Perspectives in Physiology
Published on behalf of The American Physiological Society by Springer

Jack A. Rall

Mechanism of Muscular Contraction

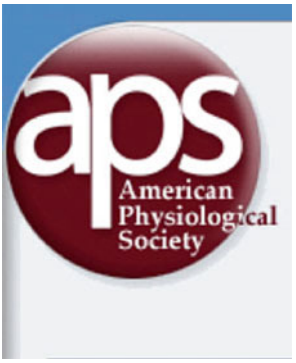
aps American Physiological Society

Springer

Perspectives in Physiology

Published on behalf of The American Physiological Society by Springer

More information about this series at http://www.springer.com/series/11779



Integrating the Life Sciences from Molecule to Organism

The American Physiological Society (APS) is a nonprofit devoted to fostering education, scientific research, and dissemination of information in the physiological sciences. The Society was founded in 1887 with 28 members. APS now has over 10,500 members. Most members have doctoral degrees in physiology and/or medicine (or other health professions).

APS is a member of the Federation of American Societies for Experimental Biology (FASEB), a coalition of 26 independent societies that plays an active role in lobbying for the interests of biomedical scientists.

APS publishes three book series, now in partnership with Springer: Physiology in Health and Disease (formerly Clinical Physiology), Methods in Physiology, and Perspectives in Physiology (formerly People and Ideas), as well as general titles.

Perspectives in Physiology
This popular series seeks to place biomedical science inside a historical framework by describing the development of a field and highlighting the contributions of prominent investigators. Published books include ***Moving Questions: A History of Membrane Transport and Bioenergetics*** and ***Exercise Physiology: People and Ideas.***

Methods in Physiology
This series describes experimental techniques in cellular, molecular or general physiology. Each book is edited by experts in the field and covers theory and history behind the method, critical commentary, major applications with examples, limitations and extensions of each technique, and vital future directions. Previous books in this series include ***Molecular Imaging FRET Microscopy and Spectroscopy*** and ***Methods in Cellular Imaging***

Physiology in Health and Disease
This series highlights the links between basic science research and clinical medicine. Each book brings together fresh ideas in physiological function and how these new models contribute to our understanding and treatment of disease states. Each book is meticulously edited by leaders in the field it covers. Books in this series include ***Physiology of Infl ammation*** and ***High Altitude and Man***.

Jack A. Rall

Mechanism of Muscular Contraction

Jack A. Rall
Department of Physiology and Cell Biology
Ohio State University
Columbus, OH, USA

ISBN 978-1-4939-2006-8 ISBN 978-1-4939-2007-5 (eBook)
DOI 10.1007/978-1-4939-2007-5
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2014948777

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

As the title denotes, the purpose of this monograph is to describe the evolution of ideas relating to the mechanism of muscular contraction since the discovery of sliding filaments in 1954. The topic has been approached in its historical development with an emphasis on ideas, techniques, experimental results, and the investigators who generated them. In order to provide perspective into the thinking about an issue at the time of its discovery, often the investigators describe in their own words an important result or conclusion as it appeared in an original paper. Also numerous figures from the original papers are included in order to allow the reader to see the data that led to important conclusions. A unique feature of the book is the inclusion of information about the scientific background of many of the investigators with the intent of providing deeper insight into their point of view on a subject. An amazing variety of experimental techniques have been employed to investigate the mechanism of muscular contraction and relaxation. Some background of these various techniques is presented in order to gain a fuller appreciation of their strengths and weaknesses. Controversies in the muscle field are discussed along with some missed opportunities and false trails.

It was difficult to decide where a history of muscular contraction should end. How can one be sure that what has recently been discovered will stand the test of time? Nonetheless to give some insight into current thinking on a particular topic, usually, a recent review is suggested. In some ways writing this book has been a daunting task. No doubt there are gaps and some topics may be overemphasized and others underemphasized. Any gaps and errors are totally my responsibility.

I am grateful to John B. West who, as chair of the history book subcommittee of the American Physiological Society (APS), encouraged me to submit a proposal for this book and to the APS for sponsorship of the project and the location of the publisher Springer Science + Business Media. My deepest appreciation goes to Nancy Curtin and Roger Woledge who have encouraged the development of the project from the beginning and have provided many comments on chapters in the book. I also would like to thank Sally Page for the photograph of Rolf

Niedergerke (Chap. 2) and for her comments on some of the book chapters. Thanks to all the investigators who have provided photographs or allowed inclusion of figures from their original papers in the book. Finally I am grateful to the department of physiology and cell biology at the Ohio State University for continuing to provide office space during my retirement.

Columbus, OH Jack A. Rall

Contents

Chapter 1
Setting the Stage: Myosin, Actin, Actomyosin and ATP

…the theory of contraction by folding of continuous filaments, which paid no attention even to the existence of the striations and which was completely wrong, came to dominate the field for half a century.

A.F. Huxley (1977)

The simple statement that contraction in muscle is essentially a reaction of actomyosin, ATP, and ions was my laboratory's main contribution to the problem of muscular contraction. (Szent-Gyorgyi 1953. With permission Elsevier)

Albert Szent-Györgyi (1953)

1.1 Introduction

It is important to understand the prevailing views of muscle contraction just before the proposals of the sliding filament model of contraction appeared in 1954. The spectacular rise of muscle biochemistry in the first half of the twentieth century has been chronicled by Dorothy M. Needham[1] in her classic book (1971): Machina Carnis: The Biochemistry of Muscular Contraction In Its Historical Development. Also Marcel Florkin has written a massive five volume history of biochemistry. The volume that is of interest here is entitled: History of the Identification of the Sources of Free Energy in Organisms (1975). We will concentrate on those aspects of research on muscle that relate closely to the contractile process itself.

[1] Dorothy Moyle Needham (1896–1987) investigated muscle biochemistry at the University of Cambridge for over 40 years. She was among the first ten females elected as a Fellow of the Royal Society (Teich 2003). She has gained lasting international acclaim for her book Machina Carnis, long out of print, that is now back in print in paperback form.

J.A. Rall, *Mechanism of Muscular Contraction*, Perspectives in Physiology,
DOI 10.1007/978-1-4939-2007-5_1

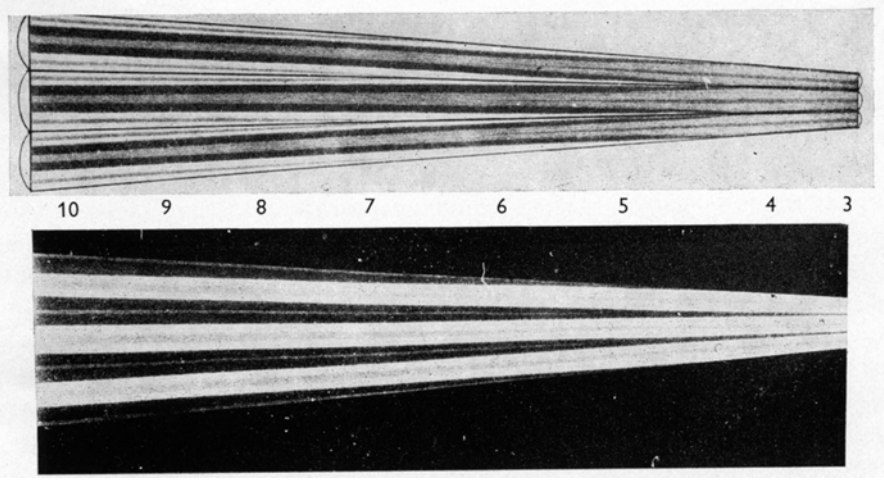


Fig. 1.1 Diagrams of the appearance of the striations in insect muscle at varying degrees of shortening from Fredericq (1876). The *upper part* shows the appearance in ordinary light and the *lower part* shows the appearance by polarized light (birefringent areas appear light). The horizontal axis represents striation spacing, whose value in micrometers is given by the numbers in the middle. The A bands are the broad dark bands in the *upper part* and the broad light bands in the *lower part*. Note that as the sarcomeres shorten the A bands maintain a constant width. See text for further details (Huxley 1977. With permission Cambridge University Press)

1.2 Muscle Structure as Observed by Nineteenth Century Microscopy

Andrew Fielding (A. F.) Huxley performed an extensive review of the nineteenth and early twentieth century literature on muscle structure as observed with the light microscope (Huxley 1957; 1977). The cross striated appearance or banding pattern of skeletal muscle fibers was well known in the nineteenth century. For example, consider Fig. 1.1 from Leon Fredericq published in 1876 and reproduced 100 years later by Huxley (1977). Fredericq measured the band widths in alcohol-fixed insect muscle as a function of striation spacing, both in ordinary light and polarized light. The upper part of Fig. 1.1, in ordinary light, shows the distribution of refractive index, i.e., protein concentration, where high refractive index appears dark. In the lower part of the figure in polarized light the birefringent[2] areas appear light. The broad dark bands in the upper diagram are the A

[2] *Birefringence* or *double refraction* is the optical property of a material in which the refractive index is different for light polarized in one plane compared to the orthogonal plane. This effect can occur only if the structure of the material is *anisotropic* (directionally dependent), as opposed to *isotropic*, which implies homogeneity in all directions. A birefringent material observed between crossed polarizers appears bright against a dark background when at an angle of 45° (or 90° multiples thereof) to the optical axis of the microscope. There are four types of birefringence. *Intrinsic birefringence* originates from the inherent asymmetry of chemical bonds. *Form birefringence* results from regular arrangement of objects which may or may not be intrinsically birefringent. *Flow birefringence* results from a preferential arrangement of structures induced by a moving stream of liquid which is a special case of form birefringence. *Strain birefringence* is produced by mechanical stress which may cause a preferential alignment of particles. The birefringence can be

bands. 'A' standing for 'anisotropic' since these high refractive index regions are also birefringent, i.e., optically anisotropic, as shown by the fact that they appear bright in the lower diagram. The paler part of the center of A is the H zone (Hensen's Streifen). The black lines midway between A bands are the Z lines (Zwischenscheiben). The pale area between adjacent A bands, bisected by Z, is the I band ('isotropic' since it is hardly at all birefringent). The gray bands within each half of the I band are the N lines (Nebenscheiben). These bands are very conspicuous in some insect muscles where they are due to regularly arranged mitochondria but faint lines of unknown composition are found in this position even when mitochondria are absent. The horizontal axis represents striation spacing, whose value in micrometers is given by the numbers in the middle of the Fig. 1.1.

As the muscle shortens, the A band remains at a constant width almost until it is met by the Z line. This crucial observation, which also was made by others and generally accepted in the nineteenth century but subsequently forgotten in the twentieth century, will be seen to be pivotal in understanding the mechanism of muscle contraction (see Chap. 2). There also is apparent an interesting reversal of striation (dark to light) during shortening in ordinary light as the dark A band becomes light just before the I band disappears. At this time a dense line, known these days as a contraction band, appears in the middle of the A band. This reversal of striation phenomenon was particularly interesting to A. F. Huxley and in part motivated his entrance into the muscle field (see Chap. 2).

Polarized light microscopy was regularly employed by the nineteenth century microscopists to gain submicroscopic structural information. An object will appear bright when placed between crossed polarizers if it exhibits a submicroscopic order as opposed to a random orientation of particles. This birefringent (see footnote 2) or anisotropic behavior of the A band suggested to some nineteenth century microscopists that submicroscopic rodlets, called Disdiaklasten, were contained in the A band (Brucke 1858). Also Engelmann (1875) concluded similarly that contractility was generally associated with the presence of birefringent particles.

One would assume that to develop a comprehensive theory of muscle contraction, a clear understanding of the ultrastructure of muscle would be required. Strangely this was not the case in the first half of the twentieth century. The prevailing theories of muscle contraction in the first half of the twentieth century paid almost no attention to the striations that were observed in muscle in the nineteenth century that gave striated muscle its name. In fact Huxley (1977) has noted that "… the theory of contraction by folding of continuous filaments, which paid no attention even to the existence of the striations and which was completely wrong, came to dominate the field for half a century." (Huxley 1977. With permission Cambridge University Press) How was it possible that a completely wrong theory could dominate the thinking of muscle scientists for so long? In part it was because new methods in the first decade of the twentieth century seemed to contradict the earlier structural results (Huxley 1977) and partly because of the spectacular rise of muscle biochemistry in the first half of the twentieth century.

---

either positive or negative depending upon the relative magnitudes of the two refractive indices. In the case of positive uniaxial form birefringence the preferential orientation of the submicroscopic particles is with their longest dimension in the direction of the optic axis. With negative uniaxial form birefringence the shortest dimension of the particles is oriented parallel with the optical axis. For further information, see Slayter (1976).

Thus the structural results of the nineteenth century microscopists were largely forgotten until rediscovered with the interference microscope and the phase contrast microscope in the 1950s. This is not the only example of important results forgotten in the history of the muscle field. (Some other examples include the existence of the transverse tubular structure of muscle and the role of calcium in muscle activation, see Chap. 4.)

1.3 Revolution in Muscle Physiology: The Pathway to ATP and the High Energy Phosphate Bond

1.3.1 Rise of Biochemistry

The emergence of biochemistry as a discipline pertinent to biology at the beginning of the twentieth century did not occur without great struggle. Looking back from the 1930s to those early years, biochemist Frederick Gowland Hopkins who received the Nobel Prize in 1929 for his discovery of the growth-stimulating vitamins summarized the feelings of many biologists toward the new biochemists in the following way: "…when the chemist touches living matter it immediately becomes dead matter…" (Florkin 1975. With permission, Elsevier) The discovery of cell-free fermentation in yeast juice was the first demonstration of biological processes outside of the living cell and thus the first major victory of the new biochemistry (see Needham 1971 or Florkin 1975). Nonetheless in as late as 1956 the distinguished cell biologist Lewis Victor (L. V.) Heilbrunn wrote a monograph entitled: "The Dynamics of Living Protoplasm Heilbrunn (1956)." In the preface of the book Heilbrunn emphasized the importance of studying protoplasm when it is alive and not after it is dead. Despite this criticism it is abundantly clear that biochemistry has led to great strides in our understanding of muscle contraction throughout the twentieth century and beyond. Nonetheless the conflict between "living" and "dead" will appear again as we follow the development of the history of thought on muscle contraction.

1.3.2 Lactic Acid Theory of Muscle Contraction

It was well known in the nineteenth century that contracting muscles produced lactic acid. Fletcher and Hopkins (1907) at the physiological laboratory at Cambridge University published a classic paper describing the first reliable measurements of lactic acid production with muscle contraction and recovery. The resting lactic acid content in amphibian muscle was small but it increased tenfold during muscle fatigue and disappeared when the fatigued muscle recovered in oxygen. Parnas and Wagner (1914) showed that the lactic acid was derived from muscle glycogen.

At about this time Archibald Vivian (A. V.) Hill (see Chap. 5) started his influential studies on the heat production of skeletal muscles during contraction and recovery.

The basis for these studies was the law of conservation of energy developed in the nineteenth century by J. R. Mayer, J. Joule and H. Helmholtz. The idea was that these thermodynamic studies would establish the framework that must be explained by any observed chemical reactions occurring in the muscle. Weizsacker in 1914 working in the physiological laboratory at Cambridge University with equipment provided by Hill found that the heat production during brief contractions of frog skeletal muscle was independent of oxygen and thus due entirely to non-oxidative processes. Hill and Hartree (1920) in a fundamental paper confirmed that the heat liberation produced during contraction of frog skeletal muscle, which they called the initial heat, was anaerobic in nature. They further showed that during recovery from contraction heat was also liberated, called recovery heat, which was similar in magnitude to the initial heat in the presence of oxygen but greatly depressed under anaerobic conditions. These experiments established the thermodynamic framework which must be explained by the chemical reactions that occurred during and after muscle contraction in the presence and absence of oxygen.

In Germany, Otto Fritz Meyerhof (1884–1951) took up this challenge and sought to determine the chemical reactions that were responsible for the observed muscle heat production. In effect he wanted to balance the thermochemical books (see Chap. 5). He showed that the conversion of glycogen to lactic acid resulted in heat production and he related this heat production to the heat produced during muscle contraction under anaerobic conditions. Meyerhof (1920) further showed that under anaerobic conditions lactic acid was produced in proportion to the duration of contractile activity.

The results of Fletcher and Hopkins in 1907 and Meyerhof in 1920 led to the lactic acid theory of muscle contraction. What was the lactic acid theory of muscle contraction? The idea was that the conversion of glycogen to lactic acid directly provided the fuel for muscle contraction. But Meyerhof went further in that he believed that lactic acid was also part of the machinery of muscle contraction as well as part of the fuel of contraction (Needham 1971). It seems reasonable to suppose that first and foremost the conversion of glycogen to lactic acid directly provided the energy for muscle contraction. This theory went unchallenged for 20 years. For their work in unraveling the relationships of muscle heat production to muscle chemistry, A.V. Hill and Otto Meyerhof shared the Nobel Prize in 1922.

But the lactic acid theory started to undergo difficulties when Embden et al. (1926) showed that under anaerobic conditions some of the lactic acid was produced after contraction and they thus rejected the lactic acid theory of contraction. Philip Eggleton and his wife Grace Palmer Eggleton (1927) working in Hill's laboratory in the department of physiology and biochemistry at University College London discovered a phosphate containing compound (which they called phosphagen) in muscle whose content decreased with muscle contraction and returned to resting levels during recovery. Independently and at the same time Cyrus Hartwell Fiske and graduate student Yellapragada SubbaRow (1927) from India in the department of biochemistry at Harvard University made similar observations and furthermore identified the phosphagen as phosphocreatine.

1.3.3 Studies on Muscle Contraction Without Formation of Lactic Acid

The real "revolution in muscle physiology" as Hill (1932) has coined it came with the beautiful experiments of the Danish physiologist Einar Lundsgaard (1899–1968) at the University of Copenhagen. In a series of five papers in 1930 and 1931, Lundsgaard showed conclusively that the conversion of glycogen to lactic could not possibly supply the direct fuel for muscle contraction nor could lactic acid be considered an integral part of the contraction machinery. von Muralt (1984) who was in the Meyerhof laboratory at that time recalled the reaction of the laboratory when the news reached them. "One has to realize today, what that meant at the time! The lactic acid cycle had been considered the principal energy source for all working muscles, mainly based on Meyerhof's research work. Suddenly this whole scientific edifice seemed to break down with Lundsgaard's discovery…All the previous work on the central role of glycolysis in working muscles seemed invalidated." (von Muralt 1984. With permission Annual Reviews).

What were the spectacular results produced by the 31 year old Lundsgaard? How did they overturn the lactic acid theory of contraction? The experiments of Lundsgaard are an example of both serendipity and exquisite pursuit of a strange observation to its natural conclusion. Needham (1971) has described the experiments in some detail. Lundsgaard was not working in the muscle field at the time of his original discovery. He was interested in the specific dynamic action of amino acids on metabolism. He decided to investigate the specific dynamic action of iodoacetate. At that time there was great interest in the metabolic effects of iodinated compounds because of the discovery of iodine in thyroxin. Lundsgaard was interested in glycine substituted with iodide, but could not get hold of the substance, so instead he used what he could get locally, iodoacetate. When iodoacetate was injected into rabbits or frogs the animals behaved normally for a while and then rather suddenly a universal muscle spasm developed ending with death of the animal and complete muscle stiffness (rigor). The animals were as stiff as a board. This totally unexpected, chance observation became the starting point for some of the most dramatic and important experiments in the history of the energetics of muscular contraction.

Lundsgaard's first classic paper (1930a) was entitled: "Untersuchungen uber muskelkontraktion ohne milchsaure" (Studies on muscle contraction without lactic acid). Lundsgaard noticed that the rigor seemed to be brought on by activity of the muscles. If the nerve to one hind limb of a frog was cut before injection of iodoacetate, the rigidity spread throughout the body with the exception of this limb. But after a short series of contractions caused by electrical stimulation of the severed nerve; this limb also became stiff. The key observation was that the poisoned muscles in rigor showed no lactic acid formation; nor was lactic acid formed when the denervated limb was stimulated until rigor was produced. Thus lactic acid formation could not be part of the fuel of muscle contraction. [We now know that iodoacetate inhibits the glycolytic enzyme glyceraldehyde-phosphate dehydrogenase.]

Clearly the lactic acid theory of muscle contraction was invalidated. What would replace it? Lundsgaard (1930a) concluded that phosphocreatine was closer to the direct energy source for contraction than lactic acid. He found that the phosphocreatine breakdown was far greater in the poisoned muscles. The content had fallen to zero in the stimulated, poisoned muscles whereas in the stimulated normal muscles there was only about a 25 % decrease in phosphocreatine content. Lundsgaard went on to suggest that in normal muscle contraction phosphocreatine breakdown directly yielded energy. Lactic acid formation provided energy for the continued resynthesis of phosphocreatine and lactic acid formation would get under way when a certain concentration of free phosphate had accumulated.

Lundsgaard informed Meyerhof of his findings prior to publication and in a striking gesture of research collaboration, Meyerhof invited Lundsgaard to visit his laboratory to continue his studies. Thus in 1930 Lundsgaard went to work in Meyerhof's laboratory in Heidelberg. There he was able to strengthen his conclusion by showing a direct proportionality between the amount of phosphocreatine hydrolysis and muscle tension development in repeated contractions (Lundsgaard 1930b). However he was careful to emphasize that phosphocreatine splitting and lactic acid formation may provide energy for a third unknown process. Lundsgaard (1930c) also observed that the working capacity of iodoacetate poisoned frog muscles was appreciably greater in aerobic than in anaerobic conditions. Moreover, the oxygenated muscle had a phosphagen content many times that of the anaerobic muscle. He drew the conclusion that resynthesis of phosphocreatine can be driven by oxidative processes in iodoacetate poisoned muscle. Thus the iodoacetate experiments furnished the first demonstration of the biological role of what would be become known as high-energy phosphates.

1.3.4 ATP and the High Energy Phosphate Bond

In 1932 A. V. Hill declared the revolution in muscle physiology to be complete. It turned out that this declaration was a premature. The lactic acid era of direct energy provision for muscle contraction lasted more than 20 years and the phosphocreatine era, it will be seen, lasted only about 5 or 6 years. Already in 1934 the first indications of the primary role of adenosine triphosphate (ATP) appeared. ATP was discovered in 1929 independently by Karl Lohmann in Otto Meyerhof's laboratory and by Fiske and SubbaRow at Harvard University. Maruyama (1991) and Florkin (1975) have described the circumstances and particularly the controversies surrounding this discovery. Lohmann's paper appeared in the August 1929 issue of Naturwissenschaften whereas the Fiske and SubbaRow paper appeared in the October issue of Science in 1929. Thus technically priority for the discovery of ATP should rest with Lohmann. But since the work was done independently and simultaneously it seems reasonable to simply state, as Maruyama and Needham have, that ATP was discovered independently by Lohmann (1929) and Fiske and SubbaRow (1929).

By 1935 Lohmann had worked out the chemical constitution of ATP and proposed the formula which is still accepted today. Needham (1971) states that the turning point in the realization of the function of ATP breakdown as the energy-yielding reaction closest to the muscle machine came with the work of Lohmann in 1934. Lohmann (1934) showed that there was no enzyme in muscle that could hydrolyze phosphocreatine but that it could only be split by an enzyme he called creatine kinase which resulted in transphosphorylation to a member of the adenyl system, principally ADP.

$$\begin{aligned} &\text{Creatine phosphate} + \text{ADP} \Rightarrow \text{Creatine} + \text{ATP} \\ &\text{ATP} \Rightarrow \text{ADP} + \text{H}_3\text{PO}_4 \end{aligned} \tag{1.1}$$

Reaction one has become known as the Lohmann reaction. Lohmann concluded that phosphocreatine breakdown in intact muscle contraction must be preceded by ATP breakdown. Needham (1960) explains the significance of these results in the following way (Needham 1960. With permission Elsevier):

> The work of Lohmann had consequences of great significance. Thus he deduced that before creatine phosphate breakdown can yield energy, ATP hydrolysis must have occurred; this latter reaction thus became the energy-yielding reaction closest to contraction. Further, this was the first observation of phosphate transfer, and involved two compounds each containing what we now call an "energy-rich phosphate bond" (Lipmann 1941). Transfer of phosphate between such molecules without formation of inorganic phosphate is a mechanism for conversation of free energy which has turned out to be of enormous biological importance.

However it was only after some 30 years more that direct proof for ATP hydrolysis during contraction in intact muscle was demonstrated by Cain and Davies (1962) (see Chap. 5).

The hydrolysis of phosphocreatine and splitting of ATP to ADP were both associated with substantial releases of heat making them potential candidates to supply energy for muscle contraction. Using calorimetry, Meyerhof and Lohmann (1928) determined that the hydrolysis of phosphocreatine is accompanied by heat output of about 12,000 cal/g mol H_3PO_4 split off. Meyerhof and Lohmann (1932) also determined that the splitting of ATP to ADP released about 12,500 cal/g mol H_3PO_4 split off. Meyerhof and Lohmann were well aware that it was free energy and not the heat of the reaction that was crucial.[3] However methods at that time were not available for measurement of the free energies, and it was assumed that the difference between the heat of reaction and the free energy available under favorable conditions was not great.

Lipmann (1941) who received the Nobel Prize in 1953 for his discovery of coenzyme A and its importance for intermediary metabolism wrote a classic review in which he introduced the term "energy-rich phosphate bond" and the "wiggle sign" (~).

[3] Meyerhof assumed that the heat of a reaction or enthalpy change, ΔH, was a guide to the free energy change of that reaction, ΔF (now usually designated as ΔG). The assumption was that the entropy change, ΔS, was insignificant or nearly so. See Chap. 5 for more information about measuring enthalpy and free energy changes in contracting muscle.

This has become known as ~P. An alternative mode of expression which he suggested was that of "high group potential". By utilization of the energy of certain metabolic processes, certain such groups can be prepared and then transferred into desired situations.

1.3.5 Meyerhof, Lundsgaard and Lohmann: The Later Years

Otto Meyerhof was a monumental figure in the study of muscle metabolism in the first half of the twentieth century. Besides being a Nobel Laureate himself, he trained four scientists who would subsequently win the Nobel Prize (Severo Ochoa, Fritz Lipmann, George Wald and Andre Lwoff). In 1929 he became the director of the newly founded Kaiser Wilhelm Institute for Medical Research in Heidelberg (now a Max-Planck Institute). He and his laboratory group published about 400 papers and his group is credited with the discovery of 6 of the 15 enzymes of the glycolytic pathway (see Florkin 1975:150 for a table of the glycolytic enzymes and their discoverers). But as a Jew in Germany life became impossible for him. In 1937 Meyerhof began making secret plans to leave the country. He arranged for a position in France. He left Germany with his wife and family in 1938. To protect the deception, Meyerhof told none of his colleagues of his departure. This also meant he was forced to leave behind all of his scientific data and personal possessions. Two years later, the German invasion of France sent the Meyerhof family on another harrowing journey ultimately leading to the United States. He and his family settled at the University of Pennsylvania where he worked as a research professor sponsored by the Rockefeller Foundation until his death in 1951. (See Meyerhof biography at Nobelprize.org for details.)

Einar Lundsgaard became a professor of physiology at the University of Copenhagen. He essentially stopped his experimental work in muscle physiology in 1934. That Lundsgaard was an exceptional scholar there can be no doubt. At the age of 27 he published a complete textbook of physiology comprising nearly 700 pages. In 1964 the book was in its eighth edition. Later in his career he worked on topics related to metabolism and glucose transport. See Lundsgaard, Kruhoffer and Crone (1972) for a biographical sketch of Lundsgaard.

Karl Lohmann joined Otto Meyerhof's laboratory in 1924. Meyerhof was a physiologist and his laboratory greatly benefited from Lohmann's chemical competence. During his stay in the Meyerhof laboratory, Lohmann generated 69 publications either as sole author or in collaboration with Meyerhof and/or other members of the laboratory. In 1937 Lohmann was promoted to professor of physiology in Heidelberg and in the same year in Berlin. Langen and Hucho (2008) in a biographical sketch of Lohmann have asked the interesting question: was the discovery of ATP and its significance in biology the most important accomplishment in biochemistry without a Nobel prize? This will not be the last time that such a question will be asked about fundamental discoveries in the muscle field.

1.4 Discovery of "Myosin" and Muscle Birefringence

The first important study of muscle proteins was conducted by Wilhelm Fredrick (Willy) Kuhne (1837–1900) (1864) in Leipzig. He extracted in high salt solution an abundant protein from frog muscle that he called "myosin". [Nearly seventy five years later, in a major discovery by Albert Szent-Gyorgyi's laboratory, it was found that this "myosin" was composed of two proteins (myosin and actin). This discovery is described below but for now "myosin" in quotes will refer to myosin with likely contamination with actin.] Thomas Henry Huxley[4] (1880) and Schipiloff and Danilewsky (1881) discovered that the birefringence of muscle disappeared when "myosin" was extracted with strong salt solutions that were known to solubilize "myosin". Thus very early, even before 1900, there appeared to be a connection between "myosin" and muscle birefringence which was located in the A band.

Wiener (1912) developed a quantitative theory of birefringence for the condition of a bundle of parallel isotropic rods (form birefringence), small compared to the wavelength of light, immersed in a medium of known refractive index. A plot of birefringence versus medium refractive index produced a U-shaped curve. The U was concave upwards in the case of particles with positive form birefringence and convex upwards in the case of particles with negative form birefringence. Positive birefringence suggested particles with their longest dimension parallel to the optical axis. Negative birefringence suggested particles with their longest dimension transverse to the optical axis (see footnote 2). The distance between the minimum (or the maximum) of the U shaped curve and the line of zero birefringence in the plot indicated the degree of intrinsic birefringence which characterized the particles. Stubel (1923) employed the Wiener theory to interpret the birefringence observed in striated muscle from the frog. Stubel's results and Wiener's theory suggested that A band birefringence in the intact muscle was due to rod-shaped particles (small compared to the wave-length of light) oriented with their long axes parallel to the axis of the muscle. Furthermore the rod-shaped particles were themselves birefringent since the minimum of the U shaped curve appeared above the zero birefringence line.

von Muralt and Edsall (1930a) at Harvard University provided evidence for a direct link between "myosin" and birefringence using the technique of flow birefringence (see footnote 2). Previously, Edsall had isolated a muscle globulin that contained, among other proteins, "myosin". Their crucial experiment is remarkable in that a qualitative conclusion was reached in one day! Quantification of the effects of various experimental conditions on the flow birefringence of the muscle globulin required a further year's worth of work. What was the observation that led to a qualitative conclusion in one day? The muscle globulin was placed in a beaker and stirred either by a glass rod or by a simple rotation. If the unstirred solution was

[4] Thomas Henry Huxley (1825–1895), the famous nineteenth century English biologist, was Andrew Huxley's grandfather. He was also considered to be Charles Darwin's "bulldog" because he championed the reclusive Darwin's theory of evolution to the general public.

observed through crossed polarizers it appeared dark. As soon as the fluid was set into motion, the field of vision become bright because the plane-polarized light changed into elliptically polarized light which could not be extinguished by the polarizer. Thus they concluded that the muscle globulin contained the basic birefringent elements of living muscle. Furthermore they concluded that these probably rod-shaped myosin particles flowing in the stream like logs in a flowing river were in a similar state of orientation in resting striated muscle fibers, producing the anisotropy of the muscle A bands. Thus evidence was mounting that the birefringence of the A band of muscle was due to the rod-shaped particles consisting of "myosin".

These experiments were a step toward the unification of the biochemistry and the physiology of muscle. Furthermore Weber (1958) emphasized that since this discovery all the authors engaged in muscular contraction field assumed, up to 1954, that muscle contraction was due to a folding or coiling of these rod-shaped molecules. There were various suggestions about how this folding or coiling might occur but no doubt that it did occur.

Weber (1935) generated an ingenious muscle model, the fibrous thread. The threads were similar to artificial silk threads of nylon. In this case, "myosin" solubilized in a high salt solution was rapidly extruded through a capillary tube into distilled water. The water diluted the KCl and "myosin" precipitated in the form of slender filaments. Under these conditions the "myosin" formed a thread which had birefringent properties. This preparation was an early model of muscle which the Weber laboratory later characterized from a mechanical point of view (see below). Of course these birefringent "myosin" threads did not exhibit striations.

A major break through occurred when Vladimir Alexandrovich (W. A.) Engelhardt and his wife Militsa Nikolaevna (M. N.) Lyubimova (1939) in Moscow published a brief one page paper in Nature in which they concluded that "myosin", the major structural protein of muscle, also exhibited ATPase activity. Under no conditions tested could they obtain a separation of the ATPase activity from myosin. Thus the ATPase activity was ascribed to myosin or at least to a protein very closely related to and indistinguishable from myosin. They further concluded that the hydrolysis of ATP, often regarded as the primary exothermic reaction of muscle contraction, proceeded under the influence and with the direct participation of the protein considered to form the main basis of the contractile mechanism of the muscle fiber. The pieces were starting to come together but there was just ahead a startling surprise when the "myosin" known for over 70 years was discovered by Albert Szent-Gyorgyi's laboratory to actually be a combination of two proteins: myosin and actin.

1.5 Albert Szent-Gyorgyi: Myosin, Actin, Actomyosin and Role of ATP

Albert Szent-Gyorgyi (Fig. 1.2) received the Nobel prize in 1937 for his work on the elucidation of the structure of vitamin C and the establishment of the groundwork of the Krebs cycle (Szent-Gorgyi 1965). Szent-Gyorgyi was a national hero in his

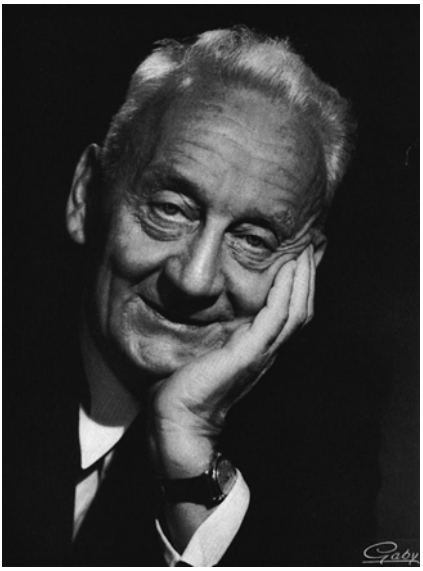


Fig. 1.2 Albert Szent-Gyorgyi (1893–1986), a Hungarian biochemist, received the Nobel prize in 1937 for his work on the elucidation of the structure of vitamin C and the establishment of the groundwork of the Krebs cycle. It was in his laboratory during the 1940s that the modern era of muscle biochemistry was established with the discovery of actin, true myosin and actomyosin and their interactions with ATP. His laboratory also elucidated the role of ATP in rigor mortis. He developed the glycerol extracted skeletal muscle preparation for mechanical studies of an ordered actomyosin. For his work on muscle he received the Lasker award in 1954. His publications span 73 years (Photo: Szent-Gyorgyi 1963. Permission to utilize this figure has been granted by Annual Reviews)

native Hungary. After receiving the Nobel prize, it was Szent-Gyorgyi's next great ambition to analyze one of the basic phenomena of life, in which chemical energy is converted into work, be it mechanical, electrical or osmotic work. He chose muscle contraction. In fact one of his many monographs (1948) describing his research was entitled: "Nature of Life: A Study of Muscle". What makes the fundamental discoveries of the Szent-Gyorgyi laboratory at the University of Szeged Medical School[5] in Hungary so compelling is that they occurred in virtually complete scientific isolation during World War II. Even the journal Nature was not accessible to the researchers in Szeged at that time.[6]

Despite, or perhaps because of, this scientific isolation, it was in Szeged where the modern biochemistry of muscle contraction was born. This ground breaking

[5] In 2000, the Faculty of Medicine and the Faculty of Pharmacy within the University of Szeged was renamed the Albert Szent-Gyorgyi Medical and Pharmaceutical Center.

[6] The German Ministry of Science and Education banned the general use in scientific libraries of the magazine Nature on November 12, 1937 for "outrageous and vile attacks on German science and the national socialist state". The journal Nature was also banned in Hungary (Hossfeld and Olsson 2013).

work was published in three volumes of the Studies of the Institute of Medical Chemistry, University of Szeged during the years 1941–1943. Essentially Szent-Gyorgyi asked the members of his research group to write up what they had been doing in the laboratory and he edited it and the results were published in English without outside peer review. For all practical purposes the rest of the world didn't know the results of the Szent-Gyorgyi group until he published a review in 1945. What were these ground breaking results and how were they discovered?

The first of the major discoveries occurred somewhat serendipitously in 1941. Ilona Banga and Albert Szent-Gyorgyi were isolating "myosin" according to well accepted procedures. "Myosin" was commonly extracted from freshly ground rabbit muscle, in the cold with 0.6 molar KCl for 20 min, separated by centrifugation, and the "myosin" precipitated by diluting the solution to 0.1 M KCl. Wilfried F. Mommaerts (1992) who was a new laboratory member from Belgium in the fall of 1941 told the story some years later. One day Banga and Szent-Gyorgyi wanted to go and listen to a lecture and the extraction mixture was left in the cold overnight. By morning, it was too thick to be centrifuged or otherwise separated. But strangely there was little more protein in the overnight extract compared to 20 min extract. The crucial observations are shown in the Banga and Szent-Gyorgyi paper (1941–1942). Figure 1.3 is a plot of relative mixture viscosity versus myosin content. The high viscosity of the 24 h extract (curve #1) could be converted to the low viscosity observed for the 20 min extract (curve #3) by adding a small amount of ATP (curve #2). But the viscosity of the 20 min extract (curve #3) was uninfluenced by ATP addition (curve #4). Banga and Szent-Gyorgyi called the low viscosity 20 min extract "myosin A" and the high viscosity overnight extract "myosin B". They further showed that when the ATP became exhausted, myosin A reverted to myosin B. They said that "myosin B" had a high "activity". By the 'activity' of the "myosin" they meant the fall of viscosity on the addition of ATP. These observations were the start of the path to the isolation and characterization of a new protein that Szent-Gyorgyi called actin in the discussion in volume 1 (Szent-Gyorgyi 1941–1942b).

Szent-Gyorgyi (1941–1942a) went on to study the contraction of myosin threads, as first described by Weber in 1935, made from myosin A or myosin B. He incubated the threads in a filtered watery extract of muscle. When myosin A was suspended in this muscle extract, no striking change in length was observed. In spectacular contrast, when a myosin B thread was suspended in this extract, a violent contraction was observed. The thread contracted to less than half of its original length in 30 s. At the same time the thread became thinner and darker as shown in Fig. 1.4. Szent-Gyorgyi determined that it was the ATP in the watery extract that caused contraction in the myosin B thread. If the extract was stored overnight, it had no activity but when ATP was added it once again caused contraction. If ATP alone was dissolved in water, no contraction occurred but if ATP was dissolved in the boiled muscle extract, a violent contraction was observed. Thus Szent-Gyorgyi concluded that three factors were involved in the production of the contraction of the myosin B thread: ATP, K^+ and Mg^{2+}.

There was a feeling amongst the Szent-Gyorgyi laboratory members that "life" had been created in the test tube. After all, motion is a fundamental feature of life

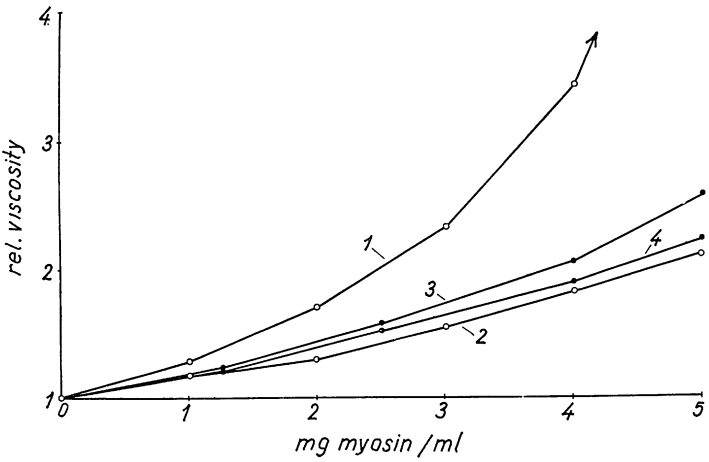


Fig. 1.3 Relative viscosity of "myosin" extracted from rabbit muscle versus protein concentration. "Myosin" extracted for 24 h (curve #1) was more viscous than "myosin" extracted for 20 min (curve #3) even though there was little more protein extracted with the longer extraction time. The viscosity of the 24 h extract (curve #1) was drastically reduced in the presence of ATP (curve #2) whereas the viscosity of the 20 min extract was insensitive to the presence of ATP (curve #4). The low viscosity, 20 min "myosin" extract was called myosin A and the high viscosity, 24 h "myosin" extract was called myosin B. Myosin B turned out to be a combination of two proteins, myosin and actin, that were combined in the absence of ATP and dissociated in the presence of ATP (Banga and Szent-Gyorgyi 1941–1942. With permission S. Karger AG)

and they just observed it in a test tube. Szent-Gyorgyi has described his excitement on various occasions (for example in a 1963 autobiographical essay): "The threads contracted. To see them contract for the first time, and to have reproduced in vitro one of the oldest signs of life, motion, was perhaps the most thrilling moment of my life." (Szent-Gyorgyi 1963. With permission Annual Reviews)

One other important observation was described in this paper (Szent-Gyorgyi 1941–1942a). Szent-Gyorgyi found that a myosin B suspension in the presence of ATP would precipitate immediately, much more rapidly than the precipitation caused by KCl alone. Szent-Gyorgyi called this process "superprecipitation" and also referred to it as "contraction without architecture" (Szent-Gyorgyi 1951). He believed that the phenomena seen in the myosin B suspension were analogous to the phenomena observed in myosin B threads. The superprecipitation of myosin B and the contraction of myosin B threads would form the basis of his future thinking about the mechanism of muscle contraction.

