Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 369 (2008) 34–42
BBRC
www.elsevier.com/locate/ybbrc

Memories of early work on muscle contraction and regulation in the 1950's and 1960's

Hugh E. Huxley

Rosenstiel Center, Brandeis University, 415 South Street, Waltham, MA 02454, USA

Received 24 September 2007
Available online 20 December 2007

Abstract

Professor Ebashi's epic work on the biochemistry of the regulation of muscle contraction began in the early 1950's, during the same period that work on the molecular basis of force production in muscle was also beginning. The latter work started in two MRC Research Units in the UK, and was continued jointly by the two workers from those Units who had, independently, gone to MIT to learn the new techniques of electron microscopy and to apply them to muscle. In a somewhat similar fashion, Professor Ebashi also spent one or two years in the USA, continuing his work on the role of calcium in muscle regulation in Lippman's laboratory, before returning to Japan to achieve the great breakthroughs in this work during the 1960's. Hanson and Huxley, after putting forward the overlapping actin and myosin filament arrays model for the striated muscle sarcomere, and subsequently the sliding filament model of muscle contraction (simultaneously with A.F Huxley and R. Niedergerke), returned to the UK to pursue detailed structural studies in separate Research Units, in a mixture of consultation, collaboration, and competition, during the later 1950's and throughout the 1960's.

However, the path to enlightenment described here in some detail was somewhat more tortuous than the standard literature perhaps reveals. Nevertheless, by the time of the Cold Spring Harbor Symposium on Muscle Contraction in 1972, the two lines of enquiry on regulation itself, and on the tilting cross-bridge model of force production, had arrived at a good deal of common ground, and indeed the identification of troponin and its periodic distribution along the actin filaments had helped resolve a long-standing puzzle in the interpretation of the low angle X-ray diagram.

Since then, an enormous amount of remarkable new work has been necessary to establish troponin regulation and the tilting cross-bridge mechanism in molecular detail, but the work in the 1950's and 1960's has provided a firm and accurate basis for our current understanding.

Keywords: Muscle contraction and regulation; Structure; Electron microscopy; Low angle X-ray diffraction; Molecular mechanisms; Troponin

I have been greatly saddened—as have many others—by Professor Setsuro Ebashi's long illness, and by his death. In my case, these feelings were particularly personal, since our ages were quite similar, and because the roots of our respective studies on muscle lay in the 1950's, and began to flower and gain more general acceptance in the 1960's. By 1972—the time of the Cold Spring Harbor Symposium on "The Mechanism of Muscle Contraction"—a fairly coherent account of the basic mechanisms of contraction and of its regulation, in vertebrate striated muscle, had been developed [1,2]. However, a great deal more direct structural evidence about the mechanism of force production was still needed to convince everyone that the earlier observations, often made with the new and unfamiliar techniques of electron microscopy and low angle X-ray diffraction, had been interpreted correctly, and to visualize the responsible structures at the atomic level.

Professor Ebashi and I began our work on very different aspects of muscle contraction—he on its regulation, and I on the structural basis of force and movement production by the actin–myosin interaction (and very basic and important parts of the latter were done in collaboration with Jean Hanson, in 1953/4). However, as our work progressed, the

E-mail address: huxley@brandeis.edu

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.11.130

areas of common interest increased, and there is a nice photograph of the two of us engaged in discussion at the CSH meeting, amongst the collection of snapshots of more informal activities, at the beginning of that volume. We could well have been talking about his work with Ohtsuki [3] showing that antibodies against troponin produced strong ~400 Å periodic axial staining of the actin filaments in stretched myofibrils, an observation which went some way towards clearing up the mystery of the origin of a periodicity of approximately this value seen in very early electron micrographs (in both A- and I-bands) by Hall, Jakus and Schmitt [4], by Jakus and Hall [5] and by Draper and Hodge [6,7]. Also, I had seen a whole series of axial X-ray reflections from live muscle at successive orders of a ~400 Å repeat in my early X-ray work [8]. And Bear and Selby [9] reported the first two orders of a ~400 Å axial periodicity in X-ray patterns from dried muscle which did not seem to fit in with the other series of reflections that came from the actin net or helix.

This turned out to be a quite complicated and interesting story, as there are in fact several different axial reflections in the 400 Å region, coming from different structural features of muscle, which only gradually were recognized and sorted out. I thought therefore that it might be of some interest (and amusement!) to give a more detailed and frank account than is usually available of all the steps and missteps along the path whereby Jean Hanson and I, and her colleagues at Kings College London—Jack Lowy, Gerald Elliott, Roy Worthington and Barry Millman,—in the 1950's and 1960's, endeavored to obtain and improve the X-ray and E/M data, to understand it fully, and to convince our scientific colleagues, and a wider scientific audience, that these techniques were providing a true picture of the basic sliding mechanism. The 400 Å periodicity and its relation to Professor Ebashi's work will provide a useful thread to lead through this little history.

Early observations of the 400 Å axial periodicity

Draper and Hodge [6,7] were the first to present good electron micrographs of metal-shadowed muscle fibrils, which showed a ~400 Å periodicity in both A- and I-bands. They reported that the exact period seemed to vary with sarcomere length, but it is clear now that this was an artefact, possibly in the estimates of magnification of different specimens. They also repeated the mistaken observation reported by Hall et al. [4] that all the filaments ran continuously through each sarcomere, with some extra component, the so-called A-substance, in the A-bands. Since Albert Szent-Györgyi and his colleagues had shown that both actin and myosin were needed to give artificial fibers that would contract in ATP, the general conclusion at that time was that the filaments seen in the micrographs were composite filaments of actomyosin. There was already evidence [5,10,11] that only actin filaments showed a long axial periodicity, so the presumption was that actin must provide the periodicity in the supposed composite filaments.

