EARLY DEVELOPMENTS IN MUSCLE RESEARCH AND THE ROLE OF NEW STRUCTURAL TECHNOLOGIES

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1. INTRODUCTION

A few years after the end of World War II, in a small Medical Research Unit located, somewhat anomalously, in the Cavendish Laboratory (a physics laboratory) in Cambridge, England, a number of scientific discoveries were made which had far-reaching effects on the subsequent course of biological research. The best known of these was of course the proposal of the double helical base-paired structure of DNA, by Watson and Crick, in 1953. But in that same year Max Perutz, the head of the Unit, discovered how to determine the phases of X-ray reflections from protein crystals and thereby how to solve the atomic structure of protein molecules, which he and John Kendrew proceeded to do for haemoglobin and myoglobin during the next few years. This paved the way for the tens of thousands of different detailed protein structures which have now been determined, and, together with the basic knowledge of life processes that flowed from understanding of how DNA functions, has revolutionized biology and medicine in the last half century.

A little earlier than these momentous discoveries, during the years from 1949 through 1952, some other new discoveries were made in the same small unit, ones of a more specialized nature, but ones which did begin to set a new direction for work on the nature of muscle contraction. These discoveries flowed from the application of the same concepts as the two very dramatic ones mentioned above.

These concepts were extremely simple, almost simple-minded ones. The first was that it was very important to know what was the detailed structure of biological molecules and tissues, in the belief that information at the molecular and atomic level would be the essential key to understanding how all these biological processes worked, and that anything else was mere speculation. The second followed directly from this, and it was that methods had to be developed and applied in biology to carry out such structural work, and that a laboratory where this was done had to equip itself to carry out that development on the premises.

The professor of physics who headed the Cavendish Laboratory at that time was Sir Lawrence Bragg, who had invented X-ray diffraction analysis (and received the Nobel Prize for it at the age of 24), and it was he who had supported Perutz for many

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years, and who was instrumental in setting up the MRC Unit, run by Perutz and Kendrew, where I was a research student, and where Crick and Watson solved the structure of DNA. The Cavendish Laboratory had a very strong experimental tradition, exemplified in the Pt II Physics Practical Glass, where we used gold-leaf electroscopes to measure radioactive decay and α-particle ranges, learned to blow our own glassware and make cathode ray tubes, and repeat Millikan's oil drop determination of electronic charge. There was a large well-equipped mechanical workshop in the Cavendish, and a small student's workshop which our group used, with plenty of odd pieces of metal sheet and tubing, and a mechanic to help us use drills and lathes. And Max always stayed close to the bench!

So when the MRC Laboratory of Molecular Biology was set upon its own a few years later, an essential feature was the provision made for technological development, with a magnificently equipped and staffed mechanical workshop, a large electronic workshop, and smaller workshops elsewhere in the lab. This recognized that the structural work would require the highest level of technical support. The success of the laboratory testified to the strength of that approach.

2. EARLY MUSCLE WORK

For my own part, fascinated by experimental atomic physics, very conversant with the importance of knowledge of atomic structure in understanding so many of the properties of matter, it was a natural and easy transition to accept that detailed structural information was essential to even begin to understand biological processes. Vague theories were no good. And it was clear that new techniques were now waiting to be exploited.

X-ray diffraction was hardly new, but its use in biology was at that time quite limited. However, some years earlier Bernal had discovered that detailed diffraction patterns could be obtained from protein crystals if -- and only if – they were kept in a fully 'native' environment, hydrated, and in their mother-liquor. This was the foundation of protein crystallography. Another pioneer, Astbury, had looked at the wide-angle X-ray diagram of muscle, and found that there was no discernible change in it during contraction, indicating that the basic polypeptide chain configurations remained the same. So when I first started thinking about how muscles might contract, I got the idea that there must be larger structural units, protein molecules or assemblies of protein molecules, still way beyond the resolution of the light microscope, which interacted with each other and re-arranged themselves in some way so as to cause the muscle to shorten. To see this type of structure, which I thought might show structural repeats upward from fifty to a few hundred angstroms in size, I would need a low-angle X-ray camera, since the reflections would lie within 1° or less of the direct beam, and that might be why they had not been noticed previously.