What remained was to determine why these spectacular results were observed only with myosin B but not with myosin A. In a separate paper entitled "discussion" at the end of volume I, Szent-Gyorgyi (1941b) provided a preview of experiments subsequently published in volume II in 1942. He stated that the experiments of F.B. Straub in his laboratory definitely showed that myosin B was a stoichiometric compound of myosin A and another substance. Szent-Gyorgyi called this other

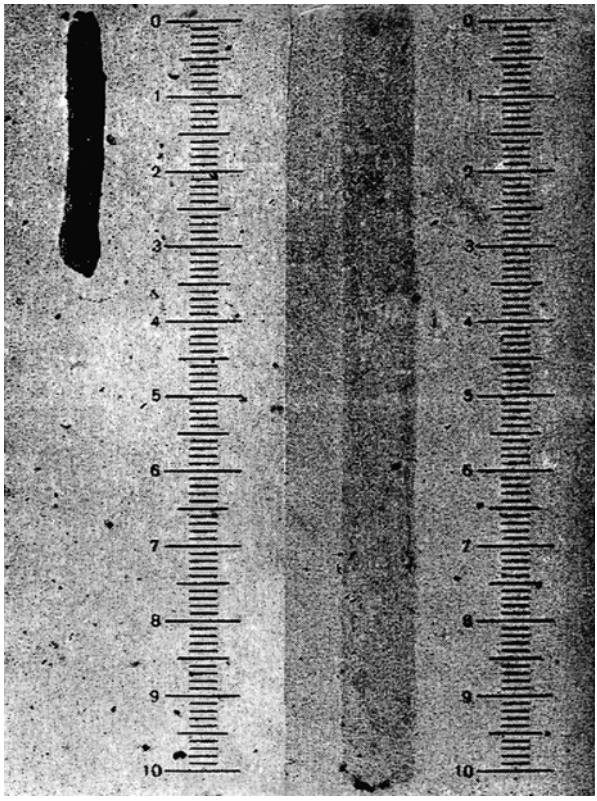


Fig. 1.4 Length and width of a myosin B thread at rest (*right*) and in the presence of a water extract of muscle (*left*). In the presence of the muscle extract, the myosin B thread contracted to greater than half its resting length and became thinner. The active agent in the water extract of muscle was determined to be ATP. This experiment was the first ever "contraction in a test tube" (Szent-Gyorgyi 1941–1942a. With permission S. Karger AG)

substance "actin" and called the myosin-actin complex "actomyosin". The name myosin was retained for the actin free protein.

F(erenc) Bruno Straub was a young Hungarian scientist who had just returned to the Szent-Gyorgyi laboratory from Cambridge University after working in the laboratory of David Keilin, the pioneer in the study of the cytochrome system and cell respiration (Moss 1988). Straub's monumental paper entitled simply "actin" appeared in volume II of the Studies from the Institute of Medical Chemistry, University of Szeged (Straub 1942). Straub proceeded by first extracting myosin A from rabbit muscle. The residue was then treated with acetone and after removal of acetone the residue was allowed to dry. Actin then was extracted from this dried residue by addition of distilled water. Straub studied the factors that brought about the conversion of myosin A into myosin B. Myosin B was formed if a certain amount of myosin A and actin were mixed. It followed that myosin A was what was termed by earlier investigators as myosin and myosin B on the other hand was a mixture of a definite amount of actin and myosin. But how did "actin" get its name? The ability of ATP to cause a decrease in the viscosity of myosin B depended on the amount of actin in the preparation. Thus the more actin in the myosin B extract, the greater the decrease in viscosity in the presence of ATP, i.e., a greater "activity" of

the myosin B extract. The name actin apparently results from its ability to "act" to cause a fall in viscosity of myosin B in the presence of ATP. The ability of ATP to cause a decrease in viscosity of myosin B or artificially prepared actomyosin was due to the splitting of the complex into constituent components.

In volume III of the series, Straub (1943) determined that actin consisted of two forms. Each form could react with myosin, but only one could give the highly viscous actomyosin of myosin B. He called these two forms active and inactive actin. It was concluded that the inactive form was a globular protein and the active form was a rod shaped fibrous protein. Szent-Gyorgyi (1945) introduced the terms G- and F-actin for the globular and fibrous forms of the protein respectively. Straub and Feuer (1950) went on to show that polymerization of G-actin to F-actin involves the conversion of actin-bound ATP to ADP. Tamas Erdos (1943b) demonstrated that the presence of actin as well as myosin in the protein threads was necessary for contraction.

Thus the modern era of muscle biochemistry was established but most of the rest of the world knew nothing about it. This situation changed when Szent-Gyorgyi published a review of the work done during the war years in Szeged in the journal Acta Physiologica Scandinavica in 1945 (Szent-Gyorgyi 1945). Szent-Gyorgyi was fearful that he would not survive the war and he didn't want this fundamental work to be lost. But there was a problem. To publish in Acta Physiologica Scandinavica one had to be a citizen of a Scandinavian country. Swedish scientist Hugo Theorell who would win the Nobel Prize in 1955 for his work on the nature and mode of action of oxidation enzymes arranged for Szent-Gyorgyi to become a Swedish citizen and thus the manuscript was published and available to the world (Szent-Gyorgyi 1963).

But the Albert Szent-Gyorgyi story isn't just one about scientific discoveries, it is also about political intrigue. A flavor of this aspect of Szent-Gyorgyi's life can be gained from the opening of his autobiographic essay (Szent-Gyorgyi 1963. With permission Annual Reviews):

> Overlooking my case history, I find a complete dichotomy. On the one hand, my inner story is exceedingly simple, if not indeed dull: my life has been devoted to science and my only real ambition has been to contribute to it and live up to its standards. In complete contradiction to this, the external course has been rather bumpy. I finished school in feudal Hungary as the son of a wealthy landowner and I had no worries about my future. A few years later I find myself working in Hamburg, Germany, with a slight hunger edema. In 1942 I find myself in Istanbul, involved in secret diplomatic activity with a setting fit for a cheap and exciting spy story. Shortly after, I get a warning that Hitler had ordered the Governor of Hungary to appear before him, screaming my name at the top of his voice and demanding my delivery. Arrest warrants were passed out even against members of my family. In my pocket I find a Swedish passport, having been made a full Swedish citizen on the order of the King of Sweden—I am "Mr. Swenson," my wife, "Mrs. Swenson." Sometime later I find myself in Moscow, treated in the most royal fashion by the Government (with caviar three times a day), but it does not take long before I am declared "a traitor of the people" and I play the role of the villain on the stages of Budapest. At the same time, I am refused entrance to the USA for my Soviet sympathies. Eventually, I find peace at Woods Hole, Massachusetts, working in a solitary corner of the Marine Biological Laboratory. After some nerve-racking complications, due to McCarthy, things straightened out, but the internal

> struggle is not completely over. I am troubled by grave doubts about the usefulness of scientific endeavor and have a whole drawer filled with treatises on politics and their relation to science, written for myself with the sole purpose of clarifying my mind, and finding an answer to the question: will science led to the elevation or destruction of man, and has my scientific endeavor any sense?

More details of these and other aspects of Szent-Gyorgyi's life can be found in a biography written by Ralph Moss (1988).

Albert Szent-Gyorgyi moved his laboratory to the Marine Biological Laboratory at Woods Hole, Massachusetts in 1947 to establish the Institute for Muscle Research. Szent-Gyorgyi first visited Woods Hole after the 13th International Congress of Physiological Sciences meeting held in Boston, Massachusetts in 1929 (McLaughlin 1988). Szent-Gyorgyi was among 400 scientists from 22 countries that made the ten day trip from England and Europe to the United States on the ship the S. S. Minnkahda (Franklin 1968). The story of this trip which has been described with photos by Zotterman (1968) is fascinating.

Despite the excitement of the Szent-Gyorgyi laboratory, the idea that the contraction of the actomyosin thread was an appropriate model of muscle contraction was strongly criticized. First of all the threads did not exhibit the structure of skeletal muscle as observed in the light microscope. But more important it had been known for nearly 200 years, since the experiments of Jan Swammerdam published in 1758, that when an isolated frog muscle contracted there was little change of its volume. Thus muscle contraction occurred at essentially constant volume. In contrast the contraction of the actomyosin thread also led to a large decrease in thread volume (see Fig. 1.4), contrary to what is observed in muscle. Buchthal et al. (1947) reported that after partial drying and stretching of a thread, ATP caused the thread to become shorter and wider as occurs in contraction of a muscle fiber.

Because of these limitations, Szent-Gyorgyi sought to develop a more physiologically relevant model of muscle contraction to test his theories. He developed the glycerol extracted psoas muscle model of contraction (Szent-Gyorgyi 1949). The psoas muscle from the rabbit was utilized because it contained little connective tissue, exhibited long, parallel fibers that ran the length of the muscle. Because of the lack of connective tissue it was easy to separate the muscle into bundles or even single fibers. In his characteristically colorful fashion, Szent-Gyorgyi (1951) described the reason for using rabbit muscle this way: "Since the rabbit is easily obtainable, does not bite or bark, is neither too big nor too small, is rather cheap and very stupid, we will choose *Musculus psoas* as the object of our study." (Szent-Gyorgyi 1951. With permission Elsevier) Psoas muscles were made permeable and the soluble muscle proteins extracted by destroying the membranes by soaking them in a 50 % solution of glycerol. The extracted bundles consisting essentially of actomyosin could be preserved for weeks in the glycerol solution at −20 °C with undiminished ability to contract in the presence of Mg^{2+} and ATP. This preparation retained the striation pattern characteristic of intact skeletal muscle. He found that the force generated was similar to that observed in intact muscle. Furthermore the removal of ATP made the muscle inelastic and it could not be stretched without breaking. The addition of ATP made the muscle once again extensible. These results

confirmed earlier work by Erdos (1943a) in the Szent-Gyorgyi laboratory and formed the basis of the understanding of the muscle stiffness observed in rigor mortis which occurs upon ATP depletion.

Szent-Gyorgyi emphasized the dual role of ATP, on the one hand causing contraction and on the other exhibiting a plasticizing effect. First, there is loosening of the actomyosin linkages in the fiber. Then, ATP activity supplies the energy for contraction. The formation of new linkages brings about fiber shortening or force development. ATPase activity continues until all the ATP is used up and the fiber then goes into the rigor state. Under these conditions, addition of new ATP causes relaxation and the cycle repeats. These results with the glycerinated muscle preparation brought conclusive evidence that the interaction of ATP with actomyosin was the basic contractile event.

H. H. Weber pioneered the investigation of the mechanical properties of models for the study of muscle contraction and cell motility. The first comprehensive mechanical study of the glycerol extracted muscle preparation at the single fiber level was carried out in Weber's laboratory by his daughter Annamarie Weber (1951) (see Chap. 4 and Fig. 4.12). She found that compared to isolated, intact muscle, the glycerol extracted preparation exhibited a similar: (a) maximum force, (b) temperature dependency of force, (c) extent of shortening and (d) redevelopment of force after a release. The only difference observed was a much slower rate of force recovery after a sudden shortening of a fiber from a fixed length contraction. This effect was attributed to small structural disturbances in the glycerol extracted preparation. The glycerol extracted muscle preparation is still in common use today.

The development of the glycerol extracted muscle preparation was the last of the major contributions of Albert Szent-Gyorgyi to the muscle field though his younger cousin Andrew G. Szent-Gyorgyi would make fundamental discoveries relating to the myosin molecule while in Albert's laboratory and then establish his own distinguished career in the muscle field (see Chaps. 3 and 6). For his discoveries related to muscle contraction, Albert Szent-Gyorgyi received the Lasker Award in 1954. Ironically 1954 was the year that the sliding filament model of muscle contraction was proposed, a theory that Albert Szent-Gyorgyi bitterly opposed only to lose (see Chap. 2).

With his major discoveries relating to muscle biochemistry, Albert Szent-Gyorgyi (1951) wasn't beyond criticizing other approaches to the study of muscle contraction. With regard to the muscle physiologists, he believed that they had generated an enormous bulk of literature which had led to little understanding of the mechanism of muscle contraction. With regard to the X-ray diffraction technique in the muscle field, Szent-Gyorgyi (1951) claimed incompetence and didn't bother to discuss the results. Apparently to Szent-Gyorgyi this was not a technique worth understanding. Plus the early results did not fit well with his view of muscle contraction (see below). Ironically in a short period of time the X-ray diffraction and physiological approaches to muscle contraction would open an entirely new view of muscle contraction.

1.6 Early Electron Microscopic Studies of Muscle Structure

Early electron microscopic studies of muscle structure (Hall et al. 1946; Draper and Hodge 1949; Rozsa et al. 1950) seemed to confirm the prevailing view that the protein threads passed continuously throughout the entire length of the sarcomere. For example, consider the study of Hall et al. (1946). They noted the difficulty in making the muscle sections thin enough to be partially transparent to the electron beam. Therefore they examined fragmented myofibrils of frog and rabbit. They could easily confirm the nineteenth century light microscope observations of A and I bands, H zone and Z and M lines (Fig. 1.5). They concluded that myosin filaments extended continuously, and in relatively straight lines, through A and I bands. Although the filaments were usually indistinguishable within the dense Z bands, they believed that the filaments could be traced through several successive sarcomeres when the Z bands are partially disintegrated. An example of their results from strongly contracted frog muscle is shown in Fig. 1.6. The sarcomere length in these photographs is 1.2 μm. The I band is no longer visible and the Z line is wider. This widening of the Z line was suggested to be due to a migration of the A substance to the Z line. This "striation reversal" or contraction band had been noted by early light microscopists. From these results Hall et al. (1946) concluded that the relative straightness of the filaments in the sarcomeres contracted to as little as 50 % of the relaxed length must mean that the filaments themselves shorten during contraction. Thus they provided apparent visual evidence of filament shortening.

There are further points to make from this study. First, the results and interpretation confirmed the prevailing view that the muscle filaments themselves contracted with muscle shortening. Second, the authors in this 1946 paper clearly were unaware of the existence of actin in muscle as they interpreted their results solely in terms of myosin filaments. Finally, the senior author Francis O. Schmitt in whose laboratory this study was conducted at the Massachusetts Institute of Technology would soon be the host to Hugh E. Huxley and Jean Hanson who developed the concept of and evidence for the sliding filament model of muscle contraction in Schmitt's laboratory.

Rozsa et al. (1950) utilized the electron microscope to examine muscle structure using a shadow-casting technique. They concluded that the appearance of the filaments was compatible with the hypothesis that they consisted of actin threads running from Z line to Z line, covered by some second substance. They suggested that the overlying substance might consist of exceedingly fine threads of myosin in lengthwise association with the thicker threads of F-actin.

Draper and Hodge (1949) confirmed many of the observations of Hall et al. (1946). They envisioned the fibrils as collapsible tubes made up of the filaments or protofibrils and believed the A substance to be arranged on the inner surfaces of these tubes. They found the electron microscopic evidence to be in agreement with the classical picture of migration of A substance to the Z line with reversal of striation during contraction.

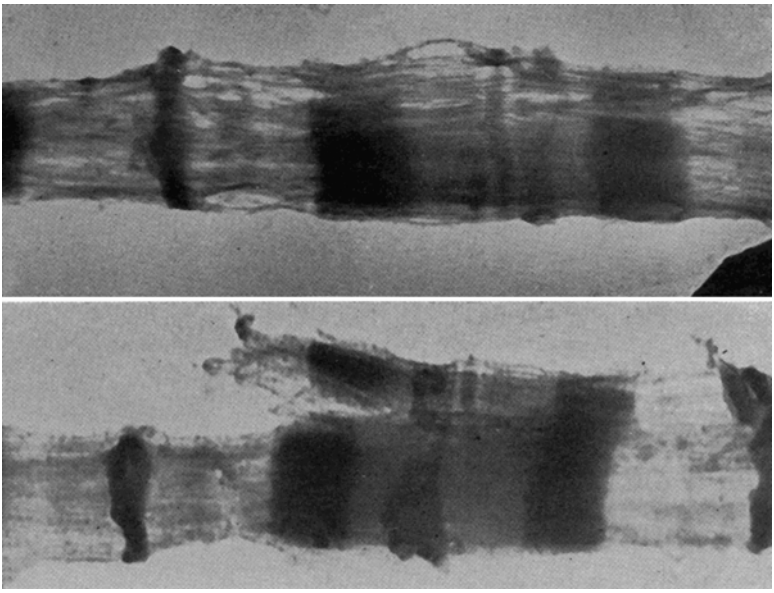


Fig. 1.5 Examples of electron micrographs of frog myofibrils fixed with phosphotungistic acid stretched by about 30 % of resting length. Note that the fibrils appear to be continuous in length throughout the sarcomere (Hall et al. 1946. With permission Marine Biological Laboratory)

Thus by the early 1950s the theories of muscle contraction based on shortening or possibly folding of filaments seemed to be confirmed by direct observation with the newest technology, i.e., the electron microscope. In light of his observations on myosin and actin, Albert Szent-Gyorgyi presumed that actin and myosin must lay side-by-side throughout the sarcomere. But this presumption seemed counter to the birefringence studies. As we have seen these studies indicated that only the A band exhibited significant birefringence and that likely this birefringence was due to the existence of myosin in the A band. In fact the I band did exhibit some birefringence but of a magnitude ten times less than observed in the A band. Possibly myosin existed in the I band but was somehow less ordered than in the A band. Furthermore it should be noted that birefringence exhibits a magnitude but also a sign (positive or negative).

Matoltsy and Gerendas (1947) in Albert Szent-Gyorgyi's laboratory observed that after extraction of myosin and actin from rat skeletal muscle that the A bands were no longer birefringent but rather that the I bands now exhibited negative birefringence. Based on these observations, they proposed that the positive birefringence of the I band was nearly cancelled out by a substance ("N-material"), possibly a nucleoprotein, exhibiting negative birefringence in the I band. Nucleic acids have negative birefringence. Thus they considered that the myofibril had a uniform positive birefringence which was compensated in the I band by the negative birefringence of a fibrous protein called the N-protein. These results fit nicely with Szent-Gyorgyi's view of actin and myosin lying side-by-side throughout the sarcomere but later these results could not be confirmed (Weber and Portzehl 1952; Dubisson 1954).

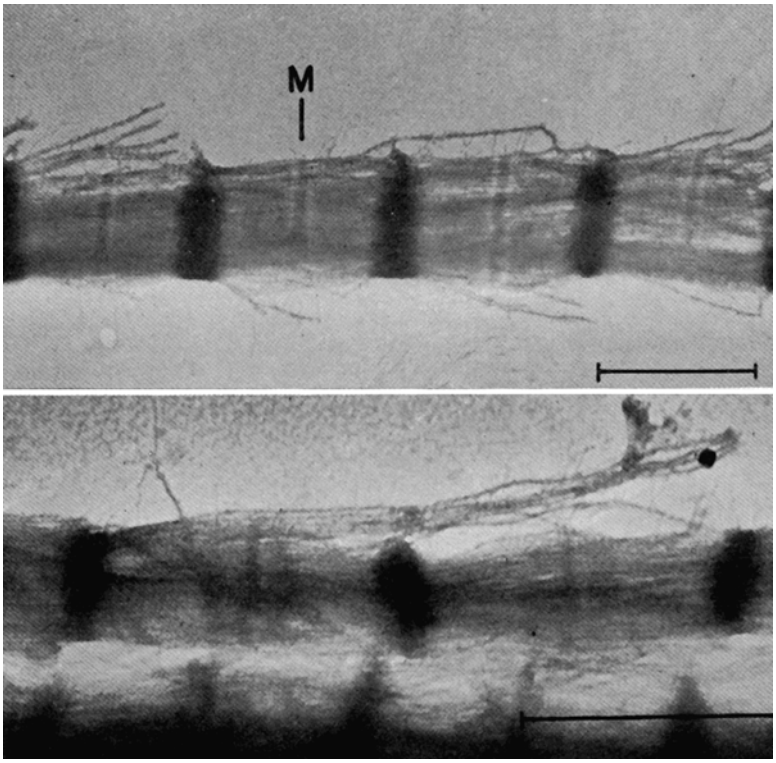


Fig. 1.6 Examples of electron micrographs of frog myofibrils strongly contracted by electrical stimulation so that the sarcomere length is above 1.2 μm. The fibrils, which appear to run the length of the sarcomere, are still straight after extreme myofibril shortening which suggested that the fibrils themselves shortened rather than folded. The widening of the Z line was similar to the "striation reversal" noted by the nineteenth century light microscopists (Hall et al. 1946. With permission Marine Biological Laboratory)

1.7 Some Theories of Muscular Contraction Prior to 1954

The first theory of the contractile thread molecule was developed by Meyer (1929) in Geneva, Switzerland. He proposed that isoelectric proteins consist of dipolar ions (or zwitterions) and not of uncharged molecules, as had been previously assumed. Meyer suggested that in the resting state the thread molecules stretched by means of the mutual repulsion of the equivalent charges along the molecule. He assumed further that at the moment of contraction metabolic processes produced an equal number of positive charges among the negative ones, thus rendering these thread molecules isoelectric and causing them to contract owing to the attractive power of the opposite charges. Many subsequent theories have shared

the concept that a shortening of thread molecules is produced by a change in the state of the electric charge. Even authors who did not ascribe muscular contraction to electric charge did not doubt that contraction was based on the contraction of thread molecules. The thread molecules of actomyosin were the contractile thread molecules.

But there were difficulties with the concept of folding molecules. The evaluation of X-ray diagrams of resting and contracting muscles were not compatible with the contraction of thread molecules. From X-ray diagrams of the muscle, longitudinal periods of the individual thread molecules can be deduced. When the whole thread molecule shortens these longitudinal periods should shorten. But the wide angle X-ray diffraction[7] results of Astbury (1947) exhibited no change in the axial pattern of the muscle filaments when a muscle shortened up to 50 % of its resting length. These results were evidence against the folding of individual protein chains. Strangely, Astbury still believed that the proteins folded upon contraction. Attempts to explain these results were made by proposing that the longitudinal periods are present only in those small areas of the muscle fiber that were oriented to a high degree. Contraction was then supposed to occur in other parts of the muscle fiber that were oriented to a low degree. These less oriented parts could not be deduced from the X-ray diagram because these parts are not observed in the X-ray diagram. This is an inherent limitation of the X-ray diffraction technique. Clearly the pieces did not all fit together to result in a comprehensive theory of muscle contraction but the idea of folding or shortening filaments was just too attractive, too obvious, to be wrong.

Szent-Gyorgyi (1952) has proposed the following theory of muscle contraction (Szent-Gyorgyi 1952. With permission National Library of Medicine):

> However, at rest, the two proteins are not joined together, but are present dissociated as free actin and free myosin. The two proteins have a great affinity but are pushed apart by electric repulsive forces. These repulsive forces are due to ions and ATP. The latter is adsorbed to myosin and gives it a charge which repels actin. Myosin is a very soft, almost liquid gel and so is actin. When they unite to form actomyosin, a new substance is formed which has new qualities. In solution the change we observe is an enormous increase in viscosity. If the two proteins are present in fairly high concentration, as is the case in muscle where there is 8 % myosin and 2 % actin, on union they form a stiff and hard gel. If ATP is present this gel goes over into a new modification in which its particles are shorter. This is muscular contraction. What brings actin and myosin together in vivo is the wave of excitation which momentarily disturbs the balance of charges and with it the balance of attractions and repulsions between actin and myosin.

This theory was strongly influenced by his observations of contraction of the actomyosin threads and superprecipitation of actomyosin.

[7] Wide angle X-ray diffraction is a technique that is used to determine the crystalline structure of polymers at the Angstrom level. In contrast small-angle X-ray scattering is a technique where the scattering of X-rays by a sample exhibits inhomogeneities in the nm-range. The small angle X-ray diffraction has been utilized brilliantly by Hugh E. Huxley to elucidate structural details of intact muscle (see Chaps. 2, 6 and 9).

1.8 Albert Szent-Gyorgyi: The Later Years

Whereas Albert Szent-Gyorgyi's scientific contributions to the muscle field were essentially complete by the time of the Lasker award in 1954, and although he moved his research interests to cancer, his intervention into the muscle field was not yet complete. In 1973 at the age of 80, Szent-Gyorgyi made the shocking announcement that Bruno Straub did not discover actin 30 years earlier in his laboratory (Mommaerts 1992; Moss 1988). He said that actin was discovered by Ilona Banga working under his supervision. Both Straub and Banga remained in Hungary after Szent-Gyorgyi left for the United States. According to Albert Szent-Gyorgyi's biographer, Moss (1988), Szent-Gyorgyi's attitude was compounded by deep-seated political differences. Banga was often on the outs with the regime because of her refusal to join the Communist Party. Straub had joined several years earlier and had prospered. Apparently Szent-Gyorgyi resented what he saw as Straub's political road to success.[8] Moss concludes that Szent-Gyorgyi's silence on the question for 30 years is the most important piece of evidence in Straub's favor. So shocking was this episode that Mommaerts (1992), who was in the Szent-Gyorgyi laboratory, during this critical period felt compelled to write a brief essay entitled: "Who discovered actin?" He confirmed that it was Straub and not Banga who discovered actin.

In the cancer field, Szent-Gyorgyi did not experience the success associated with his earlier discoveries. In an interview in 2004, Albert's cousin Andrew Szent-Gyorgyi provides insight into the scientist and the man. He states that Albert was not able to write a grant because he didn't know what he would be doing in 3 years so to write a grant he would have to lie and he wasn't prepared to do that. In an interesting comment, Andrew Szent-Gyorgyi (2004) said that Albert believed that a scientist needed to be curious and that if you ask scientists to be useful, it will "kill" science. Whereas Albert Szent-Gyorgyi stayed active in science until his death at 93 years of age in 1986, it was clear science had passed him by. His friend Edsall (1988) felt that Szent-Gyorgyi's ideas on cancer research were never promising and that he had lost his scientific brilliance.

The Profiles in Science of the National Library of Medicine contains extensive information about Albert Szent-Gyorgyi and his research career. Also an extensive series of reminiscences of Albert Szent-Gyorgyi can be found in the Biological Bulletin (volume 174: 214–233, 1988).

[8] Bruno Straub (1914–1996) was the Director of the Institute of Biochemistry in the Hungarian Academy of Sciences from 1970 to 1985. In 1985 he was elected to the Hungarian Parliament and became the President of Hungary (Chairman of the Hungarian Presidential Council), a largely ceremonial position, in 1988 a year before the declared end of Communist rule in 1989 (New York Times, February 18, 1996).

1.9 Thus the Stage Was Set

In the early 1950s muscle relaxation was still a mystery and just starting to get attention (see Chap. 4). The muscle action potential was well understood but there was no clue as to how the surface electrical activity caused muscle contraction (see Chap. 4). Well, actually there was a very good clue but the pioneering work of Victor Heilbrunn in the 1940s (Heilbrunn and Wiercinski 1947) on the role of calcium in muscle contraction was not widely appreciated (see Chap. 4). Nonetheless it was clearly understood that muscle contraction, fueled by ATP, resulted in the shortening or folding of actin/myosin filaments. So pleased was Albert Szent-Gyorgyi with the results observed with the glycerinated muscle preparation that by 1950 he was certain that within a few weeks the whole problem of muscle contraction would be cleared up (Szent-Gyorgyi 1963). But in 1954 the muscle field was "turned on its head" and revolutionized once again by the simultaneous, independent, publication of two brief papers in Nature by Hugh E. Huxley and Jean Hanson (1954) and by Andrew F. Huxley and Rolf Niedergerke (1954). The sliding filament model of muscle contraction was first proposed in these papers.

References

Astbury WT (1947) Proc Croonian Lecture: on the structure of biological fibres and the problem of muscle. Proc R Soc Lond B B134:303–328

Banga I, Szent-Gyorgyi A (1941–1942) Preparation and properties of myosin A and B. In: Studies from the Institute of Medical Chemistry, vol 1. University of Szeged, Szeged, p 5–15

Brucke E (1858) Untersuchungen uber den bau der muskelfasern mit hulfe des polarisirten lichtes. Denkschr Akad Wiss Wien, math.-naturwiss Kl 15:69–84

Buchthal F, Deutsch A, Knappeis CG, Munch-Petersen A (1947) On the effect of adenosine triphosphate on myosin threads. Acta Physiol Scand 13:167–180

Cain DF, Davies RE (1962) Breakdown of adenosine triphosphate during a single contraction of working muscle. Biochem Biophys Res Comm 8:361–366

Draper MH, Hodge AJ (1949) Studies on muscle with the electron microscope. I. Ultrastructure of toad striated muscle. Aust J Exp Biol Med 27:465–505

Dubisson M (1954) Muscular contraction. Charles C. Thomas, Springfield

Edsall JT (1988) Reminiscences on Albert Szent-Gyorgyi. Biol Bull 174:214–233

Eggleton P, Eggleton GP (1927) The inorganic phosphate and a labile form of organic phosphate in the gastrocnemius of the frog. Biochem J 21:190–195

Embden G, Hirsch-Kauffmann E, Lehnartz E, Deuticke HJ (1926) Uber den verlauf der milchsaurebildung beim tetanus. Z f physiol Chem 151:209–231

Engelhardt WA, Ljubimova MN (1939) Myosine and adenosinetriphosphate. Nature 144:668–669

Engelmann TW (1875) Contractilitat und doppelbrechung. Pflugers Arch ges Physiol 11:432–464

Erdos T (1943a) Rigor, contracture, and ATP. In: Studies from the Institute of Medical Chemistry, vol 3. University of Szeged, Szeged, p 51–56

Erdos T (1943b) On the relation of the activity and contraction of actomyosin threads. In: Studies from the Institute of Medical Chemistry, vol 3. University of Szeged, Szeged, p 57–58

Fiske CH, SubbaRow Y (1927) The nature of the inorganic phosphate in voluntary muscle. Science 65:401–403

Fiske CH, SubbaRow Y (1929) Phosphorus compounds of muscle and liver. Science 70:381–382

Fletcher WM, Hopkins FG (1907) Lactic acid in amphibian muscle. J Physiol 35:247–309
Florkin M (1975) A history of biochemistry. Part III. History of the Identification of the sources of free energy in organisms. In: Florkin M, Stotz EH (eds) Comprehensive biochemistry, vol 31. Elsevier, Amsterdam
Franklin KJ (1968) A short history of the international congresses of physiologists, 1889–1938. In: Fenn WO (ed) History of the international congress of physiological sciences 1889–1968. American Physiological Society, p 3–14
Hall CE, Jakus MA, Schmitt FO (1946) An investigation of cross striations and myosin filaments in muscle. Biol Bull 90:32–50
Heilbrunn LV (1956) The dynamics of living protoplasm. Academic
Heilbrunn LV, Wiercinski FJ (1947) The action of various cations on muscle protoplasm. J Cell Comp Physiol 29:15–32
Hill AV (1932) The revolution in muscle physiology. Physiol Rev 12:56–67
Hill AV, Hartree W (1920) The four phases of heat-production of muscle. J Physiol 54:84–128
Hossfeld U, Olsson L (2013) History of the journal Nature. Nature and Hitler. doi:10.1038/nature06242
Huxley AF (1957) Muscle structure and theories of contraction. In: Butler JAV, Katz B (eds) Progress in biophysics and biophysical chemistry, vol 7. Pergamon Press, London, pp 255–318
Huxley AF (1977) Looking back on muscle. In: The pursuit of nature: informal essays on the history of physiology. Cambridge University Press, p 23–64
Huxley AF, Niedergerke R (1954) Interference microscopy of living muscle fibres. Nature 173:971–973
Huxley HE, Hanson J (1954) Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. Nature 173:973–976
Huxley TH (1880) The crayfish. An introduction to the study of zoology. Paul Kegan, London
Kruhoffer P, Crone C (1972) Einar Lundsgaard, 1899–1968. Ergebnisse der physiologie, biologischen chemie und experimentellen pharmakologie. 65:1–14
Kuhne W (1864) Untersuchungen uber das protoplasma and die contractilitat. Engelmann, Leipzig
Langen P, Hucho F (2008) Karl Lohmann and the discovery of ATP. Angew Chem Int Ed 47:1824–1827
Lipmann F (1941) Metabolic generation and utilization of phosphate bond energy. Adv Enzymol 1:99–162
Lohmann K (1929) Uber die pyrophosphatfraktion im muskel. Naturwiss 17:624–625
Lohmann K (1934) Uber die enzymatische aufspaltung der kreatinphosphorsaure; zugleich ein beitrag zum chemismus der muskelkontraktion. Biochem Z 271:264–277
Lohmann K (1935) Konstitution der adenylpyrophosphorsaure and adenine diphosphorsaure. Biochem Z 282:120–123
Lundsgaard E (1930a) Untersuchungen uber muskelkontraktion ohne milchsaure. Biochem Z 217:162–177
Lundsgaard E (1930b) Weitere untersuchungen uber muskelkontraktionen ohne milchsaurebildung. Biochem Z 227:51–83
Lundsgaard E (1930c) Uber die einwirkung der monoiodoessigsaure auf den spaltungs- und oxydationsstoffwechsel. Biochem Z 220:8–18
Maruyama K (1991) The discovery of adenosine triphosphate and the establishment of its structure. J Hist Biol 24:145–154
Matoltsy AG, Gerendas M (1947) Isotropy in the I striation of striated muscle. Nature 159:502–503
McLaughlin JA (1988) Reminiscences on Albert Szent-Gyorgyi. Biol Bull 174:214–233
Meyer KH (1929) Uber feinbau, festigkeit und kontraktilitat tierische gewebe. Biochem Z 214:253
Meyerhof O (1920) Ueber die beziehung der milchsaure zur warmebildung and arbeitsleistung des muskels in der anaerobiose. Arch f ges Physiol 182:232–283
Meyerhof O, Lohmann K (1928) Uber die naturlichen guanidinophosphorsauren (phosphagene) in der quergestreiften muskulatur. II. Mitteilung: die physikalisch-chemischen eigenschaften der guanidinophosphorsauren. Biochem Z 196:49–72

Meyerhof O, Lohmann K (1932) Uber energetische wechselbeziehungen zwischen dem umsatz der phosphorsaureester im muskelextrakt. Biochem Z 253:431–461
Mommaerts WF (1992) Who discovered actin? Bioessays 14:57–59
Moss RW (1988) Free radical: Albert Szent-Gyorgyi and the battle over vitamin C. Paragon House Publishers, New York
Needham DM (1960) Biochemistry of muscular action. In: Bourne GH (ed) The structure and function of muscle, vol 2, Academic Press, New York, pp 55–104
Needham DM (1971) Machina carnis: the biochemistry of muscular contraction in its historical development. Cambridge University Press
Parnas JK, Wagner R (1914) Ueber den kohlehydratumsatz isolierter amphibienmuskeln und uber die beziehungen zwischen kohlehydratschwund und milchsaurebildung im muskel. Biochem Z 61:387–427
Rozsa G, Szent-Gyorgyi A, Wyckoff RWG (1950) The fine structure of myofibrils. Exp Cell Res 1:194–205
Schipiloff C, Danilewsky A (1881) Uber die Natur der anisotropen Substanzen des quergestreiften Muskeln und ihre raumliche Verheilung im Muskelbundel. Z f Physiol Chem 5:349–365
Slayter E (1976) Optical methods in biology. Robert E. Krieger Publishing Company, Huntington
Straub FB (1942) Actin. In: Studies from the Institute of Medical Chemistry, vol 2. University of Szeged, Szeged, p 3–15
Straub FB (1943) Actin II. In: Studies from the Institute of Medical Chemistry, vol 3. University of Szeged, Szeged, p 23–37
Straub FB, Feuer G (1950) Adenosinetriphosphate the functional group of actin. Biochim Biophys Acta 4:455–470
Stubel H (1923) Die ursache der doppelbrechung der quergestreiften muskelfaser. Pfl Arch f ges Physiol 201:629–645
Swammerdam J (1758) The history of insects, Pt. II, in Biblia Naturae. Trans T Floyd Seyffert, Lond, p 122–132
Szent-Gyorgyi A (1941–1942a) The contraction of myosin threads. In: Studies from the Institute of Medical Chemistry, vol 1. University of Szeged, Szeged, p 17–26.
Szent-Gyorgyi A (1941–1942b) Discussion. In: Studies from the Institute of Medical Chemistry, vol 1. University of Szeged, Szeged, p 67–71
Szent-Gyorgyi A (1945) Studies on muscle. Acta Physiol Scand 9(suppl 25):1–115
Szent-Gyorgyi A (1948) Nature of life: a study on muscle. Academic
Szent-Gyorgyi A (1949) Free-energy relations and contraction of actomyosin. Biol Bull 96:140–161
Szent-Gyorgyi A (1951) Chemistry of muscular contraction, 2nd edn. Academic, New York
Szent-Gyorgyi A (1952) The structure and chemistry of muscle. A lecture. Albert Szent-Gyorgyi Papers, National Library of Medicine
Szent-Gyorgyi A (1953) Chemical physiology of contraction in body and heart muscle. Academic, New York
Szent-Gyorgyi A (1963) Lost in the twentieth century. Annu Rev Biochem 32:1–15
Szent-Gyorgyi AvN (1965) Nobel lectures. Physiology or Medicine, 1922–1941. Elsevier, p 433–451
Szent-Gyorgyi AG (2004) Interview with Andrew Szent-Gyorgyi. Profiles in science. The Albert Szent-Gyorgyi Papers, National Library of Medicine
Teich M (2003) Dorothy Mary Moyle Needham: 22 September 1896–22 December 1987. Biogr Mems Fell R Soc Lond 49:351–365
von Muralt A (1984) A life with several facets. Annu Rev Physiol 46:1–15
von Muralt AL, Edsall JT (1930a) Studies in the physical chemistry of muscle globulin. III. The anisotropy of myosin and the angle of isocline. J Biol Chem 89:315–350
von Muralt AL, Edsall JT (1930b) Studies in the physical chemistry of muscle globulin. IV. The anisotropy of myosin and double refraction of flow. J Biol Chem 89:351–386

Weber HH (1935) Der feinbau und die mechanischen eigenschaften des myosin-fadens. Pflugers Arch ges Physiol 235:205–233
Weber A (1951) Muskelkontraktion und modelkontraktion. Biochim Biophys Acta 7:214–224
Weber HH (1958) The motility of muscle and cells. Harvard University Press, Cambridge
Weber HH, Portzehl H (1952) Muscle contraction and fibrous muscle proteins. Adv Protein Chem 7:161–252
Weizsacker V (1914) Myothermic experiments in salt-solutions in relation to the various stages of a muscular contraction. J Physiol 48:396–427
Wiener O (1912) Die theorie des mischkorpers fur das feld der stationaren stromung. I. Die mittelwertsatze fur kraft, polarisation und energie. Abd d kon Sachs Gesellsch d Wissensch Mathphys Kl 32:507.
Zotterman Y (1968) The Minnekahda voyage, 1929. In: Fenn WO (ed) History of the international congress of physiological sciences 1889–1968. American Physiological Society, p 3–14

Chapter 2
Birth of the Sliding Filament Model of Muscular Contraction: Proposal

…it is postulated that stretching of the muscle takes place, not by an extension of the filaments, but by a process in which the two sets of filaments slide past each other…one may note the possibility that an analogous process is involved in contraction.

Hugh E. Huxley (1953b)

Koscak Maruyama remembers Jean Hanson shouting: "I know I cannot explain the mechanism yet, but the sliding is a fact" (Maruyama 1995. With permission Oxford University Press)

K. Maruyama (1995)

The motion pictures taken by A. Huxley of living muscle can leave little doubt in the spectator's mind about the basic correctness of the theory. (Szent-Gyorgyi 1960. With permission Elsevier)

A. Szent-Gyorgyi (1960)

2.1 Introduction

The official date of the "birth" of the sliding filament theory of muscular contraction is May 22, 1954. On this day the journal Nature published two papers consecutively under the general title: "Structural Changes in Muscle During Contraction". The first paper by Andrew F. Huxley[1] and Dr. Rolf Niedergerke was entitled: "Interference microscopy of living muscle fibres". The second paper by Dr. Hugh Huxley and Dr. Jean Hanson was entitled: "Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation". But the story of sliding filaments begins before May 22, 1954. In order to understand and appreciate the experiments that were done and why they were done, it is necessary to review the scientific background of each of the investigators.

[1] Andrew Huxley did not work for a Ph.D. at Trinity College in Cambridge and thus he is the only one of the four authors on the classic 1954 papers who is not listed as "Dr.". During his time at Trinity promising young researchers would receive a research fellowship. Alan Hodgkin (1977) also did not work for a Ph.D.