This all led me to make a serious mistake in interpreting the very low angle axial X-ray reflections from living muscle (as seen on a miniaturized camera to get acceptable exposure times), as being the first orders of a ~415 Å axial period from actin [8,12]. These patterns also showed the well-established higher order reflections from actin, including the strong reflection whose spacing I measured (in separate experiments with a longer camera) as being 59.3 Å, quite close to a subsequent value of 59.4 Å by Haselgrove [13] and corresponding apparently to the 7th order of a 415 Å underlying period. In reality, the very low angle reflections were the first six orders of the 429 Å helical repeat of the myosin filaments, and the systematic error in my spacing measurements (by about 3.3%) probably arose from an underestimate of the specimen to detector distance in that shorter camera, by about 1 mm. But since there had been no previous indications of long periods in myosin preparations, only in actin, I did not look into these measurements more closely at the time, and did not get back to X-ray work again till the 1960's. Had I obtained a correct value for the spacings, it would have been clear that those reflections were very unlikely to be coming from the actin filaments, but rather from the myosin filaments. I had already drawn the correct conclusion (from the behavior of the equatorial reflections as between rest and rigor) that actin and myosin were present as separate sets of filaments in a double hexagonal array. So a finding that both sets of axial reflections were invariant to passive stretch might have led me to think of sliding filaments a year or two earlier than Jean and I actually did [14]. Instead, I thought that the myosin filaments must be extensible, unless cross-linked to actin by rigor bonds. It was a very vexing error!

Work at MIT and the origins of sliding filaments

After my Ph.D., I went on a two year Commonwealth Fund Fellowship to the USA, in September 1952, to work in F.O. Schmitt's lab at MIT and learn electron microscopy from the pioneers there. I was soon able to obtain electron micrographs of cross-sections of plastic-embedded muscle (a very new technique then) and was delighted to see clearly the double hexagonal arrays of thick and thin filaments [14] in end-on view, presumably myosin and actin respectively, just as I had deduced from the equatorial X-ray diffraction patterns of living and rigor muscles.

However, it was not until early 1953 that Jean Hanson arrived there too, and we began our very fruitful collaboration. This soon led to our finding, by phase-contrast light microscopy, supported by electron microscopy, that the myosin filaments were confined to the A-band (and did in fact constitute the A-substance), whereas arrays of actin filaments, attached to the Z-lines, ran through the I-bands and continued on into the A-bands, interdigitating there between the myosin filaments and terminating at the edges

of the H-zone. Because of the existing evidence for the double hexagonal array (which we could now recognize as coming from the region of overlap of the thick and thin filaments), we could put forward the partially-overlapping arrays of filaments model with some confidence, in a letter to 'Nature' in 1953 [15].

Clearly, the very nature of the structure we had discovered, particularly the robustness of the array of ~120 Å diameter myosin filaments, and the constancy of the actin periodicities in passive stretch, suggested some form of sliding filament mechanism. This might also be involved in a contraction, driven by the cross-bridges which I had already postulated, and which I believed could be seen in the e/m cross-sections. Also, we had noticed that in Jean's phase-contrast light micrographs of isolated glycerinated myofibrils, while there was considerable variation in sarcomere length, the A-band lengths always appeared to be constant. Indeed, constancy of A-band lengths during active contraction had been reported by one or two microscopists in the middle of the 19th century, based on personal visual observation only, which was subjective and controversial. However, they were not able to obtain the essential photographic evidence of the band pattern changes during contraction, and when such photographic evidence was obtained during the first half of the 20th century, it appeared to show that they were mistaken, as has been recounted by Needham and by A.F. Huxley [16,17].

Naturally, we were very enthusiastic and excited about our findings, which finally seemed to have solved a century of conflict and confusion about the structural basis of the cross-striations in muscle. We did our best to convince everyone who might be interested of the correctness of our model, not always with complete success. Our otherwise very amiable and supportive Department Head at MIT, Professor Schmitt, remained quite skeptical and forbade us to say anything about possible contraction mechanisms in our 1953 paper about the overlapping filament model—"Do not spoil a good experimental paper with a lot of speculation"! Professor Albert Szent-Györgyi was completely unconvinced and indeed published a paper with Dan Mazia [18] to try to refute our findings.

However, I did find a more receptive audience in Dr. A.F. Huxley (no relation and considerably senior to me) who happened to be visiting Woods Hole that summer, and had been unaware of our observations. He told me of a new type of interference light microscope that he had built and was using with Ralph Niedergerke in Cambridge (England) to try to measure band pattern changes on single fibers in a way which was free of the optical artefacts that formerly had plagued such measurements on these relatively thick specimens. He said that they too had preliminary indications of constancy of A-band length. Since Jean and I had already embarked on similar studies of ATP-induced contraction in isolated myofibrils (which were so thin that a true picture of the bands was given by the phase-contrast image), he and I agreed that we would coordinate our submission of papers, assuming our conclusions were similar.

By early in the New Year (1954) Jean and I had the required photographic evidence for the sliding mechanism, including data which not only showed the approximately constant A-band length during contraction, but also showed changes in the position of the boundaries of the H-zone which indicated that the actin filaments, as well as the myosin filaments, remained approximately constant in length during contraction [19]. We learnt that A.F. Huxley and Niedergerke had also observed constant A-band length in intact muscle fibers, and so 'Nature' was contacted, and they agreed to publish the two papers together. Since we had already published the essential first part of the story there, and since that paper was a direct extension of our earlier published work (including the X-ray and e/m evidence), we rather naturally assumed that our paper would lead. In the event, the paper by A.F. Huxley and R. Niedergerke came first [20], whereas ours seemed (to us) to be tacked on as supporting evidence. Presumably 'Nature' decided that observations on intact muscle fibers would trump ones made on isolated myofibrils contracting in ATP, irrespective of previous publication history This seemed an important matter at the time, and was somewhat of a disappointment for us. We were, however, pleased to have our ideas confirmed by the intact muscle data.