Also, such cameras had to employ very narrow slits to collimate the X-ray beam, and usually needed to be quite long, to allow the pattern to spread out sufficiently. This meant that the total X-ray flux tended to be small, and the flux per unit area at the detector – film – was even smaller. Furthermore, I thought it was imperative to examine muscle in its native hydrated state, rather than dried down into a more concentrated state, as had been done in some previous work. So all this meant that I would only get extremely weak patterns and have impossibly long exposure times – even some protein crystals were then needing up to a month -- unless I could drastically increase the camera speed.

At that time, in the year 1949, there was a lot of interest at the laboratory in increasing the intensity available from X-ray tubes. One way to do this was to use a rapidly rotating anode, since the basic limitation was the rate at which heat could be dissipated from the incident electron beam, to avoid melting the copper target. This

could be partly overcome by continuously presenting to the beam a fresh copper surface which had had time to cool during the cycle of rotation since its last exposure. Such machines were not easy to construct, since they operated in vacuums, with rotating seals, usually at 40,000 volts potential difference between anode and cathode, needed relatively large currents, and had to have water cooling. The ones which had been built previously were too unreliable for routine use. So a good electronic engineer was hired to construct a usable device, but it became clear that it would not be ready soon enough to help my thesis work.

Another way of increasing the available x-ray flux per unit area emitted by the target was to use a much smaller focal spot for the electron beam. Cooling of the irradiated area takes place laterally around the periphery of the spot, as well as vertically, into the depth of the target. So cooling is much more efficient for smaller or narrower spots, and the permissible flux per unit area in fact increases approximatly linearly as the inverse of the diameter of the focus. Ehrenberg and Spear, in Bernal's lab, in London, had built such a microfocus tube, in part for other reasons, and I was fortunate to obtain a prototype through my supervisor, John Kendrew, who had been a wartime colleague of Bernal (and who had been drawn into protein crystallography by him). This tube operated with a 50μ spot, which would be effectively forshortened to 5μ using a shallow viewing angle (5°). So I had an extremely bright, very narrow source, ideal for low angle diffraction, and was able to construct a camera with correspondingly narrow slits and only a few centimeters specimen to film distance which still gave me order-to-order resolution of several thousand angstroms, and a first order resolution well over 500 Å with relatively high (then!) recording speed. The patterns, recorded on film, needed to be viewed through a low-power microscope.

Looking for equatorial reflections in living muscle from filaments whose presence was indicated by very early electron micrographs, I soon found a clear set of reflections coming from a hexagonal array of filaments, spaced out about 400Å apart (Huxley, 1951). Moreover, in muscles in rigor, a second set of filaments seemed to be present, arranged in a regular pattern symmetrically between each set of three of the original filaments. Since it was known then that the muscle proteins actin and myosin seemed to form some kind of complex in the absence of ATP (the condition in a rigor muscle), and to dissociate in its presence, I concluded – an inspired guess, I suppose – that myosin and actin must be present in separate, stable filaments in muscle, the myosin filaments forming a permanent regular hexagonal array, and the actin filaments becoming regularly positioned in the array when they became attached by cross-linkages to the myosin filaments, who centers would be about 250Å away from those of the actin filaments. I presumed that it was interaction through those cross-links that produced muscle contraction, but because I assumed that this double array extended continuously through each sarcomere of the muscle, I did not envisage a sliding filament mechanism at this time. Instead, I wondered if actin depolymerization might be involved, because, even then, such depolymerization was recognized as a possibly biologically important process.

Additionally, I found that there was a clear set of axial reflections (I only had a slit camera), with a period of about 415Å – only approximate because the patterns I could record were so small – and a very strong third order at around 140Å. Remarkably, when a living muscle was passively stretched, this periodicity remained constant! (Huxley, 1953a) So I speculated in my thesis that the periodicity must come from one of the sets of filaments which was not attached to the Z-lines, probably the actin filaments, using the myosin filaments to transmit the force generated by their depolymerization during contraction!