J.A. Rall, *Mechanism of Muscular Contraction*, Perspectives in Physiology,
DOI 10.1007/978-1-4939-2007-5_2

2.2 The Investigators: Andrew Huxley and Rolf Niedergerke, Hugh Huxley and Jean Hanson

Andrew Fielding (A. F.) Huxley[2] (1917–2012) (Fig. 2.1) has described his research in physiology as "the mechanical engineering of living machines" (Huxley 2004a). A substantial part of his work has been the design and construction instruments needed for his research. Huxley conducted his first research with Alan L. Hodgkin (1914–1998) at the laboratory of the Marine Biological Association at Plymouth, England, in the summer of 1939. At that time the 22 year old Huxley had just finished his undergraduate education at Trinity College, University of Cambridge. Hodgkin invited him to join in an attempt to measure the transmembrane resting and action potential in the squid giant axon. The squid giant axon was discovered by the anatomist John Zachary (J. Z.) Young (1936). It is a single axon which is actually a syncytium of many cell bodies and it could reach a diameter of 500 μm or more. Huxley devised a method of inserting an electrode down the center of the vertically mounted axon. This worked at once, but the experiment often failed because the capillary scraped against the surface membrane. Huxley rectified this problem by introducing two mirrors which allowed one to steer the electrode down the middle of the axon by simultaneously viewing the position of the capillary through a horizontally mounted microscope from right to left and front to back. Hodgkin (1992) has commented that Huxley was a "wizard with scientific apparatus" and that he solved technical problems in an incredibly short period of time. This assessment is the first of many examples of Huxley's wizardry in the design of equipment to solve experimental problems. During the summer of 1939, they recorded for the first time an intracellular action potential that exhibited an overshoot above zero potential. This observation was fundamental because it disproved the then prevailing view developed by Bernstein (1902) that the action potential consisted of a disappearance of the resting potential due to a general increase in permeability, allowing all kinds of ions to freely enter or leave the axon. This result was published in a brief letter to Nature (Hodgkin and Huxley 1939). Soon after these experiments were completed there was a stoppage of research because of the start of World War II during which Huxley worked on anti-aircraft gunnery for the next 5 years. Hodgkin and Huxley (1945) eventually published this work in-full.

Andrew Huxley returned to Cambridge and to research in late 1945/early 1946. He was joined in Alan Hodgkin's laboratory by Robert Stampfli (1914–2002) from Alexander von Muralt's institute in Berne. Together they published a series of papers that provided strong evidence for the saltatory conduction of the action potential in single myelinated nerve fibers from the nerves of frogs (Huxley and

[2] Andrew Huxley is a member of the famous Huxley family. His grandfather was Thomas Henry Huxley, the well known nineteenth century biologist who was Charles Darwin's "bulldog" (see footnote #4, Chap. 1). Andrew Huxley's half-brothers were the famous writer Aldous Huxley (1894–1963), author of the book Brave New World, and evolutionary biologist Julian Huxley (1887–1975), the first Director General of United Nations Educational, Scientific and Cultural Organization (UNESCO). For a biography on the Huxley family, see Clark (1968).

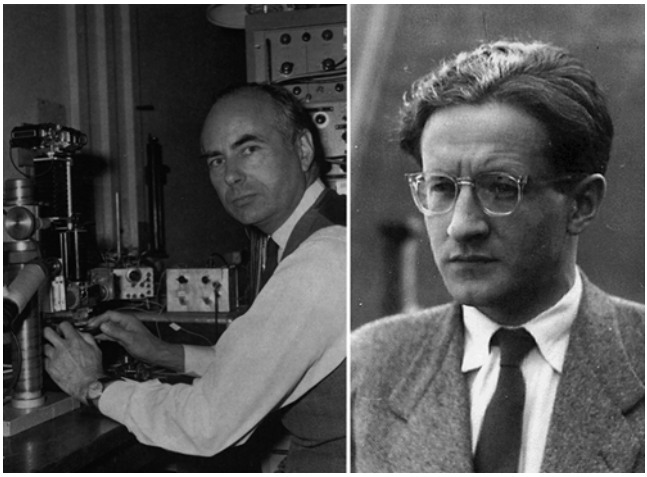


Fig. 2.1 The investigators: Andrew Fielding Huxley (*left*) and Rolf Niedergerke (*right*). Andrew Huxley (1917–2012) received the Nobel Prize along with Alan Hodgkin in 1963 for the elucidation of the ionic mechanism of the nerve action potential. He switched his research field to muscle in 1951 and thereafter made fundamental discoveries relating to muscle structure, activation and cross-bridge function over a period of 40 years. He became Sir Andrew Huxley in 1974. Huxley was the President of the Royal Society (1984–1995) and Master of Trinity College Cambridge (1984–1990). He gave up his laboratory after nearly 60 years of research in 1998. In 2005 the Andrew Huxley building at University College London opened. The building houses researchers from the departments of physiology and pharmacology. He was not related to Hugh Huxley. See an autobiographical sketch (Huxley 2004a). (Photo: Huxley 1974. With permission John Wiley & Sons Inc.) Rolf Niedergerke (1921–2011), born in Germany, came to the Andrew Huxley's laboratory from Berne, Switzerland, in the fall of 1952. After the completion of their collaboration, Niedergerke moved into the cardiac muscle field where he worked for over 40 years as a faculty member in the Department of Biophysics at University College London. His classic paper with Hans-Christoph Luttgau initiated the study of Na–Ca exchange in cardiac muscle (Luttgau and Niedergerke 1958). (Photo: courtesy of S. Page)

Stampfli 1949, 1951a, b). Stampfli remembered this collaboration (Stampfli 1992. With permission Cambridge University Press):

> …many who had difficulties getting their problem straight used Huxley as a human computer. Working with him was thus a great privilege. Not only I, but Hodgkin and Katz, appreciated his unfailing logic and mathematical talent. On such occasions, Huxley not only proved to be a brilliant thinker, but also showed an amazing knowledge of biology, physics, and chemistry and an excellent memory as well.

Hodgkin and Huxley wanted to test their hypotheses related to the ionic mechanism of the action potential. But there was a major problem. The major problem was that the action potential was changing with voltage and time as it traveled down an axon. During 1948 Hodgkin visited Kenneth S. Cole at the University of Chicago and learned that he and George Marmont had developed promising approaches to solving these problems. Marmont (1949) eliminated the propagation of the action

potential in the giant squid axon by developing a "space clamp" wherein the membrane voltage changes occur over an isolated part of the membrane, thus avoiding the complications introduced by spread of current in a cable-like structure. Cole (1949) succeeded in applying electronic feedback to control the membrane current or voltage, "voltage clamp", at a fixed value during an action potential [also see Cole (1968) for a historical perspective]. Hodgkin could see that these techniques would allow a test of their ideas about the ionic mechanism of the action potential. Once back in England in 1948, he and Huxley, along with Bernard Katz (1911–2003), made modifications to the voltage clamp technique. In 1949 they performed the experiments elucidating the ionic mechanism of the action potential and the roles of Na^+ and K^+ in squid giant axons. Amazingly the data that led to the 5 classic papers, 128 pages in all, in the Journal of Physiology (Hodgkin et al. 1952; Hodgkin and Huxley 1952a, b, c, d), and the eventual Nobel Prize for Hodgkin and Huxley in 1963[3], was collected in approximately 1 month on 20 or so squid axons! Hodgkin (1977) believed that they were able to obtain the results so quickly because they had spent a long thinking and making calculations about the kind of system which might produce an action potential of the kind seen in squid nerve. This method of "thinking ten experiments and doing one" was typical of Andrew Huxley's later approach to muscle research.

After collecting the data in 1 month, it took another 2 years to completely analyze the results. Hodgkin (1977) describes numerous reasons for the delay. One of the reasons was that the Cambridge computer was inoperative for 6 months and Andrew Huxley had to use a hand calculator to solve numerically the nonlinear differential equations used to fit the data for the time course of potential change if there were no feedback. It took up to 3 weeks to generate a simulated propagated action potential! Even though the simulations fit the data beautifully, they were disappointed with the results because no mechanism could be found. Hodgkin (1963) believed that no real progress at the molecular level could be made until much more was known about the chemistry and fine structure of the membrane. So they settled for the "more pedestrian aim" of finding a set of mathematical equations which might plausibly represent the movement of electrically charged gating particles (Hodgkin 1977). Even that was not easy. Their formulation is still considered useful today (see Hille 2001).

There are a number of themes that emerged from this research that influenced Andrew Huxley's subsequent approach to solving scientific problems. These include: (1) working with the simplest living tissue possible, preferably single fibers, (2) making time resolved measurements, (3) developing new experimental tools needed to do the best possible experiments, (4) thoroughly analyzing data, (5) generating mathematical relationships to quantitatively explain the data, and (6)

[3] The Nobel Prize in Physiology or Medicine in 1963 was awarded jointly to John Carew Eccles, Alan Lloyd Hodgkin and Andrew Fielding Huxley "for their discoveries concerning the ionic mechanisms involved in excitation and inhibition in the peripheral and central portions of the nerve cell membrane". Hodgkin and Huxley did not work with Eccles, an Australian scientist, who investigated the physiology of synapses (Eccles 1964). Hodgkin (1992) has described the "near-miss" of the Nobel Prize in 1962 and the ceremony in 1963.

thinking carefully about the possible experiments and results before actually doing the critical experiment.

Rolf Niedergerke (1921–2011) (Fig. 2.1) joined Andrew Huxley's laboratory in the autumn of 1952 (Niedergerke and Page 1992). He was born in Germany and at the time was working in Alexander von Muralt's Institute in Berne. Huxley had asked Robert Stampfli to recommend someone who would be capable of dissecting single skeletal muscle fibers. Niedergerke had worked previously on single myelinated nerve fibers. Besides having taught himself the dissection of single muscle fibers, Niedergerke introduced Andrew Huxley to the nineteenth century German literature on light microscopy of muscle (Huxley 2004a). This introduction encouraged Huxley to thoroughly evaluate the nineteenth and early twentieth century literature on muscle structure. This evaluation led to his conclusion that much that was known and accepted in the nineteenth century with regard to muscle striations was subsequently forgotten in the twentieth century (see Sect. 1.2 and Huxley 1957, 1977).

Hugh Esmor (H. E.) Huxley (1924–2013) (Fig. 2.2), who is not related to Andrew Huxley, received his undergraduate education in physics at the University of Cambridge (Huxley 1996, 2004b). After service in the Royal Air Force as a radar officer, he started graduate work at the University of Cambridge in the Medical Research Council (MRC) unit for research on the molecular structure of biological systems in 1948. This unit evolved from the famous Cavendish Laboratory headed by the eminent crystallographer W. Lawrence Bragg and eventually became in 1962 the world famous MRC Laboratory of Molecular Biology which by the early twenty-first century could claim 13 Nobel Prize winners and 14 Nobel Prizes (Fred Sanger won two Nobel Prizes). During Hugh Huxley's time as a graduate student, the laboratory members included John Kendrew (Huxley's Ph.D. advisor), Max Perutz, Francis Crick and James D. Watson. It must have been an incredibly stimulating atmosphere as all four of these scientists would go on to win Nobel Prizes in 1962[4]. Whereas no doubt a stimulating environment, it was not always pleasant to have lunch with Francis Crick who was notorious for challenging the ideas of colleagues. Watson (1968) remembered that Hugh Huxley found it difficult to enjoy Crick's continuous "inquisitive lunchtime attacks". Hugh Huxley was totally immersed in the Cambridge scientific environment from 1948 to 1987 with the exception of a few crucial years on leave at MIT (1952–1954) and time at University College London (1955–1962).

[4] The Nobel Prize in Chemistry in 1962 was awarded jointly to Max Ferdinand Perutz (1914–2002) and John Cowdery Kendrew (1917–1997) "for their studies of the structures of globular proteins". Perutz solved the so-called phase problem and this solution proved to be the breakthrough that opened up the whole field of protein crystallography. Perutz elucidated the 3D structure of hemoglobin and Kendrew the 3D structure of myoglobin. The Nobel Prize in Physiology or Medicine in 1962 was awarded jointly to Francis Harry Compton Crick (1916–2004), James Dewey Watson (b. 1928) and Maurice Hugh Frederick Wilkins (1916–2004) "for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material". They discovered the famous double helix of DNA. See Watson's (1968) entertaining account of the race to the double helix.

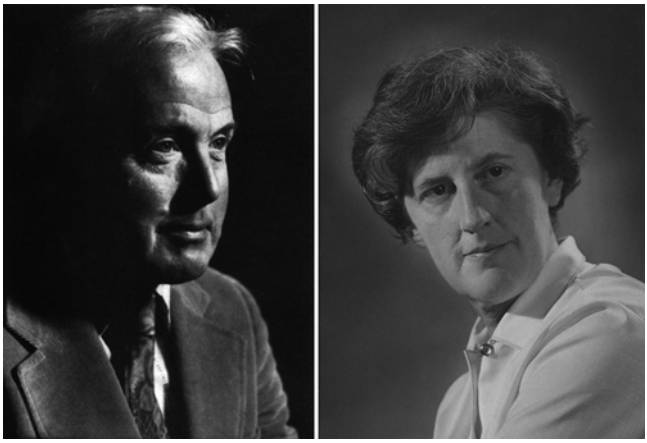


Fig. 2.2 The investigators: Hugh Esmor Huxley (*left*) and Jean Hanson (*right*). Hugh Huxley (1924–2013) investigated the structural mechanism of skeletal muscle contraction for 60 years. He pioneered many advances in utilization of X-ray diffraction and electron microscopy, including ultrathin sectioning and negative staining, for the study of muscle contraction. He was the first person to propose the sliding filament mechanism of muscle contraction (Huxley 1953b) and the titling cross-bridge theory of contraction (Huxley 1969). His demonstration of "arrowheads" in "decorated" thin filaments (actin filaments plus myosin in the absence of ATP) (Huxley 1963) helped open up the field of nonmuscle motility. After retirement from the MRC Laboratory of Molecular Biology in Cambridge in 1988, he continued his research at the Rosenstiel Basic Medical Sciences Research Center, Brandeis University. A fellow of the Royal Society at the age of 36, he received the Copley Medal, the society's highest honor, in 1997. He was elected into the National Academy of Sciences in 2003. (Photo: Huxley 1996. With permission Annual Reviews.) Jean Hanson (1919–1973) pioneered the utilization of phase contrast microscopy to investigate structural changes in contracting myofibrils. She began her collaboration with Hugh Huxley when they were both at MIT in 1953. They collaborated from 1953 to 1960, alternating first authorship on each publication. Thereafter she concentrated on investigating the generality of the sliding filament model of contraction by examining insect and smooth muscles. Along with Jack Lowy, she elucidated the first structure of actin filaments (Hanson and Lowy 1963). Hanson became director of the Biophysics Research Unit at King's College London in 1970 and was at her scientific peak when in 1973 she died suddenly of a meningococcal infection. See a biographical memoir by Randall (1975, photo) and papers in her honor in the J Muscle Res Cell Mot. 25: 2004. Photo: with permission of The Royal Society)

For his graduate student research, Hugh Huxley employed the X-ray diffraction technique to examine the structure of isolated living skeletal muscle. Earlier work by Astbury (1947) utilized wide angle X-ray diffraction to examine muscle structure at the level of a few Angstroms. In contrast Huxley employed low or small angle X-ray diffraction to probe muscle structural repeats in the 100–400 Å range. Since the diffraction angles were small[5] and thus the reflections of interest were close to

[5] The X-ray diffraction pattern is recorded in reciprocal space which means that reflections farther from the origin (wide angle reflections) are due to repeating structures that are close together and reflections near the origin (low or small angle reflections) are due to repeating structures that are further apart.

the undiffracted X-ray beam, a narrow slit in the X-ray camera was required to be able to measure intensities and positions close to the undiffracted beam. This criterion created problems with low X-ray intensity, especially with hydrated biological specimens, like muscle. The net effect is that it required many hours, sometimes days, of illumination to get sufficient intensity of the diffracted X-rays. Thus results were limited to states in which the muscle would be stable for long periods of time, i.e., resting muscle and muscle put in rigor. Huxley was able to record a diffraction pattern from live relaxed muscle isolated from frogs in a few hours for equatorial patterns and a couple of days for axial patterns. Equatorial reflections arise from transverse structural repeats in the muscle and axial reflections (also called meridional reflections) are due to structures that repeat along the length of the muscle. Huxley was unsuccessful in obtaining results from contracting muscle and indeed it would be years before X-ray intensity was sufficient to probe changes in the intensity and positions of reflections associated with muscle contraction (see Chaps. 3 and 6).

On the equator in resting muscle from the frog there were reflections whose relative spacings and intensities suggested that they came from a hexagonal array of rods about 450 Å apart (Fig. 2.3, bottom) (Huxley 1951, 1953a). Huxley speculated that these rods were composed of myosin molecules. On stretching the resting muscle, the transverse distance between rods decreased according to the inverse square root of the muscle length as would be expected from the known constant volume properties of muscle. A X-ray diagram from a muscle in rigor (either from frog muscle or Szent-Gyorgyi's glycerinated rabbit psoas muscle) showed about the same lattice spacings as in the resting muscle but very different relative intensities of the first two lines of the pattern (Fig. 2.3, top) (Huxley 1953a). Huxley speculated that the pattern could be accounted for by the presence of a second set of filaments composed of actin, located at the trigonal positions of the original lattice. The idea was that the existence of myosin-actin linkages in the absence of ATP stabilized the secondary array of filaments in the interstices of the primary array, thus enabling them to be detected by the X-ray diffraction method. This was an important observation and the first time that changes in the X-ray pattern could be related to a change in the state of the muscle (rest to rigor). Based on the early electron microscopic observations of muscle (Hall et al. 1946), Huxley assumed that both sets of filaments ran the total length of the sarcomere.

In contrast the axial X-ray patterns showed a pattern of reflections based on an approximately 420 Å axial repeat which remained unchanged in rigor. Intriguingly, the axial period did not change when the relaxed muscle was stretched. This work which appeared in abstract form (Huxley 1951, 1953a) and in his Ph.D. dissertation was never published in full because the intervening work at the Massachusetts Institute of Technology with Jean Hanson was all-consuming (see below).

The X-ray diffraction technique has both major advantages and disadvantages. A major advantage is that the muscle structure is probed in a living state, at least at rest, whereas electron microscopy requires fixation, staining and embedding which could lead to artifactual changes in structure. In a sense the X-ray technique provides a control for these possible artifacts. There are two major disadvantages.

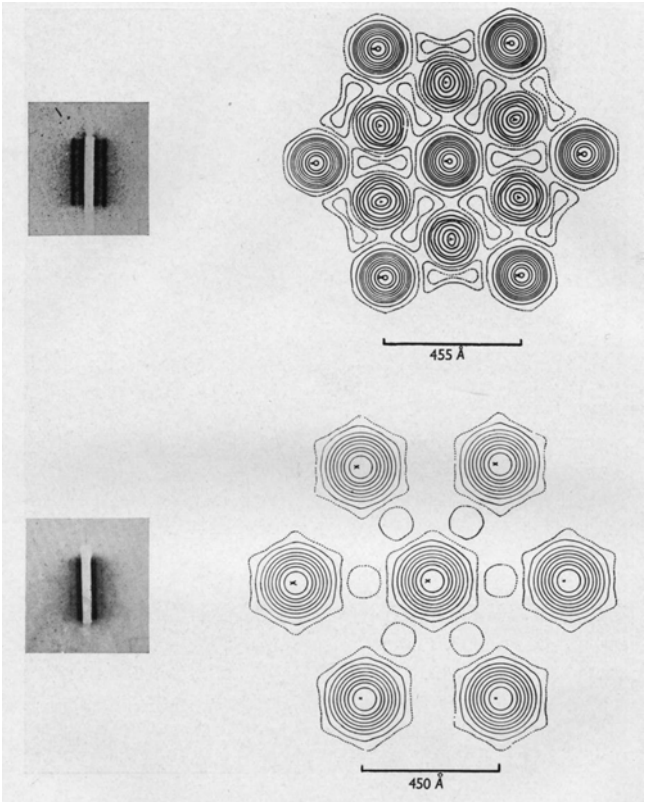


Fig. 2.3 Low angle X-ray diffraction diagrams of muscle at rest or in rigor. Reflections and electron-density distribution from: *Bottom*: resting living muscle isolated from frog and *Top*: glycerol-extracted psoas muscle from the rabbit in rigor. On *left*: transverse (equatorial) reflections. Muscle axis vertical. Two reflections (the one nearer to origin is designated 10 and the outer one 11) are visible as closely space vertical lines on either side of the center slit. These reflections are expected from a hexagonal lattice of filaments separated by about 450 Å. On *right*: possible electron-density distributions in the fiber, seen end-on, based on X-ray reflections. In the resting muscle the primary hexagonal array of filaments is visible and the region in between the filaments is of rather uniform density, suggesting that the material there is randomly arranged. In the rigor muscle, the lattice dimensions are not significantly different, i.e., the reflections are in the same position, but the 11 reflection is now much more intense. This result strongly suggested the presence of a second set of filaments (seen end-on) possibly occurring at specific sites in between the basic hexagonal array of primary filaments (Hanson and Huxley 1955. With permission Elsevier)

The first is the long exposure times required to observe a pattern which, at that time, precluded measurements on contracting muscle. The second disadvantage, at least to the outsider, is the "enigmatic" nature of the X-ray pattern. One measures spacings and intensities and then must deconvolute this information by building a molecular model that can reproduce the observed X-ray pattern. Clearly direct observation by electron microscopy would greatly enhance the conclusions reached

from the X-ray data. In order to learn the electron microscopic technique, Hugh Huxley took a temporary leave of absence from Cambridge and went to Francis O. Schmitt's laboratory at the Massachusetts Institute of Technology in September of 1952 for 2 years.

Throughout his on-going 60 year career, Hugh Huxley has pioneered improvements in the X-ray diffraction and electron microscopic techniques in search for the structural mechanism of striated muscle contraction.

Jean Hanson (1919–1973) (Fig. 2.2) was trained as a zoologist. She joined the Biophysics Research Unit at King's College, London, in 1948 (Randall 1975). Schick and Hass (1950) and Perry (1951) had recently shown that it was possible to isolate myofibrils, from mammalian skeletal muscle, which exhibited unimpaired function (ATPase activity) and normal structure (striation pattern). Hanson (1952) employed phase contrast microscopy[6], which was a relatively new technique to biology at that time, to examine changes in the striation pattern of these "living" skeletal muscle myofibrils. These myofibrils isolated from various skeletal muscles were "living" in the sense that they were not fixed or stained as was the convention for microscopic observation at that time. Also the myofibrils were excellent microscopic objects since they were only 1–2 μm in diameter. She examined, in a phase-contrast microscope, the changes in band-pattern that took place when myofibrils contracted during treatment with ATP. The experiment was ingeniously simple: a drop of dilute ATP solution was placed at one edge of a coverslip and drawn through the preparation by means of filter paper placed at the opposite edge. The myofibril contracted slowly (about 10 s) to about 60 % of its original length. Before treatment, the myofibril had well-marked A and I bands (respectively black and white in positive phase-contrast illumination), with black Z lines (2.6 μm apart), and with a white line in the middle of each A band. During the earliest phase of contraction, the I bands rapidly disappeared; the myofibril became uniformly dark grey in color and no bands could be distinguished in it. Then a series of sharply defined black lines, the contraction bands, appeared. The zones between the contraction bands became progressively paler, and further contraction brought the contraction bands closer together. The fully contracted myofibril had a simple pattern of narrow contraction bands, 1.5 μm apart.

[6] Phase contrast microscopy. There is little absorption of light rays passing through living cells and thus they are essentially transparent. The cells do contain constituents that exhibit small differences in refractive index. These inclusions do not affect the amplitudes of the light rays but do cause the light waves to differ in phase according to the path that they have taken through the cell. The image formed by such rays consists of a pattern of phase differences of uniform brightness, and as such is essentially invisible. Frederick Zernike, of Groningen, produced a visible image in these circumstances by deliberately advancing or retarding the main beam, after it traversed the specimen, by one-quarter of a wavelength, without disturbing the diffracted rays. Consequently, when the whole beam was reunited, conditions for interference existed, and the transparent specimen produced an image where refractive index differences are now observed as differences in transparency. Thus changes in phase became changes in intensity. Zernike received the Noble Prize for this discovery in 1953. For more information on phase contrast microscopy, see Slayter (1976).

Even though Jean Hanson was an expert using phase contrast microscopy to examine muscle striations, she, like Hugh Huxley, wanted to extend her vision of muscle by learning electron microscopy. Thus Jean Hanson also went to Francis Schmitt's laboratory in February of 1953. There she met Hugh Huxley for the first time and a remarkable collaboration began. Hugh Huxley saw the banding pattern of muscle for the first time while looking through the microscope at Jean Hanson's myofibrils. It wouldn't be the last time.

Thus we see that the investigators came from very different scientific backgrounds and brought different but in the end complementary approaches to the problem of measuring and interpreting the changes in the striation pattern during skeletal muscle contraction and stretch.

2.3 Overlapping Arrays of Filaments and the First Proposal of Sliding Filaments

The first paper from the Hugh Huxley and Jean Hanson collaboration appeared in September of 1953 in Nature (Hanson and Huxley 1953). It was in this paper that the first evidence was provided for the overlapping arrays of filaments containing actin and myosin. Using phase contrast and polarized light microscopy and electron microscopy, they examined structural changes in myofibrils at various stages of extraction of myosin. In isolated myofibrils irrigated with a myosin extraction solution containing 0.6 M KCl the "A-substance" disappeared in 1–2 s. This result was confirmed with electron microscopy as the thick filaments were no longer visible and only thin filaments remained in the A band. From these results, they concluded that myosin is primarily concentrated in the A band in muscle and that it is responsible for the high density and the birefringence of the A bands and furthermore that actin is present as long filaments which extend continuously through the A and I bands. That myosin was concentrated in the A band and accounted for the myofibril birefringence was a confirmation of earlier, more indirect, studies (see Sect. 1.4). This observation also eliminated the possibility that myosin filaments extended the whole length of a sarcomere as was earlier proposed. Thus there are two sets of filaments and these myosin and actin filaments overlap. This was a crucial observation.

The official birth of the sliding filament model of muscle contraction is associated usually with the classic papers of Huxley and Niedergerke (1954) and Huxley and Hanson (1954). In fact the model was first proposed by H. E. Huxley in the August of 1953 (Huxley 1953b) based on electron micrographs generated in F. O. Schmitt's laboratory. Huxley employed a thin sectioning technique developed in the Schmitt laboratory by Alan Hodge, David Spiro and himself (Hodge et al. 1954). Huxley examined transverse sections of frog sartorius and rabbit glycerinated psoas muscle in the electron microscope. He described what he called "a most remarkable compound array of filaments". Two different types of filaments were present. The

larger filaments formed a very regular hexagonal array. They were spaced 200–300 Å apart[7] and their diameter was about 110 Å. The smaller filaments, whose diameter was about 40 Å were also arranged in a regular manner. Each one was located symmetrically in between three of the primary filaments, which it shared with the six nearest neighbors. In the H zone a simple hexagonal array of filaments was observed and no secondary filaments were ever observed. Huxley also claimed to observe "bridges" extending between the filaments but the results are unconvincing on this point. Thus the electron microscopy results confirmed Huxley's speculation based on the X-ray diffraction results that there are two arrays of filaments. Furthermore these results and those of Hanson and Huxley (1953) indicated that these filaments *partially overlapped* rather than running the whole length of the sarcomere as Huxley and others had previously assumed.

In the last paragraph of the discussion, Huxley (1953b) introduced the concept of sliding filaments into the literature for the first time (Huxley 1953b. With permission Elsevier):

> This phenomenon finds a ready explanation in terms of the arrangement of actin and myosin filaments described above, if it is postulated that stretching of the muscle takes place, not by an extension of the filaments, but by a process in which the two sets of filaments slide past each other; extensibility will then be inhibited if the myosin and actin are linked together. In terms of the distribution of actin and myosin described above, this process clearly involves 'I-band filaments' being pulled out of the A-band during stretch…It is not considered appropriate to discuss here the various models which may be devised to describe the details of such a mechanism…However, one may note the possibility that an analogous process is involved in contraction.

With regard to the statement that it is *not considered appropriate* to discuss various models of contraction, Huxley (2008) has said: "Our otherwise very amiable and supportive department head at MIT, Professor Schmitt, remained quite skeptical and forbade us to say anything about possible contraction mechanism in our 1953 paper about the overlapping filament model-'Do not spoil a good experimental paper with a lot of speculation'!" (Huxley HE 2008. With permission Elsevier) Thus Huxley only noted the "possibility that an analogous process is involved in contraction" in the last line of the paper. From a historical point of view, this restriction might have resulted in an unintended injustice to Hanson and Huxley. Nonetheless these fundamental observations and this hypothesis strongly influenced the thinking of Hugh Huxley and Jean Hanson as they devised future experiments with the phase contrast and electron microscope to test this hypothesis.

Over 50 years later, Hugh Huxley expressed some regret that Jean Hanson's name wasn't associated with the first proposal of the sliding filament model of contraction. Huxley (1996) stated: "Looking back on it now, it might have been fairer to have associated Jean somehow with this suggestion at that time because a vital part of its genesis was our discovery of the partially overlapping filament arrays."

[7] Hugh Huxley noted (1953b) that the differences in spacings of the elements in the hexagonal array as observed in the X-ray pattern (450 Å) of living muscle isolated from frogs and in the electron micrographs (200–300 Å) must be indicative of shrinkage of the tissue in preparation for electron microscopy.

(Huxley 1996. With permission Annual Reviews) It is clear that they were equal partners in the development of the hypothesis and collecting supportive data. In support of this conclusion, they had agreed to alternate first authorship on successive joint papers (Huxley 2008). Their research collaboration resulted in six major publications (four with original research and two major reviews) from 1953 to 1960 with first authorship alternated on successive publications.

So it is clear that before the publication of the classic 1954 papers, Hanson and Huxley had the idea of partially overlapping, sliding filaments already in their mind. Andrew Huxley and Rolf Niedergerke also came to the same conclusion in 1953.

2.4 Andrew Huxley and the Development of an Interference Microscope

After analyzing and publishing the work with the voltage clamp, Andrew Huxley (2004a) could not see how to carry the analysis of excitation and conduction to a deeper level. He and Hodgkin predicted the existence of "gating currents", currents that would control ion permeability, but could not detect them[8]. So Huxley was looking for new experimental challenges. He became interested in muscle after giving lectures to first-year students in Trinity College in 1948. More accurately he became interested in the light microscopy of muscle. It was the so-called "reversal of striations" and formation of "contraction bands" observed with muscle shortening described by the nineteenth century microscopists that caught his attention. Huxley (2004a) thought that this observation might give a clue to the mechanism of contraction. Plus it was of interest to him because of his interest in microscopy.

The challenge as Huxley saw it was to determine the changes in the striation pattern of living, vertebrate skeletal muscle fibers during activation, force development and shortening. The experimental preparation of choice was the single skeletal muscle fiber of the frog for two primary reasons. First, much was known about the mechanical and energetic properties of frog skeletal muscle from the work of Hill (1965) and others. Second, and possibly more important, Frank W. Ramsey and his wife Sibyl F. Street (1940) showed that it was possible to dissect frog muscle fibers in the living state and do elegant mechanical experiments with them.

These proposed experiments presented numerous challenges but foremost in Huxley's mind (Huxley 2004a) was the fact that with the ordinary light microscope it was virtually impossible to obtain a satisfactory image of the refractive index differences associated with the striations of frog muscle fibers since these fibers exhibit large diameters ranging from about 50 to more than 100 μm. Polarized light microscopy would give a satisfactory image but the nineteenth century work had shown that the phenomenon of reversal of striations does not show up with polarized light. Phase

[8] The gating current was not actually detected until the 1973 by C. M. Armstrong and F. Bezanilla in sodium channels of the squid giant axon and by M. F. Schneider and W. K. Chandler (1973) in frog skeletal muscle excitation-contraction coupling.

contrast microscopy shows refractive index differences well on thin specimens, like the myofibrils employed by Jean Hanson and Hugh Huxley, but not on thick specimens such as frog muscle fibers. Andrew Huxley concluded that what was needed was an interference microscope. He envisioned an interference microscope in which the light that passed through the muscle fiber was combined with coherent light that had bypassed the fiber; the path differences due to the refractive index differences in the fiber would then be converted to intensity differences by interference and could be observed unambiguously by eye or recorded photographically. It is clear that a main attraction to this aspect of muscle research was that it allowed Andrew Huxley the opportunity to pursue his deep interest in light microscopy, an interest that he had harbored since boyhood. So Andrew Huxley, the optics expert and the wizard at solving experimental problems, decided to build his own interference microscope.

The high powered version of this interference microscope (Huxley 1954) was operational when Rolf Niedergerke arrived in Cambridge in the autumn of 1952. During the months of March, 1953 to January, 1954 Huxley and Niedergerke did the experiments that constituted the results that appeared in the classic 1954 paper in Nature (Huxley and Niedergerke 1954) and in the later full publication (Huxley and Niedergerke 1958). In March of 1953 they made a cine film of muscle fiber shortening in response to a slowly increasing current (Huxley 1977). During the shortening, the A band remained at a constant length and contraction bands appeared. Huxley has described what they saw (Huxley AF 1977. With permission Cambridge University Press):

> The contraction bands we were looking for did appear, but not where we expected: as the fibre shortened below it's slack length the first 'contraction band' to appear was a narrow dense line at the middle of the A band, not opposite the middle of I. On more extreme shortening, however, a second set of dense lines did appear at the latter position. These bands would be nicely explained if, in addition to the rodlets needed to explain the constancy of the A-band width, there was a second set of filaments in each repeat of the striation pattern, crossing the I band and overlapping with the A-band rodlets. The first set of dense lines would then be due to collision between successive sets of these I filaments, and the second set of dense lines would be due to collision between successive sets of A-band rodlets.

This film suggested to Huxley and Niedergerke that muscle shortening occurred via a sliding filament system. Thus Hugh Huxley, Jean Hanson, Andrew Huxley and Rolf Niedergerke were all thinking in terms of muscle shortening occurring via a sliding filament system before the summer of 1953. But neither group was aware of the results of the other group until the summer of 1953.

During that summer, Hugh Huxley and Jean Hanson exchanged experimental results and interpretations with Andrew Huxley when they met for the first time at the Marine Biological Laboratory at Woods Hole. Andrew Huxley spent the summer of 1953 at the Marine Biological Laboratory at Woods Hole. There he learned of the experiments of Hasselbach (1953) who had dissolved away the myosin from fragmented muscle and examined the residue with the electron microscope, finding that the actin was in the form of filaments held together at their centers by the Z line. This result suggested to Andrew Huxley that the second set of filaments that he and Niedergerke postulated were composed of actin. Later during that visit, he met Hugh Huxley and Jean Hanson. He told them of their observations with the interference

microscope and the idea that length changes in muscle took place by relative sliding movements of two interdigitating sets of filament. They showed him the electron micrographs of transverse sections of muscle that established the existence of two sets of filaments that subsequently were published by Huxley (1953b) later that same year with the brief mention of the sliding-filament theory. They agreed to communicate again when papers were nearing the publication stage (Huxley 1977) and in 1954 agreed to publish the papers together in the journal Nature.

2.5 Birth of Sliding Filaments: Nature, Volume 173, Pages 971–976, May 22, 1954[9]

The investigators, their backgrounds, experimental tools and experimental evidence generated before 1954 have been described. Now it is time to consider the two classic papers that represent the "birth" of the sliding filament model of muscle contraction. But in what order should the papers be considered? Surely it makes little difference. Just as it makes little difference in what order the papers were published in Nature in 1954 since they were published back-to-back. But it turned out that the order of publication made a great deal of difference to Hugh Huxley and Jean Hanson. Many years later Hugh Huxley remembered (Huxley HE 2008. With permission Elsevier):

> 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 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, 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 pleased to have our ideas confirmed by the intact muscle data.

In a sense this discussion relates to the one considered in Chap. 1 concerning the relevance of data collected from "dead" muscle pieces, i.e., myofibrils, to that collected from intact, living muscle. Nonetheless history has declared that because the data and conclusions in the two papers so strongly reinforced each other that the order of publication or discussion makes little difference. In fact we will go from Andrew Huxley/Rolf Niedergerke to Hugh Huxley/Jean Hanson not because one paper is more important than the other but because the observations on living muscle in the first paper were extended in the second paper on myofibrils in crucial ways that led to a deeper understanding of and support for the basic hypothesis.

[9] In these classic papers, Andrew Huxley and Rolf Niedergerke referred to the "widths" of the A and I bands whereas Hugh Huxley and Jean Hanson referred to the "lengths" of the A and I bands. In this context "width" and "length" meant the same thing. The quotations from these papers will be unchanged but elsewhere in the chapter, reference will be made to the "lengths" of the A and I bands. Also the H band and H zone were sometimes referred to interchangeably. We will refer to the H zone.

2.6 Interference Microscopy of Living Muscle Fibres by A. F. Huxley and Dr. R. Niedergerke. Nature. 173: 971–973, 1954

The rationale for the experimental approach in this first publication by Huxley and Niedergerke in the muscle field has been described. Huxley and Niedergerke chose to employ interference microscopy to monitor changes that might occur in striation spacing during passive stretch, activation, isometric contraction, and isotonic contraction of single muscle fibers, diameters 30–80 μm, isolated from frogs. To make the time resolved measurements of striation spacing, they employed cine photography with light flashes that were synchronized with the stimulation pulses that occurred at intervals of about 20 ms. These were technically demanding experiments since the experiments were conducted at room temperature where the isometric twitch reached a peak tension in about 40 ms (Huxley and Niedergerke 1958).

Passive stretch When the sarcomere length of the fiber was changed either slowly or rapidly from about 2.0–4.2 μm almost the whole of this change of length took place in the I-bands (Fig. 2.4). The length of the A band remained constant at 1.4–1.5 μm except for a fall to about 1.3 μm as the sarcomere length was reduced in the range of 2.5–2.0 μm. Huxley and Niedergerke felt that this fall may not be real since its amount was less than the resolving power of the optical system.

Isometric twitches No changes were observed in the lengths of the A and I bands during the time course of an isometric contraction.

Isotonic contractions During active muscle shortening in an isotonic twitch or short tetanus starting at an initial length of about 3.2 μm, the I band became narrower and the width of A band remained constant down to a sarcomere length of 2.5 μm and thereafter falling slightly down to a sarcomere length of 2.0 μm (Fig. 2.5). On further shortening below 2.0 μm, Huxley and Niedergerke observed a shortening of the A bands in all cases but there were additional phenomena which were not the same in every experiment. The full meaning of the statement that the phenomena were not the same in every experiment becomes clearer in the full publication of this work that appeared in 1958. This point will be taken up under "*loose ends*" below.

Summary and conclusions In summary these results showed that the changes in the ratio of widths of the A and I band depend simply on the length of the fiber and are unaffected by activation or by tension development. Huxley and Niedergerke went on to conclude (Huxley and Niedergerke 1954. With permission Nature Publishing Group):

> The natural conclusion, that the material which gives the A-bands their high refractive index and also their birefringence is in the form of submicroscopic rods of definite length, was put forward by Krause, and receives strong support from the observations reported here. The identification of the material as myosin (Hasselbach 1953 and Hanson and Huxley 1953), and the existence of filaments (presumably actin) extending through the I-bands and into the adjacent A-bands, as shown in many electron microscope studies, makes very attractive the hypothesis that during contraction the actin filaments are drawn into the A-bands,

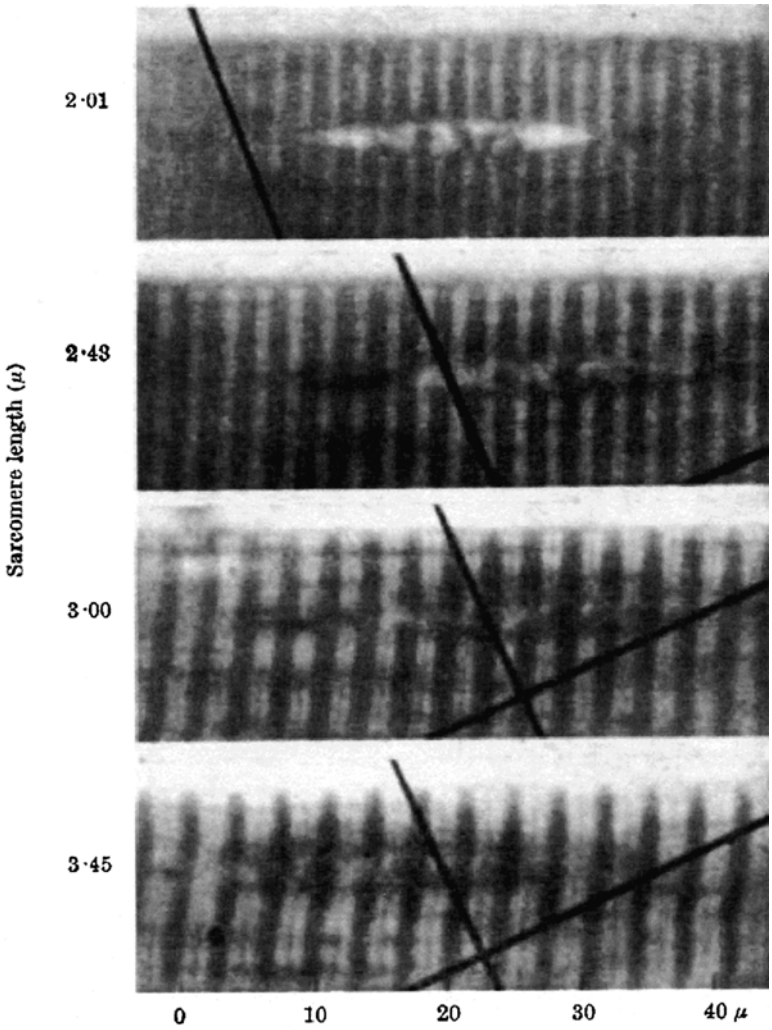


Fig. 2.4 Changes in the band pattern during passive stretch of a single living muscle fiber. Sarcomere lengths are indicated beside the photographs. Almost all the change of sarcomere length is in the I band (*light*) with little change in A band (*dark*) length (Huxley and Niedergerke 1954. With permission Nature Publishing Group)

> between the rodlets of myosin. (This point of view was reached independently by ourselves and by H. E. Huxley and Jean Hanson in the summer of 1953. It has already been mentioned by one of those authors [Huxley, 1953] and is further discussed by them in the accompanying article.)