The constant filament length result did not cast any immediate light on the origin of the ~400 Å axial X-ray periodicity in muscle—the reflections could still come from actin filaments or myosin filaments, or both, although the ~400 Å periodicity in the I-bands in Draper and Hodge's pictures [6,7] could now be seen to indicate that actin filament structure was at least partly responsible for it. On the other hand, Philpott and Andrew Szent-Györgyi had observed a clear ~420 Å periodicity in paracrystals of light meromyosin [21], which now we recognized must form the backbone of the thick filaments.

However, by this time Jean and I were so convinced that both actin and myosin filaments remained essentially constant in length during contraction that the exact identification of the origin of the periodicities seemed less important than finding out how the sliding force was generated.

Searching for more evidence—ultra-thin sections for electron microscopy

By summer of 1954 both Jean and I were back in our respective laboratories in London and Cambridge, and we wrote up a long review of all our work for an SEB Symposium held in Leeds in September and published the next year [22]—we alternated the sequence of authorship between successive joint papers! We continued to collaborate on some projects; and to avoid conflicts on others, the general arrangement was that I would concentrate on the details of vertebrate striated muscle, while Jean—who was a zoologist by training—set to work to see how our model applied to muscles in the rest of the animal king-

dom, including insects [23] and all the invertebrates. We remained close colleagues and good friends, and her early and tragic death in 1973 from a rare brain infection was a bitter blow.

Back in the MRC laboratory in Cambridge, my work did not prosper. I did not have easy access to an electron microscope, so tried to improve on the previous X-ray diffraction work using one of the early rotating anode X-ray tubes designed by Broad, which the still small MRC Unit was now bringing into service for the single crystal work. However, for low angle work these did not give a significant gain over my original fine focus tube set-up and so hopes of getting patterns from contracting muscles were still out of reach. Max Perutz and John Kendrew were very supportive, and a plan was developed whereby the Physiology Department (headed by Alan Hodgkin) would try to get a grant to purchase an electron microscope, since they had space for it and were thought more likely to raise the money than the small MRC Group. They were also interested in exploring this new field, so I would help them run the microscope, and have considerable use of it myself.

But this plan was overtaken by my recognition, by June of 1955, that for personal reasons not relevant to this scientific history, I would have to leave Cambridge and find a way of continuing work elsewhere, and I told Alan Hodgkin of this. At first, I thought of the possibility of moving to the MRC Unit at King's College London, where Jean was working, but Max had a very low opinion of Randall (Director of the King's Unit), and instead recommended and arranged that I join Professor Bernard Katz' Biophysics Department at University College London. This was a very fortunate outcome for me, because Katz was a first-class working scientist and an extremely nice man. Also, Professor A.V. Hill and Dr. D.R. Wilkie were working on muscle in the Physiology Department, one floor below Biophysics, and the MRC agreed to go on supporting me, as an external member of the MRC staff. Most important of all, Sir Harold Himsworth, Secretary (i.e. Head) of the MRC, having been appraised of the situation and my need for an electron microscope, and (presumably) having had good recommendations from Max and John, interviewed me and almost immediately obtained, through personal contacts, a grant from the Wellcome Foundation to purchase a new Siemens microscope, then the absolute top-of-the-line instrument, for my use in Katz' Department. I moved there in the late autumn of 1955, a move which also involved giving up my Research Fellowship at Christ's College, Cambridge.

I was now able to devote almost my whole time and energy towards trying to get better electron micrographs of muscle, i.e. better preserved tissue, thinner sections and more details of the cross-bridges. Many people had instinctive doubts about filament sliding, which to them seemed a very unlikely mechanism for contraction. Two of the leading electron microscopists at that time were Fritiof Sjöstrand and Alan Hodge. They had already made excellent contributions including some previous work on muscle, and they were both convinced that our model was wrong, and that only a single set of filaments ran through both A- and I-bands [24,25]. They considered that the thin filaments I had seen in cross-sections, lying in between the thick filaments in the A-bands, were artefacts, probably parts of cross-bridges joining thick filaments together in the hexagonal array, which happened to have been caught within the thickness of the cross-sections. And they continued to insist that they could always trace continuity between filaments in the A-band and a continuing filament in the adjoining I-band.

The basic problem was that the "thin" sections that electron microscopists were using at that time were 600 Å or more in thickness (grey/silver in reflected light), much thicker than was generally recognized. Thus, such a longitudinal section would only show a confused image of superimposed filament layers (the hexagonal lattice having shrunk somewhat in fixation and dehydration before embedding), which sometimes might appear like a single layer when the superposition of thick filaments was exact: thin filaments could not then be distinguished in the A-bands, but in the I-bands would appear often to be continuous with one of the prominent A-band "filaments". It was essential to have sections only about 100–150 Å in thickness, perfectly oriented to be parallel to a well-preserved and regular muscle filament lattice, and containing only a single layer of filaments.

The aftermath of the problems that had driven me from Cambridge continued to weigh heavily for some time, and so having a good problem to work on in the lab was my salvation. I built an improved version of the thin-sectioning microtome that Hodge, Spiro and I [26] had designed and used at MIT, and, with glass knives and a lot of patience, found that by 1956 I was able to cut sections which, floating on the water surface, behind the knife edge, appeared very dark grey to almost black by reflected light. These showed clearly single layers of thick and thin filaments lying side by side, with the thick (myosin-containing) filaments terminating at the ends of the A-bands, and the thin (actin-containing) filaments continuing on into the I-bands and attaching to the Z-lines [27]. For such sections, much stronger metal staining than usual was needed, and I found that 1% or 2% phosphotungstic acid dissolved in the alcohol used in dehydration was very effective. It was also advantageous to keep the muscles under slight tension during the entire preparative procedure.