3. WORK AT M.I.T.

The next step was first to find out whether my double array model was correct and then look for further evidence of how such a system might work. The best way to do this seemed to be electron microscopy, just beginning to be used in biology in a few labs in the world, one of which was F.O. Schmitt's lab at the Massachusetts Institute of Technology, where Dick Bear and Cecil Hall also worked. I was lucky enough to get a nice two-year fellowship (Commonwealth Fund) to do this, and arrived there in September 1952. Alan Hodge was also there as a postdoctoral fellow, from Australia, and he taught me how to operate the electron microscope. Together with Dave Spiro, we designed a simple microtome for ultra-thin sectioning (Hodge et al, 1953), just coming into use then, which Alan and Dave used for their own projects and I used to look at cross-sections of muscle, for my double array. I was very thrilled to soon find I could see it. The thicker filaments formed the basic hexagonal array, as I had supposed, and presumably contained myosin, since that was the major protein species, and the thinner filaments were actin, located at the trigonal positions of the lattice, as expected from the X-ray patterns. This convinced me that the combination of the two techniques was a very powerful tool indeed, and this became one of the main themes of the Structural Studies Division which Aaron Klug and I later directed at the much enlarged MRC Laboratory in Cambridge.

By Christmas 1952 I was ready to move on with this work, and by great good fortune, Jean Hanson, from the MRC Laboratory at King's College London, then arrived at M.I.T., also to learn electron microscopy. One of the specialties of the King's Lab had been different types of light microscope, including the phase-contrast light microscope. Jean was a zoologist, and had already studied a range of muscle types, but she had obtained particularly striking phase-contrast light micrographs of separated myofibrils from vertebrate striated muscle (rabbit psoas), which showed the sarcomere band pattern extremely clearly. This was a revelation to me, since I had never seen the muscle band pattern in the light microscope before, though of course I had seen it in electron micrographs. Jean was equally excited to see my EM and X-ray results, and we immediately decided to join forces, and to work together using both phase and electron microscopy.

4. MYOSIN FILAMENTS IN THE A-BAND

At that time it was generally assumed that the characteristic high density of the A-bands in striated muscle was due to the presence of some additional component other than actin and myosin. Smooth muscles contracted perfectly well, if more slowly, without such striations, and since sarcomeres could shorten down to much below the A-band length, it was assumed that filaments of the actin-myosin complex must extend continuously from one Z-line to the next. So perhaps this extra A-substance just enabled muscles to shorten faster?

We were absolutely astounded, therefore, when, in the phase contrast light microscope, we saw that myosin-extracting solutions removed the A-substance, but left behind a 'ghost' fibril, with a band of density on either side of each Z-line, extending in towards where the boundaries of the H-zone had originally been. (H-zone is a less-dense region in the center of the A-band). Within a day we realized what the explanation must be, and were soon able to confirm this by electron microscopy. Myosin-extracting solutions removed the thick filaments, leaving behind the sets of thin filaments (attached to the Z-lines), which had previously partially overlapped the thick filaments. When I had seen the double array in an end-on view, I had been looking at sections through the overlap region of the sarcomere, and what I thought was poor preservation or sectioning were just the I-regions!

This was a remarkable finding, which we published in Nature that year (Hanson & Huxley, 1953), though it took a long time to come out. We were told by Schmitt that we should not contaminate a perfectly good experimental paper with any speculation about mechanisms until we had further evidence. However, I did manage to slip some phrases to the effect that my constant X-ray axial periodicity, plus the overlapping arrays, suggested a sliding mechanism, into my write-up of the electron microscope cross-sectioning results (Huxley 1953b).

By January of 1954 we had good phase microscope data showing that the A-bands remained essentially constant in length during contraction of isolated myofibrils in ATP, and that the arrays of thin filaments, also of constant length, were drawn further and further into the A-bands as contraction proceeded. And so the sliding filament model was published in Nature in May of 1954, in two papers side by side, one from us, and one from A.F. Huxley and Niedergerke, who had been making similar observations on intact muscle fibers in the interference light microscope (Huxley & Hanson, 1954; A.F. Huxley and Niedergerke, 1954).

Subsequent development of the work took place in England again, and continued to be very dependent on new technical developments.

5. DEVELOPMENTS IN ELECTRON MICROSCOPY

Some of these were fairly straightforward. Thin sectioning for electron microscopy was still a very new technique, and I only slowly realized that most sections were over a thousand angstroms in thickness, and relatively lightly stained, so that, in longitudinal sections of muscle, several layers of filaments were superposed, obscuring the filament arrangement. By various incremental improvements of the microtome and of specimen preparations, I managed to reduce the minimum section thickness to 100-150Å, and to have sufficiently intense metal staining for single filaments to show up with good contrast. Remarkably, the double hexagonal lattice was often preserved with great regularity, so that single filament layers could be seen with thick and thin filaments lying side by side in the expected arrangement, within the thickness of the section. This began to persuade people that the overlapping filament structure really did exist. But the sliding filament mechanism still took many years of work to gain more converts.

