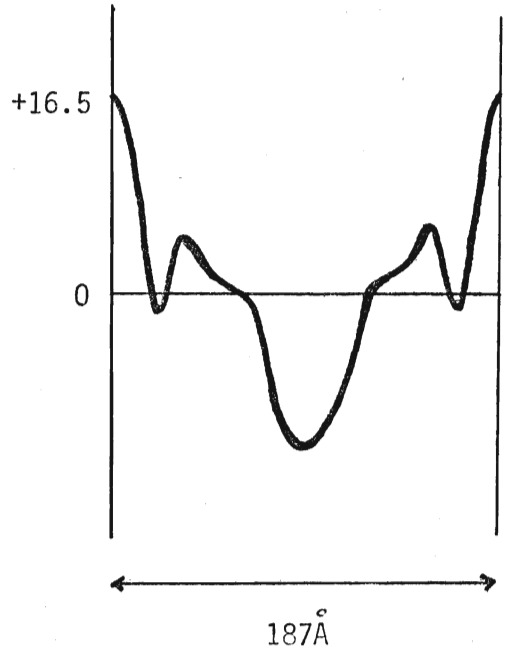


Figure 12-2

Patterson Function applied to the ring reflexions

intensity of the orders. Again, the first order reflexion was not observed due to masking by scattered radiation near the backstop. The values 8, ½, 2, and 6 were assigned respectively to the first, third, fourth and fifth orders. The resulting Patterson function (Figure 12) was in general agreement with that expected from a double membrane, or crista structure. These observations support the proposed arrangement of cristae.

The glutaraldehyde fixed and dried specimens gave a ring at 40.9Å and arc reflexions at 98.7Å and 148Å. These reflexions were interpreted as a crista thickness of 204.5Å, and the crista to crista repeat distance of 296.1Å. The separation between adjacent cristae, by the above model would be 91.6Å. Therefore, it would appear that the glutaraldehyde had increased the thickness of individual crista, by approximately 9%; however, it seemed to have little effect on the separation between adjacent cristae. These observations were from one experiment and additional experiments would be required to confirm these indications. It would appear that the insect flight muscle from Sarcophaga bullata provides a good subject for the X-ray investigation of cristae in the mitochondria.

To review, X-ray diffraction patterns from the insect flight muscle of Sarcophaga bullata (Flesh Fly) were in close agreement with previous observations of insect flight muscle by other reseachers. The X-ray diffraction patterns were consistent with the interpretations by Reedy (1965, 1968). A meridional reflexion was observed in the relaxed type pattern. This reflexion was suggested to arise from diffraction by the mitochondria. Diffraction from the mitochondria, recorded from dried specimens of flight muscle, indicated an ordered arrangement of the cristae in the mitochondria.

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APPENDIX

A-1 Table 6 ABRM Experimental Data: Extension experiments (without 5-HT)

Index	Approx Muscle Length in cm	Revised muscle length in cm	Percentage increase in muscle length	Equatorial Reflexion A°	Percentage change in equatorial spacing
5 2 70					
A	2.1	2.07	0	124.1	0
B	2.25	2.23	7.73	133.1	+7.25
C	2.35	2.3	15.46	125.4	+1.05
7 2 70					
A	2.6	2.57	0	121.9	0
B	2.7	2.73	6.23	122.3	+0.33
C	2.8	2.89	12.45	124.9	+2.46
D	2.7	2.73	6.23	118.3	-2.95
E	2.7	2.57	0	124.4	+2.05
9 2 70					
A	2.5	2.54	0	136.3	0
B	2.7	2.70	6.30	130.4	-4.33
C	2.9	2.86	12.60	133.6	-1.98
7 3 70					
A	1.5	1.84	0	137.1	0
B	2.0	2.00	8.70	137.3	+0.15
C	2.2	2.16	17.39	133.9	-2.33
D	2.25	2.32	26.10	134.1	-2.19
E	2.2	2.16	17.39	134.1	-2.19
10 3 70					
A	1.8	1.75	0	135.9	0
B	1.9	1.91	9.14	134.3	-1.18
C	2.1	2.07	18.29	132.6	-2.43
D	2.3	2.23	27.43	133.4	-1.84
E	2.0	2.07	18.29	131.5	-3.24
F	1.8	1.91	9.14	134.3	-1.18
G	1.8	1.75	0	129.5	-4.71
13 3 70					
A	1.8	1.87	0	142.9	0
B	2.1	2.03	8.56	134.6	-5.81
C	2.2	2.19	17.11	136.6	-4.41
17 3 70					
A	2.2	2.05	0	135.8	0
B	2.2	2.21	7.80	135.4	-0.29
C	2.3	2.37	15.61	140.0	+3.09
D	2.45	2.53	23.41	140.5	+3.46
19 3 70					
A	1.9	1.84	0	133.4	0
B	2.0	2.00	8.70	133.1	-0.22
C	2.1	2.16	17.39	131.1	-1.72
D	2.3	2.32	26.09	135.4	+1.50
E	2.1	2.08	13.04	132.5	-0.67
22 3 70					
A	1.7	1.67	0	142.4	0
B	1.8	1.83	9.58	137.0	-3.79
20 4 70					
B	1.9	1.79	0	130.0	0
C	2.0	1.95	8.94	128.9	-0.85
D	2.1	2.11	17.88	126.5	-2.69
E	2.1	2.27	26.82	124.8	-4.00
F	2.1	2.11	17.88	126.7	-2.54
G	1.9	1.95	8.94	131.9	+1.46