I had been somewhat pessimistic about the degree of accurate preservation of the relationship between thick and thin filaments by the osmium tetroxide fixative then in use, even though the double hexagonal lattice was visible in places in the cross-sections. It was most unexpected, therefore, to find that actin and myosin filaments could still be seen to run straight and parallel to each other, with a constant separation, through each half A-band within the sections, when the orientation of the structures in the section relative to the knife edge was within the required regular range of about $\pm 1/6°$. The fixation and embedding

procedures evidently worked much better than I had dared to hope! The best pictures were obtained when sections were parallel to the 1120 lattice planes and these showed pairs of thin filaments between thick filaments which were spaced relatively widely apart, as expected from the hexagonal lattice. Cross-bridges could be seen very clearly between the myosin and actin filaments; these occurred at an average axial spacing of about 400 Å between a given thick and thin filament, and there appeared to be three sets of cross-bridges arranged helically on the myosin filaments within the ~400 Å interval (as indeed is the case). The cross-bridges appeared to be attached over a range of angles of tilt (the glycerinated psoas muscles used in these experiments were of course in rigor). However, these very thin sections had to be cut with the fiber axis moving accurately perpendicular to the knife edge and perpendicular to the surface of the water in the trough and a large amount of axial compression took place (by about a factor of 2) during the cutting process. I could not be sure that the mechanical properties of the embedding material were sufficiently uniform that no local distortions were occurring, and therefore could not be sure that the variable attachment angle of the cross-bridge was genuine (though I was sufficiently emboldened to include a diagram showing that they might operate by a tilting mechanism in a subsequent Scientific American article [28]). In fact the structures visible in those micrographs were almost certainly the lever arm domains of the myosin heads, with the catalytic domains imaged as part of the actin filaments.

I think these micrographs convinced a great many people of the correctness of our overlapping filament model, but a lot of skepticism remained as to whether the actual sliding model driven by cross-bridge movement was valid, and in particular whether filament lengths did stay constant during contraction, despite the evidence that had already been presented, admittedly only with light microscope accuracy. And although I had virtually full-time access to an electron microscope, I did not have any X-ray equipment, nor was there in fact any way then in which existing equipment could have obtained X-ray diagrams from contracting muscle.

Negative staining of muscle filaments for electron microscopy

I spent a great deal of time trying to find a way that would show me more details of the internal structure of the thick and thin filaments in sectioned tissue, but I was not successful, which was very frustrating. It was not until I tried an entirely different approach that I made progress.

As a sideline, I had been examining the appearance of one or two virus preparations, beginning with TMV (tomato mosaic virus) which Jim Watson brought from Rosalind Franklin's group nearby at Birkbeck College, hoping that I could find a way of visualizing the RNA in the rod-shaped particles, when partially depolymerized. This was not possible, but in the course of trying out different electron stains, I found that an aqueous solution of phosphotungstic acid (PTA), gave a remarkable outlining effect when a small amount of it was allowed to dry on the preparation, delineating the surface of the virus very clearly and showing a central channel in the particles. At the time, as it was not my main interest, I just described the finding and the technique at an electron microscope meeting (which was published in 1957 [29]). A couple of years later Aaron Klug, also then in the Birkbeck group, gave me some preparations of a spherical virus (TYMV, turnip yellow mosaic virus) to see whether I could visualize the exact arrangement of the surface subunits which constituted the protein shell of the virus for which his X-ray evidence indicated 532 symmetry. By this time Brenner & Horne [30] had improved my original technique by neutralizing the PTA solution, and by using a 2% solution directly rather than diluting a much stronger one and allowing it to dry on the particles. Zubay and I made the further improvement of using carbon films with small circular holes over which a solid film of negative stain, containing the particles under study, could form without the noisy features given by the surface of the carbon films [31,32]. We also began using 2% uranyl acetate solution (aqueous) as a much better negative stain.

Encouraged by the amount of fine detail revealed by this technique, I wanted to apply it to muscle filaments. I found that these could be released from muscle in a relatively intact state simply by gently blending muscle in a Waring blender in a relaxing medium. These provided excellent preparations for negative staining [33,34], and showed several very important structural features. In the case of the actin filaments, addition of heavy meromyosin (HMM, the soluble actin-binding part of myosin), produced a remarkable "decorated" actin (or "arrowheads") structure which revealed the structural polarity of actin filaments and how it was organized in muscle. In the case of myosin, comparison with synthetic myosin filaments (which form when myosin solutions at higher salt concentrations are diluted) showed a reversal of polarity at the center of each filament (and hence in muscle at the H-zone) which was a requirement of the sliding filament, moving cross-bridge mechanism we had proposed.

Although the projecting myosin heads were clearly visible, their regular arrangement was not preserved, and indeed was not seen in myosin filament preparations from muscle until many years later [35,36]. So no further light was cast on their possible contribution to the ~400 Å axial periodicity. In the case of the actin filaments, however, Hanson and Lowy [37] were able to see the helically-arranged subunits directly in molluscan thin filaments (following our arrangement, I had given Jean early details of my technique for separating filaments from muscle and negatively staining them). They measured the repeat of the cross-over points as approximately 350 Å and suggested, with remarkable insight, that the ~400 Å visible in the I-bands of muscle might arise from the way tropomyosin molecules were arranged as part of the overall thin filament structure.

Separation of the myosin and actin X-ray patterns

In the meantime, Worthington [38] and Elliott and Worthington [39] had improved upon some of my X-ray measurements of axial spacings in muscle. They found that the strongest reflection, the third in the low angle series, was meridional and had a measured spacing of 145 Å, suggesting an underlying periodicity of 435 Å (current data puts the value at 429.6 Å). Their value was much closer than my original estimate of 415 Å, and meant that it could not be related to actin filament periodicities, and must very likely come from the myosin filaments. Worthington also reported a meridional reflection at about 400 Å, whose spacing varied depending on the treatment of the muscle. Glycerinated psoas muscle examined in the wet state gave a 410 Å spacing, similar to that seen by Bear and Selby in molluscan adductor muscle. However, after formalin fixation and air-drying, the spacing changed to 380 Å. It is not clear whether the same structure was involved in each case, or whether the two treatments accentuated different pre-existing periodicities, as seems likely from evidence that I will discuss later.

So by 1963 there was general agreement that the actin and myosin filaments have distinctly different long periodicities; myosin filaments have a helical pitch around 430–435 Å, whereas the cross-over points in the (two stranded) actin filaments occur at spacings near to 360 Å. Some other structure(s) in those filaments, possibly associated with tropomyosin, gives an axial repeat of about 400 Å (actually, slightly less).