Thus Andrew Huxley and Rolf Niedergerke and Hugh Huxley and Jean Hanson independently and essentially simultaneously developed the concept of the sliding filament model of muscle contraction. But Andrew Huxley and Rolf Niedergerke

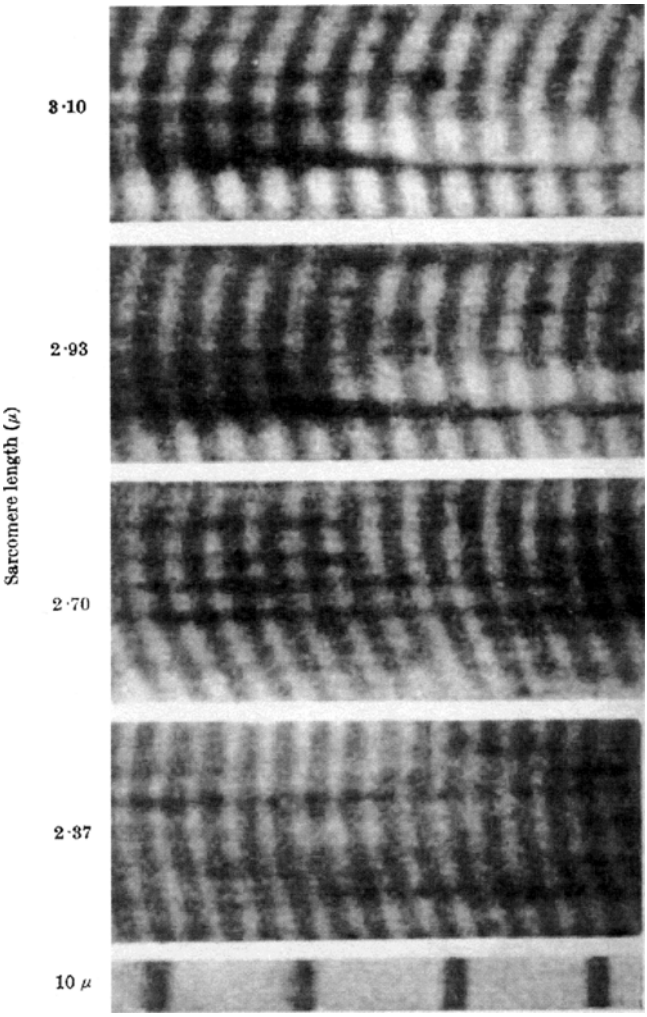


Fig. 2.5 Changes in the band pattern when a single living muscle fiber shortens during a brief isotonic tetanus. Sarcomere lengths shown beside the photographs. As observed with passive stretch (Fig. 2.4) the A bands (*dark*) remain almost constant in length as the muscle fiber shortens (Huxley and Niedergerke 1954. With permission Nature Publishing Group)

clearly acknowledged that Hugh Huxley was the first to propose in the literature a sliding filament model of muscle contraction (also see Huxley 1977).

Predictions They went on to make three important predictions concerning muscle behavior if the sliding filament model is correct (Huxley and Niedergerke 1954). The first prediction was the most crucial prediction. If a relative force between actin and myosin is generated at each of a series of points in the region of overlap in each sarcomere, then the tension per filament should be proportional

to the number of these points, and therefore to the width of the zone of overlap. If the myosin rods are 1.5 μm long and the actin filaments 2.0 μm, the isometric tetanus tension should fall linearly as the fiber is stretched over the range of sarcomere lengths from 2.0 to 3.5 μm. This prediction was in "fair agreement" with observation. The "fair agreement with observation" refers to the experiments of Ramsey and Street (1940) who observed a linear fall of isometric force as a muscle fiber was stretched beyond rest length. But Huxley and Niedergerke no doubt realized that the "fair agreement" may not be a precise agreement. In fact analysis of the mechanical results of Ramsey and Street, who did not measure sarcomere lengths, suggested that a muscle fiber may still generate appreciable isometric tension at lengths beyond 3.5 μm. This discrepancy was real and it turned out to be associated with a difficult technical problem that was solved in two classic papers by Gordon et al. (1966a) (see Chap. 3).

The second prediction was that the speed of contraction should be correlated with the resting sarcomere length. Muscles with long sarcomeres should shorten more slowly than muscles with short sarcomeres. This would be expected if the relative sliding velocity between actin filaments and myosin rods in any one zone of overlap was the same for muscles of different sarcomere lengths since the number of sarcomeres shortening in series per unit length is inversely proportional to sarcomere length. This prediction has not been adequately tested, in part, because only later was it realized that myosin molecules came in various isoforms that split ATP at different rates and thus resulted in different velocities of muscle shortening (Barany 1967). The third prediction was that a muscle with longer sarcomeres would be capable of producing a greater isometric tension. This prediction is based on the idea that there would be more overlap of thick and thin filaments and thus more tension generating structures in parallel which would lead to greater tension development. This prediction has been verified in arthropod muscles which exhibit long sarcomeres and thus increased overlap of thick and thin filaments (Jahromi and Atwood 1969).

Loose ends The full publication of these results didn't appear until 1958 (Huxley and Niedergerke 1958). Whereas it isn't completely clear why there was such a long delay between the initial publication in Nature and the full publication in the Journal of Physiology, long delays to full publication would be a recurring characteristic of the Huxley laboratory. Numerous reasons may have contributed to the delay in publication. First, there were other important experiments on-going [see Huxley and Taylor (1958) in Chap. 4]. Second, considerable time was spent developing an important theoretical model of muscle contraction (Huxley 1957). Third, the analysis of the data was exceedingly thorough with micro-densitometer tracings of the photographic negatives to obtain an unbiased measure of band lengths. Finally there were some loose ends.

These experiments were originally planned to determine whether contraction bands (and reversal of striations) were formed and if so under what conditions. As it turned out their results were equivocal on this point. When fiber shortening was induced by a current that increased gradually over a period of a few seconds, two different types of changes were observed on different occasions. In March and April

of 1953, upon shortening from 2.0 to 1.8 μm, the A bands became gradually shorter until they appeared as thin lines and then on shortening to about 1.7 μm another thin dense line (corresponding to a contraction band) appeared midway between each two members of this series. These results suggested a sliding filament model of contraction, i.e., the first set of dense lines being formed by folding of the ends of the secondary filaments when they meet at the centers of the A bands and the second set (contraction bands) by folding of the primary filaments when they meet at the Z lines (Huxley and Niedergerke 1958).

But in later experiments (January 1954), the ratio between the lengths of the A and I bands did not change noticeably as the sarcomeres shortened from 2 μm down to 1.5 μm (Huxley and Niedergerke 1958). In other words the second series of experiments indicated a progressive shortening of the A bands without disappearance of the I bands, a result inconsistent with the sliding filament model. This discrepancy must have been very troubling to them but since they couldn't see how either experiment was wrong, they had no choice but to report both results in the full 1958 paper even though they were in conflict with each other. They concluded that the formation of contraction bands was observed only in extreme shortening, and then only occasionally.

It was only some years later that Andrew Huxley and postdoctoral fellow Albert M. Gordon (1962) discovered the reason for this discrepancy. With the slowly increasing current of stimulation, the myofibrils near the surface of the fiber remained straight while shortening whereas the myofibrils in the interior became wavy in appearance. In the straight fibrils the contraction-band pattern developed, while in the wavy fibrils narrow I bands remained and the A bands shortened progressively. It was concluded that the contraction-band pattern corresponded to active shortening whereas the pattern with shortened A band corresponded to passive shortening due to contraction of the surface myofibrils. Thus they concluded that these results supported the sliding-filament theory of muscular contraction. Nonetheless there is still some mystery related to the original results. Why did Huxley and Niedergerke get the correct answer in March and April of 1953? Many years later, Andrew Huxley (1977) commented that there was still no explanation why that preparation in early 1953 gave contraction bands, indicating active shortening, over its whole cross-section while later preparations appeared to contract only very locally unless they gave action potentials. If the January 1954 results were obtained in March and April of 1953, Andrew Huxley would have gone to Woods Hole in the summer of 1953 with a very different interpretation of the band pattern changes with extreme muscle shortening than that reached by Hugh Huxley and Jean Hanson and history might have turned out differently.

With regard to the sliding filament model of contraction, Huxley and Niedergerke (1958) emphasized that their results established a number of points relevant to the theory of contraction which could only be demonstrated on whole fibers. These points included: (a) the constancy of A band length, and other changes of the striation pattern required by the theory, were demonstrated in fibers in which the contractile system was in a normal condition, (b) the A band length was shown to be

independent not only of fiber length but also of tension and of activation in twitches and tetani and (c) the A band length was shown to be unaffected by rapid stretch.

Thus living muscle fibers exhibited changes in the striation pattern with changes in length that were consistent with a sliding filament model of contraction. But were these results sufficient to prove the correctness of the proposed model? The results were certainly consistent but not really sufficient to prove the theory. There was a crucial missing piece to the puzzle. Huxley and Niedergerke made no comment about changes in the H zone length with changes in fiber length in their 1954 paper but did state in the 1958 paper that the H zone was not sufficiently sharply outlined to justify measurement. If there is truly relative sliding of filaments with changes in muscle length, there must be predictable changes in the length of the H zone. Hugh Huxley and Jean Hanson were very aware of this fact and explored this point, and many others, carefully.

2.7 Changes in the Cross-Striations of Muscle During Contraction and Stretch and Their Structural Interpretations by Dr. Hugh Huxley and Dr. Jean Hanson. Nature. 173: 973–976, 1954

Hugh Huxley and Jean Hanson employed the isolated myofibril preparation that she had developed earlier (Hanson 1952) and that they were currently using (Hanson and Huxley 1953). With their 2 μm diameter, the rabbit myofibrils were ideal objects for phase contrast microscopy. The contraction of the myofibrils in the presence of low levels of ATP was much slower than would have occurred in intact muscle and thus allowed them to photograph the changes in band pattern as they occurred. Plus they often worked at 2 °C to further slow contraction. They also developed an ingeniously simple technique to stretch the myofibrils. A suspension of myofibrils, mounted as a very thin layer on a slide under a coverslip, was examined in the microscope until a myofibril was found with one end embedded in a fiber fragment adhering to the coverslip, and its other end in a fragment attached to the slide. Movement of the coverslip in the appropriate direction produced the desired stretch or would permit the myofibril to shorten if ATP was present. Whereas these experiments were not demanding in the technical sense that the Andrew Huxley and Rolf Niedergerke experiments were demanding, they were demanding in another sense. Huxley (2004c) remembered that it was grueling work with long hours peering through the microscope in the cold room, searching for the ideal myofibril and the ideal contraction series. They took turns, a few days at a time, because of the eye strain and both worked very long hours.

Myofibrils contracting freely at room temperature in ATP Using cine photography, they found that the I bands shortened from a resting length of about 0.8 μm until they disappeared completely (Fig. 2.6). During this shortening the A bands remained constant at about 1.5 μm. Importantly, they observed changes in density

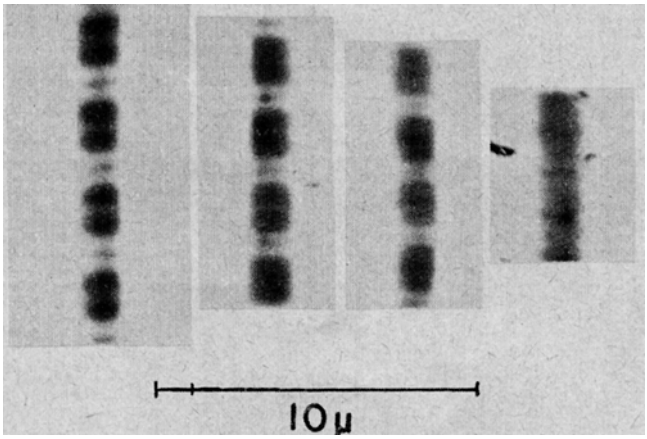


Fig. 2.6 Myofibril contraction photographed in the phase contrast microscope. The same four sarcomeres of one myofibril photographed during contraction (from *left* to *right*) induced by ATP from rest length down to 50 % of rest length where contraction bands formed. Note almost no change in A band length despite dramatic change in sarcomere length. Also note on the extreme left the light region in the A band (H zone) and its disappearance with sarcomere shortening. Magnification: ×4,000 (Huxley and Hanson 1954. With permission Nature Publishing Group)

within the A band during shortening. The H zone, originally of low density, first became indistinguishable from the rest of the A band and was then replaced by a narrow zone which was more dense than the rest of the A band. At a slightly shorter sarcomere length, a very dense line became visible at either end of the A band. When the I bands disappeared at about 65 % rest length, contraction bands formed at the lines of contact of adjacent A bands. Thus their results provided the missing piece of the puzzle that Huxley and Niedergerke (1958) could not resolve in their experiments. In fact they acknowledged this in the their 1958 paper when they pointed out that the crucial demonstration of variation of the H band width with sarcomere length was given by Huxley and Hanson (1954) on isolated myofibrils. Also the results of Hugh Huxley and Jean Hanson, somewhat ironically, provide a more consistent demonstration of the contraction bands that Andrew Huxley sought to study than did the Huxley and Niedergerke results.

Isometric contraction of myofibrils in ATP The A and I band lengths were unchanged during isometric contraction.

Passive stretch Only the I bands changed in length. The A bands remained at constant length but the central region become somewhat less dense, as though the H-zone were lengthening; the length of the less dense region increases as stretch proceeds. The process was reversible.

Myosin extraction Using the myosin extraction procedure developed by Hasselbach (1953), they found that the "ghost" myofibrils contained a faint backbone structure with a density which was about the same as that of the original I bands and thus they believed that the backbone was actin (Fig. 2.7). In stretched

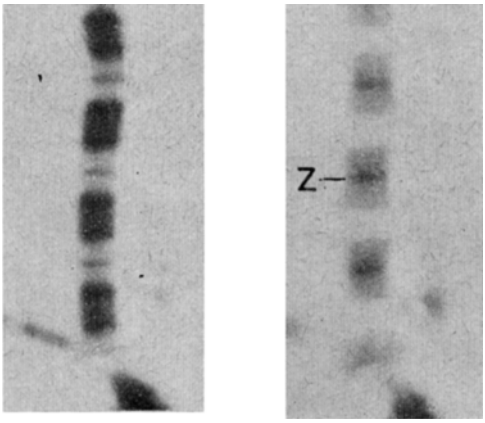


Fig. 2.7 Stretched myofibril (115 % of rest length) before (*left*) and after (*right*) extraction of myosin. Photographs of extracted fibrils were printed by Huxley and Hanson so as to give adequate contrast and the myofibrils were in fact less dense than they appear here (Huxley and Hanson 1954. With permission Nature Publishing Group)

myofibrils, where there was originally a longer zone of low density in the center of the A band, the length of the gap was correspondingly greater than in an extracted myofibril at rest length. Myosin extraction of shortened myofibrils did not change the contraction bands. Surprisingly, the ghost myofibrils were still structurally intact. Although no material was visible in this gap in extracted myofibrils, they concluded that some structures must bind to the actin filaments and cross the gap because these myofibrils could be stretched elongating the gap and shortened spontaneously when released. This was a reversible process. They provisionally called these structures S-filaments because they could be stretched.

Electron microscopy They also described preliminary results with electron microcopy of stretched and contracted myofibrils before and after myosin extraction that were consistent with the light microscopic results but showed no images.

Discussion and conclusions They concluded that a "fairly simple model" could explain the results. In their words (Huxley and Hanson 1954. With permission Nature Publishing Group):

> The backbone of the muscle fibril is made up of actin filaments which extend from the Z-line up to one side of the H-zone, where they are attached to an elastic component… which for convenience we will call the S-filaments. The S-filaments provide continuity between the set of actin filaments associated with one Z-line and that associated with the next…Myosin filaments extend from one end of the A-band, through the H-zone, to the other end of the A-band, and their length is unaltered by stretch or by contraction down to the point where the sarcomere length is equal to the length of the A-band; when contraction beyond this point takes place, the ends of the myosin filaments fold up and contraction bands form. Thus myosin and actin filaments lie side by side in the A-band and, in the absence of adenosine triphosphate, cross-linkages will form between them; the S-filaments are attached to the myosin filaments in the centre of the A-band by some more permanent cross-linkages.
>
> In this model, plastic stretch takes place when the actin filaments are partly withdrawn from the A-band, leaving a long lighter central region and stretching the S-filaments. Only the I-bands and the H-zones increase in length, the length of A-band remaining constant…

> Contraction takes place in this model when the actin filaments are drawn into the A-band (until the H-zone is filled up) and are then folded up in some way to produce more extensive shortening. Thus, when the model is allowed to shorten, only the I-bands decrease in length until adjacent A-bands are pulled into contact with the Z-lines…

They speculated further that the driving force for contraction might be the formation of actin-myosin linkages when ATP, having previously displaced actin from myosin, was enzymatically split by the myosin. Thus it is clear that Hugh Huxley and Jean Hanson had developed a very deep vision, with strong supporting evidence, of a sliding filament theory of muscle contraction.

By the autumn of 1954 Jean Hanson and Hugh Huxley had returned to their respective laboratories in London and Cambridge. They soon followed up on their 1954 publication with a publication (Hanson and Huxley 1955) emanating from a presentation given by Jean Hanson at a Symposium of the Society for Experimental Biology held at Leeds, England, in September of 1954. In this paper she and Hugh Huxley reviewed their previous results and the results of others and added to them with more electron microscopic images and more extraction experiments where now not only myosin but also actin was extracted from myofibrils. Also they took the opportunity to speculate more freely than was possible in the earlier papers. It is a very important review and it deserves wide recognition.

Hanson and Huxley did further extraction experiments and for the first time reconstitution experiments with the myofibrils. Actin extraction with potassium iodide solution (Szent-Gyorgyi 1951) after myosin extraction left a backbone of virtually zero optical density in the myofibrils (Fig. 2.8). The Z lines, however, were not removed. In an addendum to the paper, Hugh Huxley described an interesting experiment that indicated that when the myosin extracted ghost myofibrils were irrigated with myosin, these "reconstituted ghosts" were capable of contraction in the presence of ATP.

They put all of these results together in Fig. 2.9. The length of the A bands (i.e., the length of the myosin filaments) remains virtually constant until the I bands have disappeared (~65 % rest length). The ends of the A bands are then in contact with the Z lines, and further shortening is accompanied by the formation of contraction bands at these lines, presumably by crumpling of the ends of the myosin filaments.

The paper also contains interesting speculation about how "cross-linkages" between actin and myosin might operate (Hanson and Huxley 1955. With permission Elsevier):

> …each myosin-actin linkage can pull the actin filament along a distance of, say, 132 Å, by the contraction of a branch of the myosin molecule; the branch would not, of course, give rise to any overall change in length of the myosin filaments (this type of model was mentioned both by ourselves and A.F. Huxley & R. Niedergerke in previous papers).

The 132 Å step size came from their estimate of the relative numbers of actin and myosin molecules in the filaments. They furthermore envisioned that at each step, a considerable proportion of all the actin-myosin linkages on the filament, if not all of them, were broken and reformed again. This speculation is remarkably similar to current day beliefs concerning how the filaments slide (see Chap. 9).

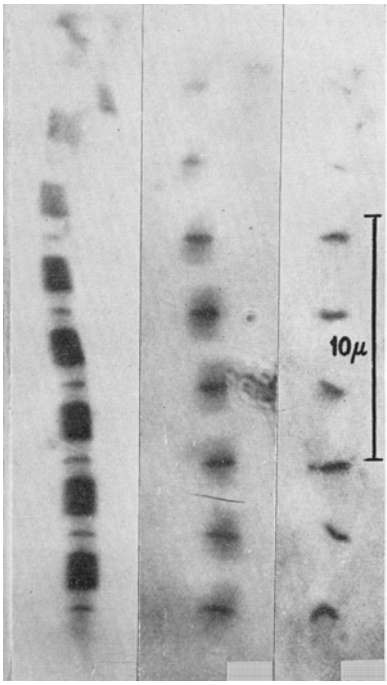


Fig. 2.8 Myofibril band patterns after extraction of myosin and then actin. One stretched myofibril photographed before treatment (*left*), after extraction of myosin by pyrophosphate treatment (*middle*), and then after treatment with potassium iodide solution to remove actin (*right*). After both myosin and actin have been removed only the Z lines and a 'backbone' of virtually zero optical density remain (Hanson and Huxley 1955. With permission Elsevier)

Loose ends What about the invisible S-filaments? Maruyama (1995) noted in a review describing the birth of the sliding filament model of muscle contraction that neither Hugh Huxley nor Jean Hanson mentioned S-filaments after 1955. In fact in a brief review in the journal Endeavour in 1956, Hugh Huxley (1956) described the sliding filament model of contraction and showed a diagram with filaments connecting the ends of the thin filaments in the middle of the sarcomere but he did not mention S-filaments in the review. After a while the S-filaments were dropped all together from the typical diagram showing the structure of the sarcomere (Huxley 1965). We know now that there is an elastic filamentous protein called titin (also known as connectin) that links the myosin filaments to the Z line. When myosin is dissolved away, most titin filaments retract toward either side of the Z line but a few opposing filaments from both Z lines appear to bind each other keeping continuity of the sarcomere (Maruyama 1995). (See Chap. 7 for more on the titin molecule and its role in sarcomeric structure and function.)

There is a historical curiosity associated with the observations in the classic 1954 work. Hanson and Huxley (1955) in their review point out that similar changes also were recorded by Harman (1954) using cine photography of myofibrils contracting and relaxing while in contact with active mitochondria. John W. Harman, at the University of Wisconsin, was investigating the relationship of mitochondrial oxidative capacity and structure to myofibril contractility and structure using phase contrast microscopy and electron microscopy (Harman and Osborne 1953). He exhibited a cine film in 1954 at the Federation meeting in Atlantic City, New Jersey,

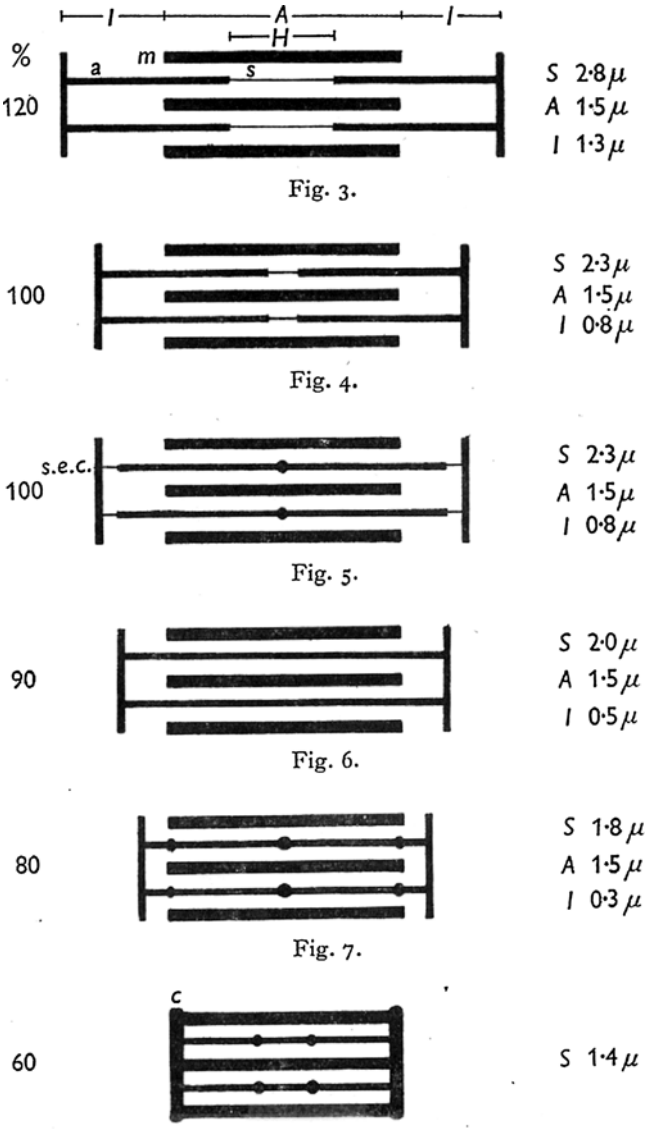


Fig. 2.9 Schematic representation of the sliding filament model of contraction. Proposed arrangement of filaments in one sarcomere of a myofibril at different sarcomere lengths. In order from top down: stretched to 120 % rest length while relaxed; at rest length; in isometric contraction; contracted to 90 % rest length; to 80 % rest length; to 60 % rest length. Sarcomere lengths (S) and A and I band lengths are given to the right of the diagrams, and lengths of sarcomeres expressed in % of rest length are given to the left. *s* "S filament", *a* actin filament, *m* myosin filament, *s.e.c.* series elastic component, *c* contraction band (Hanson and Huxley 1955. With permission Elsevier)

that showed that the A band stayed constant in length during contraction of myofibrils. Since his interest was in mitochondrial structure and function, he did not carry this observation further and thus his observation became a "footnote" to the birth of the sliding filament theory of contraction.

Should one of the classic 1954 papers be given greater weight in the development of the sliding filament model of contraction than the other? Certainly Hugh Huxley and Jean Hanson provided more crucial details, particularly with regard to changes in the H zone with stretch and contraction. But remember, their work was done on "dead" pieces of muscle whereas Andrew Huxley and Rolf Niedergerke worked on living muscle. One can only conclude that each paper provided crucial information that the other didn't and as such both should be honored. With regard to publishing the two papers together, Huxley (2004b) has remarked: "Fortunately, we did, and these papers gave the basic description of the sliding filament model, which has remained essentially unchanged since then."

2.8 Scientific Reception of the Sliding Filament Model of Contraction

Despite the fact that the basic description of the sliding filament model has remained essentially unchanged since the classic papers of 1954, the papers received a mixed reception at the time of their publication. There are various reasons for this less than enthusiastic reception. First, the results "flew in the face" of dogma (see Chap. 1). For example, Albert Szent-Gyorgyi was not convinced that the myosin filaments were confined to the A-band (Huxley 1996). Second, the weight of the results and interpretations were based on light microscopy, sophisticated light microscopy to be sure, but still based on an old fashion technique compared to the emerging exotic technology of electron microscopy. Also it is true that the light microscopy observations were at the very limit of what was possible. Third, and probably most important, even though Hugh Huxley's electron micrographs supported the light microscopic results, his electron micrographs were not completely convincing. In fact his interpretation was not in agreement with the earlier results and interpretations generated in the same laboratory in which he was working at MIT (Hall et al. 1946). Thus his mentor there, F. O. Schmitt, was understandably skeptical (Huxley 2008). And there was more skepticism (Hodge 1956; Spiro 1956; Sjostrand and Andersen 1956). Alan Hodge and David Spiro also worked in the Schmitt laboratory at the same time as Huxley. The three investigators developed a thin sectioning technique together (Hodge et al. 1954). But neither one of them agreed with the Huxley and Hanson interpretations. Hodge (1956) felt that the data on balance favored the 'classical' model, in which a continuous skeletal framework of myofilaments traversed all bands of the sarcomere with the band pattern arising from interstitial materials. Hodge was critical of the sliding filament model. First, he argued that Huxley had not demonstrated directly that the thick filaments were not continuous with the thin filaments. Second, it was not shown that the secondary array of

dots seen in Huxley's (1953b) transverse electron micrographs actually represented filaments or that these presumed filaments were continuous with the I-bands. Third, the postulated S-filaments had not been observed. Spiro (1956) interpreted his electron micrographs to indicate that the thin filaments *became* thick filaments as they went in the A-band. In his view shortening of muscle beyond equilibrium length was accompanied by a progressive transformation of the thin filaments into thick filaments, i.e, on shortening some mechanism caused filaments to aggregate in the form of thick filaments. There must have been some lively discussions in the Schmitt laboratory during this period.

The 1954 Nature papers would become classic and the sliding filament model of contraction would eventually become the new dogma. Nonetheless in the view of many investigators in the mid 1950s it was far from proven that muscle contracted via the sliding of two sets of filaments. Much more work would have to be done to prove the point.

References

Armstrong CM, Bezanilla F (1973) Currents related to the movement of the gating particles of the sodium channels. Nature 242:459–462

Astbury WT (1947) Croonian lecture: on the structure of biological fibres and the problem of muscle. Proc R Soc Lond B B134:303–328

Barany M (1967) ATPase activity of myosin correlated with speed of muscle shortening. J Gen Physiol 50(Pt 2):197–216

Bernstein J (1902) Untersuchungen zur thermodynamik der bioelektrischen strome. Erster Theil Pflugers Archiv Ges Physiol 92:521–562

Clark RW (1968) The Huxleys. McGraw-Hill, New York

Cole KS (1949) Dynamic electrical characteristics of the squid axon membrane. Arch Sci Physiol 3:253–258

Cole KS (1968) Membranes, ions and impulses: a chapter of classical biophysics. University of California Press, Berkeley

Eccles JC (1964) The physiology of synapses. Springer, New York

Gordon AM, Huxley AF, Julian FJ (1966a) Tension development in highly stretched vertebrate muscle fibres. J Physiol 184:143–169

Gordon AM, Huxley AF, Julian FJ (1966b) The variation of tension with sarcomere length in vertebrate muscle fibres. J Physiol 184:170–192

Hall CE, Jakus MA, Schmitt FO (1946) An investigation of cross striations and myosin filaments in muscle. Biol Bull 90:32–50

Hanson J (1952) Changes in the cross-striation of myofibrils during contraction induced by adenosine triphosphate. Nature 169:530–533

Hanson J, Huxley HE (1953) Structural basis of the cross-striations in muscle. Nature 172:530–535

Hanson J, Huxley HE (1955) The structural basis of contraction in striated muscle. In: Brown R, Danielli JF (eds) Symposia of the society for experimental biology. Number 9: fibrous proteins and their biological significance. Academic, New York, pp 228–264

Hanson J, Lowy J (1963) The structure of F-actin and actin filaments isolated from muscle. J Mol Biol 6:46–60

Harman JW (1954) Contractions of skeletal muscle myofibrils by phase microscopy. Fed Proc 13:430

Harman JW, Osborne UH (1953) The relationship between cytochondria and myofibrils in pigeon skeletal muscle. J Exp Med 98:81–98
Hasselbach W (1953) Elekronmikroskopische untersuchungen an muskelfibrillen bei totaler und partieller extraktion des L-myosins. Z Naturforsch 8b:449–454
Hill AV (1965) Trails and trials in physiology. The Williams & Wilkins Company, Baltimore
Hille B (2001) Ionic channels of excitable membranes, 3rd edn. Sinauer Associates, Sunderland
Hodge AJ (1956) The fine structure of striated muscle: a comparison of insect flight muscle with vertebrate and invertebrate skeletal muscle. J Biophys Biochem Cytol 3:131–142
Hodge AJ, Huxley HE, Spiro D (1954) Electron microscope studies on ultra thin sections of muscle. J Exp Med 99:201–206
Hodgkin AL (1963) The ionic basis of nervous conduction. Nobel Lecture, December 11. nobelprize.org
Hodgkin AL (1977) Chance and design in electrophysiology: an informal account of certain experiments on nerve carried out between 1934 and 1952. In: The pursuit of nature: informal essays on the history of physiology. Cambridge University Press, Cambridge, p 1–21
Hodgkin A (1992) Chance & design: reminiscences of science in peace and war. Cambridge University Press, Cambridge
Hodgkin AL, Huxley AF (1939) Action potentials recorded from inside a nerve fibre. Nature 144:710–711
Hodgkin AL, Huxley AF (1945) Resting and action potentials in single nerve fibres. J Physiol 104:176–195
Hodgkin AL, Huxley AF (1952a) Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. J Physiol 116:449–472
Hodgkin AL, Huxley AF (1952b) The components of membrane conductance in the giant axon of *Loligo*. J Physiol 116:473–496
Hodgkin AL, Huxley AF (1952c) The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. J Physiol 116:497–506
Hodgkin AL, Huxley AF (1952d) A quantitative description of membrane current and its application to conduction and excitation in nerve. J Physiol 117:500–544
Hodgkin AL, Huxley AF, Katz B (1952) Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*. J Physiol 116:424–448
Huxley AF (1954) A high-power interference microscope. J Physiol 125:11–13P
Huxley AF (1957) Muscle structure and theories of contraction. In: Butler JAV, Katz B (eds) Progress in biophysics and biophysical chemistry, vol 7. Pergamon Press, London, pp 255–318
Huxley AF (1974) Muscular contraction. J Physiol 243:1–43
Huxley AF (1977) Looking back on muscle. In: The pursuit of nature. Informal essays on the history of physiology, Cambridge University Press, Cambridge, p 23–64
Huxley AF (2004a) Andrew F. Huxley. In: Squire LR (ed) The history of neuroscience in autobiography, vol 4. Elsevier, Amsterdam, pp 284–318
Huxley AF, Gordon AM (1962) Striation patterns in active and passive shortening of muscle. Nature 193:280–281
Huxley AF, Niedergerke R (1954) Interference microscopy of living muscle fibres. Nature 173:971–973
Huxley AF, Niedergerke R (1958) Measurement of the striations of isolated muscle fibres with the interference microscope. J Physiol 144:403–425
Huxley AF, Stampfli R (1949) Evidence for salutatory conduction in peripheral myelinated nerve fibres. J Physiol 108:315–339
Huxley AF, Stampfli R (1951a) Direct determination of membrane resting potential and action potential in single myelinated nerve fibres. J Physiol 112:476–495
Huxley AF, Stampfli R (1951b) Effect of potassium and sodium on resting and action potentials of single myelinated nerve fibres. J Physiol 112:496–508
Huxley AF, Taylor RC (1958) Local activation of striated muscle fibres. J Physiol 144:426
Huxley HE (1951) General discussion. In: Faraday society symposium on the size and shape factor in colloidal systems, vol. 11. p 148–149

Huxley HE (1953a) X-ray analysis and the problem of muscle. Proc R Soc Lond B Biol Sci B141:59–62
Huxley HE (1953b) Electron microscope studies of the organization of the filaments in striated muscle. Biochim Biophys Acta 12:387–394
Huxley HE (1956) Muscular contraction. Endeavour 15:177–188
Huxley HE (1963) Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. J Mol Biol 7:281–308
Huxley HE (1965) The mechanism of muscular contraction. Sci Am 213:18–27
Huxley HE (1969) The mechanism of muscular contraction. Science 164:1356–1366
Huxley HE (1996) A personal view of muscle and motility mechanism. Annu Rev Physiol 58:1–19
Huxley HE (2004b) Fifty years of muscle and the sliding filament hypothesis. Eur J Biochem 271:1403–1415
Huxley HE (2004c) Jean Hanson's legacy. J Muscle Res Cell Mot 25:443–445
Huxley HE (2008) Memories of early work on muscle contraction and regulation in the 1950's and 1960's. Biochem Biophys Res Commun 369:34–42
Huxley HE, Hanson J (1954) Changes in the cross-striations of muscle during contraction and stretch and their structural interpretations. Nature 173:973–976
Jahromi SS, Atwood HL (1969) Correlation of structure, speed of contraction, and total tension in fast and slow abdominal muscle fibers of the lobster (Homarus americanus). J Exp Zool 171:25–37
Luttgau HC, Niedergerke R (1958) The antagonism between Ca and Na ions on the frog's heart. J Physiol 143:486–505
Marmont G (1949) Studies on the axon membrane. I. A new method. J Cell Comp Physiol 34:351–382
Maruyama K (1995) Birth of the sliding filament concept in muscle contraction. J Biochem 117:1–6
Niedergerke R, Page S (1992) Hypodynamic tension changes in the frog heart. In: Simmons RM (ed) Muscular contraction. Cambridge University Press, Cambridge, pp 83–106
Perry SV (1951) The adenosinetriphosphatase activity of myofibrils isolated from skeletal muscle. Biochem J 48:257–265
Ramsey RW, Street SF (1940) The isometric length-tension diagram of isolated skeletal muscle fibers of the frog. J Cell Com Physiol 15:11–34
Randall J (1975) Emmeline Jean Hanson. 14 November 1919–10 August 1973. Biogr Mems Fell R Soc 21:312–344
Schick AF, Hass GM (1950) The properties of mammalian striated myofibrils isolated by an enzymatic method. J Exp Med 91:655–664
Schneider MF, Chandler WK (1973) Voltage-dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. Nature 242:244–246
Sjostrand FS, Andersen E (1956) The ultrastructure of striated muscle myofilaments at various conditions of shortening. Exp Cell Res 11:493–496
Slayter EM (1976) Optical methods in biology. Robert E. Krieger Publishing Company, Huntington
Spiro D (1956) The ultrastructure of striated muscle at various sarcomere lengths. J Biophys Biochim Cytol 2(4):157–162
Stampfli R (1992) A. F. Huxley: an essay on his personality and his work on nerve physiology. In: Simmons RM (ed) Muscular contraction. Cambridge University Press, Cambridge, pp 19–42
Szent-Gyorgyi A (1951) Chemistry of muscular contraction, 2nd edn. Academic, New York
Szent-Gyorgyi A (1960) Remarks on muscle. In: Bourne GH (ed) The structure and function of muscle: pharmacology and disease, vol 3. Academic, New York, pp 445–451
Watson JD (1968) The double helix. A personal account of the discovery of the structure of DNA. Atheneum, New York
Young JZ (1936) The giant nerve fibres and epistellar body of cephalopods. Q J Microsc Sci 78:367–386

Chapter 3
Glory Days: Establishment of the Sliding Filament Model of Muscular Contraction in the 1950s and 1960s

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… (Huxley 2008. With permission Elsevier)

Hugh E. Huxley (2008)

Clearly, the most likely seat of the force-developing mechanism is the globular part of heavy meromyosin and its attachment to the actin filament…Changes in orientation of the cross-bridge relative to the actin filament to which it is attached will then give rise to a relative sliding force between the filaments in the manner required (Huxley 1969. With permission American Association for the Advancement of Science)

Hugh E. Huxley (1969)

3.1 Introduction

The late 1950s and the 1960s turned out to be the "glory days" for the sliding filament model of muscle contraction. But these years started with a mixed reception to the sliding filament model. There were at least two issues that required careful further consideration. The first issue related to dogma and the criticism by Albert Szent-Gyorgyi that it had not been proven that myosin was located *only* in the A band. The dogma was that myosin was distributed throughout the sarcomere and that the filaments folded on muscle shortening. The second and more fundamental problem was that neither light microscopy nor Hugh Huxley's electron micrographs from longitudinal muscle sections were good enough to trace a single thin filament from the Z line into the A band where it was presumed to overlap with the thick filaments.

J.A. Rall, *Mechanism of Muscular Contraction*, Perspectives in Physiology,
DOI 10.1007/978-1-4939-2007-5_3

3.2 1957 Was a Very Good Year for the Sliding Filament Model of Muscle Contraction

3.2.1 Myosin Limited to the A band

It was well accepted that myosin existed in the A band of striated muscle but Szent-Gyorgyi believed that it also existed in the I band. Hugh Huxley and Jean Hanson set out to perform quantitative studies on isolated myofibrils from rabbit psoas muscle to determine if all of the myosin could be ascribed to the A band. They measured the amount of myosin extracted from the myofibrils and determined that myosin constituted about 50 % of the total protein of the myofibrils (Hanson and Huxley 1957). In order to determine where myosin existed in the sarcomere, they utilized interference microscopy[1] to quantify the amount of material removed from the A bands of myofibrils with myosin extraction (Huxley and Hanson 1957). With the extraction of myosin, all of the A band was removed along with about 60 % of the total protein in the myofibrils. Of this 60 %, about 10 % was attributed to thin filaments in the A band. These results suggested that about 50 % of the A band material was myosin. Thus they concluded that the most straightforward interpretation of the results was that all of the myosin in muscle is located in the A bands. Subsequently this conclusion was confirmed by Corsi and Perry (1958). This issue was essentially settled: myosin exists only in the A band.

3.2.2 Double Hexagonal Array of Filaments

With regard to the criticism that the electron micrographs from longitudinal muscle sections, published by Hugh Huxley and others, did not convincingly show two separate sets of overlapping filaments, Huxley deduced that much thinner longitudinal muscle sections were required to critically test this point. He worked to develop the technique to obtain these ultra-thin sections. He published the results in a relatively new journal called The Journal of Biophysical and Biochemical Cytology (Huxley 1957b). (The journal is now known as the Journal of Cell Biology). What resulted can only be described as spectacular.