I was disappointed that the staining methods that I had developed for thin sections showed little internal detail of the structure of the actin and myosin filaments, although it was possible to see crossbridges very clearly. However, I had accidentally discovered the so-called 'negative staining' technique in some work I was doing on tobacco mosaic virus, as a sideline (Huxley, 1957b), and I improved it further by using uranyl acetate as a negative stain, in work on another virus, and on ribosomes (Huxley & Zubay, 1960a & 1960b). The technique could only be used on small isolated objects, which were submerged or outlined in the stain, but it occurred to me that muscle might fragment easily if mechanically blended in a relaxing medium, and that turned out to be the case. The product was a nice suspension of separated and often unbroken myosin and actin filaments, which showed up excellently in negative stain (Huxley, 1963). This enabled me to recognize that both types of filament were constructed with a defined structural polarity, appropriate for the directions in which relative sliding forces needed to be developed in an overlapping filament system. This was further strong evidence in support of such a mechanism, and it also prompted me to suggest that similar directed movements might be involved in other forms of cell motility. It also enabled Jean Hanson and Jack Lowy (1963) to elucidate the helical structure of F-actin.

6. X-RAY DEVELOPMENTS IN THE 1960'S

The major developments came next from the X-ray field. I was now back in the new MRC laboratory in Cambridge and had access again to rotating anode X-ray tubes, several of which were operating routinely there. Ken Holmes and Bill Longley had also moved to the laboratory from Birkbeck College, in London, and had made a big improvement to such a tube by grafting on the cathode from a Boudoin X-ray tube, which provided a much smaller focus and hence light brilliance, very appropriate for the focusing quartz monochromator they were using in studies on Tobacco Mosaic Virus. Ken and I thought it would be interesting to combine this with a focusing mirror I had previously been using in conjunction with a commercial version of the Ehrenberg-Spear tube. In the course of playing around with the rotating anode-mirror-monochromator set up, I discovered that it was possible to use the entire input aperture of the monochromator, rather than a narrow collimated region (as was conventional), without excessive background scattering, with an enormous increase in total X-ray intensity and all the advantages of a monochromatic beam.

This made it possible for the first time to record the low angle meridional and layer-line patterns from live, contracting frog muscles, to show that the axial spacings from both myosin and actin remained essentially constant between rest and contraction (Huxley, Brown & Holmes, 1965), and to show, amongst other things, that the myosin layer line reflections from the helical arrangement of crossbridges around the myosin filaments, became very much fainter during contraction, showing that the crossbridiges must move when they developed the sliding force between the filaments (Huxley and Brown, 1967).

Holmes and I were able to continue to improve the technology because of the excellent workshop facilities and technicians in the lab. In a successful effort to achieve higher X-ray output, we constructed a large diameter (about 20 ins) rotating anode X-ray set, which eventually developed into the commercial Elliott 'Big Wheel'. But we realized that this was essentially the end of the line as far as increases X-ray intensity from these types of sources was concerned, since we had reached the limit of what the mechanical strength and the melting point of copper would allow, and other metals would give X-rays with a much less suitable wavelength, and present much bigger fabrication problems. Nevertheless, we still needed greatly increases X-ray fluxes, since there was potentially so much detailed information available in the diagrams from contracting muscle, and we needed to be able to record them with high time resolution to follow the changing patterns satisfactorily.

7. SYNCHROTRON RADIATION

Ken Holmes had always been thinking about more exotic X-ray sources, particularly for use in his work on insect flight muscle, and had become interested in the radiation emitted by electron synchrotrons, particle accelerators used by physicists to produce very high velocity electrons for collision experiments. Initially, it appeared that the available machines would not produce enough X-rays to be useful sources, but later, after he had moved to Heidelberg, and had learned from Gerd Rosenbaum about the characteristics of the DESY synchrotron in Hamburg, things appeared more hopeful, and he, Rosenbaum, and Witz carried out the crucial test experiment there in 1971 (Nature, Rosenbaum, Holmes, & Witz, 1971). This showed that a substantial gain was available over the best that could ever be obtained from a rotating anode X-ray tube, and that potentially enormous factors of improvement might be possible in the future from the electron storage rings being planned, in which much larger, and continuous, circulating currents of electrons and positrons would be present.