Work by Professor Ebashi during the same time period

By 1961, Professor Ebashi had returned to Japan after his stay in Lippman's lab in New York. There he had followed up his earlier work with Kumagai [40] on the ability of a vesicular fraction from muscle to cause relaxation in skinned muscle fibers in the presence of ATP. He had shown that the Ca^{2+}-binding ability of a series of chelating agents was rather accurately correlated with their ability to produce relaxation in glycerinated muscle fibers [41,42]. Moreover, he and Lippman [43] had shown that the so-called vesicular relaxing factor (derived from the sarcoplasmic reticulum) had ATP-dependent calcium-binding activity comparable to that of the chelating agents, and that this accounted for its relaxing ability with muscle. Annemarie Weber had also shown [44] that calcium was the activator of actomyosin ATPase. Back in Japan, Ebashi set to work to discover the mechanism by which calcium removal caused muscles to relax.

In 1963 he showed [45] that while actomyosin reconstructed from purified actin and myosin in the presence of ATP was insensitive to the relaxing action of calcium chelating agents and would superprecipitate quickly [46], the preparation could be re-sensitized by the addition of a factor very similar to Bailey's tropomyosin [47] but not by tropomyosin purified by Bailey's original procedure. (Weber and Wincur [48] had also found variability in the relaxing ability of synthetic actomyosin which depended on the type of actin preparation used). He pointed out that this type of actin–myosin interaction "undoubtedly represents the basic mechanism of muscle contraction", and in 1964, in work with Fumiko Ebashi [49], showed further evidence that this essential extra component needed to re-sensitize purified actomyosin was a special form of tropomyosin ("native tropomyosin"). This was all before the advent of SDS gels, and so isolating and identifying particular protein components was a very difficult and lengthy form of art.

Recognition of the role of troponin

Nevertheless, in 1965, Ebashi and Kodama [50] showed that the "native tropomyosin" system contains an additional protein component, more globular than tropomyosin itself, and that it was the combination of the two that would re-sensitize actomyosin to calcium. The new protein, which was called troponin [51], was shown to be the calcium-receptive component [52] and was shown to be present along with the entire length of the thin filaments [53]. Furthermore, it was shown by Ohtsuki et al. [3] that antibodies to troponin bound to the thin filaments in chicken myofibrils, showing 24 bands with an approximately 400 Å periodicity, as also seen by Cohen and Langley in tropomyosin paracrystals [54]. Nonomura et al. [55] found that extra material could be seen in such paracrystals, adding to the same periodic pattern when troponin was present in the preparations. This was strong evidence that the position of troponin in the thin filaments of muscle was being determined by the tropomyosin repeat in its arrangement along the actin filaments. This view was further strengthened by Jean Hanson's work on actin paracrystals [56] where she found that neither actin alone nor actin with purified tropomyosin would form paracrystals with a visible axial periodicity (though of course the cross-over points in the individual actin filaments in the paracrystals did occur, with a periodicity of about 350 Å). However, in the presence of troponin, a prominent axial banding appeared in the paracrystals, with spacings in the range of 370–390 Å. (It is interesting that Nonomura et al. also reported spacings somewhat less than the 400 Å period commonly mentioned as the I-band period: a figure of 390 Å is given for many of the measurements, but in some types of preparation the spacing was measured as 360–380 Å.)

So Professor Ebashi's discovery of troponin had the additional and very welcome side effect of accounting for one of the low angle meridional X-ray reflections whose origin had been so puzzling for many years! In 1967, Brown and I measured the spacing of this reflection (from live, resting frog muscle) as being approximately 385 Å, but noted that it sometimes appeared as a close-spaced doublet. Study of the behavior of this reflection (and its higher orders) during excitation can give significant information

about the behavior of troponin during the muscle activation process. It is also worth noting that since the function of troponin seems to be to change the position of the tropomyosin strands relative to actin during activation, some part of the troponin molecule must be anchored both to a specific site on tropomyosin and also to the actin filament, presumably by specific bonds to an actin monomer. Troponin must therefore be positioned at integral multiples of the actin subunit repeat. Given that the axial repeat of monomers along each of the two actin strands is 54.6 Å, the most likely tropomyosin repeat would be seven actins, giving a troponin spacing of 382.2 Å, reasonably close to the observed values. This still left unexplained some of the other somewhat longer spacing X-ray meridional reflections which had been reported in the region of 410–415 Å, and a strong one which Brown and I [57] later measured as 442 Å. These were also accounted for in due course.

X-ray patterns from contracting muscles

I had returned to the MRC lab in Cambridge in February 1962, when it moved from the temporary hut outside the Cavendish (Austin Wing) to a splendid new building on the new Addenbrooke's hospital site on Hills Road, the University having been unable to provide suitable accommodations within the university area for the now much enlarged group. This meant that I had access to X-ray facilities again, as well as an electron microscope. Ken Holmes had also moved there with Aaron Klug's virus group, and he and Longley modified one of the now fully developed rotating anode X-ray tubes so that it was fitted with the cathode assembly of a Beaudoin X-ray tube of the type they had used at Birkbeck College and which provide a very fine focus suitable for use with a quartz crystal focusing monochromator. This had become standard practice in their work on TMV gels, and gave much clearer X-ray diffraction diagrams because of the absence of the continuous X-ray spectrum present along with the copper Kα line in the raw X-ray output directly from the copper target. Ken and I joined forces to further improve the camera design, and added a second focusing element, a bent glass mirror, giving total (external) reflection to X-rays, usually with a thin gold coating to increase the acceptance angle. The two focusing elements were set at right angles, so that a point focus of X-rays was obtained, with very high resolution in the monochromator focusing direction. Initially, we thought it was necessary to have very tight collimation by the monochromator input and output slits so as to cut down background scattering and to get a sharp focus, but one evening I found I could open up almost the whole length of the bent crystal to the X-ray beam, and still record good patterns, with a very large increase in intensity. This meant that my exposure time for axial X-ray patterns from muscle came down from many hours to 10–15 min i.e. less than 1000 s. It was at last practical, though still time consuming, to record axial patterns from contracting muscles—1000 one-second tetani, with 1 or 2 min in between the contractions to allow the muscle to recover, giving a total time for the experiment of 17–34 h. This basic camera design has become widely used, being more or less standard at most synchrotron radiation facilities.

