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THE ROYAL SOCIETY

Past, present and future experiments on muscle

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Since the basic outline of the sliding filament mechanism became apparent some 45 years ago, the principal challenge, an experimental one, has been to produce definitive evidence about the detailed molecular mechanisms by which myosin cross-bridges produce force and movement in a muscle. More recently, similar questions could be posed about other molecular motors, in non-muscle cells. This problem proved unexpectedly difficult to solve, in part because of the technical difficulty of obtaining the structural and mechanical information required about rapid events within macromolecules, especially in a working system, and this triggered many remarkable technical developments. There is now very strong evidence for a large change in shape of the myosin heads during ATP hydrolysis, consistent with a lever-arm mechanism. Whether this does indeed provide the driving force for contraction and movement—and, if so, exactly how—and whether some other processes could also play a significant role, is discussed in the light of the experimental and theoretical findings presented at this meeting, and other recent and long-term evidence.

Keywords: contraction; molecular mechanism; structure; X-ray

1. INTRODUCTION

The problem of how muscles contract has been a surprisingly long-lived one. It is a remarkable fact that, continuously over the past 50 years, since The Royal Society Discussion Meeting organized by A. V. Hill in 1949 (Hill 1950), the field has been one of considerable intellectual excitement, with important and rewarding progress being made all the time in experiments and understanding. Yet the ultimate objective of our work, though its nature was clearly identified 45 years ago, turned out to be much further away than was originally realized. There was much more that needed to be discovered than first met the eye. However, the intervening pathways have been full of interesting challenges, and have led to insights into a much broader range of motile phenomena than one had originally dared to hope. So the momentum and motivation have been maintained.

This present meeting, and the situation in the field that it reflects, has some of the same characteristics. We have all this marvellous new crystallographic evidence, at high resolution, about the structural changes that can take place in the head region of the myosin molecule during ATP hydrolysis, yet the task of proving that these occur in the actomyosin complex in muscle—and must develop force—is still not completed, despite a wealth of circumstantial evidence. Indeed, even though the tilting lever-arm mechanism is now remarkably well supported, there still seems to me to be room for some additional process to be involved as well. And reducing all this to basic physics and an even approximate set of equations is clearly some distance away.

As to the relationship between the linear motor mechanisms in myosin and kinesin, and the remarkable rotary motors now being characterized, there is little I can add to what has been discussed at this meeting, and the detailed mechanisms do not seem to be closely related. However, I would like to comment on the lengthy chain of experimental evidence on the muscle mechanism and the reasoning that has led us to the present position, and the further evidence that we need to obtain.

2. THE EARLY YEARS

At the time of the 1949 discussion meeting on muscle, it was recognized in a general way that contraction involved the interaction of actin and myosin, two proteins that were still poorly characterized, and that this interaction was expressed through the shortening of a longitudinally orientated filament structure in muscle. It was suspected that ATP hydrolysis was involved, but exactly how was still uncertain. There was no concept that individual myosin molecules might function as individual molecular motors. Indeed, as an outsider coming into the field from physics, I was amazed at how little was known—or even thought—about the underlying molecular events.

The overlapping filament model, with actin and myosin each in their own filaments, in separate but interacting arrays, provided the first clear picture of the structure, from a combination of X-ray diffraction, phase-contrast light microscopy and electron microscopy (Hanson & Huxley 1953; Huxley 1951, 1952, 1953*a*,*b*). (Electron microscopy was then just beginning to reveal the remarkable structures of the submicroscopic world, but evidence from such observations was sometimes regarded rather sceptically!) The overlapping filament

rray model soon developed into the sliding filament odel, based on changes in the visible band pattern uring contraction and stretch (Huxley & Niedergerke 954; Huxley & Hanson 1954) and on other evidence that ndividual filaments were approximately constant in ength. It was suggested that sliding was produced by the ction of cross-bridges between actin and myosin fila- ents, structural elements whose existence had been ostulated a couple of years earlier on the basis of the -ray results and the inextensibility of muscle *in rigor* Huxley 1952), and which were detected in muscle ross-sections by electron microscopy the next year Huxley 1953*b*). They were clearly demonstrated using he electron microscope during 1955–1957 (Huxley 1957; Huxley & Hanson 1956) and were identified as projec- ons from the myosin filaments; in one model they ere thought to move backwards and forwards in some yclical way (Hanson & Huxley 1955).