In any event, it took nearly ten years more before all the component of a satisfactory system were available. It took some years before a storage ring came on line, and was operating smoothly enough (not very smoothly!) for the muscle enthusiasts to begin to collect some really useful data. In the meantime, we gained a lot of essential experience with electronic data collection and analysis, and with the operation of remotely controlled cameras, at the synchrotron NINA, in Daresbury, U.K. (because of the high flux of radiation in the immediate environment of such a beamline, remote operation was mandatory). Once again, in the MRC lab, we were very fortunate that nearly all the equipment could be designed by excellent electronics and mechanical engineers and put together in our workshops (Haselgrove et al, 1977; Faruqi & Bond, 1980).

And then, in the early 1980s, working at the EMBL Outstation, in Hamburg (specially built to exploit synchrotron radiation), we were able to obtain good time-resolved patterns of the myosin and actin layer-lines with 5-10 msec time resolution, so that the time course of the changes in them during the onset of contraction could be compared with the time course of tension development. They were very closely related as we expected. More importantly, in measurements of the meridional 145Å reflection from the myosin crossbridge repeat, now with 1 msec time resolution, we found that a large drop in intensity was closely synchronized with a small quick length decrease, or increase, applied to a previously isometrically contracting muscle. This strongly indicated a tilting movement of actin-attached crossbridges, if they were approximately perpendicularly oriented in the isometric muscle, and provided the first direct demonstration of a direct relationship between crossbridge configuration and tension generation (Huxley et al 1981, 1983).

8. RECENT ADVANCES

Later development of new structural techniques in other laboratories have led to further remarkable extensions of our ability to obtain direct information about molecular motility processes. Perhaps the most striking of these has been the introduction of in vitro motility measurements in which force and movement can be measured by direct optical means on single myosin molecules interacting with single actin filaments, as exemplified, for instance, by Finer et al (1994), following the pioneering work of Kron and Spudich (1986) and Kishino and Yanagida (1988).

Another great step forward depended on extensive computerization of protein crystallographic analysis, to make the solution of very large structures possible, plus the use of synchrotron radiation and cryo techniques to collect data from very small and sensitive protein crystals. This made possible the solution of the high resolution structure of myosin subfragment one, the motor part of myosin, by Rayment et al. (1983a & 1983b), a very great help to our understanding of the details of the tilting movement of myosin crossbridges in the sliding filament mechanism.

More recently still, the latest generation of electron-positron storage rings provide extremely small X-ray sources with very high total flux. These make it possible to record X-ray patters from muscle at extremely high spatial resolution and at a time resolution of 1 msec or better, making use of imaging plates and improved CCD X-ray detectors (Linari et al, 2000; Huxley et al, 2000). Such patterns enable axial crossbridge movements to be measured with an accuracy of a few angstrom units.

9. CONCLUSION

Over the last fifty years or so, then, it has been very remarkable, and encouraging, to see how the well-directed scientific efforts of many people and many

laboratories have been so successful in providing the tools for what were originally almost unimaginable opportunities to explore molecular reality.

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DISCUSSION

Gonzalez-Serratos: In your early experiments with Jean Hansen, you showed that, after extraction of myosin when only actin was left, the sarcomere decreased in length. How could they shorten without myosin?

Huxley: No. In the experiments I showed, myosin had been extracted after the contraction had taken place, so as to show more clearly the location of the I-segments in the shortened sarcomeres.

Pollack: In one of your EM slides showing two thin filaments between two thick filaments, we can see two kinds of bridges: between thick and thin and between thin and thin. We found the thin-thin connections even in the I-band (cf. Pollack, Muscles and Molecules, Ebner & Sons, Seattle, 1990). How do you interpret the thin-thin bridges that you see in the EM, and why do they also appear in the I-band?

Huxley: In the I-band, the thin-thin bridges may be some of the glycolytic enzymes which bind quite strongly to actin. In the A-band, they may also represent myosin crossbridges from myosin filaments above and below the actual plane of sectioning.