Meanwhile, Gerald Elliott at King's College had started using a Franks double glass mirror focusing system with a fine focus X-ray tube, similar to the prototype tube I had used earlier. This gave a point focus of increased intensity, and made it possible to record the strong equatorial reflective during contraction, and to make the important observation that the side spacing between the filaments varied with sarcomere length just as it did in resting muscle [58,59]. It also enabled Elliott to separate the meridional and off-meridional components of the myosin layer lines [60] in patterns from relaxed frog muscles, though not at first from contracting muscle. With the mirror-monochromator camera and the fine focus rotating anode X-ray tube, I was in a somewhat better position intensity-wise and my hope was to see a new pattern of intensities along the myosin and actin layer lines in muscles during contraction. This could give information about the configuration of the myosin cross-bridges when they were attached to actin and developing tension, since strong labeling of actin layer lines was readily visible in patterns from rigor muscles. I was very disappointed to find that this was not the case—the myosin off-meridional layer lines merely became extremely faint, and there was no perceptible change in the actin pattern or in its spacings. The myosin meridional reflections remained quite strong, also with their spacing essentially unchanged, though later work revealed a very small (~1½%) increase in spacing in contraction. Elliott and his colleagues had obtained similar results, and we published them in adjoining papers in Nature in 1965 [58,61]. The constancy of the axial spacings during contraction did provide strong support for our view that the filaments themselves remained essentially constant in length during contraction, and the large decrease in the off-meridional myosin layer line intensities did show that some considerable movement of the myosin cross-bridges must be taking place. We made an extensive study of all the features of the resting, rigor and contracting patterns [57,62], in the course of which we were able to get much clearer pictures of the long-spacing meridional reflections. These showed the ~385 Å close-spaced doublet identified as coming from the long repeat in the thin filaments, i.e. the troponin-tropomyosin repeat, and we realized that the splitting of the reflection must come from interference between the two half I-bands, through we don't seem to have actually said this in print at the time, only talked about it. The patterns also showed a strong meridional reflection at ~442 Å and a weaker one at ~416 Å, which we identified as coming from some then unknown extra component(s) in the thick filaments.

Putting together the X-ray results with all the previous work, I was able to formulate the swinging–tilting cross-bridge model [2] whereby the sliding force was developed by elongated myosin heads attached to actin, either forc-

ibly tilting about their attachment point, or else undergoing an equivalent change in shape. The myosin heads (now referred to as S1) were attached to the LMM backbones of the thick filaments via the rod-shaped S2 domain, which I envisaged as providing axial rigidity so as transmit axial force efficiently, but with hinge-like joints to S1 and to LMM to accommodate changes in side spacing between the filaments which occur as sarcomere length changes. This would allow S1 heads to search radially and azimuthally for a suitably positioned actin monomer to form the necessary stereo-specific attachment.

So this structural model reached maturity at just about the same time that Professor Ebashi was able to write his major review [1] of the entire process of calcium control of contractile activation and relaxation, a parallelism of which I am very proud to have been a part.

Later developments before Cold Spring Harbor

By the time of the Cold Spring Harbor meeting (Summer 1972) there had been many interesting further developments, one of them relating to regulation [63,64]. I had observed [65] a large intensification of the outer part of the second actin layer line during muscle contraction—a striking effect because it is so different from the weakening of the myosin layer lines. John Haselgrove and I showed that this was consistent with there being an azimuthal movement of the tropomyosin strands in the thin filaments, produced in response to calcium binding by troponin, and resulting in the unblocking of the attachment sites on all the actin monomers for incoming myosin heads—the "steric blocking mechanism". This idea was very much in line with Professor Ebashi's thinking, and was quickly supported by results on molluscan muscle by Lowy and Vibert [66] and by Parry and Squire [67]. The other development was that Starr and Offer [68] identified C-protein as an additional component in the thick filaments, and in work with Pepe and Craig [69,70] found that antibody to C-protein bound to nine transverse bands separated axially by about 430 Å in each half A-band of muscle, corresponding closely to the transverse periodicity seen in the H-zones of stretched muscle (e.g. [71]) Moreover, Elizabeth Rome showed that 442 Å and 418 Å X-ray reflections could arise from interference effects between the sets of transverse bands in the two halves of each A-band when the actual periodicity of the bands was 429 Å [72]. The two reflections were intensified when antibody to C-protein was present.

So, many puzzling and interesting features of the X-ray diagram were accounted for, and good general models of the structural basis of muscle contraction, and the biochemical and structural basis of its regulation had been developed and had received a considerable general level of acceptance by other workers in the field. At that time, muscle contraction was one of the outstanding examples of a major biological mechanism whose workings had been elucidated by the application of modern biophysical and biochemical techniques.

Coda

Despite all these relatively rapid advances, it took a surprisingly long time to obtain conclusive structural evidence as to how the postulated change in shape of the myosin head occurred, involving the lever arm structure revealed eventually by X-ray crystallography. It was also necessary to obtain direct evidence from single molecule studies using nanometric techniques that a single myosin head could impart a 5–10 nm axial movement to an actin filament to which it was attached with a force of up to 10 pN: and to find that in a live muscle, making use of the intense X-ray beams now available from synchrotron radiation facilities, one can obtain extremely strong evidence that the tilting lever arm mechanism is indeed operating during contraction. Moreover, the same mechanisms as were found in muscle contraction were discovered to be used in a wide variety of other intracellular motile processes, often involving closely similar protein molecules, but organized in a different way. Many people have shared in this work, and it is fortunate that the many powerful new techniques required did in fact become available, even though they once might have seemed impossible dreams. Professor Ebashi and I were fortunate to see so much of what we had begun come to fruition, and to continue to participate in the work for many years.