Very thin longitudinal sections required To guide his investigation, Huxley relied on a clear hypothesis that he developed based on his previous results from X-ray diffraction of living muscle (see Fig. 2.3) and electron microscopy of muscle cross-sections (Huxley 1953a, b). This hypothesis stated that there was a double hexago-

[1] With interference microscopy it is possible to determine the solid content of a cell or portion of a cell from the refractive index measurement. Cell interferometry can give approximate dry weights of cells and cell constituents, *independent of composition and independent of concentration* (Davies and Wilkins 1952). This form of interference microscopy, sometimes called quantitative interference microscopy, is to be distinguished from Nomarski differential interference contrast (DIC). See Slayter (1976) for a chapter on the interference microscope.

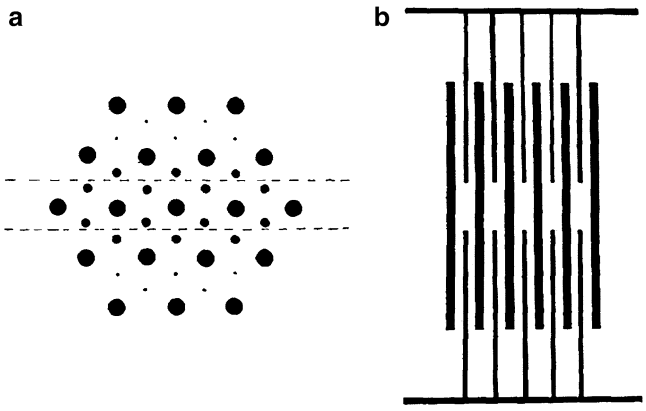


Fig. 3.1 (**a**) Diagram showing end-on view of a double hexagonal array of filaments. The *two dotted lines* indicate the outline of a longitudinal section, about 250 Å in thickness, parallel to the 1010 lattice planes, at the appropriate level to include one layer of thick filaments, and two layers of thin filaments. (**b**) Diagram showing predicted appearance of longitudinal section, cut as indicated in (**a**). Note one thin filament between two thick filaments. The two thin filaments will be vertically above each other in the section (H. Huxley 1957b. With permission Rockefeller University Press)

nal array of overlapping, longitudinally oriented filaments as shown in cross-section in Fig. 3.1a. Furthermore from the electron microscopy measurements in fixed and embedded material, he was able to deduce the separation between the filaments. The separation between adjacent layers of thick filaments in this lattice depends on the plane in which the particular set of layers is drawn relative to the hexagonal axes of the lattice. The maximum separation of primary (thick) filament layers will be found in the direction indicated by the dashed lines in Fig. 3.1a (the 1010 crystallographic direction), and will be approximately 250 Å. Figure 3.1b shows the predicted appearance of a longitudinal section cut as indicated in Fig. 3.1a. The next largest layer separation is found in the direction indicated in Fig. 3.2a (the 1120 crystallographic direction). The separation of these thick filament layers is about 150 Å. In the thickness of this section only one primary and one secondary (thin) filament exists. Figure 3.2b shows the predicted appearance of a longitudinal section cut as indicated in Fig. 3.2a. Huxley pointed out that if the section thickness exceeded the layer separation in that particular direction, then the section will contain two or more superimposed layers of filaments. When two or more filaments lie vertically above each other in the sections, they appear as a single dense filament. Thus to see the predicted arrangements of filaments, the longitudinal sections must be cut in a defined orientation and must be strictly parallel to the filaments and be about 250 and 150 Å in thickness.

The electron microscopic results are a beautiful confirmation of Huxley's predictions. Rabbit psoas muscle, fresh and glycerinated, was fixed in osmium tetroxide, dehydrated, embedded and stained. Figure 3.3 shows an example of a longitudinal section cut in the 1010 crystallographic direction which clearly exhibits the arrangement

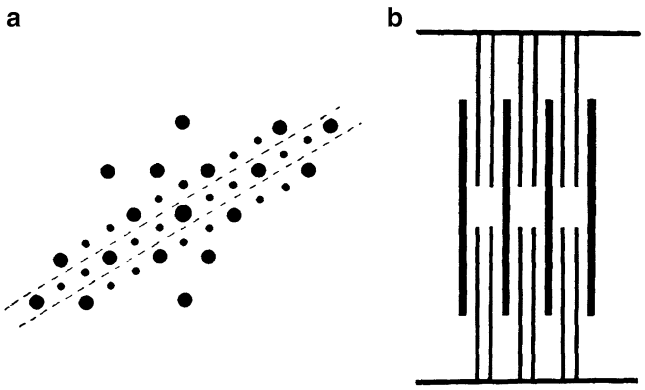


Fig. 3.2 (**a**) As in Fig. 3.1a except with *dotted lines* showing a longitudinal section about 150 Å in thickness, cut parallel to the 1120 planes of the lattice. The thick and thin filaments in this case are all in the same layer. (**b**) Diagram showing expected appearance of a longitudinal section, cut as in (**a**). Note the characteristic appearance of two thin filaments between each pair of thick filaments (H. Huxley 1957b. With permission Rockefeller University Press)

of thick and thin filaments predicted in Fig. 3.1b. The thick filaments run the whole length of the A band. The H zone is clearly seen. There appears to be some extra material in the middle of the A band where the M line has been observed. Even though the thin filaments are not well preserved, it is possible to trace a single thin filament from the Z line into the overlap region. When the section is cut in the 1120 crystallographic direction as shown in Fig. 3.4, two thin filaments lay between each pair of thick filaments as predicted in Fig. 3.2b. Thus the number of thin filaments observed between two thick filaments in these very thin longitudinal sections depended on the orientation of the sections relative to the double hexagonal lattice. These images were a spectacular confirmation of the existence of a double hexagonal array of overlapping filaments in the muscle sarcomere. There could be no doubt now.

Muscles slightly stretched or contracted When a muscle was slightly stretched or contracted, the only changes that were observed were in the length of H zone. The thick filaments did not change length. There was only a change in the amount of overlap between the thick and thin filaments, i.e., with stretch the overlap decreased and with contraction the overlap increased. Huxley felt that these large differences in the length of the H zones which were unaccompanied by any appreciable changes in the over-all length of the A bands provided the most direct and compelling argument for the sliding filament model.

Muscles contain "*cross-bridges*" In this paper, Huxley provides the first comprehensive description of what he called "cross-bridges". The cross-bridges between thick and thin filaments are prominent in these longitudinal sections (Figs. 3.3 and 3.4). These cross-bridges likely arise from the thick filaments since they are not seen in the thin filaments in the I band. Figure 3.5 clearly shows cross-bridges

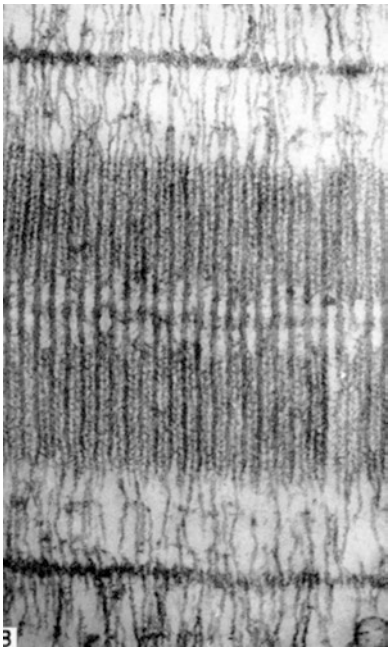


Fig. 3.3 A longitudinal section through one sarcomere, parallel to 1010 planes. As predicted a simple alteration of thick and thin filaments is observed with the thin filaments terminating at the edge of the H zone. Magnification, 150,000 (H. Huxley 1957b. With permission Rockefeller University Press)

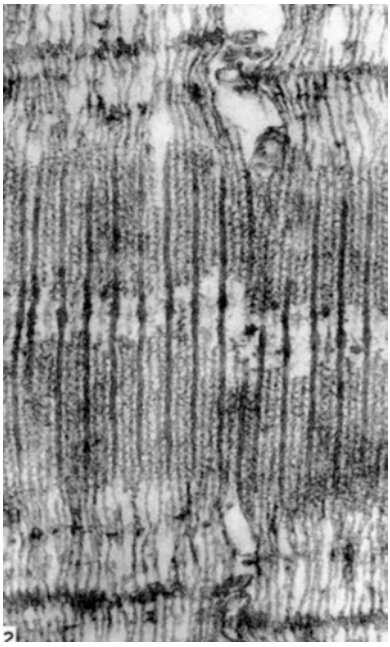


Fig. 3.4 A longitudinal section through one sarcomere, parallel to 1120 planes, showing thick filaments and the predicted pairs of thin filaments in between them. The interruption of the thin filaments at the edges of the H zone is readily visible. The thick filaments are thickened in the H zone, suggesting extra material there. Magnification, ×175,000 (H. Huxley 1957b. With permission Rockefeller University Press)

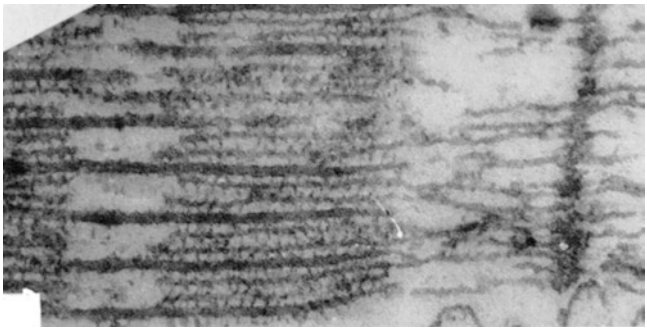


Fig. 3.5 A longitudinal section showing thick and thin filaments at the A-I boundary and at the H-A boundary. The tapering of the thick filaments at the A-I boundary is readily seen and in places they may be observed to terminate, while the thin filaments continue on into the I bands. Note cross-bridges protruding from the thick filament at the bottom edge of the section. Magnification, ×150,000 (H. Huxley 1957b. With permission Rockefeller University Press)

emanating from the last thick filament at the bottom of the image. By counting the number of cross-bridges in one half of an A band and dividing by the length of one half of an A band as seen in the light microscope, Huxley avoided the artifacts associated with electron microscopy and concluded that the cross-bridges occurred about every 400 Å along a thick filament. He went on to describe the cross-bridge orientations. The bridges did not seem to have any precisely fixed form. They were more or less at right angles to the filaments, and there did not appear to be any preferential direction of tilt, even in muscle fixed under continuous tension.

Implications of a double hexagonal array of filaments. Huxley discussed some of the implications of the results in terms of the sliding filament hypothesis of contraction (Huxley 1957b. With permission Rockefeller University Press):

> One simple feature of the structures seen in these longitudinal sections is that, no matter whether the muscle is stretched, at rest-length, or shortened, the cross-bridges between primary and secondary filaments have the same form, i.e., they are, more or less, at right angles to the filaments. The only difference between the muscles at different lengths is that the two sets of filaments overlap to different extents. This must mean that the filaments slide past each other during contraction, the bridges between them remain attached for a short distance only, and they must then detach from the secondary filament and reattach at a point a little further along. We will refer to the separation between these points of attachment on the secondary filament as the 'step distance'…One complete contraction of the muscle will represent a number of cycles of operation of the contraction mechanism associated with the bridges…If the bridges represent sites of actin-myosin interaction and ATPase activity, if they represent the sites at which chemical energy is transformed into a mechanical deformation and hence into external work, then each site will be able to operate a number of times during a single contraction of the muscle.

Huxley speculated that the step distance was of the order of 50 Å, the postulated distance between actin monomers. Besides considering the new positive results in the discussion of this paper, Huxley detailed how the other investigators got it wrong.

The main conclusion was that the previous investigators employed longitudinal sections that were much thicker than they realized and this led to erroneous conclusions.

Even though there could be no doubt now about the existence of a double hexagonal array of overlapping filaments in muscle, these results did not prove the existence of a sliding filament mechanism of contraction. The results are necessary but not sufficient. Looking back on this paper, Huxley (2008) has observed: "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." Some of this skepticism could be traced to the deeply ingrained idea that the muscle filaments themselves must become shorter during muscle contraction. Plus the light microscopy results had limited resolution, the electron microscopy results were subject to artifacts and the X-ray results were enigmatic to the non expert. Clearly there was much more work to be done before a sliding filament model of muscle contraction could be accepted. Nonetheless this paper is one of the most important in the history of the muscle field because it clearly set out the structural context in which any proposed mechanism of contraction must fit. These and other results supporting a sliding filament mechanism of contraction were reviewed comprehensively by Huxley and Hanson (1960). Hugh Huxley also wrote a major review (Huxley 1960).

3.2.3 A Hypothesis for the Mechanism of Contraction

In the summer of 1954 Andrew Huxley spent a lot of time "brooding" on the nature of the mechanism by which the independent force generators produce force or sliding movement (Huxley 2004a). He generated a kinetic model in which a "side-piece", attached to the thick filament by an elastic connection, was able to attach to a site on the thin filament with a rate constant that was moderate when the relative positions of the filaments were such that the connection gave a positive contribution to the overall tension, and the rate constant for detachment was large after sliding motion had brought it to a position where its contribution was negative. Generating a kinetic model for muscle contraction was analogous to Andrew Huxley's role in generating a kinetic model for the propagation of an action potential with Alan Hodgkin (Hodgkin and Huxley 1952). Andrew Huxley used the term 'side-piece' and not 'cross-bridge' as described by Huxley (1957b) because his model was generated in 1954 and was submitted for publication before Hugh Huxley introduced the term cross-bridge into the muscle literature. Andrew Huxley's side-piece is equivalent to Hugh Huxley's cross-bridge. This theory gave a good approximation to the classic mechanical and energetic data of Hill (1938) (see Chap. 5). Andrew Huxley was "very disappointed" that the article did not appear until nearly 2 years

later in 1957 (Huxley 2004a). He should not have worried. The theory has remained useful to this day because its main kinetic assumptions are still considered to be approximately correct. Even though more recent evidence suggests that the generation of force is likely due to more specific changes in the cross-bridge than merely attaching in a position where the elastic element is stretched, this does not alter the kinetics greatly. Whereas this model has been modified and expanded over the years, it is still considered to be the starting point for any serious consideration of the kinetic mechanism of muscle contraction.

What mechanism did Andrew Huxley propose in 1957 to cause the filaments to slide during muscle shortening? What data was the model designed to fit? Huxley begins by assuming that shortening and the development of force are produced by independent force generators (the side-pieces or cross-bridges) which can be effective only in the region of overlap of the thick and thin filaments. With each contraction, each force generator undergoes repeated cycles of activity: attachment, pulling, and detachment, followed by reattachment (perhaps at a different site) and a new cycle. The filaments themselves do not change length but rather slide relative to each other in a direction leading to muscle shortening. Furthermore the side-pieces cycle asynchronously thus resulting in a smooth force development or shortening during contraction. An essential point of the proposal is the postulate that the probabilities of attachment and detachment of the side-pieces are determined by their position.

The basic reactions Huxley envisioned a side-piece M connected to the thick filament via a 'spring' (Fig. 3.6). This side-piece oscillates by thermal agitation backwards and forwards along the length of the filament. The extent of oscillation is limited by the elastic connection to the thick filament. The equilibrium position is denoted by O. There is an active site A on the actin filament at a longitudinal distance x from O. The A and M sites spontaneously attach to each other and this reaction has a rate constant f:

$$\mathrm{A} + \mathrm{M} \xrightarrow{\mathrm{f}} \mathrm{AM} \tag{3.1}$$

Since the AM link was formed spontaneously, this link can only be broken by a reaction requiring energy supplied from metabolic sources, i.e., a high-energy phosphate compound XP. The reaction proceeds with a rate constant g:

$$\mathrm{AM} + \mathrm{XP} \xrightarrow{\mathrm{g}} \mathrm{AXP} + \mathrm{M} \tag{3.2}$$

Finally the system is reset so that reaction (3.1) can occur again by dissociation of AXP and splitting of the high-energy phosphate bond (which supplies the energy needed to bring about this dissociation):

$$\mathrm{AXP} \rightarrow \mathrm{A} + \mathrm{X} + \mathrm{Phosphate} \tag{3.3}$$

Thus one high-energy phosphate bond is split for each side-piece cycle. If the muscle is in rigor, reaction 2 cannot occur because of the lack of XP and as a consequence the AM links remain and the muscle becomes inextensible.

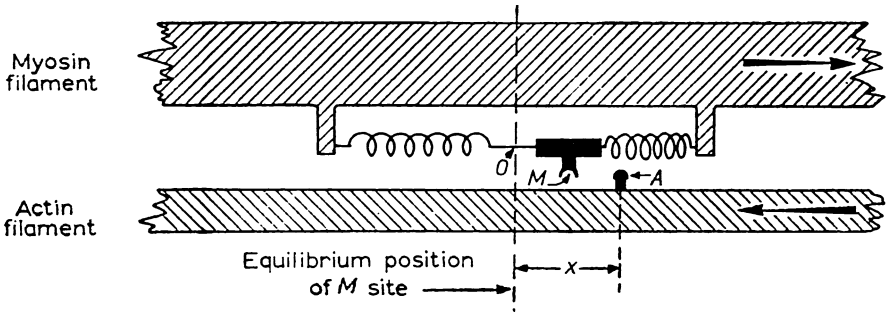


Fig. 3.6 Diagram illustrating the proposed kinetic mechanism of force generation in muscle. The part of a myofibril which is shown is in the right-hand half of an A band so that the actin filament is attached to a Z line which is out of the picture to the right. The *arrows* give the direction of the relative motion between the filaments when the muscle shortens. The side-piece, M, is attached to the myosin filament via an elastic connection. M oscillates along the myosin filament around its equilibrium position, O. The active site on the actin filament is designated A. The distance of the A site from the equilibrium position O is labeled as x (A. Huxley 1957a. With permission Elsevier)

Rate constants depend on side-piece position In this two state model the side-piece is either detached or attached and generating force. Rate constants of attachment, f, and detachment, g, are assumed to be dependent on x, the position of the A site with respect to O (Fig. 3.7). Their dependency on x was chosen in such a way to attempt to fit quantitatively the mechanical and energetic data in a classical study by Hill (1938) (see Chap. 5). Hill's results included:

1. During the maintenance of force in an isometric contraction, the muscle liberates energy as heat at a steady rate called the maintenance heat rate.
2. During muscle shortening, the rate of energy liberation increases linearly with the speed of shortening.
3. During muscle shortening, the force generated by a muscle decreases as the velocity of shortening increases resulting in the characteristic force versus velocity relationship.

Muscles shorten, not lengthen, when activated First of all, the attachment rate constant f is taken as zero when x is negative (A to the left of O). A side-piece will not attach when it is in a position to generate negative force or in a position to impede positive force. This assumption assures that a muscle will shorten rather than lengthen when activated. The rate constant f increases linearly with increasing x up to the point h. Force is generated by the attached side-pieces pulling the actin filament in the direction of muscle shortening. The amount of force generated by an attached side-piece is presumed to also increase linearly as a function of x. Beyond h, f becomes zero again. This postulate dictates that a side-piece will detach if the muscle is stretched beyond h during contraction. Thus there is a limited range of attachment in the positive force range. Huxley calculates a range of attachment for the side-pieces of about 100–150 Å.

During an isometric contraction The rate constant g is presumed to be small when x is positive, increasing slowly with increasing x. The side-pieces will thus cycle slowly in the positive force range. This cycling of side-pieces is necessary to

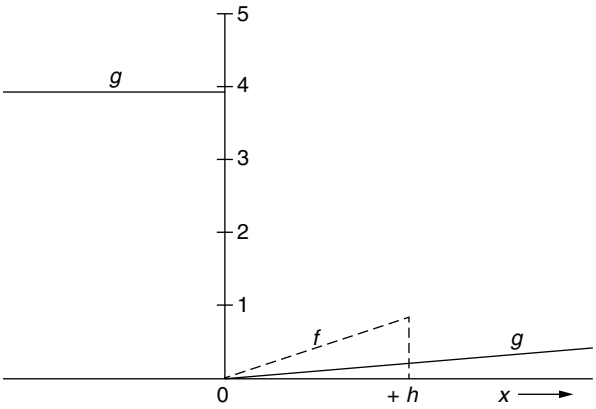


Fig. 3.7 Dependence of rate constants of attachment, f, and detachment, g, on position x. The *dashed line* indicates that the dependence of f on x for formation of links between actin and myosin. The attachment cannot occur when the side-piece is to the left of the equilibrium position O which assures that a muscle will shorten, not lengthen, when activated. The *solid line* represents the dependence of g on x. The probability of detachment, g, is low but finite when the attachment is to the right of the equilibrium position, O. This feature results in slow cycling of side-pieces and low rate of energy liberation during an isometric contraction. When the side-piece moves to the left of O during muscle shortening, the rate constant for detachment becomes very large and energy liberation increases (A. Huxley 1957a. With permission Elsevier)

explain the continual liberation of energy as heat during a maintained isometric contraction as observed by Hill (1938).

During muscle shortening In contrast, rate constant g is very high (and constant) when x is negative, but zero at O. Thus side-pieces will quickly detach when they move into the negative force range. During muscle shortening, the average value of g will rise, since the A sites are continually moving to the left of O. Therefore the rate at which the reaction cycle occurs will also increase and this will result in an increase in energy liberation during shortening as found by Hill (1938). Another consequence of shortening is that the time available for an A site to react with an M site will decrease as the velocity of muscle shortening increases. Since the rate constant of attachment (f) is finite, the probability of an attachment occurring between any one pair of sites will decrease as the velocity of shortening increases. As the shortening velocity increases, the total number of links formed at any one time will decrease. Thus the model predicts that the force during shortening will decrease with increases in the shortening velocity. This prediction fits with the characteristic force versus velocity relationship shown by Hill (1938). Huxley was able to adjust values of f and g to fit the mechanical and energetic data generated by A. V. Hill remarkably well (Huxley 1957b).

Maximum velocity of muscle shortening (V_{max}) The model also provides a mechanistic explanation for the maximum velocity of muscle shortening (V_{max}) which

occurs when the force generated during shortening or load is zero. In the model, the V_{max} occurs when the opposing force, due to side-pieces that have moved to negative values of x is equal to the force due to side-pieces that are still attached at positive values of x. The value of V_{max} is primarily dependent on the value chosen for the rate of detachment, g, during shortening. Thus V_{max} is predicted to be dependent on a balance of the positive and negative forces and not on their magnitudes. This prediction implies that V_{max} should be independent of the number of attached side-pieces and thus independent of overlap of thick and thin filaments. This postulate was verified some years later by Gordon et al. (1966b) and by Edman (1979).

Thus Andrew Huxley showed that a cyclic mechanism resulting in filament sliding could account quantitatively for a great deal of experimental data. This model was a major advance because it provided a plausible mechanism of how a sliding filament model of muscle contraction might operate. Along with Hugh Huxley's (1957b) definitive structural experiments indicating that muscle contained a double array of hexagonal filaments, the stage was set for an explosive effort in testing the sliding filament model of muscle contraction. 1957 was indeed a very good year for the sliding filament mechanism of muscle contraction. Nonetheless to gain a truly molecular understanding of muscle contraction, much more needed to be discovered about the myosin and actin molecules and their arrangement into filaments.

3.3 Myosin, Actin, Thick and Thin Filaments

3.3.1 The Myosin Molecule

All of the myosin exists in the thick filaments of the A band whereas actin exists in the thin filaments. During muscle contraction, myosin and actin interact using the energy of ATP to generate force and do work. Thus the myosin molecule has multiple functions: thick filament formation, ATPase activity and reversible binding with actin. Because of the central role of myosin in muscle structure and function, there was and still is a great interest in elucidating its molecular properties. Work on elucidating the fundamental properties of myosin started in earnest in 1950 and continues to this day.

Light meromyosin and heavy meromyosin Myosin is a fibrous protein of high molecular weight. In the early 1950s there was considerable uncertainty about the molecular weight of myosin, values ranged from 400,000 to 1,000,000 Da (Szent-Gyorgyi 2004). No matter what the real molecular weight was, a molecule this large presented considerable challenges to the biochemists. In order to simply the molecule, John Gergely (1950) and Samuel V. Perry (1951) (Fig. 3.8) independently initiated the study of the properties of myosin in the presence of the proteolytic enzyme trypsin. The resulting preparations split ATP but lost their ability to bind to actin. The first break through came when Elemer Mihalyi found that trypsin split myosin into two fragments of varying molecular weight (noted by Gergely 1953, in a footnote). Mihalyi and Szent-Gyorgyi (1953a, b) separated and characterized these

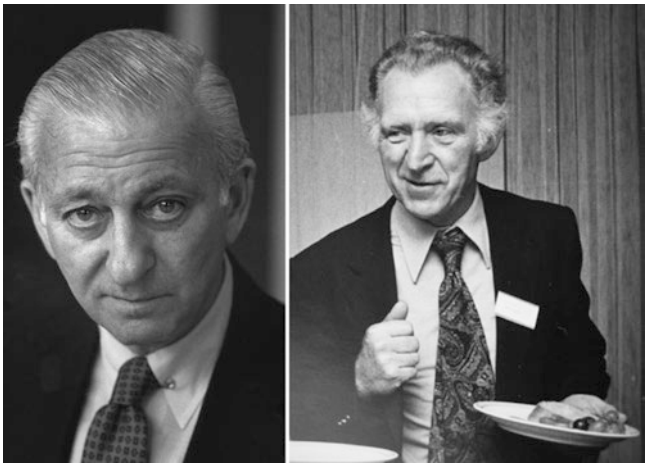


Fig. 3.8 (*Left*) John Gergely (1919–2013) is best known for his studies on the regulation of muscle contraction, in particular the interaction among the subunits of the troponin complex and their relation to tropomyosin and actin (see Chap. 4). He was a member of Albert Szent-Gyorgyi's research group in Hungary during World War II and came to the United States in 1948. He was a co-founder, and former director, of the Boston Biomedical Research Institute. Gergely's publications span more than 50 years (Photo: Moss 1997. With permission Springer) (*Right*) Samuel (Sam) Victor Perry (1918–2009), English biochemist and long-time department chair at the University of Birmingham, England made fundamental discoveries relating to various muscle proteins, including myosin, tropomyosin and particularly the subunits of the troponin molecule, namely troponin I and troponin T (see Chap. 6). He pioneered investigations into the mechanisms and role of protein phosphorylation in the regulation of muscle contraction, especially phosphorylation of myosin in skeletal muscle and troponin I in cardiac muscle. He led an exciting, sometimes harrowing, non-scientific life (Perry 1997). During World War II, he was captured and escaped multiple times, only to be re-captured once again. Also he was an avid athlete and played ruby for England in international matches. He liked to state that he was the only Fellow of the Royal Society (1974) who also played international ruby (Photo: Schaub 2010. With permission Springer)

fragments of myosin. Andrew Szent-Gyorgyi (1953) (Fig. 3.9) named the heavier fragment heavy meromyosin (HMM) and the lighter fragment light meromyosin (LMM). The key was to control the duration of digestion of myosin by trypsin. If the duration of digestion was over hours, multiple fragments of myosin appeared and ATPase and actin binding ability was lost. If the digestion was brief (10 min or so), two fragments were observed during sedimentation in the ultracentrifuge. Importantly they found that HMM retained the ATPase activity and actin binding activity of the original myosin and furthermore was soluble at all ionic strengths. In sharp contrast LMM exhibited no ATPase or actin binding ability and it precipitated at low ionic strength similar to the original myosin. Also LMM formed long needle shaped crystals (Szent-Gyorgyi 1953). These crystals exhibited birefringence in

Fig. 3.9 Andrew G. Szent-Gyorgyi, Albert's younger cousin, preparing a sample for X-ray exposure at the Marine Biological Laboratory at Woods Hole, circa 1954. Andrew Szent-Gyorgyi is a distinguished muscle scientist noted for his investigations of the basic properties of the myosin molecule and the discovery of the dual regulation of contraction in invertebrate muscle (Photo: Profiles in Science. National Library of Medicine. The Albert Szent-Gyorgyi Papers [on-line])

polarized light as does the A band of muscle. Cohen[2] and Szent-Gyorgyi (1957) determined that LMM was a fully coiled α-helix. These important studies started an intensive characterization of myosin using proteolytic enzymes including, beside trypsin, also chymotrypsin and papain.

Myosin is made up of HMM and LMM but how is it constructed? Szent-Gyorgyi (2004) has stated that there were difficulties in determining the myosin structure because of uncertainties in: (a) molecular weight, (b) number of parallel peptide chains and (c) estimates of the length of myosin. These uncertainties led to missteps. For example, Szent-Gyorgyi (1953) incorrectly hypothesized that myosin

[2] Carolyn Cohen, at the Rosenstiel Basic Medical Sciences Research center at Brandeis University, has concentrated her more that 50 year career on the investigation of the molecular architecture of **α**-helical proteins using X-ray crystallography, molecular biology and biochemical tools. Besides myosin, she has also investigated the **α**-helical coiled coil structure of the proteins tropomyosin and paramyosin with the goal of elucidating their roles in muscular contraction at the molecular level. Cohen was elected to the National Academy of Sciences in 1996.

was made of a linear arrangement of four LMMs attached to one HMM. Also it was uncertain how many α-helical chains made up LMM. Proposals ranged from one to three α-helical chains in LMM of myosin.

Myosin subfragment 1 (*S-1*) *and subfragment 2* (*S-2*) Further dissection of myosin was carried out by Mueller and Perry (1962). They showed that exposure of HMM to trypsin for an extended period of time converted it to a single major component that sedimented slowly in the ultracentrifuge which they called HMM subfragment 1 (S-1) and even further digestion yielded a component called HMM subfragment 2 (S-2). HMM S-1 retained the ATPase and actin binding ability of myosin. HMM subfragment 2 did not bind actin and exhibited little ATPase activity.

But still the question remained. How was myosin put together? Electron microscopy played a crucial role here. Hall (1956) at the Massachusetts Institute of Technology showed that it was possible to observe single protein molecules in the electron microscope by using a shadow casting[3] technique. Huxley (1963) applied this technique to myosin molecules and concluded that the myosin was a long rod-shaped molecule with a single globular end. A major advance was made by Slayter and Lowey[4] (1967) when they showed in shadow cast images (Fig. 3.10) that the myosin molecule ended in two globular heads. These images have become classic in the muscle literature. They noted that the globular heads of myosin appeared to possess considerable flexibility. They speculated that each head is attached to a single strand of the rigid rod portion of myosin. HMM is therefore a two-headed fragment of myosin connected to LMM via HMM S-2. Each head represents a HMM S-1. This result also proved that a myosin molecule was made up of two parallel peptide chains. LMM + S-2 formed the rod portion of the molecule which for most of its length is a coiled-coil[5]. It was natural then to speculate that HMM S-1 was equivalent to the cross-bridge as seen by Huxley (1957b) and proposed

[3] The shadow casting technique involves the production of exaggerated contrast by deposition of an electron dense material at an angle over molecules on a substrate. The molecules acquire a shadow on the uncoated side. Molecules covered with the deposited film have a three-dimensional appearance. This technique produces a topographical representation of the surface of the molecule. When examined in the electron microscope the coated molecules appear dark and the shadow which is more electron-transparent appears bright. The micrographs are printed as negatives so that the shadows appear dark and give the impression that the observer is looking down on the molecules which are obliquely illuminated with white light.

[4] Susan Lowey has played a major role in the biochemical elucidation of myosin structure and function with publications spanning more than 50 years. She has been particularly instrumental in the characterization of myosin isoforms from fast and slow contracting skeletal muscle, smooth and cardiac muscle and non-muscle myosins. She was part of the research group at the Rosentiel Basic Medical Sciences Research Center at Brandeis University before moving to the University of Vermont in 1998.

[5] A coiled coil is a structural motif in proteins in which two or more **α**-helices are rapped or coiled together like the strands of a rope. This concept dates back to 1951 when Linus Pauling and colleagues at the California Institute of Technology discovered the **α**-helix. Pauling suggested that in complex fibrous proteins the **α**-helices would tend to twist around each other and form a coiled coil. Francis Crick in 1953 predicted this tight packing and it is often referred to as "knobs into holes packing". Thus LMM is a coiled coil as is the muscle protein tropomyosin. See Cohen (1975) for further details about tropomyosin as a coiled coil.

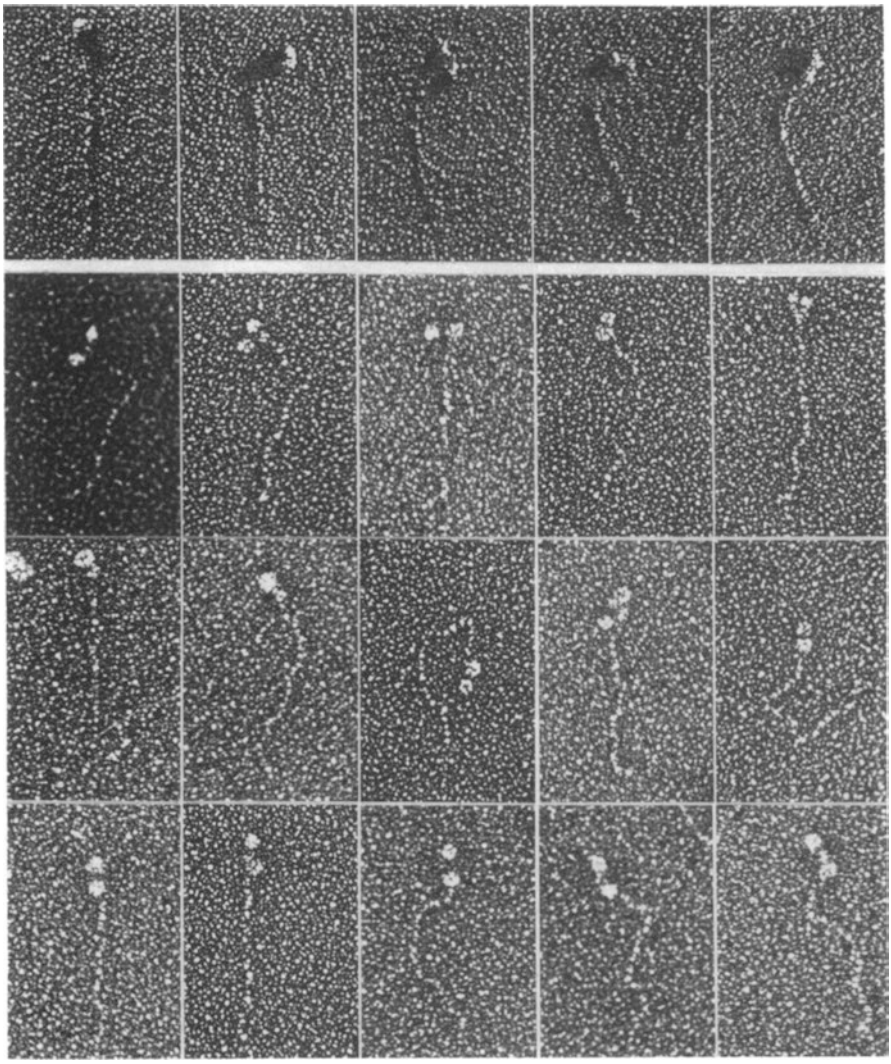


Fig. 3.10 Individual myosin molecules observed in the electron microscope by the shadow casting technique show that myosin is a long rod with two globular regions at one end. The globular heads exhibit considerable flexibility. Magnification ×175,000 (Slayter and Lowey 1967. With permission of S. Lowey)

by Huxley (1957a). As such, HMM S-1 would prove to be a very suitable experimental preparation for the study of many aspects of the cross-cycle in solution. Also HMM S-2 would garner special attention as a possible "hinge" allowing the cross-bridge to reach out to actin (Sutoh et al. 1978).

Lowey et al. (1969) put it all together when they isolated all the subfragments of myosin generated by trypsin and papain digestion and exhibited them in electron

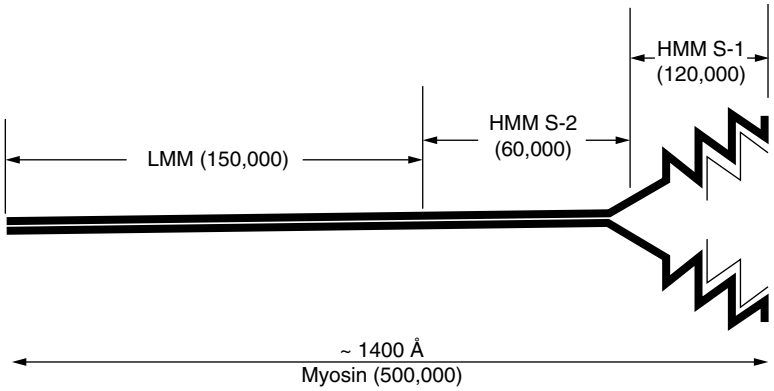


Fig. 3.11 A schematic representation of the myosin molecule. The molecular weight and approximate length of a myosin molecule is shown as are the molecular weights of the subfragments of myosin. Note the presence of a low molecular weight peptide in each myosin head in recognition of then on-going studies on the myosin light chains (Lowey et al. 1969. With permission Elsevier)

microscopic images. Thus it was now possible to give a reasonably accurate picture of a myosin molecule (Fig. 3.11). But there was more to come. This figure from Lowey et al. (1969) clearly shows an extra, low molecular weight, peptide chain associated with each HMM S-1. This was an introduction to their soon to be published results on what would become known as the myosin light chains. Even here the picture was incomplete.

In many ways this first two decade era of the study of myosin structure-function with proteolytic enzymes was remarkable. After all, proteolytic enzymes might be expected to attack a large molecule like myosin in many places and destroy its function entirely. That was indeed true if the digestion was prolonged in time. But if the digestion was carefully controlled, the rules of which had to be painstakingly worked out, myosin could be cleaved without loss of function into fragments amenable to biochemical and biophysical analysis. See Szent-Gyorgyi (2004) and Needham (1971) for further details of this era in the study of the myosin molecule.

Light chains of myosin Using proteolytic enzymes is one effective way to dissect the myosin molecule but another approach is to apply denaturing agents that would dissociate possible subunits from myosin that are not covalently attached. With prolonged urea treatment, small subunits from myosin, of about 16,000 molecular weight, were first observed by the Chinese investigator Tien-chin Tsao[6] (1920–1995)

[6] From 1944 to 1946, Tien-chin Tsao (Tianqin Cao) worked with Joseph Needham (husband of Dorothy Needham), the famous British scholar and author of China's Science and Civilization, in Chongqing at the Sino-British Co-operation Office to help investigate the development of science and education in China. In 1946 he went to Cambridge on a British Council Scholarship to study in Kenneth Bailey's laboratory with the recommendation of Joseph Needham. He obtained his Ph.D. from Cambridge University in 1951. He then went to the Institute of Physiology and Biochemistry in Shanghai, China. For a biographical recollection of Tianqin Cao (Tien-chin Tsao) describing his life and contributions to the development of biochemistry in China see Zhang (2010).

in 1953. Tsao was working in Kenneth Bailey's laboratory at the University of Cambridge. Tsao found it difficult to believe that a fibrous molecule of the dimensions and complexity of myosin could be synthesized fully formed from its template. Nonetheless, it was possible that these small proteins could be impurities tightly bound to myosin. To determine if these were true subunits of myosin, it was necessary to demonstrate: (a) their effects on myosin ATPase, (b) their location within the myosin molecule and (c) their stoichiometry on myosin. Two classes of small polypeptide subunits, called light chains, were reported. One class of possible subunits was removed from myosin under severe alkaline conditions, i.e., pH 11 (Stracher 1969; Dreizen and Gershman 1970). Removal of these light chains resulted in loss of the ATPase activity of myosin. Since these light chains seemed to be essential for the myosin ATPase activity, they were called "essential light chains".

Because the ATPase activity of myosin was known to be dependent on specific sulfhydryl groups within the globular region of the molecule, a procedure was developed based on reaction of all of the sulfhydryl groups of the protein with a blocking reagent. A second class of light chains was removed from myosin with the sulfhydryl blocking agent 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB). Full ATPase activity was recovered upon regeneration of the thiol groups with dithiothreitol in the residual DTNB-treated myosin. Therefore these DTNB light chains were not essential for myosin ATPase activity (Weeds 1969; Gazith et al. 1970). Weeds concluded that since these light chains were inessential to the ATPase activity of myosin, they should be regarded as contaminants in the preparations, at least until a specific function could be ascribed to them. Thus there was controversy as to whether the light chains were integral parts of the myosin molecule or contaminants. Also there was uncertainty as to the molecular weights of the light chains.

Much of the controversy was clarified by Weeds and Lowey (1971). In a very thorough study of myosin and its subfragments from rabbit skeletal muscle, they were able to isolate and characterize three light chains. Two chemically related light chains were isolated under alkaline conditions (A1 and A2). A1 had a higher molecular weight (25,000 Da) than A2 (16,000 Da). They decided to study the light chain content of HMM and HMM S-1 because they are water-soluble proteins and because of their lower molecular weight as opposed to the parent molecule, it was simpler to determine the stoichiometry of the light chains. The alkali light chains were present in both HMM and HMM S-1. There was a single alkali light chain in each HMM S-1 "head". These light chains were very similar in amino acid sequence and the possibility was considered that A2 might be a fragment of A1. Another possibility was that A1 and A2 could be associated with a single myosin molecule which would mean that the myosin "heads" were not identical. They suggested that most likely A1 and A2 were associated with different myosin populations in rabbit skeletal muscle.