It took a long time (about 15 years, until around 1970) nd a lot of further work for this general model to ecome moderately well accepted (though not universally). The crucial factors were the elucidation of a great eal of detail about the structure of the actin and myosin laments, which all fitted in with the requirements of the odel (Worthington 1959; Hanson & Lowy 1963; Huxley 963; Elliott 1964; Elliott *et al*. 1967; Huxley & Brown 967), the recognition that many general properties of uscles could be explained by the model (Huxley 1960; Huxley & Hanson 1960) and particularly the theoretical emonstration (A. F. Huxley 1957), that a particular ersion of the model—essentially a thermal ratchet—ould account in a very convincing way for a good deal of he detailed behaviour of vertebrate striated muscle.

In the early days, we had thought in terms of cross- ridges tilting back and forth on the myosin filaments, i.e. eing firmly attached laterally to the myosin filament ackbone. However, the X-ray finding that filament eparation could vary without apparently interfering with he actin–myosin interaction led us to think of a rather ifferent model (Huxley 1969). In this, the force- enerating part of the structure is located in the ttachment of the myosin head to the actin filament, and ongitudinal force generated by tilting of the head on ctin is transmitted to the myosin filaments via the S2 ortion of myosin. The S2, while longitudinally rigid nder stretch, was thought to provide the required radial nd azimuthal flexibility.

This model was taken up, and developed further in the . F. Huxley & Simmons (1971) mechanism, with several epwise changes in the angle of attachment of the myosin ead to actin (for example), and an elastic component in ach cross-bridge, possibly in the S2 element. This model ould account for the remarkable rapid mechanical tran- ents, which they had characterized in single muscle bres, and which were not easy to explain satisfactorily n the original thermal ratchet mechanism.

Around the same time, Lymn & Taylor (1971) showed, y ingenious enzyme kinetic and stopped flow experi- ents, that the hydrolysis of ATP by actomyosin followed very unexpected pathway, but one in which dissociation nd reassociation of myosin and actin took place during ach ATPase cycle, exactly as was required by the sliding lament model.

3. THE MATURE MODEL

Thus the status of the problem then (1969–1971) was that we had, as a result of the previous 20 years' work, a very good idea of the overall structure of a muscle, which proteins were involved and approximately how they were arranged; and we knew how the overall structure behaved in contraction—basically, sliding at constant filament length. We had plausible models involving cyclically operating myosin cross-bridges tilting backwards and forwards and attaching to and detaching from actin at appropriate points in the ATPase cycle. But we had absolutely zero experimental evidence that such cross-bridge movement actually did take place. Clearly, such evidence was essential.

The obstacles to obtaining this kind of information have taken a long time to overcome—about 30 years so far, and we have still a little way further to go—in large part because the necessary technology, both physical and biological, has taken many years to reach the required level.

The first step was to show that the cross-bridges moved at the onset of activity. This was accomplished by X-ray diffraction using laboratory X-ray sources (rotating anode tubes) in the late 1960s and early 1970s (Haselgrove & Huxley 1973; Huxley & Brown 1967; Huxley & Haselgrove 1976), when it was shown that the myosin heads moved towards actin as soon as a muscle was activated, and that they also lost the regular helical arrangement present in resting muscle during contraction and became much more disordered. However, it was surprising and disappointing to find that no striking 'labelling' of the actin pattern developed as the myosin layer-line pattern disappeared during contraction. The relatively small changes that were seen in the visible actin reflections (at 59 Å and 51 Å) could not be interpreted in the absence of detailed knowledge of the structures of the actin and myosin molecules.

But the real problem was to see what changes (if any) there were during the postulated working strokes of the cross-bridges. These are normally completely asynchronous during contraction, and this makes informative measurements much more difficult to perform. The Huxley & Simmons (1971) quick-release manoeuvre showed us how to partially synchronize the movements (if indeed they took place), but the difficulty was how to detect them externally in a working muscle and how to do so during the millisecond or two during which some synchrony can be maintained.

Simmons and I were able to see changes in the intensity of the 143 Å meridional X-ray reflection (which comes from the cross-bridge repeat) using synchrotron radiation as an intense X-ray source (Huxley *et al*. 1981, 1983), and these changes were found to be closely synchronized with 1 ms releases or stretches of an intact contracting muscle. This experiment showed that some type of major structural change, in an axial direction, was happening in the cross-bridges during their working strokes; this could be the postulated tilting, but that was not the only possible explanation. The change in intensity was a large one, despite the relatively small proportion (20% or less) of myosin heads that were believed (from X-ray and other evidence) to be generating force at any

noment. Presumably, the other heads were too disordered o contribute.