References

[1] S. Ebashi, M. Endo, I. Ohtsuki, Control of muscular contraction, Q. Rev. Biophys. 2 (1969) 351–384.
[2] H.E. Huxley, The mechanism of muscle contraction, Science 164 (1969) 1356–1366.
[3] I. Ohtsuki, T. Masaki, Y. Nonomura, S. Ebashi, Periodic distribution of troponin along the thin filament, J. Biochem. 61 (1967) 817–819.
[4] C.E. Hall, M.A. Jakus, F.O. Schmitt, An investigation of the cross-striations and myosin filaments in muscle, Biol. Bull. 190 (1946) 32–41.
[5] M.A. Jakus, C.E. Hall, Studies of actin and myosin, J. Biol. Chem. 167 (1947) 705–714.
[6] M.H. Draper, A.J. Hodge, Studies on muscle with the electron microscope, Austr. J. Exp. Biol. Med. 27 (1949) 465–476.
[7] M.H. Draper, A.J. Hodge, Submicroscopic localization of minerals in skeletal muscle by internal micro-incineration within the electron microscope, Nature 163 (1949) 576–578.
[8] H.E. Huxley, X-ray diffraction and the problem of muscle, Proc. R. Soc. B 141 (1953) 59.
[9] R.S. Bear, C.C. Selby, The structure of actin-rich filaments of muscles according to X-ray diffraction, J. Biophys. Bichem. Cytol. 2 (1956) 71–85.
[10] G. Rozsa, A. Szent-Gyorgyi, R.W.G. Wyckoff, The electron microscopy of F-actin, Biochim. Biophys. Acta 3 (1949) 561–569.
[11] G. Rozsa, A. Szent-Gyorgyi, R.W.G. Wyckoff, The fine structure of myofibrils, Exp. Cell Res. 1 (1950) 194–205.
[12] H.E. Huxley, Investigations of biological structures by X-ray methods. The structure of muscle, Ph.D. thesis, University of Cambridge, 1952.
[13] J.C. Haselgrove, X-ray diffraction studies on muscle, Ph.D. thesis, University of Cambridge, 1970.
[14] H.E. Huxley, Electron-microscope studies of the organization of the filaments in striated muscle, Biochim. Biophys. Acta 12 (1953) 387–394.
[15] J. Hanson, H.E. Huxley, The structural basis of the cross-striations in muscle, Nature (Lond.) 172 (1953) 530–532.