Weeds and Lowey (1971) also found that there was one DTNB light chain of molecular weight 18,000 Da per HMM S-1. Even though this light chain was not essential for myosin ATPase activity, they concluded that the stoichiometric relationship of the DTNB light chain to myosin "head" argued against the interpretation that this light chain was a tightly bound contaminant of myosin. In this regard they turned out to be correct. The DTNB light chain is now known as the regulatory light chain because it regulates contraction in smooth muscle (via phosphorylation) and

Light Chain Molecular Weights and Nomenclature

Molecular Weight (Daltons)			
25,000	**LC1**	**Alkali 1**	**Essential LC**
18,000	**LC2**	**DTNB**	**Regulatory LC**
16,000	**LC3**	**Alkali 2**	**Essential LC**

Fig. 3.12 Nomenclature and molecular weights of the myosin light chains

regulates contraction in molluscan muscle by binding calcium (Szent-Gyorgyi 1975) (see Chap. 6). The molecular weights and destinations of the light chains are listed in Fig. 3.12.

Thus the myosin molecule is a hexamer with two heavy chains and four light chains. The molecule can be broken into subfragments by proteolytic enzymes. These subfragments include LMM and HMM. LMMs aggregate and likely form the backbone of the thick filaments. HMM can be further dissected into HMM S-2 and two HMM S-1s. Each HMM S-1, or myosin "head", splits ATP, binds to actin and contains one essential light chain and one regulatory light chain.

Myosin ATPase activity correlated with the speed of muscle shortening By the mid-1960s rapid progress was being made in the biochemical investigation of the contractile proteins but this work was going on more or less independently of studies in the physiology of muscle contraction. There wasn't a great deal of cross-talk between fields. This situation changed in 1967 when Michael Barany[7] published a very influential paper in the muscle field. He asked the following question: can the large differences in the known speeds of muscle contraction across the animal kingdom be attributed to the ATPase activity of the myosin molecule in these muscles? He approached this issue by comparing the ATPase activity of myosin isolated from muscles of various mammals, lower vertebrates and invertebrates for which the contraction times were known. He measured the actin- and Ca^{2+}-activated ATPase activities of myosin at the same temperatures at which the mechanical experiments were conducted. He first established that the source of actin had no effect on the actin-activated myosin ATPase activity. He compared the myosin ATPase activities to two mechanical parameters: isometric twitch contraction time and maximum velocity of

[7] Michael Barany (1921–2011) and his wife Kate Barany (1929–2011) were distinguished muscle biochemists. They have written an autobiographical chapter (Barany and Barany 2000) describing their scientific and personal lives. They describe their "strife and hope" and ultimate triumph over great odds. Both Hungarian Jews, they were Nazi concentration camp survivors (Michael from Buchenwald and Kate from Auschwitz). They came to the United States in 1960. They settled at the University of Illinois, Chicago. Michael's publications in the muscle field covered 50 years. Each year the Biophysical Society awards a gift to a young investigator who has not yet reached the rank of full professor. In 1998 that award was named the "The Michael and Kate Barany Award for Young Investigators". They died within one month of each other.

muscle shortening. Barany's conclusion was the same in each case. The results suggested a role for the ATPase activity of myosin in determining the speed of muscle contraction. Consider the case for the maximum velocity of muscle shortening. The values for the maximum velocity of shortening from 14 different muscles studied at temperatures ranging from 0 to 36 °C varied by 240-fold. Myosin was isolated from the muscles of rabbits, rats, mice, cats, humans, frogs, dogfish, scallops and mussels. The actin-activated myosin ATPase activity also varied by 200-fold and the Ca^{2+}-activated ATPase by 130-fold. More importantly, when the value for the velocity of muscle shortening was divided by the value for the myosin ATPase activity for each muscle, the ratios were very nearly the same. Generally they varied by less than a factor of 2. This result meant that nearly all of the variation in the maximum velocity of muscle shortening could be attributed to variation in the myosin ATPase activity. Even though this was merely a correlation, it did suggest a cause and effect relationship. These results led to obvious questions: what's different about the myosin molecules in different muscles? How does the myosin ATPase activity determine the speed of muscle contraction? Now the biochemists and physiologists were starting to talk to each other.

This paper was part of a symposium of the New York Heart Association but it was not actually presented at the meeting. Just before the lecture a fire broke out in the hotel and the audience left the hall. Thus, the results were not known to the scientific community for sometime. But the scientific community did find out about the investigation. As of 1997, the paper had been cited 1,700 times (Barany and Barany 2000).

3.3.2 The Actin Molecule and Thin Filaments

Actin was discovered and characterized in Albert Szent-Gyorgyi's laboratory by F. Bruno Straub (1942) (see Sect. 1.5). It is stable as a monomer (G-actin). Unlike myosin, it is a small globular shaped molecule of about 50,000 molecular weight. In the presence of salt, actin polymerizes to form actin filaments (F-actin). F-actin accelerates the ATPase of myosin. G-actin contains ATP and F-actin contains bound ADP. In a classic study in 1963, Jean Hanson and Jack Lowy examined the structure of actin filaments with electron microscopy. True to Jean Hanson's interest in the generality of the contractile mechanism, the actin filaments were isolated from striated and smooth muscles of a staggering array of animals, including mammals (rabbit and guinea pig), insects (beetles and blow flies), mollusks (scallops, oysters, mussels, squid) and earth worms. These native filaments were compared to synthetic F-actin filaments and to each other. The filaments were negatively stained and examined in the electron microscope. The negative staining technique was developed for muscle proteins by Huxley (1963) and he provided advice to Hanson and Lowy on its application to actin filaments. In this technique the actin filaments are unstained and embedded within an electron dense material. Using this technique, they were able for the first time to provide detailed structural information about the actin filaments.

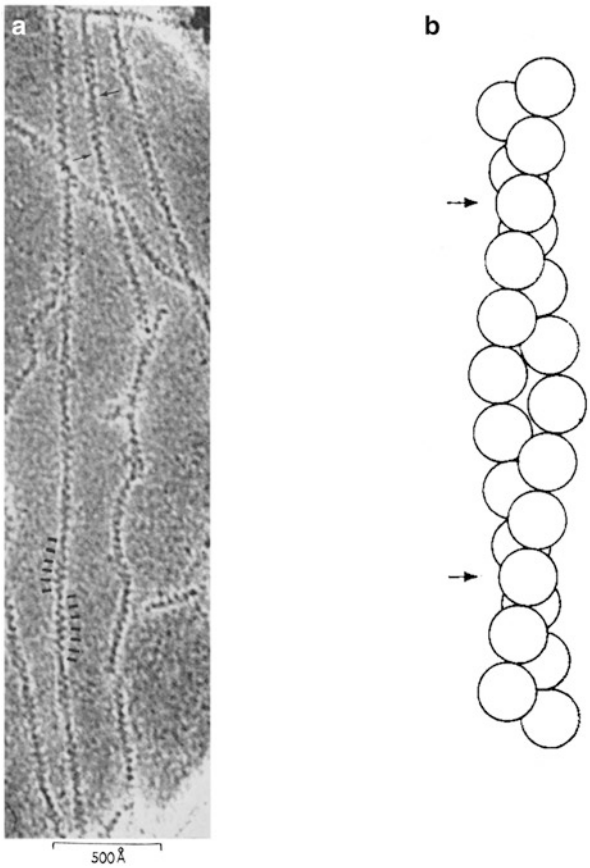


Fig. 3.13 (**a**) Electron micrograph of a preparation of negatively stained F-actin. (**b**) A possible arrangement of the globular subunits in an actin filament. The subunits are drawn as spheres of diameter 55 Å. The centers of the subunits are helically arranged on the circumference of a cylinder. There are two twisted strands of subunits. The *arrows* indicate the cross-over points of the two strands. (**a**) Magnification ×525,000 (Hanson and Lowy 1963. With permission Elsevier)

They found that the native actin filaments isolated from striated and smooth muscles are indistinguishable from each other and from filaments of synthetic F-actin preparations. The actin filament consists of two strands which are helically-wound around each other. Each strand is composed of subunits which are alike and approximately spherical (see Fig. 3.13a). The number of globular subunits per turn of helix is 13 (Fig. 3.13b). The spacing of the subunits along each strand is 56.5 Å. The positions where the two strands are seen to cross over one another are regularly spaced along the filament at intervals of 349 Å. The overall diameter of the filament is about 80 Å. They concluded that each globular subunit seen in the electron microscope represents one actin monomer. Since the length of a thin filament in a striated muscle is about 1 μm, they estimated that a muscle thin filament would contain about 370 actin monomers.

Because of the potential introduction of artifacts with the preparation of the filaments for electron microscopy, Hanson and Lowy wanted to compare their results with those from intact muscle. Selby and Bear (1956) at the Massachusetts Institute of Technology published a paper on the structure of actin-rich filaments from intact

dried muscle using X-ray diffraction. In that study, they could not decide whether the X-ray scattering centers lied on a planar two dimensional net or on a helix. But the measured dimensions agreed with the helical model proposed by Hanson and Lowy (1963). Hanson and Lowy also compared their results with those from electron microscopy of striated muscle. Draper and Hodge (1949) had found that the I-bands of striated muscle contain transverse striations spaced axially at intervals of approximately 400 Å. No such striations were seen in the actin filaments isolated from muscles or in the synthetic actin filaments. Hanson and Lowy realized that the thin filaments in muscle likely contain another protein called tropomyosin B (Perry and Corsi 1958). They proposed a model in which there were two strands of tropomyosin which followed the actin helix and lied on the *outside* of the actin filament in the two "grooves" between the strands of actin monomers (Hanson and Lowy 1963). They speculated that such tropomyosin strands might easily be removed while preparing material for the microscope. But they admitted that there was no evidence available at time either for or against this model. The idea was that tropomyosin molecules might exhibit the 400 Å repeat. Their proposal that tropomyosin might lie in the "grooves" of the actin filament turned out to be remarkably prophetic but still this didn't explain the 400 Å repeat. There was yet another protein complex unknown to them that had to be considered, namely troponin (see Chap. 4 for details of tropomyosin and troponin association with the thin filaments and their functions there).

3.3.3 Myosin and Actin Filaments and Filament Polarity

Eight months after the classic paper by Hanson and Lowy (1963) appeared, Huxley (1963) produced another paper considered to be a classic in the muscle field. Like his paper in 1957, this publication can only be described as spectacular. The evidence produced not only was crucial in strengthening the proposal of a sliding mechanism of muscle contraction but also suggested that sliding filaments might be involved in movement of nonmuscle cells. His most important results derived from the examination of the structure of native and synthetic myosin filaments and actin filaments in combination with heavy meromyosin (HMM). These preparations were negatively stained and examined in the electron microscope.

Thick and thin filaments isolated from skeletal muscle The thick filaments isolated from rabbit or chicken skeletal muscle were 100–120 Å in diameter, 1.5–1.6 μm in length and tapered at each end. The filaments exhibited large numbers of irregular-looking projections on their surfaces. These were present right out to the tips of the filaments but they appeared to be absent from a central zone about 0.15–0.2 μm in length. The length of these filaments was independent of the length of the muscle before extraction. The isolated thin filaments were 60–70 Å in diameter and often 0.5–1.0 μm long. Some of the thin filaments were present as bundles of filaments attached together at a central plate (Z-line) and extended about 1 μm on either side of it. Huxley called these structures I-segments.

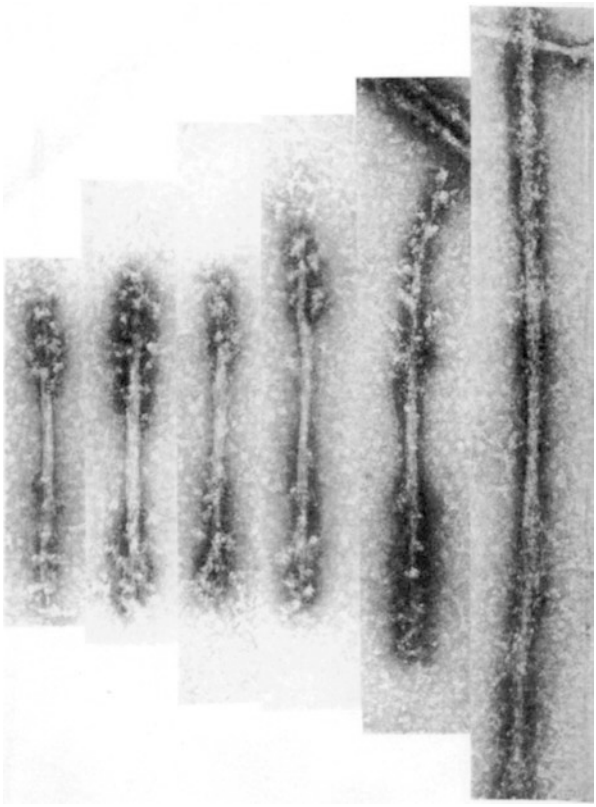


Fig. 3.14 Synthetic myosin filaments of varying lengths. All of the myosin filaments show the same characteristic pattern of a bare central shaft and projections all the way along the rest of the length of the filament. The myosin molecules are packed in an anti-parallel fashion with their tails of LMM overlapped in the center of the filament. Magnification: ×145,000 (Huxley 1963. With permission Elsevier)

Synthetic thick filaments Thick filaments composed of purified myosin were spindle shaped, variable in length and exhibited rough surfaces due to projections as seen in naturally occurring thick filaments (see Fig. 3.14). Throughout the whole range of lengths the bare shaft was always about the same length, i.e. 0.15–0.2 μm, and was situated near to the center of the filament, never at one end. From these results, Huxley concluded (Huxley 1963. With permission Elsevier):

> …the myosin molecules could have an HMM at one end only, could be 1500 Å long, and could form a short filament with the molecules at one end all pointing in one direction (with the HMM end outwards) with those at the other end pointing in the reverse direction, and with the anti-parallel molecules partially overlapped in the centre. In this way the projections due to the HMM could be separated by a bare central shaft whose length could be up to twice the length of the straight part (as opposed to the 'HMM projection') of the myosin molecule. All the LMM would be in the core of the filaments…This model …seems entirely satisfactory.

Actin filaments treated with HMM and with myosin In earlier experiments with myofibrils from which myosin had been extracted (Hanson and Huxley 1955), Huxley showed that incubation with HMM resulted in HMM binding to the myofibril thin filaments. He wanted to do this same experiment with isolated thin filaments

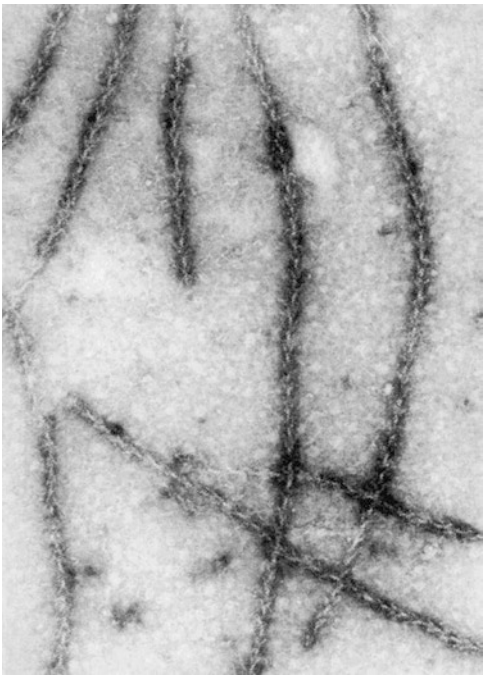


Fig. 3.15 Filaments of F-actin that have been treated with a solution of heavy meromyosin (HMM) in the *absence* of ATP. A very characteristic compound structure is formed. These "decorated" filaments have a well-marked structural polarity exhibiting "arrowheads" of HMM which point in the same direction all the way along any given filament. Magnification ×155,000 (Huxley 1963. With permission Elsevier)

from muscle and F-actin filaments. What he found has become famous in the muscle and motility literature (Fig. 3.15). Huxley's (1963) observed that the filaments had a very definite structural polarity with the appearance of "arrowheads" which all pointed in the same direction along the whole length of the filament. This complex was dissociated in the presence of ATP. Huxley interpreted these observations to indicate that a HMM subfragment (or myosin molecule) combined with each of the G-actin units in the F-actin structure, giving rise to a double helix of HMM subfragments wound around the outside of the original actin filament. The structural polarity must be imposed by the underlying actin structure and thus the actin filament itself must have a structural polarity. This meant that the monomeric units in the F-actin structure in both chains must all be oriented in the same direction. The question then became how are these thin filaments arranged in a muscle? Since some of his isolated preparations contained so called I segments consisting of a Z-line with bundles of thin filaments attached on either side, Huxley exposed these preparations to HMM. Huxley observed that all the filaments on the one side of the Z-line were structurally polarized in the same direction and all the ones on the other side in the reverse direction with the "arrowheads" pointing away from the Z-line.

These were remarkably exiting experiments but at first Huxley didn't fully recognize their importance. In 1996 he described his thoughts upon seeing these results (Huxley 1996. With permission Annual Reviews):

> After looking at my pictures of these 'decorated' actin filaments for about two days, the significance of what I was seeing suddenly dawned on me! They were structurally polarized, which meant that all the attached myosin heads and all the underlying actin sites must be

> oriented in the same direction. Clearly, such a polarity is what one would expect in a sliding filament system, but somehow the requirement had never occurred to me before- nor to anyone else, as far as I know! After that, it did not take very long to show that actin filaments were indeed attached to the Z-line with the appropriate polarities and that each half of a myosin filament had a corresponding structural polarity, reversing at the M-line. So it all made a great deal of sense in terms of the cross-bridge mechanism for muscle contraction.

More than making sense, it was actually a requirement that the sliding filament model of muscle contraction be able to explain the fact that unconstrained muscles when activated shorten, never lengthen. Thus there must be a polarity built into the muscle that any theory of contraction would have to explain. The results of these experiments must have been very gratifying to Hugh Huxley.

Huxley (1963) went on to speculate that the polarity of the actin filaments might be involved in nonmuscle motility. He suggested that an oriented gel of actin in which the filaments were polarized in one direction might be involved in cytoplasmic streaming. Thus he introduced the concept of "arrowheads" and oriented actin filaments into the nonmuscle motility field.

Huxley (1963) put it all together when he speculated about the mechanism of muscle contraction based on these and earlier results (Huxley 1963. With permission Elsevier):

> Two types of filaments, built up of different proteins, lie side by side in an aqueous medium of ionic strength 0.1 to 0.2 μ. The filaments have diameters of about 65 Å and 110 Å and their surfaces are about 170 Å apart. The thick filaments contain myosin, the thin ones actin. There are projections, 30 to 40 Å in diameter, on the thick filaments which extend out sideways and touch the thin filaments. These projections probably contain the enzymic site of the myosin molecule, and also the site responsible for combination with actin…The sites on all the projections are oriented in the same sense. It is not known whether the projections are rigid or flexible, nor whether they move along during activity. There is a projection between a given pair of myosin and actin filaments at regular intervals, and the interval is probably 435 Å. The actin filaments contain two helical chains of actin monomers…The actin monomers in each chain are about 55 Å apart; they are all oriented in the same direction and each of them can interact with the cross-bridge from the myosin filaments. Each actin filament has three myosin filaments around it, with which it interacts, and probably the cross-bridges from these filaments touch the actin filament at intervals of 435/3 = 145 Å…The cross-bridges provide the only apparent mechanical contraction between the two types of filament, and so they are likely to be responsible in some way for the relative force which can be developed between the filaments in an axial direction. A relative force is developed between the actin and myosin filaments when ATP is split by the system; the filaments can slide past each other by distances which are very large compared to the separation of the active sites on the actin and on the myosin and continue to generate tension.

This is a beautiful paper and is still exciting to read a half century after publication. Besides providing important information relative to the sliding filament mechanism of contraction, other important unanswered questions arose. For example, the synthetic myosin and actin filaments were variable in length but in muscle their length is precisely controlled. What determines the length of these filaments in vivo? What other proteins might be associated with the thick and thin filaments? And the nagging question of how might the preparative procedures alter the filaments and affect the conclusions reached? Finally, of course, the ultimate question is how does the myosin/actin interaction lead to muscle contraction? Much more would need to

be accomplished but this paper was an important building block in understanding the mechanism of muscle contraction.

Thick and thin filaments lengths in skeletal muscle With regard to the nagging question about how preparative procedures of muscle for electron microscopy might affect the conclusions related to filaments lengths, Sally G. Page, a graduate student from New Zealand, did a detailed study of this question in Hugh Huxley's laboratory at University College London (Page and Huxley 1963; Page 1964). The questions that she considered included: (a) do the preparative procedures affect the thick and thin filament lengths, (b) what are the likely thick and thin filament lengths in living resting muscle, (c) do these filament lengths change with isometric contractions at various sarcomere lengths? Results from light microscopy provided approximate values for the filament lengths but the resolution was limited to approximately 0.2 μm. This limitation implies that changes of filament length up to 10 % or so would likely be undetectable. The electron microscope provides much greater resolution but preparations had to be fixed, dehydrated, stained and embedded before observation. Any or all of these steps could in principle alter filament length. Page considered all the possibilities.

She concluded that the best estimate for the I-filament length in resting, living, muscle was derived from observation of isolated I-segments in the electron microscope *before* fixing and dehydration. Isolated I-segments are bundles of I-filaments attached to either side of a Z-line. The measured value of the I-segment length was 2.05 μm. Taking this value as the "gold standard", she found that the methods of fixation and dehydration could cause I-filament shortening in resting muscles. Osmium tetroxide (OsO_4) fixation could cause up to a 10 % decrease in I-filament length. The smallest effect was associated with glutaraldehyde fixation. She thus assumed that the measured A-filament length in glutaraldehyde fixed resting muscle represented the true length in living muscle. The value for the A-filament length was 1.6 μm. OsO_4 also caused up to 6–7 % decrease in A-filament length. So the best values for the living, resting frog muscle were 2.05 μm for the I-segment length and 1.6 μm for the A-filament length.

Page went on to determine the A- and I-segment lengths in frog muscles contracting isometrically at various sarcomere lengths greater than 2.1 μm. She found that the filament lengths in these fixed contracting muscles were the same as in the resting muscles and also were unchanged at sarcomere lengths from 2.2 to 3.7 μm, within an experimental error of less than 2 %. These muscles were fixed in OsO_4 but the filaments did not change length. Page speculated that this was because the cross-links formed between thick and thin filament during contraction prevented filament length changes.

But still these observations were not on living muscles but rather on muscles fixed for electron microscopy. So the nagging question was reduced but not completely eliminated. Page added credence to her conclusions by comparing the axial periodicity seen in living muscles with X-ray diffraction that was attributed to the thin filament periodicity observed in the electron microscope. This periodicity is seen in the electron micrographs of muscle as transverse striations in the I band. The value of the periodicity from X-ray diffraction studies in living muscle was 410 Å

(Worthington 1959). Page measured values from 350 to 420 Å in the I-filaments of muscles in the electron microscope. Correction for changes in length of the I-filaments due preparative procedures was done by multiplying the measured periodicity by the factor, 2.05 μm/measured I-segment length. When this was done, the value of the periodicity averaged about 406 Å. This value was in excellent agreement with the results from living muscle.

If the period of the transverse striations in the I-filaments is assumed to be 400 Å in various muscles, one could determine the I-filament length by counting the number of striations and multiplying by 400 Å. When this is done, the following results were found for I-filaments lengths in: (a) frog and chicken: 2.05 μm; (b) rabbit: 2.24 μm; (c) rat: 2.3 μm; and human: 2.55 μm. Page and Huxley (1963) speculated that these differences in I filament lengths in different muscles may have measurable physiological consequences. Finally Page and Huxley concluded that at a sarcomere length of 3.65 μm (2.05 + 1.6 μm) in frog skeletal muscle there would be no overlap of thick and thin filaments and thus, according to the sliding filament model, there should be no active force generated by a stimulated muscle. This number of 3.65 μm would turn out to become important in testing the sliding filament model of muscle contraction in living frog muscle (see below).

3.3.4 Cross-Bridge Orientation in Muscle at Rest and in Rigor

Hugh Huxley showed in 1957 that in resting muscle, the cross-bridges were "more or less at right angles" to the thick and thin filaments (see Fig. 3.5). In 1963 he demonstrated the "arrowhead" appearance when HMM became attached to actin filaments in the absence of ATP. It was natural to wonder if the arrowhead appearance could be observed in muscles that had transitioned into the rigor state when ATP was absent. Michael K. Reedy examined this question during a period of postdoctoral research in Hugh Huxley's laboratory at Cambridge. John W. S. Pringle of Oxford University suggested that Reedy use the flight muscle from the giant tropical waterbug, *Lethocerus*, for his studies because its structure is exceptionally well ordered, almost crystalline-like. Reedy et al. (1965) examined glycerinated muscle in the resting and rigor states using electron microscopic and X-ray diffraction techniques. In the resting state, the cross-bridges were essentially perpendicular to the filaments as seen by Huxley (1957b). In contrast the transition from the resting to the rigor state was accompanied by a re-orientation of cross-bridges between actin and myosin filaments (Fig. 3.16). The bridges were slanted at a mean angle of about 45° to the filament axis in rigor. The bridges joined the actin in symmetrical pairs, forming actin-centered "chevrons" which pointed away from the Z band. This orientation resembled the polarized "arrowhead" pattern observed by Huxley (1963) in actin filaments which had been complexed with heavy meromyosin. Also the polarization was the same as that seen in heavy meromyosin-treated I filaments which remained attached to the Z band. This study was the first to show a re-orientation of cross-bridges in the transition from one state of the muscle to another state. Also

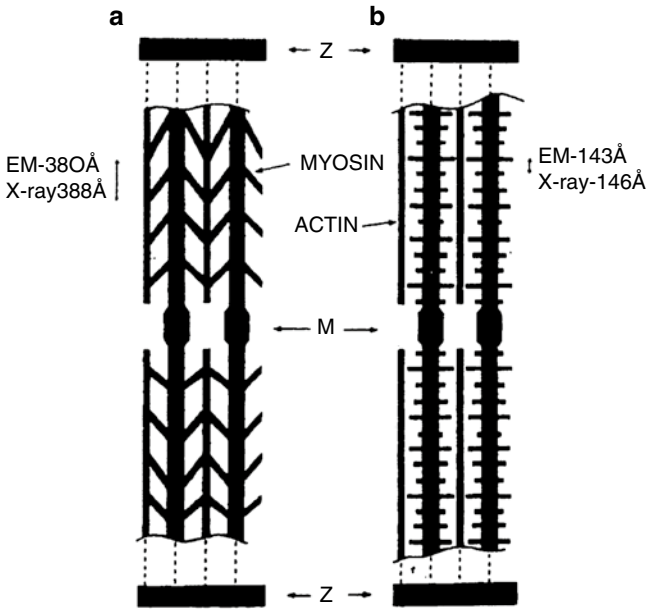


Fig. 3.16 Diagrams of single layers of actin and myosin filaments showing cross-bridge positions found in (**a**) rigor and (**b**) relaxed states of glycerinated insect flight muscle. Thick filaments represent myosin from which cross-bridges extend towards actin (thin) filaments. In (**b**) the variation in length of projections represents varying azimuthal positions of cross-bridge pairs around myosin filaments. At rest the cross-bridges project at an angle of about 90° from the thick filaments and that in rigor the cross-bridge angle is about 45°. Note that the polarity of the "chevrons" is reversed in each half of the A band. This appearance is reminiscent of the *arrowheads* observed when HMM attaches to F-actin (see Fig. 3.15) (Reedy et al. 1965. With permission Nature Publishing Group)

this study re-enforced the importance of filament polarity in muscle contraction. Huxley's "arrowheads" in isolated actin filaments are the same as Reedy's "chevrons" in glycerinated muscle. Reedy et al. (1965) went on to suggest that the changes in the position of cross-bridges might cause the active oscillation of the flight muscle. The tacit assumption here is that the rigor state represents a transient state in the cross-bridge cycle in living muscle. This is a common assumption that will re-appear throughout the muscle literature.

3.4 X-Ray Diffraction Studies of Muscles at Rest and During Contraction

The King's College London research group followed up on Hugh Huxley's pioneering X-ray studies on muscle with important contributions of their own. Elliott et al. (1963) provided evidence that the equatorial X-ray patterns seen in living muscle at rest and in rigor came from the A band with little contribution from the I band. As a

muscle was stretched the X-ray pattern changed in a way consistent with the decrease in overlap between thick and thin filaments and thus consistent with the sliding filament model of contraction. They noted that Knappeis and Carlsen (1962) at the University of Copenhagen showed that that the actin filaments were in a square array at the Z-line. Thus Elliott, Lowy and Worthington suggested that the actin filaments must be distorted through the I band in order occupy trigonal positions in the A band. This distortion would reduce the contribution of the actin filaments in the I band to the equatorial diffraction.

In Huxley's (1953a) pioneering low angle X-ray diffraction studies of muscle, he was not able to obtain useful X-ray patterns from contracting muscle because of the long exposure times that were required. Despite the "enigmatic" nature of the X-ray patterns, Huxley (1987) has argued that they "must be the truth". The X-ray diffraction technique provides the only way to examine structure in the living muscle on the nanometer scale. In many ways the electron microscopic observations of muscle facilitated the interpretation of the X-ray patterns and in turn the X-ray patterns from living muscles provided a control for potential artifacts associated with electron microscopy. With improvements in X-ray intensity and with the exposure of muscles to X-rays only during intermittent contractions (either twitches or brief tetani), Elliott et al. (1965) and Huxley et al. (1965) recorded the first X-ray patterns from contracting muscle. Their papers were published back-to-back as letters to the journal Nature.

What was found in these pioneering papers of the X-ray diffraction of contracting muscles? Elliott et al. (1965) considered two important questions. First, did the lateral spacing between the filaments, which was known to increase at shorter sarcomere lengths, adjust to a constant value during muscle contraction? The answer was no. Based on the equatorial X-ray pattern, they found only a small change in lateral spacing (6–12 Å) during contraction. They concluded that the interaction between the actin and myosin filaments during contraction must then involve long-range forces, or possibly some type of variable-length projections on the myosin filaments which can be at least 50 Å longer at short sarcomere lengths than at long lengths. Second, did the axial periodicities attributed to actin and myosin change during contraction? Again, the answer was no. This result eliminated any possibility of overall structural rearrangement of the actin and myosin filaments during contraction. These important results subsequently were published in full (Elliott et al. 1967). Huxley et al. (1965) also found little change in the axial periodicities attributed to the actin and myosin filaments when the muscle contracted. Huxley and Brown (1967) followed up these initial observations in great detail.

Elliott (2007) has described how these first papers of X-ray diffraction in contracting muscles came to be published together in Nature. Elliott knew that Hugh Huxley had started to do similar experiments at Cambridge. The DNA helix events had left a "friendly rivalry" between King's College London and the Cambridge laboratory. Elliott and his colleagues wanted to be the first to publish X-ray diffraction results from contracting muscles. When they sent their paper to Nature, the editor of Nature sent it to Max F. Perutz, head of the Cambridge laboratory, to referee. Perutz was willing to recommend publication but "in the manner of the times"

he wanted a paper from the Cambridge workers to be written for inclusion in the same issue. Thus the earliest low-angle X-ray diffraction studies of living contracting striated muscle by Elliott et al. (1965) and Huxley et al. (1965) were published back to back in a single issue of Nature. Gerald Elliott refers to the "friendly rivalry" that resulted from the famous incidence involving King's College investigator Rosalind Franklin's crucial data in elucidating the double helix of DNA and Max Perutz's role in sharing that unpublished information with his colleagues Watson and Crick as Elliott suggests was the "manner of the times". See James Watson's book (1968) for the story. Nonetheless, as an apparent gesture of good will, Huxley et al. (1965) acknowledged that Elliott, Lowy and Millman pioneered the study of the equatorial reflexions during contraction. In turn, years later Elliott (2007) noted that many other aspects of the diffraction patterns from contracting striated muscle were described in detail in a "wonderful paper" by Huxley and Brown (1967) that has become a classic of the field. The rivalry may not in fact have been friendly but it was at least respectful.

In the Huxley and Brown (1967) paper, they described two-dimensional axial X-ray patterns from relaxed and contracting frog muscle. These X-ray patterns were two-dimensional in the sense that both meridional and off-meridional reflections were recorded. An example of the X-ray diagram from a resting frog muscle is shown in Fig. 3.17a. This X-ray pattern was interpreted in terms of two helices: the helical arrangement of myosin cross-bridges in the thick filaments (Fig. 3.17b) and the helical arrangement of actin monomers in the thin filaments (Fig. 3.17c).[8] This interpretation was facilitated by the already published electron micrographs suggesting these helical arrangements (Hanson and Lowy 1963; Huxley 1963).

During muscle contraction with substantial shortening, the axial periodicities hardly changed, confirming that the thick and thin filaments remained essentially constant in length. However, the myosin layer lines which were derived from the helical arrangement in the resting muscle of the myosin cross-bridges around the thick filaments (Fig. 3.17), almost completely disappeared during contraction. Huxley and Brown (1967) interpreted this result as an indication that the cross-bridges moved during contraction. A meridional reflection remained at about 145 Å, suggesting that the distance between cross-bridges was unaltered. In what must have been somewhat of a disappointment, there were no new reflections observed during contraction. Thus the X-ray diffraction studies were moving into the realm of contracting muscle but clearly they had a long way to go before it would be possible to observe changes in the X-ray patterns on the same time scale as the time course of force development during contraction.

Huxley (1968) followed up these X-ray studies by examining the equatorial X-ray pattern of muscles at rest and in rigor. He concluded that the changes in the X-ray pattern seen in rigor could be explained if approximately 30 % of the original mass of the thick filaments moved from the vicinity of the thick filaments to the thin

[8] John M. Squire (1981) provides an introduction to X-ray methods in muscle research in his comprehensive book on the structural basis of muscular contraction. He describes how a helix will give axial x-ray pattern with repeats in intensities on and off the meridian.

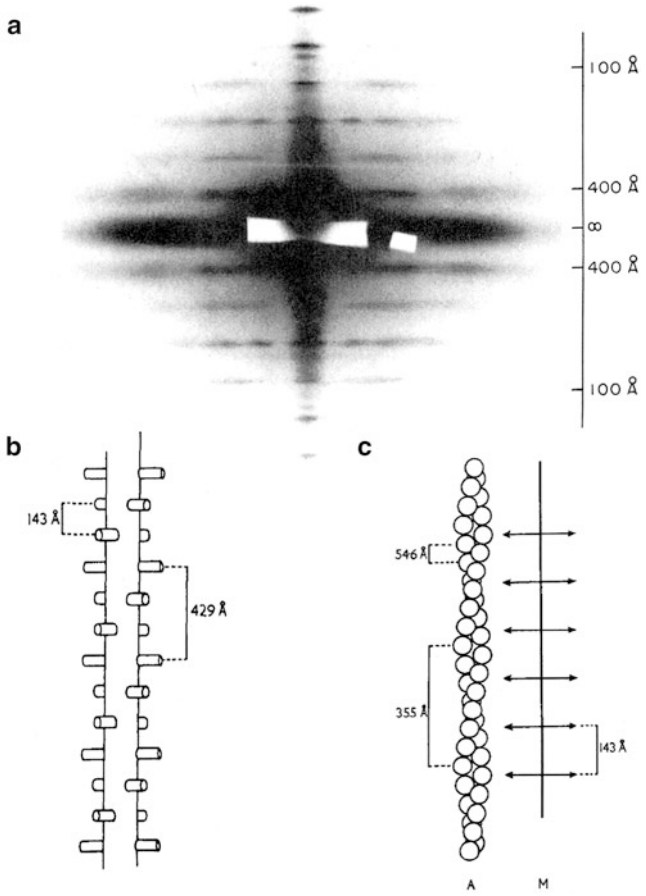


Fig. 3.17 (**a**) Axial X-ray diffraction pattern from living frog sartorius muscle: fiber axis vertical. The pattern of layer-line reflections corresponds to a repeat of 429 Å and is believed to arise from the helical arrangement of cross-bridges on the myosin filaments. (**b**) Schematic diagram showing the arrangement of cross-bridges in a thick filament based on X-ray diffraction experiments. The helical repeat is 429 Å and the true meridional repeat is 143 Å. Thus cross-bridge pairs are separated by 143 Å along a thick filament. (**c**) Diagram illustrating actin monomers in an actin filament in relation to the longitudinal positions of cross-bridges in a myosin filament (Huxley and Brown 1967. With permission Elsevier)

filaments. He went on further to speculate that the change in the equatorial diagram could be accounted for if the cross-bridges moved outwards from the thick filaments and attached themselves to the actin filaments when the muscle passed into rigor. Huxley was thinking in terms of a cross-bridge that could reach out and thus could accommodate the variations in thick and thin filament lateral spacings associated with changes in sarcomere length.

In the 1970s the synchrotron became the X-ray source of choice for muscle studies, largely through the work of Ken Holmes and Hugh Huxley. The early work

using laboratory X-ray generators became part of the history and lore of the muscle field. Looking back on these early X-ray studies, Huxley has commented on the fundamental problem (Huxley 2004a. With permission John Wiley & Sons Inc): "The problem was (and still is) that billions of individual crossbridge events happen asynchronously in a contracting muscle, so that all one normally sees is an X-ray pattern averaged over the whole crossbridge cycle, even in the shortest exposures." In the future, attempts would be made to minimize this problem by synchronizing cross-bridge movements during contraction.

3.5 Muscle Length Versus Isometric Force: A Critical Test of the Sliding Filament Hypothesis

In their classic paper, Huxley and Niedergerke (1954) predicted that if the sliding filament model of contraction is valid, the force developed during an isometric contraction should decrease as the overlap between the thick and thin filaments decreases and should be zero when the filament overlap is zero. This prediction is based on the assumptions that each cross-bridge is an independent force generator and that the cross-bridges are uniformly distributed throughout the filament overlap region. They noted that this prediction was in "fair agreement with observation". The "fair agreement with observation" referred to the elegant experiments of Robert W. Ramsey and his wife Sibyl F. Street (1940) at the University of Rochester. They observed a linear fall of isometric force when a single muscle fiber from the frog was stretched beyond rest length (Fig. 3.18). Huxley and Niedergerke didn't call the agreement "excellent". Andrew Huxley was always careful with his words and he certainly was careful here. There was a problem. Huxley and Niedergerke's results did not fit quantitatively with those of Ramsey and Street. From Huxley and Niedergerke's light microscope observations, the thick filament length could be estimated as 1.5 μm and the length of the thin filament as 1.0 μm. Thus Huxley and Niedergerke expected that zero filament overlap in frog skeletal muscle fibers would occur at a sarcomere length of about 3.5 μm (1.5 μm + 2 × 1.0 μm). In fact analysis of the mechanical results of Ramsey and Street, who did not measure sarcomere lengths, suggested that a muscle fiber may still generate appreciable isometric tension at lengths beyond 3.5 μm (Gordon et al. 1964). This discrepancy was real and it was not trivial. According to the analysis of the results of Ramsey and Street, the muscle would be expected to generate not zero force at 3.5 μm but rather about 40 % of its maximal force! Isometric force would not become zero until the fiber was stretched to a sarcomere length greater than 4.0 μm. There was a serious problem somewhere.

Was the problem with theory or experiment? Huxley (1980) admired the results of Ramsey and Street and thought that these experiments were among the first really satisfactory experiments of any kind performed on isolated muscle fibers. Does this mean the problem was with the theory? Maybe, but it seemed that the linear fall-off

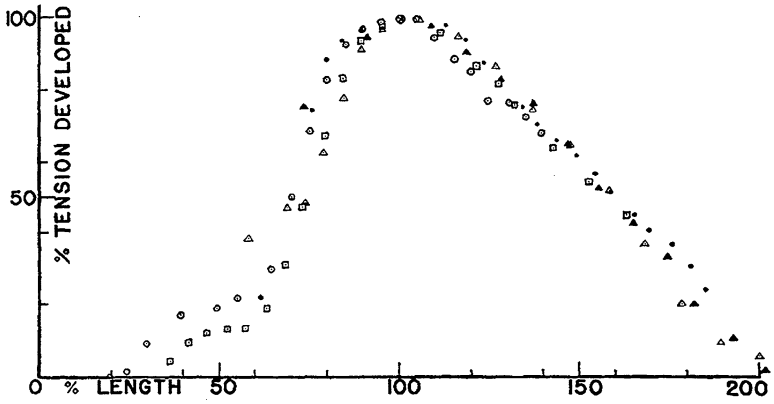


Fig. 3.18 Average isometric force versus muscle fiber length for frog semitendinosus fibers. The active force decreases linearly and reaches zero at 200 % of rest length. Assuming that rest length occurs at a sarcomere length of 2.0–2.1 μm, active force would reach zero at a sarcomere length greater than 4.0 μm (Ramsey and Street 1940. With permission John Wiley & Sons Inc)

of isometric force at fiber lengths greater than the length where force was optimum fit too well *qualitatively* with the notion of the sliding filament theory to be wrong. What then was the problem? A clue to the problem can be seen in the results of Ramsey and Street (1940). They observed that when a fiber was stimulated to produce an isometric contraction at long lengths, force increased in two phases, a fast phase similar to that observed at shorter fiber lengths followed by a very much slower phase. The slower phase became more prominent as the fiber lengths became longer. This result was not predicted by the sliding filament model of muscle contraction. Lee Peachey and Andrew Huxley discovered what the problem was in 1959 (Huxley and Peachey 1959, 1961) and Gordon et al. (1966a, b) solved the problem (or nearly solved the problem) in two classic papers in 1966.