At that time, this was the only useful reflection intense nough to study adequately with the necessary high time esolution, and the equatorial reflections showed virtually o change, during a quick release.

The other general approach was to attach spectroopic labels of various kinds to the myosin and look or preferred orientations and changes between different tates and in muscle transients (Thomas & Cooke 1980; anagida 1981). Unfortunately, for many years the esults from these techniques were somewhat misleading, ecause whatever label was used, they seemed to show nly a single orientation in the attached myosin heads, nd no change of orientation in a quick release. This as bad news for the tilting cross-bridge model and owed a certain amount of doubt about the whole iding filament theory, despite all the earlier evidence. here was even a scurrilous article in *Nature*, jeering at he supposed imminent demise of yet another outdated heory!

4. THE MODERN ERA

The situation was partially rescued by the arrival of he *in vitro* motility systems, in which actin filaments ould be clearly seen, in the light microscope, sliding over olated myosin heads on a surface (Kron & Spudich 986; Toyashima *et al*. 1987). The problem was further larified when the high-resolution myosin head structure as published in 1993 (Rayment *et al*. 1993*a*), and it was pparent that all of the previous spectroscopic labels had een placed on the so-called 'catalytic domain', which ould remain bound to actin in a fixed orientation, while he long protruding 'regulatory domain', which was learly likely to act in some way as a tilting lever arm, ad not been labelled. The atomic structure of the actin lament had now also been solved (Holmes *et al*. 1990), nd so it was possible to have a very good idea of how the yosin head would bind to it (Rayment *et al*. 1993*b*).

Since then, very elegant experiments by Irving, oldman and Trentham, and their collaborators Hopkins *et al*. 1998; Irving *et al*. 1995), have attached uorescent labels to specific sites on the lever arms by ery specific techniques, and have measured changes in eir orientation, which vary qualitatively in exactly the xpected way in the course of rapid releases and stretches f the labelled, single muscle fibres during ATP-induced ontraction. However, the average change in angle is uite small, just 3–5°. The problem is that the signals om all the labelled cross-bridges in a muscle are being veraged together, and if, as seems likely from internal vidence in these experiments and from other work, only small proportion of them are actively developing ension at any given moment, then one is having to look t a small signal on a high background and the actual hange in angle could be much greater.

Also in recent years, Irving and Lombardi, and their olleagues (Dobbie *et al*. 1998; Irving *et al*. 1992; ombardi *et al*. 1995), have been able to make superb high me-resolution measurements on the 143 Å meridional -ray reflection from single muscle fibres during mechanal transients, and have shown in great detail that a tilting lever-arm model with a change in angle of 30° or more can account for all the effects seen in this reflection in a variety of mechanical transient manoeuvres. The advantage of this X-ray method, as mentioned above, is that it appears (and this is supported by other recent evidence) that in a contracting muscle, most of the 143 Å signal comes from the tension-generating cross-bridges, and that the ones that are 'between engagements' are axially disordered and contribute very little to the measured signal.

However, the corresponding disadvantage is that this is a myosin periodicity, presumably visible because myosin heads need only move a relatively small distance axially (probably $< \pm 27$ Å) from their average 143 Å repeating position to find an actin monomer to which to attach. In reality, however, it is the myosin heads that are specifically attached to the actin periodicity that we are interested in, so it is the actin reflections, especially the off-meridional layer-lines, which should in principle be the more direct source of information, and which should show which part of the myosin head is moving. However, the corresponding disadvantage is that the actin signal will be diluted by the unoccupied actin sites.

Until recently, these actin reflections were too weak to measure at millisecond time resolution. However, due to continued improvements in technology, and the use of an undulator beam line on the APS Storage Ring at Argonne, we have now been able to get good two-dimensional data on these reflection with millisecond time resolution (Huxley *et al*. 1999). Use of charged-coupled device cameras, and also imaging plates, makes it possible to employ the full available flux, and particularly strong recordings can be made using a synchronized succession of 2 ms time windows and quick release and restretch cycles during each tetanic contraction. The patterns do indeed show characteristic changes in several of the actin reflections during a quick release, which is a significant advance. However, getting a good match between the observed layer-line profiles, and those calculated from the full atomic structures of actin and myosin inserted into the 'decorated actin filament' structures believed to be present in contraction, is not straightforward, and will keep us occupied for some time.