A-2 Table 7 ABRM Experimental Data: Extension Experiments (with 5-HT)

Index	Approx muscle length in cm	Revised muscle length in cm	Percentage increase in muscle length	Equatorial Reflexion A°	Percentage change in equatorial spacing
24 3 70					
A	1.9	1.76	0	131.1	0
B	2.0	1.96	11.36	139.8	+6.64
C	2.0	2.16	22.73	133.9	+2.14
25 3 70					
A	2.1	2.1	0	136.3	0
B	2.3	2.3	9.52	137.1	+0.59
30 4 70					
A	2.0	2.04	0	145.8	0
B	2.2	2.24	9.80	149.4	+2.47
C	2.5	2.44	19.61	146.5	+0.48
D	2.3	2.24	9.80	151.0	+3.57
E	2.0	2.04	0	144.1	-1.17
3 5 70					
A	2.25	2.23	0	146.0	0
B	2.5	2.43	8.97	146.9	+0.62
C	2.65	2.63	17.94	148.4	+1.64
D	2.8	2.83	26.91	146.4	+0.27
E	2.6	2.63	17.94	144.8	-0.82
F	2.4	2.43	8.97	140.2	-3.97
G	2.2	2.23	0	143.1	-1.97
6 5 70					
A	2.0	2.0	0	145.9	0
B	2.2	2.2	10.0	147.8	+1 30
C	2.4	2.4	20.0	140.9	-3.43
8 5 70					
A	2.0	1.98	0	136.4	0
B	2.2	2.18	10.10	136.4	0.00
C	2.4	2.38	20.20	136.2	-0.15
D	2.6	2.58	30.30	137.8	+1.03
E	2.7	2.78	40.40	137.2	+0.59
11 5 70					
A	2.6	2.55	0	139.2	0
B	2.9	2.75	7.84	137.0	-1.58
C	3.0	2.95	15.69	138.2	-0.72
D	3.0	3.15	23.53	136.8	-1.72
E	2.9	2.95	15.69	139.1	-0.07
13 5 70					
A	2.5	2.47	0	132.2	0
B	2.6	2.67	8.10	138.4	+4.69
C	2.9	2.87	16.19	132.2	0
D	3.1	3.07	24.29	135.7	+2.65

Statistical Analysis of the Anterior Byssus Retractor Muscle Data

As the data consisted of relative changes, the population could not be considered normally distributed. Non-parametric tests were used to analyse the data (see Introduction to Probability and Statistics, Alder and Roessler, Chapter 11).

A-3 Statistical Analysis of ABRM Extension Data (without 5-HT)

(see appendix A-1, p 101)

The Wilcoxon test was applied to the extension experiment to test the following hypothesis: there was no change in the equatorial spacings due to the extension of the muscle.

In this test:

n_1 and n_2 respectively, were the number of variates of each sample.

$n = n_1 + n_2$

W_1 and W_2 respectively, were the sums of the ranks of the two samples.

m_w was the mean of the sample.

σ_w was the standard deviation of the sample.

z was the normal deviate.

A was the area between the line of symmetry and the given z value, under the normal probability curve.

P represented the probability.