3.5.1 "Creep" and Sarcomere Length Irregularity in Contracting Muscle

The two phases of the rate of rise of force at long muscle lengths observed by Ramsey and Street (1940) were reminiscent of similar observations of Hill (1953) with isolated whole muscles from the frog. Hill observed that at greater initial extensions the rise of tension during an isometric tetanus was much slower than at smaller initial extensions. He speculated that at an initial extension not greater than that at which the developed tension was maximum the system was stable and the tension reached its full value sharply but at greater extensions the system was unstable and a long slow "creep" of rising tension occurred. Hill further speculated that the instability which caused the slow rise of force which he called 'creep' was due

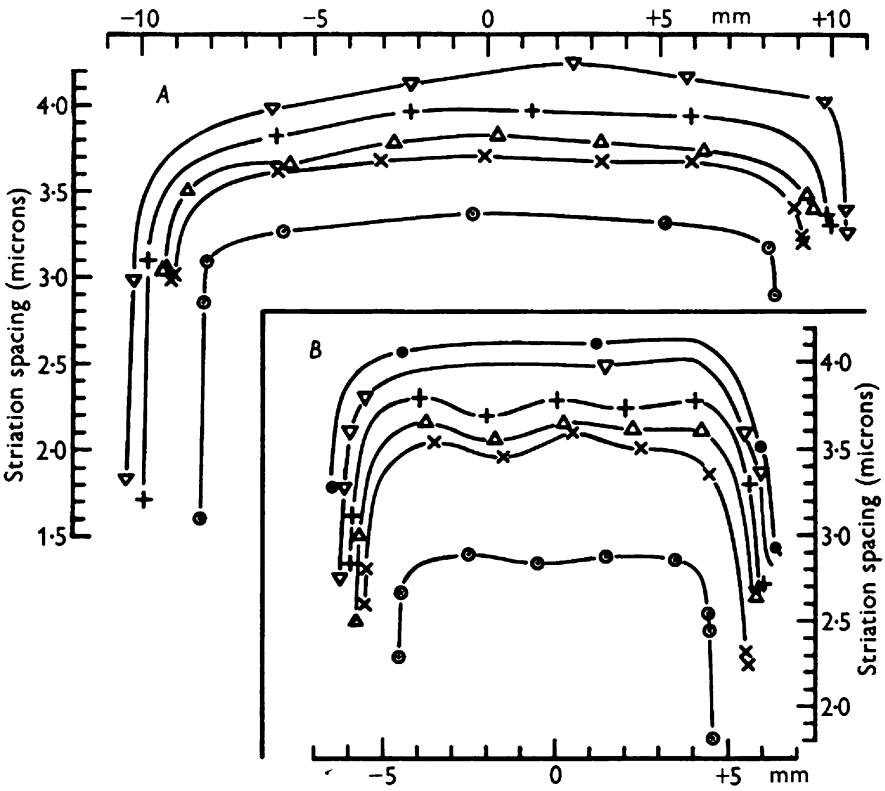


Fig. 3.19 Variation of sarcomere length along isolated muscle fibers at rest. (**a**) Fiber from semitendinosus muscle. (**b**) Fiber from iliofibularis muscle. Abscissa: distance from the center of the fiber; ordinate: sarcomere length. The various curves for each fiber were obtained at different degrees of elongation. Note the uniformity of sarcomere length in the middle 90 % of the fibers and the dramatic decrease in sarcomere length at each end of the fibers (Huxley and Peachey 1961. With permission John Wiley & Sons Inc)

to non-uniformities in the muscle that are inherent at long muscle lengths. Because of these non-uniformities those parts of the muscle which happened to be less extended than other parts would generate more force, and would shorten, and stretch the more extended parts, thus increasing any initial inequality in the degree of extension.

Lee D. Peachey came to the Huxley laboratory at the University of Cambridge in 1958 and he and Huxley examined the possibility that sarcomere length non-uniformities might explain the discrepancy between the results of Ramsey and Street and the sliding filament theory (Huxley and Peachey 1959, 1961). What they found was startling! When a resting muscle fiber from the frog was stretched, the sarcomere length was substantially shorter at the ends of the fiber than in the main part of the fiber (see Fig. 3.19). For example, the sarcomere length might be 3.6 to 3.7 μm over the middle 90 % of the fiber but only 3.0 μm at the ends (Huxley and

Peachey 1959). When such a fiber was stimulated to produce an isometric (fixed ends) contraction, the force rose slowly and the ends shortened greatly, stretching the middle part of the fiber (Huxley and Peachey 1961). Because of the inequality of sarcomere lengths and the resulting creep during contraction, the fixed-end contraction was not truly isometric from the viewpoint of the sarcomeres. This result made the determination of a truly isometric force versus sarcomere length relationship impossible. Because of this complication, to use a favorite expression of Andrew Huxley (Simmons 1992), the experiment would be a "bog" and thus was to be avoided.

So Peachey and Huxley took another approach. Rather than stimulating the fiber to produce force at a constant length, they stimulated the fiber to shorten under a light load, i.e., to produce an isotonic contraction. Under these conditions the force is the same in all sarcomeres. They examined the change of sarcomere length during contraction with cine-micrography. They wanted to determine the critical sarcomere length at which the middle of a fiber would not shorten when stimulated. Would that critical sarcomere length be near 3.5 μm as predicted by the sliding theory or greater than 4.0 μm as suggested by the results of Ramsey and Street? What they found was that if the sarcomere length in the middle of the fiber was less that 3.5 μm, the middle sarcomeres shortened vigorously upon stimulation. But if the middle sarcomere length was greater than 3.5 μm, no shortening occurred. Thus they concluded (Huxley and Peachey 1961) that contraction ceased to occur if a fiber was stretched to such an extent that there was no overlap between the two sets of filaments. This was taken as evidence that contraction depended on an interaction between filaments of the two types in the regions where they overlap, as postulated in theories of contraction based on the idea that length changes take place by a relative sliding motion of the filaments.

Even though the results were consistent with the sliding filament model of muscle contraction, they are not completely satisfying. The problem was clearly defined but not completely solved. The determination of the full relationship of muscle length to isometric force development was critical for any comprehensive test of the validity of the sliding filament model of contraction. Andrew Huxley had no choice but to wade into the "bog' and he did so with colleagues Albert M. Gordon and Fred J. Julian. What resulted is considered to be one of the main functional cornerstones of the sliding filament theory of muscle contraction.

3.5.2 The Spot Follower Device and the Muscle Length Versus Isometric Force Relationship

What was needed was a way to generate a truly isometric contraction from the viewpoint of the sarcomeres at various short and long muscle lengths. This project was initiated in 1960 when postdoctoral fellow Al Gordon came to the Huxley laboratory, now at University College London, from Cornell University after receiving a Ph. D. in solid state physics. The basic idea was to develop a feed-back apparatus that would keep the sarcomeres in the middle of the muscle fiber at a constant length

during an isometric contraction even though the sarcomeres at the ends were shortening. Analogous to the voltage clamp apparatus, this would be a segment length clamp apparatus. Designing and developing such a machine would be very complicated indeed. This project was just the kind of challenge that Andrew Huxley relished. What was developed was the famous "photo-electronic spot follower device". This apparatus gave an electric signal proportional to any change in the separation between two markers (pieces of gold leaf) stuck on the surface of a fiber. This signal was fed back to a servo-motor which pulled on one of the tendons so as to hold the marker separation at a constant value. Thus the servo system continuously adjusted the total fiber length in such a way as to hold constant, during an isometric contraction, the length of a uniform region in the middle of the fiber. The assumption here is that if the surface markers stay at a fixed length, the underlying sarcomeres will also remain at a fixed length. A complete description of the apparatus can be found in Gordon et al. (1966a) and a lucid description is given by Aidley (1998).

After helping to develop the apparatus and performing the initial experiments, Al Gordon left the Huxley laboratory in 1962 to accept a position in the department of physiology and biophysics at the University of Washington. Fred Julian arrived in the Huxley laboratory soon thereafter in 1962. Julian, a M.D. (1930–2010), was an experienced electrophysiologist who had performed voltage clamp experiments at the National Institutes of Health (Julian et al. 1962). He and Huxley made improvements to the apparatus and did the classic experiments described in the 1966 papers (Gordon et al. 1966a; b)[9]. What were the results of these experiments and why were they critical to the establishment of the sliding filament model of muscle contraction?

In the first paper (Gordon et al. 1966a), they concentrated on testing the apparatus and determining the critical sarcomere length where isometric force reached zero. When a part of a fiber with uniform sarcomere length was stretched so far that there was no overlap of filaments (3.92 μm), the force developed during an isometric tetanus with the spot follower activated was very small (about 3–5 % of the force developed at optimum length). If the tendon ends were held stationary, a fiber with the same initial length developed a large amount of force (of the order of 30–40 % of force at optimum length) with a slow time course. This additional force is due to shortening of the end parts of the fiber where the sarcomere length is shorter and overlap of filaments still exists. This shortening increased the distance between the surface markers which indicated that the central sarcomeres were stretched.

But the spot follower did not eliminate all of the sarcomere length inequalities, since a slow phase of contraction, though much reduced, was still present at the long sarcomere lengths. They attributed this slow phase to local "residual irregularity" of sarcomere length. To eliminate these residual irregularities, they manually extrapolated the force traces back to the start of the contractions. After this correction, the critical sarcomere length where force was zero was found to occur at a sarcomere length of 3.65 μm. This sarcomere length was precisely the length where thick and thin filament overlap became zero according to the results of Page and Huxley (1963).

[9] The names of authors on papers published in the Journal of Physiology appeared in alphabetical order until 1990. From 1990 onward the authors themselves determined their order on a publication in the Journal of Physiology.

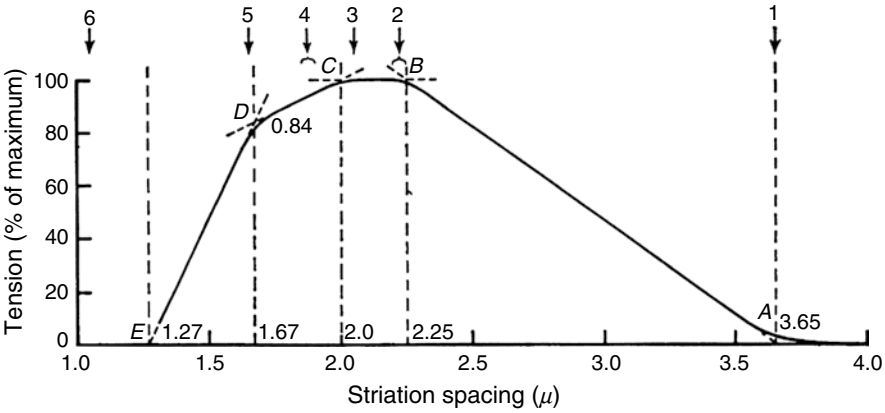


Fig. 3.20 A schematic summary of results describing the striation spacing versus isometric tension relationship in frog fibers. The *arrows* along the top are placed opposite the striation spacings at which the critical stages of overlap of the filaments occur (see Fig. 3.21). Note the plateau of optimum force production, the linear fall of active force at long sarcomere lengths and the dramatic decrease of force at short sarcomere lengths (Gordon et al. 1966b. With permission John Wiley & Sons Inc)

In the second paper (Gordon et al. 1966b), they put it all together and determined the full sarcomere length versus active force (length-tension) relationship. In summarizing their results, they produced two figures that have become classic in the muscle literature and that have found their way into many textbooks (Figs. 3.20 and 3.21). For technical reasons they could not construct the full curve on a single fiber. They split the curve into three parts: (a) the plateau, (b) lengths beyond the plateau and (c) lengths shorter than the plateau. Concerning the plateau where maximum force was generated, they found that it occurred over a sarcomere length range of 2.05–2.2 μm (Fig. 3.20). Over this sarcomere length range, isometric force varied by less than 2 %. This result is predicted by the sliding filament theory of contraction. Huxley (1963) had shown that because of the polarity of the thick filaments, there was a region in the middle of the filament where there are no cross-bridges (see Fig. 3.14). This bare zone was 0.15–0.2 μm wide. Given this information and the known length of the filaments (Page and Huxley 1963), one would predict that going from a sarcomere length of 2.2–2.05 μm (stage 2 to 3 in Fig. 3.21), would not result in any change in the number of cross-bridges that could generate force. Therefore there should be a plateau region in the length versus force relationship as was found. With regard to sarcomere lengths above the optimum (going from stage 2 to 1 in Fig. 3.20), isometric force decreased in a linear fashion and was zero at 3.65 μm. Again this result was in excellent agreement with the filament length measurements of Page and Huxley (1963).

The situation was more complex for the sarcomere lengths less than the lengths where optimum force was generated. The drop in force which begins when the

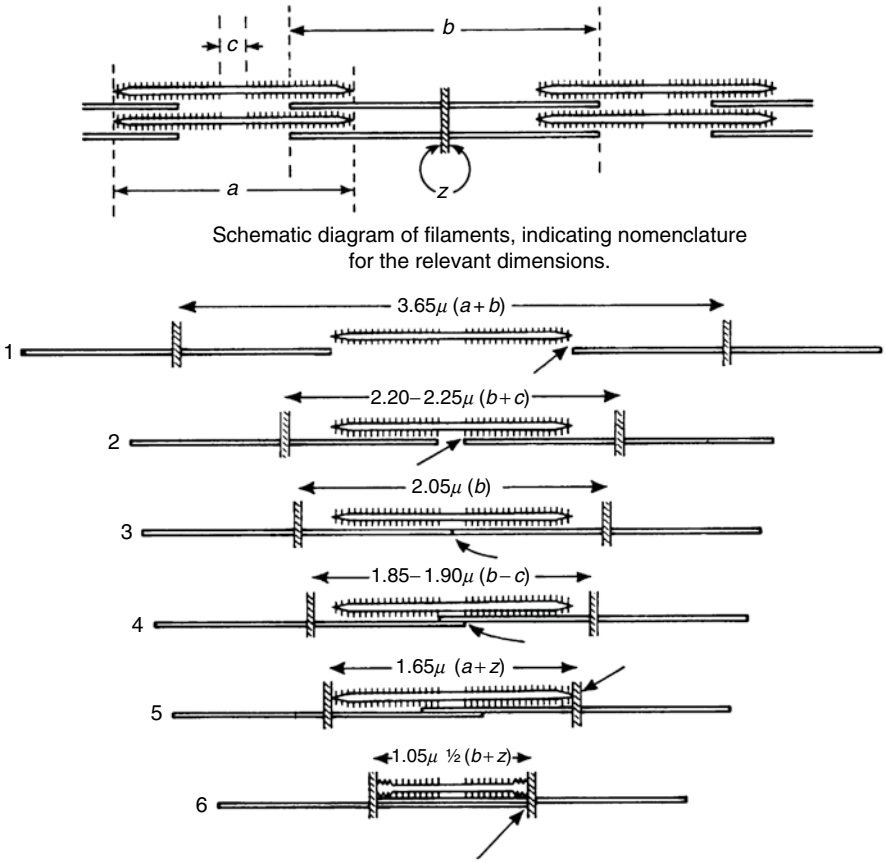


Fig. 3.21 Schematic diagram of filaments, indicating nomenclature for the relevant dimensions (*top*). Critical stages in the increase of overlap between thick and thin filaments as a sarcomere shortens from a long sarcomere length (*bottom*) (Gordon et al. 1966b. With permission John Wiley & Sons Inc)

sarcomere length is reduced below about 2.0 μm might be related to meeting of thin filaments at the center of the sarcomere (stage 3) and/or to the beginning of overlap of thin filaments with cross-bridges in the opposite half of the sarcomere (stage 4). The precipitous fall in force that starts at a sarcomere length of about 1.65 μm coincides with the position (stage 5 in Fig. 3.21) where the thick filaments would collide with the Z-lines in the sarcomere. In all, these beautiful results remain the strongest physiological evidence in support of the sliding filament attached cross-bridge theory of muscle contraction in living fibers.

Implications, *loose ends and curiosities* The story of the sarcomere length versus isometric force relationship doesn't end here. There are some implications, loose ends and curiosities that have to be mentioned. First, Gordon, Huxley and Julian assumed naturally that a tetanus would lead to maximum activation of the muscle

fiber at all sarcomere lengths. It has turned out that this is not true at the short sarcomere lengths since Taylor and Rudel (1970), in Andrew Huxley's laboratory, have shown that the fiber is not completely activated at short lengths. This inactivation decreases the amount of force generated at these lengths. Also since the lengths of the filaments were not determined in living muscle [see Page and Huxley (1963) discussed above], there was still a possibility of some slight uncertainty here. More serious from the viewpoint of the sliding filament model of contraction was the issue of *assuming* that the residual, manually corrected, creep was due to residual sarcomere irregularity. With regard to this manual correction of creep, Gordon et al. (1966b) stated that they had no direct evidence that sarcomere length irregularity was the cause of the residual 'creep'. Nonetheless they regarded it as "sufficiently probable" and thus extrapolated the force trace back to the beginning of the tetanus. This uncertainty, which Huxley (1980) called "a very tiresome complication", allowed "wiggle room" for some critics of the sliding filament theory (Noble and Pollack 1977). Despite these issues, the results of Gordon, Huxley and Julian and their interpretations have stood the test of time.

There is an important implication of these results that must be considered. Muscle is known to be a constant volume system with respect to changes in muscle length. This property implies that the lateral spacing of the thick and thin filaments would change as the length of the muscle changes. This prediction was verified in resting frog muscle using X-ray diffraction by Huxley (1953a) and Elliott et al. (1963) and in contracting muscle by Elliott et al. (1965; 1967, see above). The spacing could vary by as much as 70 Å when a frog muscle goes from a sarcomere length of 3.6–2.1 μm. Elliott et al. (1963) concluded that a regular lattice is maintained, even though the separation of the filaments varies over a wide range. Clearly these facts must be taken into consideration in any theory which attempts to explain the nature of the forces set up during contraction between actin and myosin filaments. These observations coupled with the results of Gordon et al. (1966b) imply that a cross-bridge is able to generate the same force even though the filament spacing may vary widely. It became important to develop a model of cross-bridge action that could satisfy this requirement and then to test the model.

There are some curious stories related to these experiments. One is that Al Gordon and Fred Julian were never in the Huxley laboratory at the same time and in fact never met until some 5 years after the classic papers were published (Simmons 1992). When they did meet, Gordon was at the University of Washington and Julian was an investigator at the Boston Biomedical Research Institute. Nevertheless their names are forever linked in the history of the validation of the sliding filament model of muscle contraction. Finally it should be noted that since muscles in the body operate around the plateau of the sarcomere length versus force relationship, they will not exhibit the sarcomere instabilities associated with the long, non-physiological, lengths. One might then ask: why do the experiments under non-physiological conditions? Of course the answer is that these experiments proved that muscles operate by a sliding filament mechanism of contraction also in the physiological length range and that force is developed at all sarcomere lengths by independent force generators.

3.6 Swinging-Tilting Cross-Bridge Model of Muscle Contraction

The late 1950s and 1960s was a period of great advances in the biochemical, structural and functional understanding of muscle contraction. In some ways, it was truly a "golden age". By the end of the 1960s, there could be little doubt that striated muscle contracted by a sliding filament mechanism involving independent force generators, i.e., the cross-bridges. But how did the cross-bridges work? Hugh Huxley (1969b) proposed the first comprehensive model of cross-bridge action. This proposal was called the "swinging-tilting cross-bridge" model of muscle contraction. A crucial experimental fact that had to be incorporated into any viable cross-bridge model was the observation that the lateral spacing between the thick and thin filaments varied substantially with muscle length without influencing the force generating capabilities of the cross-bridges. Huxley incorporated into the model the understanding of the myosin molecule that came from the enzymatic digestion experiments. There was the light meromyosin (LMM) subfragment that formed the backbone of the thick filaments and the soluble heavy meromyosin subfragment 1 (HMM S-1) that bound actin and hydrolyzed ATP. HMM S-1 was taken to represent the cross-bridge seen in the electron micrographs. In between these subfragments was a soluble heavy meromyosin subfragment 2 (HMM S-2) that attached to LMM and HMM S-1. Huxley (1969b) reasoned that there might be two "hinges" in the myosin molecule (Fig. 3.22). Since the junction between heavy and light meromyosin was susceptible to tryptic digestion, he speculated that it might represent a less perfectly α-helical region of the molecule and hence a region of greater flexibility. Similarly the junction between the linear part of heavy meromyosin and the globular part of the molecule was also susceptible to enzymatic digestion and so it too might be a flexible coupling. With two flexible joints, the orientation of the head of the molecule could be maintained when the link swung further out from the backbone of the filaments. Thus Huxley envisioned that the cross-bridge could "swing" out a variable distance as the filament spacing changed during contraction. This concept fit with his equatorial X-ray data that suggested that 30 % of the thick filament mass seemed to move toward the thin filaments when a muscle went from rest to rigor (Huxley 1968). He also incorporated into the proposal the concept of a change in orientation of the cross-bridge as seen by of Reedy et al. (1965) when a muscle went from rest to rigor when he suggested that "Clearly, the most likely seat of the force-developing mechanism is the globular part of heavy meromyosin and its attachment to the actin filament…Changes in orientation of the cross-bridge relative to the actin filament to which it is attached will then give rise to a relative sliding force between the filaments in the manner required" (Fig. 3.23). Thus in this formulation the cross-bridge swung out, attached and tilted utilizing the chemical free energy of ATP to generate force and do work. Huxley summarized the evidence leading to this proposal in his Croonian Lecture given in 1970 (Huxley 1971). In the ensuing decades much attention would be focused on characterizing cross-bridge behavior and unraveling the molecular mechanism of contraction.

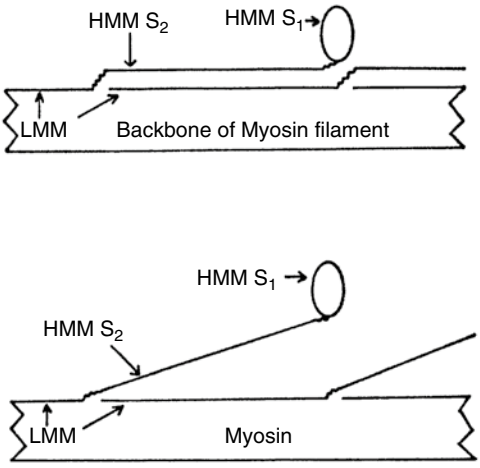


Fig. 3.22 Proposed behavior of myosin molecules in the thick filaments. The light meromyosin (LMM) part of the molecule is bonded into the backbone of the filament while the linear portion of the heavy-meromyosin (HMM S-2) component can tilt further out from the filament by bending at the HMM-LMM junction allowing the globular part of HMM (HMM S-1) to attach to actin over a range of different side-spacings, while maintaining the same orientation (Huxley 1969b. With permission American Association for the Advancement of Science)

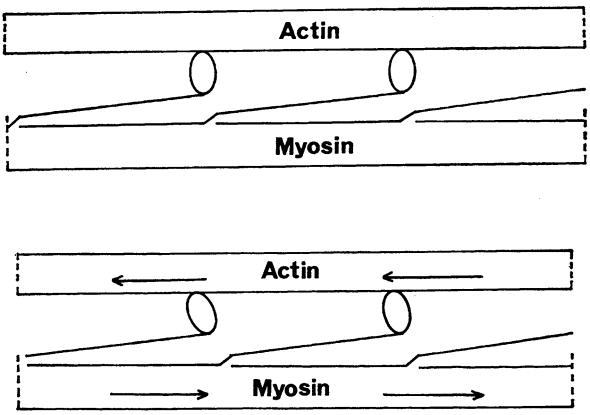


Fig. 3.23 Diagram illustrating a possible mechanism for producing relative sliding movement by tilting of cross-bridges toward the center of the sarcomere as shown in Fig. 3.16 (Huxley 1969b. With permission American Association for the Advancement of Science)

Toward the end of this classic paper, Huxley (1969b) made the perceptive observation that a full understanding of the molecular mechanism of muscle contraction would likely require the solution of the three-dimensional structure of the molecules by crystallographic techniques. He and others would have to wait two decades for these structures to be solved (see Chap. 9).

3.7 The Investigators: The Later Years

Many of the investigators who made fundamental discoveries during this period continued distinguished careers and some of their work will be described in later chapters. Of the four proposers of the sliding filament theory of contraction, Rolf Niedergerke moved immediately into the cardiac muscle field after the 1954 Nature paper with Andrew Huxley. In fact his classic paper with Hans-Christoph Luttgau initiating the study of Na-Ca exchange in cardiac muscle appeared earlier in 1958 than the full write-up of the sliding filament paper with Andrew Huxley. He continued his investigations of cardiac muscle contractility over a period of 40 years in the department of biophysics at the University College London.

Andrew Huxley continued to make fundamental discoveries relating to cross-bridge mechanics and muscle activation that will be considered in later chapters. He often spoke and wrote about the history of research and the pitfalls of ignoring the past. Also throughout his career he continued to design and make elegant devices for carrying out experiments. Probably the best known device is the Huxley micromanipulator which he designed (Huxley 1961). It is still commercially available as the Huxley-Goodfellow micromanipulator. Huxley (2004a) has commented that making these devices provided additional income for paying fees for the education of their six children. In a surprise, Huxley (2004a) has stated that he considered leaving the muscle field in the late 1950s and doing research on the mechanism of cochlear function. He did publish a theoretical paper on cochlear function (Huxley 1969b). Fortunately for the advancement of research in the muscle field he changed his mind.

Hugh Huxley continued to maintain his focus on elucidating the molecular mechanism of muscle contraction and his further contributions will be considered in later chapters. His research career has been remarkable. His contributions to understanding muscle contraction are fundamental and his work also opened up the field of non-muscle motility. One can't help but ask the question: Why no Noble Prize for Hugh Huxley? Some insight is gained from an email that Hugh Huxley sent to Max Perutz's biographer Ferry (2007). Apparently Perutz's strategy was to try to achieve separate Nobel Prizes for the Laboratory of Molecular Biology in the same year as had happened previously in 1962 by nominating Aaron Klug for a Noble Prize in chemistry and Hugh Huxley for a Noble Prize in physiology or medicine rather than making a joint nomination. This strategy turned out to be a tactical error as Aaron Klug won the Noble Prize in chemistry in 1982 and Hugh Huxley didn't win. In a interview with Alan Macfarlane for the King's College Cambridge history anthropology in 2007 (Alanmacfarlane.com), Klug stated that Hugh Huxley was the best electron microscopist of his time and it was a mystery why he did not get a Noble Prize for his work on muscle.

Jean Hanson and Hugh Huxley agreed to divide their future focus on muscle contraction (Huxley 2008). Huxley would concentrate on the details of the mechanism of contraction in vertebrate striated muscle and Hanson, the zoologist by training, would work to see if the sliding filament model applied to muscles across the

animal kingdom. In this regard her results on insect flight muscle were consistent with the sliding filament model (Hanson 1956). She and Jack Lowy published a major review on the contractile apparatus of invertebrates (Hanson and Lowy 1960). Even her classic paper with Lowy describing the actin filament structure utilized filament preparations from throughout the animal kingdom (Hanson and Lowy 1963). Tragically her flourishing career was cut short. Jean Hanson, whose phase contrast microscopy of isolated myofibrils and myosin extraction procedures, were a cornerstone of the sliding filament hypothesis of contraction (Huxley and Hanson 1954) died in 1973 at the age of 53 (see Fig. 2.2 for biographical details). She became the director of the Biophysics Research Unit at King's College London in 1970 and was at her scientific peak when in 1973 she died suddenly of a meningococcal infection. Bennett (2004) poignantly tells the tragic story (Bennett 2004. With permission Springer): "At a garden party in the summer of 1973 she was full of life and vigour and at the height of her powers. She had this wonderful unit going for her and she was really in charge. A week later she was dead…It was a great tragedy and left a great hole in the life of all of us." Praise for her as a scientist was only exceeded by praise for her as a person (Randall 1975). John Randall noted that Jean Hanson was a "big-hearted person" who was generous to her colleagues and always willing to collaborate. Hugh Huxley paid this tribute to Jean Hanson (Huxley 2004b. With permission Springer):

> She was a remarkable person, very modest and unpretentious, but with great and growing abilities in many directions. She was always absolutely fair, full of common sense, tolerant and forgiving of other people's weaknesses and trespasses, but very strong in what she thought was right, and extremely tenacious in getting to the bottom of a scientific problem. She also had an enormous capacity for hard work and long hours.

She did not seem to mind being the "moon" to Hugh Huxley's "sun" when it came to recognition of their discoveries relating to the proposal of the sliding filament model of muscle contraction. In 2004 Jean Hanson and her work was honored in an issue of the Journal of Muscle Research and Cell Motility (volume 25) on the fiftieth anniversary of the publication of the classic papers describing the sliding filament model of muscle contraction. (Also see the King's College London Archives on the pioneering work of professor Jean Hanson [www.kcl.ac.uk/depsta/iss/archives//hanson/index.htm].)

Thus there now could be no doubt that muscle contraction occurred via a sliding filament mechanism. Was the filament sliding caused by swinging cross-bridges attaching to actin and then tilting? That idea certainly seemed promising. But it is important to remember that the fact the filaments slide does not in and of itself specify a mechanism that causes the sliding. Much more work needed to be done and it wouldn't easy. There would be setbacks and doubts ahead.

Throughout this chapter on the description of the tests of the sliding filament hypothesis of muscle contraction, there was no consideration of how the muscle was turned on and off or how force was regulated. There were spectacular advances in these areas also during the 1950s and 1960s and those advances will be considered next.

References

Aidley DJ (1998) The physiology of excitable cells, 4th edn. Cambridge University Press, Cambridge

Barany M (1967) ATPase activity of myosin correlated with speed of muscle shortening. J Gen Physiol 50(Pt 2):197–216

Barany M, Barany K (2000) Strife and hope in the lives of a scientist couple. In: Semesnza G, Jaenicke R (eds) Selected topics in the history of biochemistry, personal recollections, VI. Comprehensive biochemistry, vol 41. Elsevier Science, Amsterdam, pp 91–167

Bennett PM (2004) Jean Hanson—a woman to emulate: leading research into the molecular basis of contractility in muscle. J Muscle Res Cell Motil 25:451–454

Cohen C (1975) The protein switch of muscle contraction. Sci Am 233:36–45

Cohen C, Szent-Gyorgyi AG (1957) Optical rotation and helical polypeptide chain configuration in α-proteins. J Am Chem Soc 79:248

Corsi A, Perry SV (1958) Some observations on the localization of myosin, actin and tropomyosin in the rabbit myofibril. Biochem J 68:12–17

Davies HG, Wilkins MHF (1952) Interference microscopy and mass determination. Nature 169:541

Draper MH, Hodge AJ (1949) Studies on muscle with the electron microscope. I. Ultrastructure of toad striated muscle. Aust J Exp Biol Med 27:465–505

Dreizen P, Gershman LC (1970) Relationship of structure to function in myosin. II. Salt denaturation and recombination experiments. Biochemistry 9:1688–1693

Edman KAP (1979) The velocity of unloaded shortening and its relation to sarcomere length and isometric force in vertebrate muscle fibres. J Physiol 291:143–159

Elliott G (2007) X-rays, twitching muscles, and burning anodes. Physiol News 67:6–10

Elliott GF, Lowy J, Worthington CR (1963) An x-ray and light-diffraction study of the filament lattice of striated muscle in the living state and in rigor. J Mol Biol 6:295–305

Elliott GF, Lowy J, Millman BM (1965) X-ray diffraction from living striated muscle during contraction. Nature 206:1357–1358

Elliott GF, Lowy J, Millman BM (1967) Low-angle x-ray studies of living striated muscle during contraction. J Mol Biol 25:31–45

Ferry G (2007) Max Perutz and the secret of life. Chatto & Windus, London

Gazith J, Himmelfarb S, Harrington WJ (1970) Studies on the subunit structure of myosin. J Biol Chem 245:15–22

Gergely J (1950) On the relationship between myosin and ATPase. Fed Proc 9:176

Gergely J (1953) Studies on myosin-adenosinetriphosphatase. J Biol Chem 200:543–550

Gordon AM, Huxley AF, Julian FJ (1964) The length-tension diagram of single vertebrate muscle fibres. J Physiol 171:28–30P

Gordon AM, Huxley AF, Julian FJ (1966a) Tension development in highly stretched vertebrate muscle fibres. J Physiol 184:143–169

Gordon AM, Huxley AF, Julian FJ (1966b) The variation of tension with sarcomere length in vertebrate muscle fibres. J Physiol 184:170–192

Hall CE (1956) Method for the observation of macromolecules with the electron microscope illustrated with micrographs of DNA. J Biochem Biophys Cytol 2:625–628

Hall CE, Jakus MA, Schmitt FO (1946) An investigation of cross striations and myosin filaments in muscle. Biol Bull 90:32–50

Hanson J (1956) Studies on the cross-striation of the indirect flight myofibrils of the blowfly *Calliphora*. J Biophys Biochem Cytol 2:691–710

Hanson J, Huxley HE (1955) The structural basis of contraction in striated muscle. In: Brown R, Danielli JF (eds) Symposia of the society for experimental biology. Number 9: fibrous proteins and their biological significance. Academic, New York, pp 228–264

Hanson J, Huxley HE (1957) Quantitative studies on the structure of cross-striated myofibrils. II. Investigations by biochemical techniques. Biochem Biophys Acta 23:250–259

Hanson J, Lowy J (1960) Structure and function of the contractile apparatus in the muscles of invertebrate animals. In: Bourne GH (ed) The structure and function of muscle, vol 1. Academic, New York, pp 265–335
Hanson J, Lowy J (1963) The structure of F-actin and actin filaments isolated from muscle. J Mol Biol 6:46–60
Hill AV (1938) The heat of shortening and the dynamic constants of muscle. Proc R Soc Lond B 126:136–195
Hill AV (1953) The mechanics of active muscle. Proc R Soc Lond B 141:104–117
Hodgkin AL, Huxley AF (1952) A quantitative description of membrane current and its application to conduction and excitation in nerve. J Physiol 117:500–544
Huxley AF (1957a) Muscle structure and theories of contraction. In: Butler JAV, Katz B (eds) Progress in biophysics and biophysical chemistry, vol 7. Pergamon Press, London, pp 255–318
Huxley AF (1961) A micromanipulator. J Physiol 157:5–7P
Huxley AF (1969a) Is resonance possible in the cochlea after all? Nature 221:935–940
Huxley AF (1980) Reflections on muscle. Liverpool University Press, Liverpool
Huxley AF (2004a) Andrew F. Huxley. In: Squire LR (ed) The history of neuroscience in autobiography, vol 4. Elsevier, Amsterdam, pp 284–318
Huxley AF, Niedergerke R (1954) Interference microscopy of living muscle fibres. Nature 173:971–973
Huxley AF, Peachey LD (1959) The maximum length for contraction in striated muscle. J Physiol 146:55–56P
Huxley AF, Peachey LD (1961) The maximum length for contraction in vertebrate striated muscle. J Physiol 156:150–165
Huxley HE (1953a) X-ray analysis and the problem of muscle. Proc R Soc Lond B B141:59–62
Huxley HE (1953b) Electron microscope studies of the organization of the filaments in striated muscle. Biochim Biophys Acta 12:387–394
Huxley HE (1957b) The double array of filaments in cross-striated muscle. J Biophys Biochem Cytol 3:631–648
Huxley HE (1960) Muscle cells. In: Brachet J, Mirsky AE (eds) The cell: biochemistry, physiology, morphology: specialized cells—Part 1, vol 4. Academic, New York, pp 365–481
Huxley HE (1963) Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. J Mol Biol 7:281–308
Huxley HE (1968) Structural difference between resting and rigor muscle; evidence from intensity changes in the low-angle equatorial x-ray diagram. J Mol Biol 37:507–520
Huxley HE (1969b) The mechanism of muscular contraction. Science 164:1356–1366
Huxley HE (1971) The structural basis of muscular contraction. Proc R Soc Lond B 178:131–149
Huxley HE (1987) Double vision reveals the structure of muscle. New Scientist 21:42–45
Huxley HE (1996) A personal view of muscle and motility mechanism. Annu Rev Physiol 58:1–19
Huxley HE (2004b) Fifty years of muscle and the sliding filament hypothesis. Eur J Biochem 271:1403–1415
Huxley HE (2004c) Jean Hanson's legacy. J Muscle Res Cell Motil 25:443–445
Huxley HE (2008) Memories of early work on muscle contraction and regulation in the 1950s and 1960s. Biochem Biophys Res Comm 369:34–42
Huxley HE, Brown W (1967) The low-angle x-ray diagram of vertebrate striated muscle and its behavior during contraction and rigor. J Mol Biol 30:383–434
Huxley HE, Brown W, Holmes KC (1965) Constancy of axial spacings in frog Sartorius muscle during contraction. Nature 206:1358
Huxley HE, Hanson J (1954) Changes in the cross-striations of muscle during contraction and stretch and their structural interpretations. Nature 173:973–976
Huxley HE, Hanson J (1957) Quantitative studies on the structure of cross-striated myofibrils. I. Investigations by interference microscopy. Biochem Biophys Acta 23:229–249

Huxley HE, Hanson J (1960) The molecular basis of contraction in cross-striated muscles. In: Bourne GH (ed) The structure and function of muscle: structure, vol 1. Academic, New York, pp 183–227
Julian FJ, Moore JW, Goldman DE (1962) Current-voltage relations in the lobster giant axon membrane under voltage clamp conditions. J Gen Physiol 45:1217–1238
Knappeis GG, Carlsen F (1962) The ultrastructure of the z disc in skeletal muscle. J Cell Biol 13:323–335
Lowey S, Slayter HS, Weeds AG, Baker H (1969) Substructure of the myosin molecule. I. Subfragments of myosin by enzymic degradation. J Mol Biol 42:1–29
Luttgau HC, Niedergerke R (1958) The antagonism between Ca and Na ions on the frog's heart. J Physiol 143:486–505
Mihalyi E, Szent-Gyorgyi AG (1953a) Trypsin digestion of muscle proteins. I. Ultracentrifugal analysis of the process. J Biol Chem 201:189–196
Mihalyi E, Szent-Gyorgyi AG (1953b) Trypsin digestion of muscle proteins. III. Adenosinetriphosphatase activity and actin-binding capacity of the digested myosin. J Biol Chem 201:211–219
Moss RL (1997) Towards the understanding of muscle contraction and regulation. J Muscle Res Cell Motil 18:115–118
Mueller H, Perry SV (1962) The degradation of heavy meromyosin by trypsin. Biochem J 85:431–439
Needham DM (1971) Machina carnis: the biochemistry of muscular contraction in its historical development. Cambridge University Press, Cambridge
Noble MIM, Pollack GH (1977) Molecular mechanisms of contraction. Circ Res 40:333–342
Page S (1964) Filament lengths in resting and excited muscles. Proc R Soc Lond B 160:460–466
Page SG, Huxley HE (1963) Filament lengths in striated muscle. J Cell Biol 19:369–390
Peachey LD (1983) (Section Editor). In: Peachey LD, Adrian RH, Geiger SR (eds) Handbook of physiology, section 10, skeletal muscle. American Physiological Society, Bethesda, p v–ix
Perry SV (1951) The adenosinetriphosphatase activity of myofibrils isolated from skeletal muscle. Biochem J 48:257–265
Perry SV (1997) Fate has smiled kindly. In: Semesnza G, Jaenicke R (eds) Selected topics in the history of biochemistry, personal recollections, V. Comprehensive biochemistry, vol 40. Elsevier Science, Amsterdam, pp 383–462
Perry SV, Corsi A (1958) Extraction of proteins other than myosin from the isolated rabbit myofibril. Biochem J 68:5–12
Ramsey RW, Street SF (1940) The isometric length-tension diagram of isolated skeletal muscle fibers of the frog. J Cell Com Physiol 15:11–34
Randall J (1975) Emmeline Jean Hanson. 14 November 1919–10 August 1973. Biogr Mems Fell R Soc 21:312–344
Reedy MK, Holmes KC, Tregear RT (1965) Induced changes in orientation of the cross-bridges of glycerinated insect flight muscle. Nature 207:1276–1280
Schaub MC (2010) Golden days of British muscle biochemistry. In memoriam S. V. Perry. J Muscle Res Cell Motil 31:9–11
Schneider MF, Chandler WK (1973) Voltage-dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. Nature 242:244–246
Selby CC, Bear RS (1956) The structure of actin-rich filaments of muscles according to X-ray diffraction. J Biophys Biochem Cytol 2:71–85
Simmons RM (1992) A. F. Huxley's research on muscle. In: Simmons RM (ed) Muscular Contraction. Cambridge University Press, Cambridge, pp 19–42
Slayter E (1976) Optical methods in biology. Robert E. Krieger Publishing Company, Huntington
Slayter H, Lowey S (1967) Substructure of the myosin molecule as visualized by electron microscopy. Proc Natl Acad Sci U S A 58:1611–1618
Squire J (1981) The structural basis of muscular contraction. Plenum Press, New York
Stracher A (1969) Evidence for the involvement of light chains in the biological functioning of myosin. Biochem Biophys Res Commun 35:518–525
Straub FB (1942) Actin. In: Studies from the Institute of Medical Chemistry, vol 2. University of Szeged, Szeged, pp 3–15.