5. THE CURRENT SITUATION

I think it is fair to say that there is now strong evidence, from the X-ray diffraction and polarized fluorescence measurements on functioning muscles, that a change in tilt of the so-called 'lever arm' does take place during the working stroke of the myosin cross-bridges. At the same time, there is definitive evidence from X-ray crystallography for two very different orientations of the crystallography of the lever arm relative to the catalytic subunit depending on which nucleotide or analogue is occupying the binding site (see review by Holmes 1998). When these orientations are assigned to their expected position in the ATPase cycle, and are incorporated into the 'decorated' actin structure, then they predict that the actin-attached myosin heads go through the appropriately directed lever-arm tilting movement that would be required between the successive steps of the tilting cross-bridge model. Thus either that model is correct, or it is

eing mimicked by some other mechanism in a rather emarkable fashion.

Nevertheless, there is one further point that one should onsider. One needs to ask—is this the whole story, or is here perhaps some other process going on as well? Could there be an element of a thermal ratchet echanism, in addition to the lever-arm system, to take irect advantage of the initial binding energy of myosin o actin? As I, and others, have pointed out elsewhere Huxley 1998), the measurements from many (not all) of he *in vitro* motile systems seem to be converging on a gure of about 5 nm for the working stroke, and about pN (or less) for the average force. This would give 5 pN nm for the available mechanical energy, or slightly ver 6 kT. However, the amount of mechanical energy eleased in a contracting muscle by one molecule of ATP much greater than this. Even using a conservative gure of 48 kJ mol^{-1} for the free energy of ATP hydrolysis Woledge *et al.* 1985)—equivalent to about 19 kT per olecule—the observed value of 60% or more of the nergy released in contraction that can appear as echanical work leads to a figure of about 11.5 kT of work er myosin head per ATP, or almost twice the amount bserved in the *in vitro* experiments. Thus, either the force f the distance or both are being seriously underestimated n these experiments, or else a cross-bridge in a working uscle somehow manages to convert another 5 or 6 kT of ee energy into mechanical work for each molecule of TP hydrolysed.

This is an amount of energy which could feasibly be pplied within the short time required for an additional troke' of 4–5 nm by Brownian motion of the myosin eads against an elastic restoring force, as in an A. F. Huxley (1957)-type mechanism. This energy would be alanced by some of the binding energy of myosin to ctin. This additional displacement might not show up in any of the *in vitro* experiments, since the myosin lament structure may be required for the head to have he appropriate position and mobility. This may be why Or Yanagida observes larger steps than many other '*in itro*' experimenters when he uses synthetic myosin lament backbones to support individual myosin olecules, rather than having them deposited on a rface. And perhaps this type of behaviour is occurring n his recent experiments (Kitamura *et al.* 1999) in which single myosin head is held in contact with an actin lament bundle, in a mechanical system which has high igidity in a direction perpendicular to the actin filament nd very low rigidity along the filament axis, so that the ead can make particularly long Brownian excursions in earching for a preferred actin site, while being unable to iffuse away laterally immediately on each dissociation.

6. THE FUTURE

As far as future experiments are concerned, I think the ext urgent needs are (i) to find a way of crystallizing yosin heads in combination with actin, for crystallo-raphic analysis; (ii) to develop the technology and nalysis of *in vitro* motile systems even further, so that efinitive values of force and step-length can be obtained, t high time resolution and in a situation closely pproximating that in muscle; and (iii) to obtain as detailed as possible three-dimensional solutions, at high time resolution, of X-ray diffraction data that can now be obtained from intact muscle during mechanical transients.

A request to the theorists would be to look very closely at the kinds of experimental data that are now available, or could be obtained, and give advice on sophisticated ways of analysing them to distinguish between the different possible types of models that might represent the underlying mechanisms.

Finally, even when all the changes in the atomic structure of the myosin head have been characterized (and in actin too if necessary), and we can see how they are linked structurally to the cross-bridge 'stroke', that is not the end of the problem. One still has to understand quantitatively the detailed internal structural mechanics and energetics of the myosin head and actin interacting with ATP, and I think we are some distance from being able to make those kinds of calculation at the moment—and to check them by genetic engineering. So there is still much to do.

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