Data at rest length:

0, 0, 0, 0, +2.05, 0, 0, 0, -4.71, 0, 0, 0,

where $n_1 = 12$

Data at extended length:

+7.25, +1.05, +0.33, +2.46, -2.95, -4.33, -1.98, +0.15,
-2.33, -2.19, -2.19, -1.18, -2.43, - 1.84, -3.24, -1.18,
-5.81, -4.41, -0.29, +3.09, +3.46, -0.22, -1.72, +1.50,
-0.67, -3.79, -0.85, -2.69, -4.00, -2.54, +1.46

where $n_2 = 31$

Original Data:	+7.25,	+3.46,	+3.09,	+2.46,	+2.05,			
Rank:	1,	2,	3,	4,	5,			
Original Data:	+1.50,	+1.46,	+1.05,	+0.33,	+0.15,	0,	0,	
Rank:	6,	7,	8,	9,	10,	15.5,	15.5	
Original Data:	0,	0,	0,	0,	0,	0,	0,	0,
Rank:	15.5	15.5	15.5	15.5	15.5	15.5	15.5	15.5
	0,	-0.22,						
	15.5	21,						
Original Data:	-0.29,	-0.67,	-0.85,	-1.18,	-1.18,	-1.72,	-1.84,	-1.98,
Rank:	22,	23,	24,	25.5,	25.5,	27,	28,	29,
Original Data:	-2.19,	-2.19,	-2.33,	-2.43,	-2.54,	-2.69,		
Rank:	30.5,	30.5,	32,	33,	34,	35,		
Original Data:	-2.95,	-3.24,	-3.79,	-4.00,	-4.33,	-4.41,	-4.71,	
Rank:	36,	37,	38,	39,	40,	41,	42,	
Original Data:	-5.81							
Rank:	43							

where $W_1 = 202$ and $W_2 = 744$

Since n = 43 was large, the normal curve approximation was made.

$$m_W = \frac{n_1 (n + 1)}{2} = \frac{12(44)}{2} = 264$$

$$\sigma_W = \sqrt{\frac{n_1 n_2 (n + 1)}{12}} = \sqrt{\frac{12.31(44)}{12}} = \sqrt{1364} = 36.93$$

Therefore, the probability of finding a sum of ranks less than or equal to 202 was the area under the normal probability curve denoted by A.

$$z = \frac{\bar{X} - m_W}{\sigma_W} = \frac{202.5 - 264}{36.93} = -1.67$$

such that A = o.45254

$$P = 0.50000 - 0.45254 = .04746$$

Consequently, for a two-tailed test, twice this area equaled .09492.

At the 5% level of significance,

$$.095 > .025$$

Therefore, the hypothesis was accepted.

A-4 Statistical Analysis of ABRM Extension Data (with 5-HT)

(see appendix A-2, p 102)

The Wilcoxon test was applied to the extension experiment, in which 5-HT was added, to test the following hypothesis:

There was no change in the equatorial spacings due to the extension of the muscle in the presence of 5-HT.

Data at zero extension:

0, 0, 0, -1.17, 0, -1.97, 0, 0, 0, 0,

where $n_1 = 10$

Data at extended length:

+6.64, +2.14, +0.59, +2.47, +0.48, +3.57, +0.62, +1.64,
+0.27, -0.82, -3.97, +1.30, -3.43, 0, -0.15, +1.03,
+0.59, -1.58, -0.72, -1.72, -0.07, +4.69, 0, +2.65

where $n_2 = 24$

Original Data:	-3.97,	-3.43,	-1.97,	-1.72,			
Rank:	1,	2,	3,	4,			
Original Data:	-1.58,	-1.17,	-0.82,	-0.72,	-0.15,	-0.07,	
Rank:	5,	6,	7,	8,	9,	10,	
Original Data:	0,	0,	0,	0,	0,	0,	0,
Rank:	15.5,	15.5,	15.5,	15.5,	15.5,	15.5,	15.5,
	0,	0,					
	15.5,	15.5					
Original Data:	0,	+0.27,	+0.48,	+0.59,	+0.59,	+0.62,	
Rank:	15.5,	21,	22,	23.5,	23.5,	25,	
Original Data:	1.03,	1.30,	1.64,	2.14,	2.47,	2.65,	
Rank:	26,	27,	28,	29,	30,	31,	
Original Data:	+3.57,	+4.69,	+6.64				
Rank:	32,	33,	34				

where $W_1 = 133$ and $W_2 = 462$

Since n = 34 was large, the normal curve approximation was made.

$$m_W = \frac{n_1 (n + 1)}{2} = \frac{10(35)}{2} = 175$$

$$\sigma_W = \sqrt{\frac{n_1 n_2 (n+1)}{12}} = \sqrt{\frac{10.24(35)}{12}} = \sqrt{700} = 26.46$$

Therefore, the probability of finding a sum of ranks less than or equal to 133 was the area under the normal probability curve denoted by A.

$$z = \frac{\bar{X} - m_W}{\sigma_W} = \frac{133.5 - 175}{26.46} = -1.57$$

such that A = 0.44179

$$P = 0.50000 - 0.44179 = .05821$$

Consequently, for a two-tailed test, twice this area equaled .11642

At the 5% level of significance,

$$.116 > .025$$

Therefore, the hypothesis was accepted.