[16] D.M. Needham, Machina Carna, Cambridge University Press, 1971.
[17] A.F. Huxley, Reflections on Muscle, Liverpool University Press, 1980.
[18] A.G. Szent-Gyorgyi, D. Mazia, A. Szent-Gyorgi, On the nature of the cross-striation of body muscle, Biochim. Biophys. Acta 16 (1953) 339–342.
[19] H.E. Huxley, J. Hanson, Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation, Nature (Lond.) 173 (1954) 973–976.
[20] A.F. Huxley, R. Niedergerke, Structural changes in muscle during contraction. Interference microscopy of living muscle fibres, Nature (Lond.) 173 (1954) 971–973.
[21] D.E. Philpott, A.G. Szent-Gyorgyi, The structure of light-meromyosin: an electron microscopic study, Biochim. Biophys. Acta 15 (1954) 165–173.
[22] J. Hanson, H.E. Huxley, Structural basis of contraction in striated muscle, Symp. Soc. Exp. Biol. 9 (1955) 228–264.
[23] J. Hanson, Studies of the cross-striation of the indirect flight myofibrils of the blowfly Calliphora, J. Biophys. Biochem. Cytol. 2 (1956) 691–710.
[24] F.S. Sjöstrand, E. Andersen, The ultrastructure of striated muscle myofilaments at various conditions of shortening, Exp. Cell Res. 11 (1956) 493–496.
[25] A.J. Hodge, The fine structure of striated muscle, J. Biophys. Biochem. Cytol. 2 (Suppl.) (1956) 131–142.
[26] A.J. Hodge, H.E. Huxley, D. Spiro, A simple new microtome for ultrathin sectioning, J. Histochem. Cytochem. 2 (1954) 55–58.
[27] H.E. Huxley, The double array of filaments in cross-striated muscle, J. Biophys. Biochem. Cytol. 3 (1957) 631–648.
[28] H.E. Huxley, The contraction of muscle, Sci. Am. 199 (1958) 66–73.
[29] H.E. Huxley, Some observations on the structure of tobacco mosaic virus, in: Proceedings of the First European Regional Conference on Electron Microscopy, Almqvist and Wiksell, Stockholm, 1956, p. 260.
[30] S. Brenner, R.W. Horne, A negative staining method for high-resolution electron microscopy of viruses, Biochim. Biophys. Acta 34 (1959) 103–110.
[31] H.E. Huxley, G. Zubay, Electron microscope observations on the structure of microsomal particles from *Escherichia coli*, J. Mol. Biol. 2 (1960) 10–18.
[32] H.E. Huxley, G. Zubay, The structure of the protein shell of turnip yellow mosaic virus, J. Mol. Biol. 2 (1960) 189–196.
[33] H.E. Huxley, The contractile structure of cardiac and skeletal muscle, Circ. Res. 24 (1961) 328–335.
[34] H.E. Huxley, Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle, J. Mol. Biol. 7 (1963) 281–308.
[35] M. Stewart, R.W. Kensler, R.J.C. Levine, The structure of Limulus telson muscle thick filaments, J. Mol. Biol. 153 (1981) 781–790.
[36] R.W. Kensler, M. Stewart, An ultrastructural study of the cross-bridge arrangement in frog thigh muscle thick filaments, Biophys. J. 49 (1986) 343–351.
[37] J. Hanson, J. Lowy, The structure of F-actin and actin filaments isolated from muscle, J. Mol. Biol. 6 (1963) 46–60.
[38] C.R. Worthington, Large axial spacings in striated muscle, J. Mol. Biol. 1 (1959) 398–401.
[39] G.F. Elliott, C.R. Worthington, Low-angle X-ray diffraction patterns of smooth and striated muscles, J. Physiol. (Proc.) 149 (1959) 32–33.
[40] H. Kumagai, S. Ebashi, F. Takeda, Essential relaxing factor in muscle other than myokinase and creatine phosphokinase, Nature (Lond.) 176 (1955) 166.
[41] S. Ebashi, Ca-binding and relaxation in the actomyosin system, J. Biochem. 48 (1960) 150–151.
[42] S. Ebashi, Ca-binding activity of vesicular relaxing factor, J. Biochem. 50 (1961) 236–244.
[43] S. Ebashi, F. Lippman, ATP-linked concentration of Ca ions in a particulate fraction of rabbit muscle, J. Cell Biol. 14 (1962) 389–400.
[44] A. Weber, On the role of calcium in the activity of adenosine 5′-triphosphate hydrolysis by actomyosin, J. Biol. Chem. 234 (1959) 2764–2769.
[45] S. Ebashi, Third component participating in the superprecipitation of 'natural actomyosin', Nature (Lond.) 200 (1963) 1010.
[46] S.V. Perry, T.C. Gray, EDTA and the ATPase activity of actomyosin systems, Biochem. J. 64 (1956) 5.
[47] K. Bailey, Tropomyosin, a new asymmetric protein component of the muscle fibril, Biochem. J. 43 (1948) 271–279.
[48] A. Weber, S. Wincur, The role of calcium in the superprecipitation of actomyosin, J. Biol. Chem. 236 (1961) 3198–4002.
[49] S. Ebashi, F. Ebashi, A new protein component participating in the superprecipitation of myosin B, J. Biochem. 55 (1964) 604–614.
[50] S. Ebashi, A. Kodama, A new protein factor promoting aggregation of tropomyosin, J. Biochem. 58 (1965) 107–108.
[51] S. Ebashi, A. Kodama, Interaction of troponin with F-actin in the presence of tropomyosin, J. Biochem. 59 (1966) 425–426.
[52] S. Ebashi, F. Ebashi, A. Kodama, Troponin as the Ca-receptive protein in the contractile system, J. Biochem. 62 (1967) 137–138.
[53] M. Endo, Y. Nonomura, T. Masaki, I. Ohtsuki, S. Ebashi, Location of native tropomyosin in relation to the striation pattern, J. Biochem. 60 (1966) 605–608.
[54] C. Cohen, W. Longley, Tropomyosin paracrystals formed by divalent cations, Science 152 (1966) 794–796.
[55] Y. Nonomura, W. Drabikowski, S. Ebashi, The localization of troponin in tropomyosin paracrystals, J. Biochem. 64 (1968) 419–422.
[56] J. Hanson, Evidence from electron microscope studies on actin paracrystals concerning the origin of the cross-striation in the thin filaments of vertebrate skeletal muscle, Proc. R. Soc. B 183 (1973) 39–58.
[57] H.E. Huxley, W. Brown, The low angle X-ray diagram of vertebrate striated muscle and its behaviour during contraction and rigor, J. Mol. Biol. 30 (1967) 383–434.
[58] G.F. Elliott, J. Lowy, B.M. Millman, X-ray diffraction from living striated muscle during contraction, Nature (Lond.) 206 (1965) 1357–1358.
[59] G.F. Elliott, J. Lowy, B.M. Millman, Low angle X-ray diffraction studies on living striated muscle during contraction, J. Mol. Biol. 25 (1967) 31–45.
[60] G.F. Elliott, X-ray diffraction studies on striated and smooth muscles, Proc. R. Soc. (Lond.) B160 (1964) 467–472.
[61] H.E. Huxley, W. Brown, K.C. Holmes, Constancy of axial spacings in frog sartorius muscle during contraction, Nature 206 (1965) 1358.
[62] H.E. Huxley, Structural difference between resting and rigor muscle: evidence from intensity changes in the low angle equatorial X-ray diagram, J. Mol. Biol. 38 (1968) 507–520.
[63] J. Haselgrove, X-ray evidence for a configurational change in the actin-containing filaments of vertebrate striated muscle, Cold Spring Harbor Symp. Quant. Biol. 37 (1973) 341–352.
[64] H.E. Huxley, Structural changes in the actin- and myosin-containing filaments during contraction, Cold Spring Harbor Symp. Quant. Biol. 37 (1973) 361–376.
[65] H.E. Huxley, Structural changes during muscle contraction, Biochem. J. 125 (4) (1971) 85.
[66] J. Lowy, P.J. Vibert, Studies on the low-angle X-ray pattern of a molluscan smooth muscle during tonic contraction and rigor, Cold Spring Harbor Symp. Quant. Biol. 37 (1973) 353–359.
[67] D.A.D. Parry, J.M. d Squire, Structural role of tropomyosin in muscle regulation, J. Mol. Biol. 75 (1973) 33–55.
[68] R. Starr, G. Offer, Polypeptide chains of intermediate molecular weight in myosin preparations, FEBS Lett. 15 (1971) 40–44.
[69] G. Offer, C-protein and the periodicity of the thick filaments in vertebrate striated muscle, Cold Spring Harbor Symp. Quant. Biol. 37 (1973) 87–95.
[70] F.A. Pepe, The myosin filament: immunological and ultrastructural approaches to molecular organization, Cold Spring Harbor Symp. Quant. Biol. 37 (1973) 97–108.
[71] H.E. Huxley, The fine structure of striated muscle and its functional significance, Harvey Lect. 60 (1966) 85–118.
[72] E. Rome, G. Offer, F.A. Pepe, X-ray diffraction of muscle labeled with antibody to C-protein, Nature (New Biol.) 244 (1973) 152–154.