A-5 Statistical Comparison of ABRM Extension Data (with and without 5-HT)

The Wilcoxon test was also applied to the extension experiments to verify whether the data from experiments without 5-HT was consistent with the data from experiments with 5-HT (see appendix A-1, A-2).

The hypothesis, that the data from experiments without 5-HT was similar to the data from experiments with 5-HT, was tested.

The extended length data from A-1 and A-2 respectively was used, giving

$n_1 = 31$ and $n_2 = 24$

Original Data:	-5.81,	-4.41,	-4.33,	-4.00,	-3.97,		
Rank	1,	2,	3,	4,	5,		
Original Data:	-3.79,	-3.43,	-3.24,	-2.95,	-2.69,	-2.54,	
Rank	6,	7,	8,	9,	10,	11,	
Original Data:	-2.43,	-2.33,	-2.19,	-2.19,	-1.98,	-1.84,	
Rank	12,	13,	14.5,	14.5,	16,	17,	
Original Data:	-1.72,	-1.72,	-1.58,	-1.18,	-1.18,	-0.85,	
Rank	18.5,	18.5,	20,	21.5,	21.5,	23,	
Original Data:	-0.82,	-0.72,	-0.67,	-0.29,	-0.22,	-0.15,	
Rank	24,	25,	26,	27,	28,	29,	
Original Data:	-0.07,	0,	0,	+0.15,	+0.27,	+0.33,	
Rank	30,	31.5,	31.5,	33,	34,	35,	
Original Data:	+0.48,	+0.59,	+0.59,	+0.62,	+1.03,	+1.05,	
Rank	36,	37.5,	37.5,	39,	40,	41,	
Original Data:	+1.30,	+1.46,	+1.50,	+1.64,	+2.14,	+2.46,	+2.47,
Rank	42,	43,	44,	45,	46,	47,	48,
Original Data:	+2.65,	+3.09,	+3.46,	+3.57,	+4.69,	+6.64,	+7.25
	49,	50,	51,	52,	53,	54,	55

where $W_1 = 705.5$

and $W_2 = 834.5$

Since n = 55 was large, the normal curve approximation was used.

$$m_w = \frac{n_1 (n+1)}{2} = \frac{31 (56)}{2} = 868$$

$$\sigma_w = \sqrt{\frac{n_1 n_2 (n+1)}{12}} = \sqrt{\frac{31.24 (56)}{12}} = \sqrt{3472}$$

$$= 58.92$$

Therefore, the probability of finding a sum of ranks less than or equal to 705.5 was the area under the normal probability curve denoted by A.

$$z = \frac{\bar{X} - m_w}{\sigma_w} = \frac{706-868}{58.92} = -2.75$$

such that A = 0.49702

$$P = 0.50000 - 0.49702 = .00298$$

Consequently, for a two - tailed test, twice this area equalled .00596.

Since $.006 < .025$

the result was significant at the 5% level. The hypothesis was rejected.

At the 1% level of significance

$$.006 > .005$$

the hypotheses was acceptable.

Therefore, it would appear that the two sets of data do differ. However, this difference was not highly significant.

A-6 ABRM Extension Experiments - Grouping of data into regions

The average values of the change in spacing and the length change, were calculated for three separate regions. These regions were (i) at zero extension
(ii) from zero to+15% length extension
(iii) from +15% to +31% length extension

The averages for these regions were:

region (i) Data without 5-HT added to the seawater
average spacing change - 0.22%
average length change 0 %
Data with 5-HT added to the seawater
average spacing change - 0.31%
average length change 0%
region (ii) Data without 5-HT added to the seawater
average spacing change - 0.78%
average length change 9.01%
Data with 5-HT added to the seawater
average spacing change + 1.43%
average length change 9.45%
region (iii) Data without 5-HT added to the seawater
average spacing change - 1.37%
average length change 20.17%
Data with 5-HT added to the seawater
average spacing change + 0.23%
average length change 20.85%

This was graphed (see Fig. 3). The data from experiments without 5-HT added to the seawater was fitted by a line with slope -0.046, and the data from experiments with 5-HT added to the seawater was fitted by a line with slope +0.035.

A-7 Data and Statistical Analysis of the Effect of 5-HT on the Equatorial Spacing

As well as performing the sign test for paired cases on the data, the hypothesis that the addition of 5-HT to the bathing solution produced no change in the equatorial spacing was also tested, using the Wilcoxon test for the paired case.

This was a non-parametric test that takes into consideration the magnitude of the difference, as well as the sign, and therefore has a higher efficiency than the sign test.

Experimental Index	Pair	Equatorial Spacing (with 5-HT) X	Equatorial Spacing (without 5-HT) Y	D=X-Y	Rank
28/8/72	1	127.0Å	121.1Å	+ 5.9	9
29/8/72	2	125.6Å	115.4Å	+10.2	11
30/8/72	3	124.5Å	127.3Å	- 2.8	6
6/9/72	4	127.9Å	123.9Å	+ 4.0	7
7/9/72	5	113.5Å	112.7Å	+ 0.8	3
8/9/72	6	123.1Å	127.1Å	- 4.0	8
15/9/72(A&B)	7	122.2Å	133.4Å	-11.2	12
15/9/72(C&D)	8	121.5Å	123.5Å	- 2.0	5
18/9/72(B&C)	9	130.5Å	129.7Å	+ 0.8	2
18/9/72(D&E)	10	137.7Å	130.9Å	+ 6.8	10
19/9/72(A&B)	11	134.1Å	134.0Å	+ 0.1	1
19/9/72(C&D)	12	133.9Å	135.0Å	- 1.1	4

The ranks corresponding to negative values of D were underlined, so that $W_1 = 35$. The probability of obtaining any given sequence of signs for the 12 pairs (under the hypothesis of equal chance for plus or minus signs) was

$$(\tfrac{1}{2})^{12} = \frac{1}{4096}$$

Consequently, for a two-tailed test and with the information,

$$W_1 - n = 35 - 12 = 23,$$

then from Table VIIb (Introduction to Probability and Statistics, Alder and Roessler)

$$P = 2 \left(\frac{1620}{4096} \right) = .791$$

At the 5% level of significance,

$$.791 > .025$$

Therefore, the hypothesis was accepted.

A-8 Table 8 ABRM Equatorial Spacing in Varying Concentrations of Seawater

Experiment Index		Equatorial Spacing	Seawater Concentration
15/12/70	A	129.9 A°	1.5 S
	B	147.0 A°	1.0 S
	C	diffuse	0.5 S
16/12/70	A	134.7 A°	1.0 S
	B	diffuse	0.5 S
	C	120.9 A°	1.5 S
	D	diffuse	0.5 S
17/12/70	C	150.7 A°	1.0 S
	D	diffuse	0.5 S
	E	117.5 A°	1.5 S
	F	141.6 A°	1.0 S
	G	diffuse	0.5 S
	H	112.9 A°	1.5 S
13/1/71	A	105.9 A°	1.5 S
	B	145.6 A°	1.0 S
	C	diffuse	0.5 S
18/1/71	A	161.9 A°	1.05 S
	B	diffuse	0.5 S
	C	118.5 A°	1.5 S
6/9/72	B	123.9 A°	1.0 S
	C	103.1 A°	2.0 S
13/9/72	A	137.3 A°	1.0 S
	B	109.1 A°	2.0 S
14/9/72	A	131.2 A°	1.0 S
	B	102.8 A°	2.0 S

A-9 Table 9 ABRM Experiments on the tension generating properties and the relaxation of ABRM muscles in various concentrations of seawater

Muscle Index	Seawater Concentration	Tension generated in gms - electrical stimulation	Tension generated in gms - Ach stimulation	half-peak decay time (T) after electrical stimulation	$\frac{1}{T}$ X100
1/2/71	1.0 S	44.6	61.4	12.5 sec	8.0
	1.5 S	29.3	46.5	21 sec	4.8
	2.0 S	1.9	12.2	approx 200 sec	0.5
	0.5 S	7.7	33.5	12.5 sec	8.0
18/8/72	0.5 S	16.4	21.1	12.7 sec	7.9
	2.0 S	1.4	14.7	approx 145 sec	0.7
	1.0 S	12.3	37.2	12.7 sec	7.9
21/8/72	1.0 S	17.4	38.2	10.9 sec	9.2
	1.5 S	5.5	26.6	14.5 sec	6.9
	2.0 S	no measurable response	13.3	no measurable response	- -
22/8/72	1.0 S	7.5	51.2	9.09 sec	11.0
	1.5 S	6.1	45.7	14.5 sec	6.9
24/8/72	1.0 S	17.7	30.0	9.09 sec	11.0
	0.5 S	7.5	16.0	9.09 sec	11.0

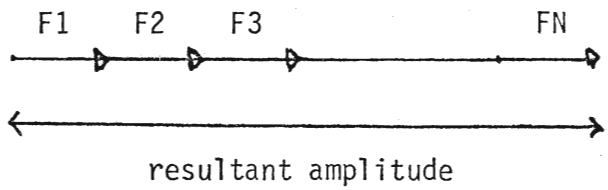
A-10 Insect Flight Muscle

Calculation of the number of planes in a diffracting unit (condensed from Molecular Biophysics, Setlow and Pollard).

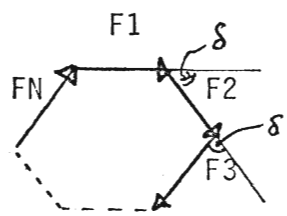
At angles which satisfy the Bragg relationship

$$n\lambda = 2d \sin\theta_B$$

the scattered radiation is in phase and the resultant amplitude is the sum of the amplitude from each plane.



If the angle of reflection is slightly greater than the Bragg angle, there will be a small phase angle δ difference between the scattered radiation from successive planes. When the path difference between adjacent planes is $\frac{\lambda}{N}$, which corresponds to $\delta = \frac{2\pi}{\lambda} \cdot \frac{\lambda}{N}$, then the resultant amplitude will be zero.



$\delta = \frac{2\pi}{N}$ and resultant = 0

When the intensity of a reflexion falls to zero, the path differences are

$$n\lambda + \frac{\lambda}{N} = 2d \sin(\theta_B + \Delta\theta)$$

$$= 2d(\sin\theta_B \cos\Delta\theta + \cos\theta_B \sin\Delta\theta)$$

---

n is the order of the reflexion
λ is the wavelength
d is the distance between planes
$2\theta_B$ is the angle between the incident and reflected waves

Now $\Delta\theta$ is small, and $n\lambda = 2d \sin\theta_B$

therefore the expression reduces to $\Delta\theta = \pm \frac{\lambda}{2Nd\cos\theta_B}$.

However, the incident beam makes an angle θ_B, and not $\theta_B + \Delta\theta$ with the planes and therefore the reflected rays are deviated through twice $2\Delta\theta$ on each side of the centre line. Hence,

$$\text{angular width} = \frac{2\lambda}{Nd\cos\theta}$$

The width of the X-ray beam has to be added to this angular width to give the total angular width. Therefore, the number of reflecting planes can be calculated from measurements of the width of a reflexion.

A-11 Insect Flight Muscle

Patterson Function Applied to the Ring and Arc Reflexions

The Patterson function[3] is the Fourier transform of the intensities. It represents the distribution of interatomic vectors in the structure.

For one dimensional analysis, as required by the ring and arc reflectors, the Patterson function may be written

$$P_x = \sum_{1}^{h} I_h \cos 2\pi(hx)$$

where I_h is the intensity of the h order reflexion and $0 \leqslant x \leqslant 1$

The Patterson function is symmetrical about $x = \frac{1}{2}$

Patterson function of the ring reflexions.

The first order (h=1) intensity was given the value 8.0
The third order (h=3) intensity was given the value 0.5
The fourth order (h=4) intensity was given the value 2.0
The fifth order (h=5) intensity was given the value 6.0

For all the other orders the intensity was assigned as 0.

---

3. For further information of Patterson functions see The Use of X-ray Diffraction in the Study of Protein and Nucleic Acid Structure by Holmes and Blow, or other textbooks on X-ray diffraction.

Applying the above formula generates:

x	P
0	+16.5
1/8	- 0.9
1/6	+ 5.5
1/4	+ 2.0
1/3	+ 0.5
3/8	- 3.1
1/2	-12.5

This is shown in Figure 12-2.

Patterson function of the arc reflexions:

The first order intensity was given the value 16.0
The second order intensity was given the value 2.0
The third order intensity was given the value 6.0
The fourth order intensity was given the value 1.0

For all the other orders the intensity was assigned as 0.

Applying the above formula generates:

x	P
0	+25.0
1/8	+ 6.1
1/6	+ 0.5
1/4	- 1.0
1/3	- 3.5
3/8	- 8.1
1/2	-19.0

This is shown in Figure 12-1.