Sutoh K, Sutoh K, Karr T, Harrington WF (1978) Isolation and physico-chemical properties of a high molecular weight subfragment-2 of myosin. J Mol Biol 12:1–22
Szent-Gyorgyi AG (1953) Meromyosins, the subunits of myosin. Arch Biochem Biophys 42:305–320
Szent-Gyorgyi AG (1975) Calcium regulation of muscle contraction. Biophys J 15:707–723
Szent-Gyorgyi AG (2004) Milestone in physiology: the early history of the biochemistry of muscle contraction. J Gen Physiol 123:631–641
Taylor SR, Rudel R (1970) Striated muscle fibers: inactivation of contraction induced by shortening. Science 167:882–884
Tsao T-C (1953) Fragmentation of the myosin molecule. Biochim Biophys Acta 11:368–382
Watson JD (1968) The double helix. A personal account of the discovery of the structure of DNA. Atheneum, New York
Weeds AG (1969) Light chains of myosin. Nature 223:1362–1364
Weeds AG, Lowey S (1971) The substructure of the myosin molecule. II. The light chains of myosin. J Mol Biol 61:701–725
Worthington CR (1959) Large axial spacings in striated muscle. J Mol Biol 1:398–401
Zhang Y (2010) In memory of professor Tianqin Cao (Tien-chin Tsao). Protein Cell 1:507–509

Chapter 4
Excitation-Contraction Coupling and the Role of Calcium in Contraction and Relaxation in the 1950s and 1960s

We shall designate the entire sequence of reactions-excitation, inward acting link, and activation of contraction-by the term excitation-contraction (E-C) coupling. (Sandow 1952. With permission Yale Journal of Biology and Medicine)

Alexander Sandow (1952)

It is quite impossible, therefore, to explain the rapid development of full activity in a twitch by assuming that it is set up by the arrival at any point of some substance diffusing from the surface: diffusion is far too slow. (Hill 1949. With permission The Royal Society)

A. V. Hill (1949)

It is ironic that recognition of the essential role of Ca ion in contraction has resulted mainly from the investigation into the mechanism of relaxation. (Ebashi and Endo 1968. With permission Elsevier)

Setsuro Ebashi and Makoto Endo (1968)

4.1 Introduction

While spectacular advances were being made in the understanding of the mechanism of contraction during the 1950s and 1960s, there were equally exciting advances during this same period in the understanding of how muscles were turned on and turned off. Alexander Sandow at New York University gave a name to this new field of study of activation of muscle contraction when he coined the term "excitation-contraction coupling" (Sandow 1952). He defined the field this way: "We shall designate the entire sequence of reactions—excitation, inward acting link, and activation of contraction—by the term excitation-contraction (E-C) coupling". The elucidation of the fundamental features of E-C coupling came from a melting of physiology, cell biology and biochemistry. Even though this is a great success story, there were mis-steps, forgotten clues and strong personalities along the way. And as pointed out by Ebashi and Endo (1968): "It is ironic that recognition of the essential role of Ca ion in contraction has resulted mainly from the investigation into the mechanism of relaxation." This period also resulted in the emergence of the influence of Japanese scientists in the muscle field. This emergence was almost single handedly due to the outstanding achievements and influence of Setsuro Ebashi.

J.A. Rall, *Mechanism of Muscular Contraction*, Perspectives in Physiology,
DOI 10.1007/978-1-4939-2007-5_4

4.2 Muscle Excitation and Onset of Contraction

At the time when Alan Hodgkin and Andrew Huxley were doing their classical experiments elucidating the ionic mechanism of the nerve action potential in 1949, William (Bill) L. Nastuk came to the Hodgkin laboratory from Columbia University to work on the excitation of skeletal muscle. In 1948 Hodgkin had visited Ralph Waldo Gerard's (1900–1974) laboratory at the University of Chicago where he learned of the possibility of making intracellular electrical recordings with microelectrodes. While there he met a Chinese investigator, Gilbert Ling, who had found that if he drew a 1 mm glass tube down to a fine tip, it would penetrate a frog muscle fiber easily, giving a stable resting potential while causing no damage to the fiber. The trick was that the tip had to be so fine that it disappeared when looked at with visible light under a high-powered objective (Hodgkin 1992).

Gerard had the original idea to develop the microelectrode. Judith Graham produced microelectrodes with tip diameters of several micrometers and they used these larger microelectrodes to measure resting membrane potentials in skeletal muscle fibers (Graham and Gerard 1946). Gilbert Ling perfected the technique by making smaller microelectrode tips of about 0.5 μm diameter which led to measurement of the first accurate values of the resting membrane potentials in frog skeletal muscle (Ling and Gerard 1949). Ling taught Hodgkin how to make and fill these microelectrodes with a potassium chloride solution for which Hodgkin was "greatly indebted" (Nastuk and Hodgkin 1950). Ling and Gerard gave Hodgkin permission to use the microelectrodes in an attempt to measure action potentials in skeletal muscles back in Cambridge. Hodgkin (1992) was particularly interested in these experiments because they helped to answer the criticisms that the work on crab or squid nerve might not be relevant to mammalian excitable tissues. What resulted (Nastuk and Hodgkin 1950) were the first intercellular recordings of action potentials in skeletal muscle.

The action potentials observed in skeletal muscle fibers of the frog with microelectrodes of about 0.5 μm tip diameter were qualitatively similar to those observed in squid axons. The action potential started from an average resting potential of −88 mV and overshot the zero potential by about 30 mV. The magnitude of the action potential was strongly depressed when the extracellular Na^+ concentration was decreased whereas little change was observed in the resting membrane potential. These results were similar to those observed in squid axons and supported the generality of the theory that the action potential was due to a specific increase in membrane Na^+ permeability.

There were some differences in the muscle action potentials compared to the previously observed nerve action potentials. Most notable was the tail or slow repolarization of the action potential, called the negative after-potential, that was always seen in the skeletal muscle action potentials but not in the squid axon action potentials. Another notable difference was that the capacitance of the skeletal muscle fiber membrane was found to be about five times greater than that observed in nerve fibers (Katz 1948). Membrane capacitance is a function of the chemical structure of the membrane and is expressed relative to the area of membrane as micro-farads per centimeter squared ($\mu F/cm^2$). Thus either the muscle membrane was fundamentally

different from the nerve membrane, which seemed unlikely given the similar nature of the action potential, or somehow the area of the muscle membrane was substantially under estimated when based on fiber diameter alone. It would be some years before this mystery was solved in favor of the latter possibility.

Contraction of the muscle follows the action potential after a very brief delay. In a very influential study in 1949, A. V. Hill showed by stretching a frog muscle rapidly immediately after an action potential that it reached peak active force within 40 ms at 0 °C. Hill (1948) estimated the time that it would take for any putative activator substance to diffuse from the cell membrane to the center of a typical frog muscle fiber of 100 μm diameter. Based on this calculation, Hill (1949) concluded that "It is quite impossible, therefore, to explain the rapid development of full activity in a twitch by assuming that it is set up by the arrival at any point of some substance diffusing from the surface: diffusion is far too slow." The situation is even more acute when a muscle contracts at a higher temperature since diffusion is relatively temperature insensitive. For example, the interval between the action potential and the start of contraction which is called the latent period is about 6 ms in a frog fiber at 20 °C (Buchthal and Sten-Knudsen 1959).

If not a chemical diffusing inward from the muscle surface, what then is the link between the action potential and contraction? One possibility is that the local currents setup by the action potential could spread rapidly throughout the fiber to directly activate contraction. Kuffler (1946) showed that it is not the local current flow that activates contraction but rather the membrane potential change. He observed that a contraction is produced even by a uniform depolarization of the fiber surface by immersion in a solution of elevated K^+ concentration where there should be no current flow. This result was confirmed by Watanabe (1958) who passed current between two intracellular microelectrodes and found that no contraction occurred. Another possibility was that the membrane potential change could directly cause contraction. But the membrane potential change would be expected to only exert a direct influence on the membrane itself and not propagate into the fiber interior. Thus there must be some other process that leads to the activation of a muscle fiber. In early 1950s this process was totally unknown.

4.3 Inward Spread of Muscle Activation

In late 1953, Andrew Huxley and Rolf Niedergerke were finishing up their famous experiments which led to the development of the sliding filament theory of contraction. At about this time Robert (Bob) E. Taylor[1] joined the Huxley laboratory from

[1] Robert E. Taylor received his Ph.D. from the University of Rochester. After a brief stay in R. W. Gerard's laboratory at the University of Chicago, he went to Andrew Huxley's laboratory from the University of Illinois College of Medicine in Chicago. He then went to the Laboratory of Biophysics at the National Institutes of Health in Bethesda, Maryland in 1956 where he spent his research career. Besides doing experiments at the NIH, he also worked at the Marine Biological Laboratory at Woods Hole for many years. He investigated electrophysiological properties of nerve fibers. In 1982 Bezanilla, White and Taylor discovered the gating current associated with the activation of K^+ channels.

the University of Illinois (Chicago). Huxley and Taylor worked together to produce some of the most famous experiments in the muscle field which established the mode of the inward spread of muscle activation. There were mis-steps and surprises along the way, starting with a hypothesis that turned out to be wrong. In 1924 the Australian zoologist Oscar Werner Tiegs (1897–1956) proposed that the Z line could provide a pathway for the inward spread of muscle activation. By this he meant not only the Z line as observed in a myofibril but also the transverse connections that linked myofibrils together and attached them to the cell membrane. Together this structure was called Krause's membrane based on the work of the nineteenth century light microscopist, Wilhelm Krause (1833–1910). It was not envisioned by Huxley and Taylor as a membrane in the usual sense but rather as solid network that might in some unspecified way be a pathway for muscle activation (Huxley 1995). Thus they started their experiments with the working hypothesis that the Z line provided a transverse pathway into the muscle fiber leading to activation of contraction.

But how does one test this hypothesis? The immediate answer is: with great difficulty. This difficulty was exactly the kind of experimental challenge that Andrew Huxley relished. The basic idea was to cause a local depolarization by applying a very small current, not enough to generate an action potential, to a micropipette whose tip was in contact with the surface of a muscle fiber at various spots along a sarcomere of the fiber. The prediction was that a local contraction would occur only when the pipette was at the Z line. This experiment was demanding for a number of reasons. A single muscle fiber from the frog had to be dissected. The glass pipette, of the Ling and Gerard (1949) type, filled with a saline solution had to have a flat tip of diameter no more than 2–4 μm and had to be placed in contact with the fiber but not penetrate the fiber membrane. A micromanipulator would have to be utilized that allowed very precise, submicrometer, positioning of the pipette. A high powered light microscope, at first a polarizing and later an interference microscope, was necessary for accurate identification of the fiber banding pattern and placement of the pipette. A controlled depolarizing pulse of various magnitudes had to be applied to the pipette. Finally cine photography, at 16 frames per second, would have to be employed to observe the results of the depolarization as a function time. To improve resolution, the frog fibers were stretched from a normal resting sarcomere length of about 2.2 μm to about 3.0 μm.

The results of the experiments were spectacular (Huxley and Taylor 1958). When the pipette was placed opposite the A band and a depolarizing pulse applied, nothing happened (Fig. 4.1a). But when the pipette was placed opposite the Z line, a "sensitive spot" was observed where a depolarizing pulse caused a local contraction (Fig. 4.1b). The I band shortened symmetrically about the Z line. The adjacent A bands did not shorten. A hyperpolarizing pulse had no effect. Most remarkably (Fig. 4.1c), with a larger pulse the I band to which this pipette was applied shortened about 10 μm inward from the surface while the adjacent I bands (1.5–3 μm from the central Z line) did not shorten but rather were passively stretched (Huxley and Taylor 1955a). It was as if some structure directed the depolarization transversely into the fiber but not longitudinally. In a paper sent to Nature in September of 1955,

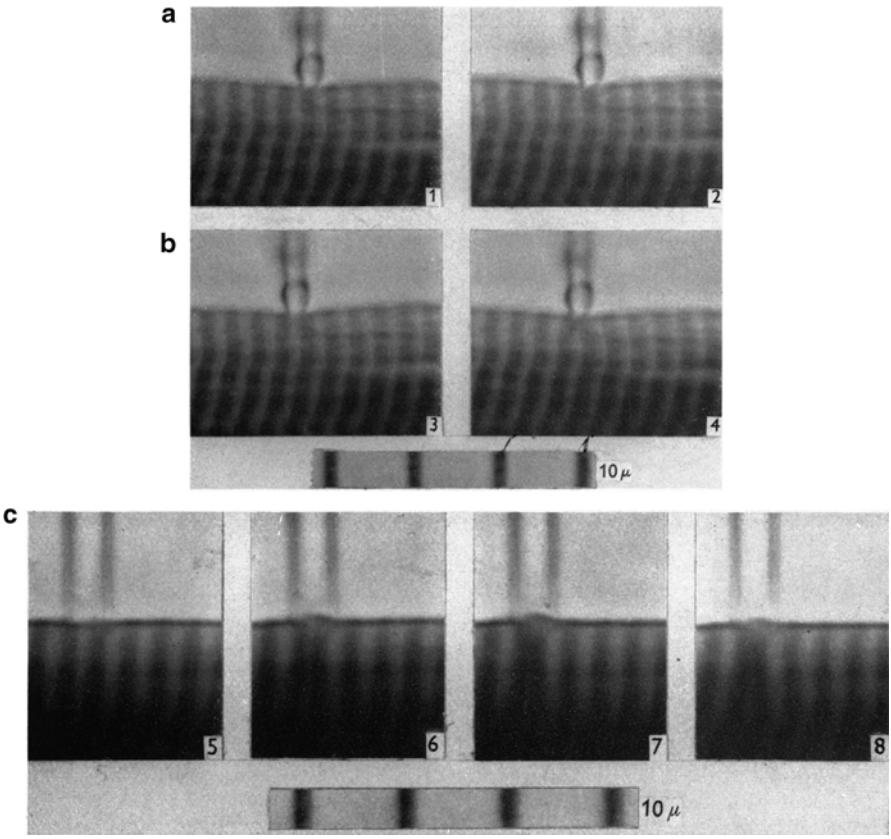


Fig. 4.1 Photographs of an isolated frog muscle fiber in contact with a micropipette. Polarized light compensated so that the A bands appear dark. (**a**) The pipette (tip diameter 2 **μ**m) is applied in images 1 and 2 to an A band and (**b**) in images 3 and 4 to an I band. In each case, the left-hand picture is taken just before, and the right-hand picture during, a negative pulse applied to the pipette. A contraction is produced only when the pipette is opposite an I band (image 4). (**c**) Images 5 through 8 are successive frames of a cine film showing the nature of shortening induced by a local depolarization with a 4 **μ**m pipette. The onset of a negative pulse applied to the pipette occurs between images 5 and 6. Note that the contraction spreads inward but not longitudinally (Huxley and Taylor 1958. With permission John Wiley & Sons Inc)

Huxley and Taylor (1955a) concluded that the results were strong evidence that the influence of membrane depolarization was conveyed to the interior of the fiber by spread along some structure in the I band. From the anatomy of the fiber, they believed that "this must almost certainly be Krause's membrane". In his classic review written in early 1955 (Huxley 1957a), Andrew Huxley was somewhat more circumspect in his conclusion when he stated that it was not actually proven that the Z membrane was the structure concerned but rather it may be that the activation was conveyed along some other structure near the middle of each I band. Nonetheless he noted that the Z membrane had the required characteristics and thus it seemed probable

that it was the structure involved in the inward spread of activation. Huxley and Taylor naturally were pleased to have their hypothesis confirmed. But it turned out that there was a problem, a big problem.

Later that year, in November of 1955, Huxley and Taylor presented their results at a meeting of the Physiological Society in London (Huxley and Taylor 1955b). In the audience was J. David Roberston[2] who at the time was working in the department of anatomy at University College London. Huxley (2004) recalled that Robertson "produced from his pocket" a slide showing an electron micrograph of a longitudinal section of muscle which clearly showed a pair of tubules penetrating the fiber on either side of each Z membrane. He therefore suggested that inward conduction took place along these tubules and that the pipettes utilized by Huxley and Taylor were not small enough to distinguish between the two members of each pair. [The slide in question subsequently appeared as figure number ten in a paper published by Robertson (1956).] This suggestion must have been galling to the technical expert Andrew Huxley, not to mention that their hypothesis regarding Krause's membrane would be invalidated. But there was another possibility other than a technical one. Since Robertson's experiment was done on a lizard skeletal muscle and not frog skeletal muscle, there might be a species differences. But given the "uniformity of nature" this possibility seemed somewhat unlikely.

Huxley (1957b) responded to this criticism by doing experiments on crab muscle fibers which have a resting sarcomere length of about 8 μm instead of the 3 μm of the stretched frog fibers. To his surprise, what he found confirmed Robertson's prediction. The sensitive spots along the sarcomere existed at the boundary between the A and I bands and not at the Z line. Only the adjacent half-I band shortened and pulled the Z line towards the A band. Furthermore Huxley confirmed the earlier results from the frog fibers. Subsequently Ralph Straub (1928–1988) joined the Huxley laboratory from Geneva and they (Huxley and Straub 1958) determined that in lizard muscle the sensitive spots appeared at the A/I boundary and not at the Z line just as Robertson predicted. Thus the inward spread could not in general be attributed to Krause's membrane.

Huxley (1957b) made another important observation in frog muscle fibers. The sensitivity did not extend continuously along the Z line but was confined to spots separated by distances of the order of 5 μm around the fiber. With regard to this observation, Huxley and Taylor (1958) concluded that each fiber contained networks

[2] J. (James) David Robertson, M.D., Ph.D. (1923–1995) was a pioneer in the use of the electron microscopy to study cell membrane structure. After receiving his M.D. from Harvard University, Robertson joined F. O. Schmidt's laboratory at MIT in the late 1940s to learn electron microscopy. He received a Ph.D. from MIT in 1952. He then went to the anatomy department at University College London in 1955 and while there Robertson described in 1957 the "unit membrane" hypothesis at a meeting of the Physiological Society to explain the structure of the cell membrane (Robertson 1958). This hypothesis became the fore runner of the "fluid mosaic" model of the cell membrane developed by S. J. Singer and G. L. Nicholson in 1972. Robertson is also credited with the discovery of the structural basis of electrical synaptic transmission. He spent most of his career at Duke University where he was the chair of the anatomy department. For more information, see an autobiographical chapter (Robertson 1987).

of tubules, electrically continuous with the external fluid, in the transverse planes corresponding to the positions at which surface depolarization was effective and that reduction of the potential difference across the walls of these tubules activated the myofibrils. The work on frog and crab muscle fibers subsequently was published in full in 1958 in the same issue of the Journal of Physiology that included the full publication of the Huxley and Niedergerke (1958) sliding filament paper. These two classic papers were published "back to back".

There was no indication that the physiological data was wrong but the picture was confusing. The sensitive spots occurred in different parts of the sarcomere in different muscle species. A no doubt somewhat frustrated Andrew Huxley (1971) noted that this was not what might be expected from phylogenetic relationships. What was needed was a deeper understanding of the internal membrane structure of muscle fibers. It turned out that this understanding also was evolving in the 1950s.

4.4 Sarcoplasmic Reticulum, Triads and Transverse Tubules

In 1953, cell biology pioneers H. Stanley Bennett and Keith R. Porter, using electron microscopy, described an intracellular membrane system in skeletal muscles of the fowl. The membrane system, located between the myofibrils, exhibited longitudinal and transverse components. They named the structure the sarcoplasmic reticulum in analogy to the endoplasmic reticulum observed in other cells. Bennett (1955) suggested that the sarcoplasmic reticulum might be responsible for conveying the inward spread of activation seen by Huxley and Taylor (1958). Bennett (1956) has stated that he and Porter actually rediscovered the sarcoplasmic reticulum with the electron microscope. He held this view because the nineteenth century light microscopists already had described such a system (Bennett 1956, 1960).

The most meticulous study of the reticular apparatus of striated muscle was conducted by Emilio Veratti (1872–1967) at the University of Pavia in 1902. Bennett (1956) believed that this paper was one of the most important ever published on muscle structure. So enthusiastic was Bennett about Veratti's results that he helped translate the paper from Italian and had it re-published in the Journal of Biophysical Biochemical Cytology (Veratti 1961). What did Veratti observe? Using the "black reaction" (precipitation of silver chromate) discovered by Camillo Golgi in 1873 (the Golgi stain) (Mazzarello 2002), Veratti observed in the light microscope transverse and longitudinal structures in fibers of muscles of various types. As impressive as this work was, it was completely forgotten along with the other early studies until the rediscovery of the sarcoplasmic reticulum in the 1950s using electron microscopy. In truth the study was ahead of its time. We will see below that Veratti's results regarding transverse structures in muscle fibers dovetail almost exactly with the location of the sensitive spots seen by Huxley and Taylor in various muscle types.

Porter and George Palade, who would go on to win a Nobel Prize, made a comprehensive study of the sarcoplasmic reticulum with the electron microscope (Porter

and Palade 1957). They examined the sarcoplasmic reticulum of amphibian muscle (myotome fibers of *Amblystoma* larvae) and rat skeletal and cardiac muscle. Myotomes of *Amblystoma* larvae received the major attention because they showed the reticulum to "excellent advantage". It was a membrane delineated system. They found that an elaborate lacework or reticulum of tubular and vesicular elements existed as a structural component of the interfibrillar sarcoplasm. This reticular component was continuous in such a way that if the myofibrils were removed, it would appear as a fine "honeycomb". They considered it to be a sleeve around each myofibril.

This sarcoplasmic reticulum was further characterized at the level of the Z line in amphibian muscle. At the Z line the reticulum became dilated. The dilated portion of one sarcomere faced an equivalent dilated portion in the next sarcomere. The dilated portions of the sarcoplasmic reticulum were called terminal cisternae by Porter and Palade (1957). A space of about 500 Å existed between the dilated pairs of the sarcoplasmic reticulum. This space contained a single row of profiles of small vesicles running transversely across the fiber. The two opposing dilated units (terminal cisternae) and the intervening space, containing smaller vesicular units, constituted a three-component structure that was named the triad. Thus one triad existed at the level of each Z line in amphibian muscle.

An important advance was made in 1959 by Ebba Andersson-Cedergren, a graduate student, working in Fritiof S. Sjostrand's laboratory in the department of anatomy at the Karolinska Institute in Stockholm. In an electron microscopic study employing serial sections of mouse muscle fibers, Andersson-Cedergren (1959) noted that one part of the network consisted of transversely oriented tubules which appeared as rows of small vesicles. This part extended from the plasma membrane across the muscle fiber close to the level of the A-I boundary and appeared to form a transversally oriented system, which was not continuous with the rest of the sarcotubular system. She referred to this system as the *transversal or T system*. The middle component of the triad, the T system, consisted of elements which in longitudinal sections usually looked like fragments of tubules or vesicles. However, in cross sections through the muscle fibers the T system consisted of tubules with a very complicated convoluted shape. The three dimensional reconstructions showed that the convoluted tubules appeared to form continuous components extending over long distances across the muscle fiber.

Thus the there is not one intracellular membrane system but rather two discontinuous intracellular membrane systems. One component is the sarcoplasmic reticulum or SR and the other the transverse tubular system or T system. The T system is not made up of a series of small vesicles as suggested by Porter and Palade (1957) but is rather a complicated tubular system running across the muscle fiber. These days, these tubules often are referred to as transverse tubules or T tubules. Finally the triads in the mouse fibers did not exist at the Z line, as in frog fibers, but were observed at the boundary between the A and I bands.

This structural information helped greatly in interpreting the data of Huxley and Taylor (1958). The pieces were starting to come together but unfortunately not all of the pieces. Based on the structural studies, the sensitive spots of Huxley and

Taylor likely represented places where the T system was located in the sarcomere. Thus the T system might convey the surface depolarization into the muscle fiber leading to muscle contraction. In a review in 1960, Bennett emphasized that the transverse structures observed by Veratti in 1902, and rediscovered in the 1950s by electron microscopy, were precisely located where the sensitive spots were observed by Huxley and Taylor in frog and lizard muscle. In fact the transverse tubules were the components that best fitted Veratti's drawings. He didn't actually detect the sarcoplasmic reticulum as defined today (Franzini-Armstrong 2002).

But there was one complication. Lee Peachey, working with Andrew Huxley, observed tubules with the electron microscope in crab fibers at the Z line (Peachey 1959) and later also at the A/I boundary (Peachey and Huxley 1964) but the sensitive spots observed in crab fibers by Huxley (1957b) were only observed at the A/I boundary! The tubules at the A/I boundary were associated with the sarcoplasmic reticulum in the form of a dyad and the tubules at the Z line were not associated with the sarcoplasmic reticulum. It turned out that the presence of the sarcoplasmic reticulum in association with the transverse tubules would make all the difference from the viewpoint of muscle activation (see below).

A major technical advance occurred in 1963 when David D. Sabatini, Klaus Bensch and Russell J. Barrnett then at Yale University introduced glutaraldehyde fixation into electron microscopic studies. Glutaraldehyde is an effective cross-linking agent for proteins and it turned out to be far less disruptive to cell membrane structures than osmium tetroxide fixation. Virtually immediately electron microscopists shifted from osmium tetroxide to glutaraldehyde fixation. One of those microscopists was Peachey (1965) at the University of Pennsylvania who undertook an extensive, quantitative study of structure of the sarcoplasmic reticulum and transverse tubules in frog skeletal muscle. He confirmed the results of Porter and Palade (1957) and Andersson-Cedergren (1959) and added quantitative information. The T system exhibited a volume of 0.3 % of the fiber volume and a surface area about seven times greater than the outer cylindrical surface area of a typical fiber 100 μm in diameter. He produced a beautiful electron micrograph showing the components of the sarcoplasmic reticulum and the transverse tubules (Fig. 4.2) and his schematic illustration of these structures is now considered to be a classic (Fig. 4.3).

4.5 Mechanism of Inward Spread of Activation

Despite the uncooperative crab, the focus now concentrated on the possible role of the T system in muscle activation. Important questions included: did the T tubules open to the muscle surface? Were the T tubules invaginations of the surface membrane or a separate system? Did electrical activity spread into the T tubules by passive electronic spread or a propagated action potential? And, of course, ultimately how does this inward spread of electrical activity lead to muscle contraction?

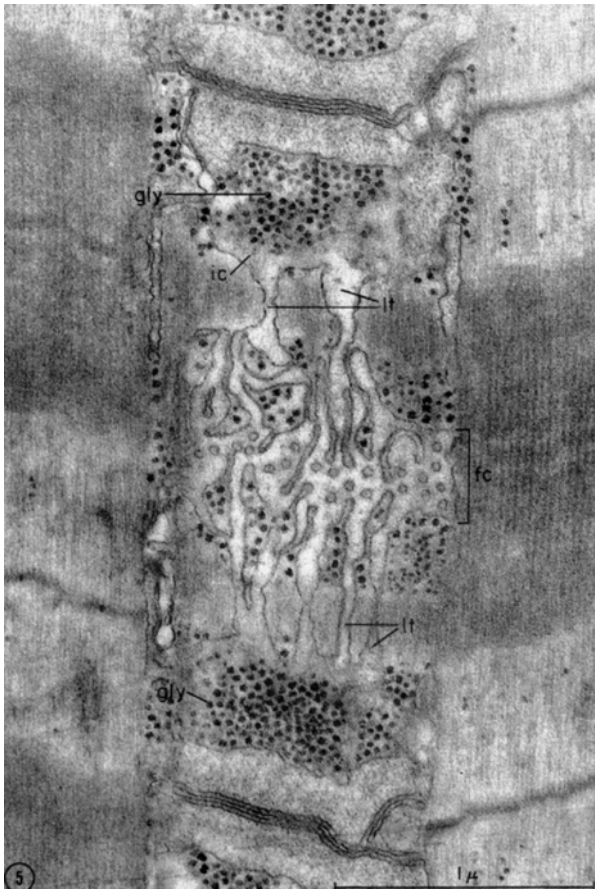


Fig. 4.2 Longitudinal electron micrograph of frog skeletal muscle showing the sarcoplasmic reticulum and transverse tubular system in relation to the myofibrils. In this very thin section, the central vertical strip of the area shown is almost entirely confined to the sarcoplasm between two myofibrils. The underlying myofibril is in register with the myofibril at the right side of the figure. Triads appear at the *top* and *bottom* of the figure, opposite Z lines. Details of the sarcoplasmic reticulum are shown. The section passes through the glycogen masses. Magnification ×60,000 (Peachey 1965. With permission Rockefeller University Press)

4.5.1 Are the Transverse Tubules Open to the Surface?

A flurry of structural and functional studies appeared in 1964 which proved that the transverse tubules were open to the surface and were likely invaginations of the surface membrane. Clara Franzini-Armstrong (Fig. 4.4) and Porter (1964) provided convincing electron microscopic evidence that the transverse tubules were invaginations of the surface membrane in fish striated muscle. They utilized glutaraldehyde fixation to fix muscles of the fish, Black Mollie, a small fish 2–3 cm in length. They did something that seems at first glance very strange. To avoid anesthesia and dissection, they immersed the whole animal in the fixative for 1.5 h! Strange as it may seem, their results are beautiful (Fig. 4.5). They concluded that the T system was a sarcolemmal derivative that retained its continuity with the sarcolemma and limited a space that was in direct communication with the extracellular environment. Furthermore they observed about 20–30 transverse tubules at each Z level

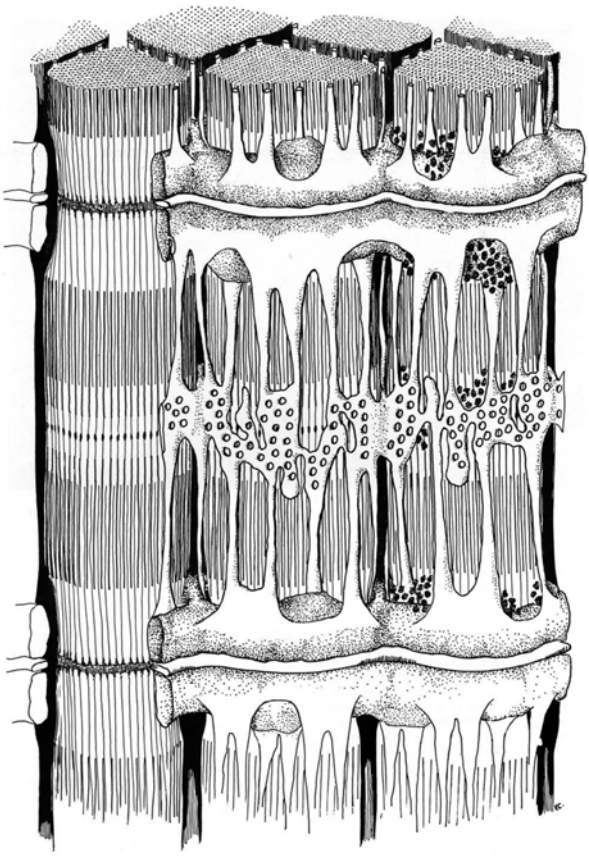


Fig. 4.3 Three-dimensional reconstruction of the sarcoplasmic reticulum and transverse tubular systems associated with several myofibrils in the frog. Magnification approximately ×40,000 (Peachey 1965. With permission Rockefeller University Press)

around the circumference of the small 12 μm diameter fish fibers. This result is reminiscent of the circumferential separation of sensitive spots at the Z line in frog fibers (Huxley 1957b). Further evidence that the transverse tubules were open to the surface came from the observations by Makoto Endo[3] (1964) in Andrew Huxley's laboratory. When a frog fiber is soaked in a solution containing a charged fluorescent dye that does not penetrate the muscle cell membrane and the solution then removed, the fiber exhibits striations in the fluorescence microscope. Upon rapidly shifting to a polarizing microscope it was observed that fluorescent striations

[3] Makoto Endo came to Andrew Huxley's laboratory in the autumn of 1962 from Hiroshi Kumagai's laboratory at the University of Tokyo. In Japan he learned biochemistry and physiology from Setsuro Ebashi. After his stay in the Huxley laboratory he returned to Japan and spent most of his distinguished research career at the University of Tokyo. He is best known for his work describing calcium-induced calcium release in muscle (Endo et al. 1970), a mechanism that is prominent in the heart. In 1957 Professor Kumagai organized the first international conference on the chemistry of muscular contraction to be held in Japan after World War II.

Fig. 4.4 Clara Franzini-Armstrong (b. 1938) (*right*): Her electron microscopic studies on the transverse tubular system and its relationship to the sarcoplasmic reticulum span more than 40 years and are considered foundational and pioneering in muscle field. She received her degree (Laurea) from the University of Pisa in 1960. She did postdoctoral training with Keith Porter at Harvard University, worked with Andrew Huxley at University College London and Richard Podolsky at the NIH. She has spent the majority of her career at the University of Pennsylvania. She was elected to the National Academy of Sciences in 1995 and as a foreign member of the Royal Society London in 2001. She received the Founders Award from the Biophysical Society. She became professor emerita in 2007 at the University of Pennsylvania. Her husband Clay Armstrong (*left*), the eminent electrophysiologist, also is a member of the National Academy of Sciences. Photo: courtesy C. Franzini-Armstrong

occurred in the middle of the I bands. The fluorescence then quickly faded suggesting that the dye was washed out of the transverse tubules without entering the main part of the fiber. Rather than a dye, Huxley (1964) and independently Page (1964) showed that ferritin could enter the transverse tubules of frog skeletal muscle. Ferritin consists of a protein shell enclosing a dense core of ferric hydroxide with each ferritin containing about 3,000 iron atoms. The electron dense ferritin was easy to detect in the electron microscope. The results (Huxley 1964) clearly showed that ferritin could enter the transverse tubules (Fig. 4.6). Huxley (1964) concluded that the transverse tubular system opened directly into extracellular space and that ferritin entered by free diffusion. He also concluded that there was continuity of the transverse system across the fiber diameter. A dramatic affirmation of this conclusion is seen in the scanning electron micrographs (Fig. 4.7) of the surface of a frog muscle fiber by McCallister and Hadek (1970). Openings on the surface occur at the Z line and circumferentially around the fiber just as predicted by the electrophysiological experiments of Huxley and Taylor. There was no disagreement now, the transverse tubules were invaginations of the surface membrane.

These experiments also re-enforced the conclusion that the T system is separate from the sarcoplasmic reticulum. The T system is an invagination of the surface membrane whereas the sarcoplasmic reticulum exists totally within the fiber. Certainly they are in close apposition at the triad but is there any connection between

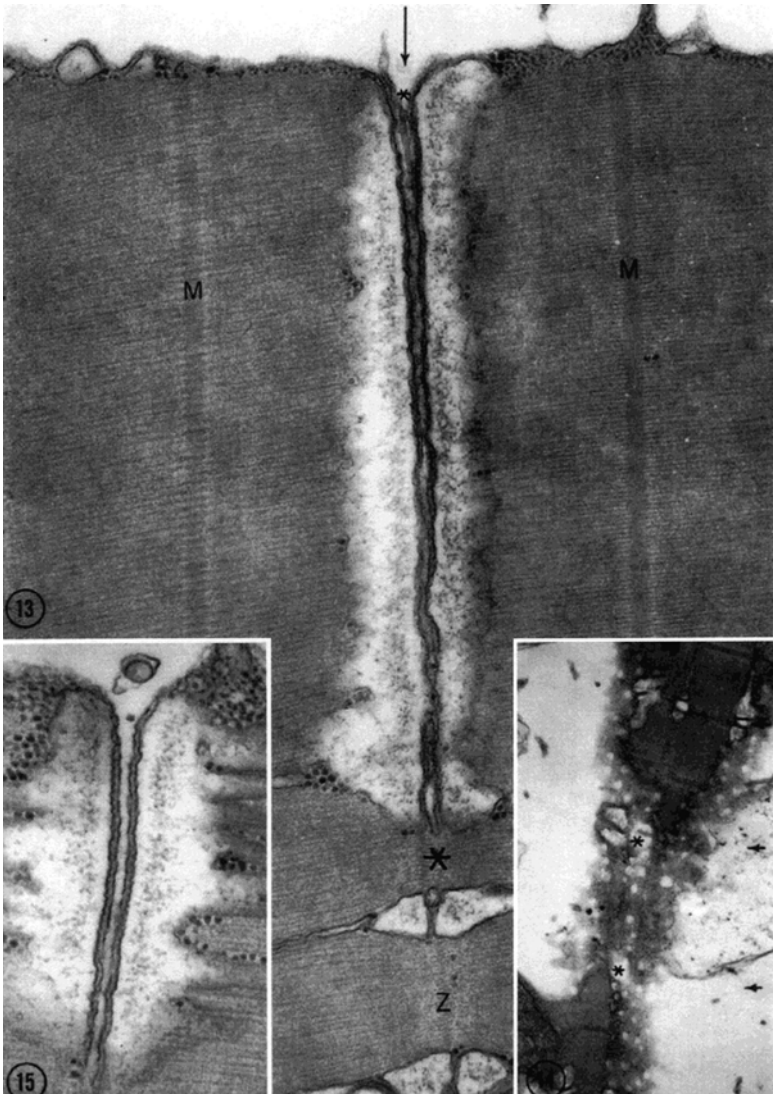


Fig. 4.5 Longitudinal electron micrographs of the T system in fish muscle fibers. These images provide a lateral view of the T system and terminal cisternae of the sarcoplasmic reticulum. Note that the T system is located at the Z line, opens to the surface and exhibits a close relationship with the sarcoplasmic reticulum. Magnification ×46,000–×60,000 (Franzini-Armstrong and Porter 1964. With permission Rockefeller University Press)

the two systems? Franzini-Armstrong (1970) showed that there were structures that reached across the 100–150 Å gap from the sarcoplasmic reticulum to the T system. At periodic intervals of about 300 Å the sarcoplasmic reticulum membrane forms small projections whose tips are joined to the T system membrane by some amorphous

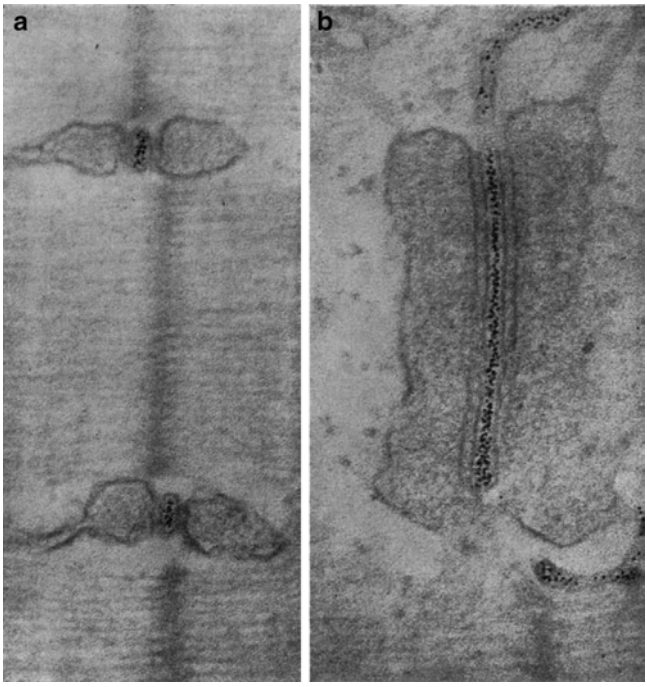


Fig. 4.6 Electron microscopic views of triads in ferritin treated frog muscle fibers. (**a**) A section perpendicular to transverse tubules filled with ferritin molecules. (**b**) A section parallel to a transverse tubule indicating penetration of ferritin molecules throughout the tubule. Magnification ×145,000 (Huxley 1964. With permission Nature Publishing Group)

material (Fig. 4.8). She called these processes "SR feet". Over time these foot processes sometimes whimsically have been called "Clara's feet". At the time of their discovery the functional significance of these structures was a mystery but they soon would become important in understanding the excitation-contraction coupling process (see Chap. 8).

Various other experiments could now be given a ready explanation. The observation by Bernard Katz (1948) of a fivefold higher capacitance in skeletal muscle fibers than in nerve fibers could now be attributed to the extra membrane area of the T system. Hodgkin and Horowicz (1960) made an extensive study of the effects of K^+ solutions on membrane potential and contraction in frog muscle fibers. When a single muscle fiber was exposed to a solution of elevated K^+ concentration, the membrane depolarized to a steady level. When that solution was rapidly removed, the membrane repolarized but at a much slower rate than the rate of depolarization. Hodgkin and Horowicz (1960) speculated that the K^+ was retained for a short time in "a special region" in the fiber. This region was most likely the T system of the fiber from which K^+ that entered would only slowly diffuse out. Freygang et al. (1964) studied the effects of repeated stimulation on the shape of the action potential of frog muscle fibers. Skeletal muscle action potentials exhibit a tail or negative

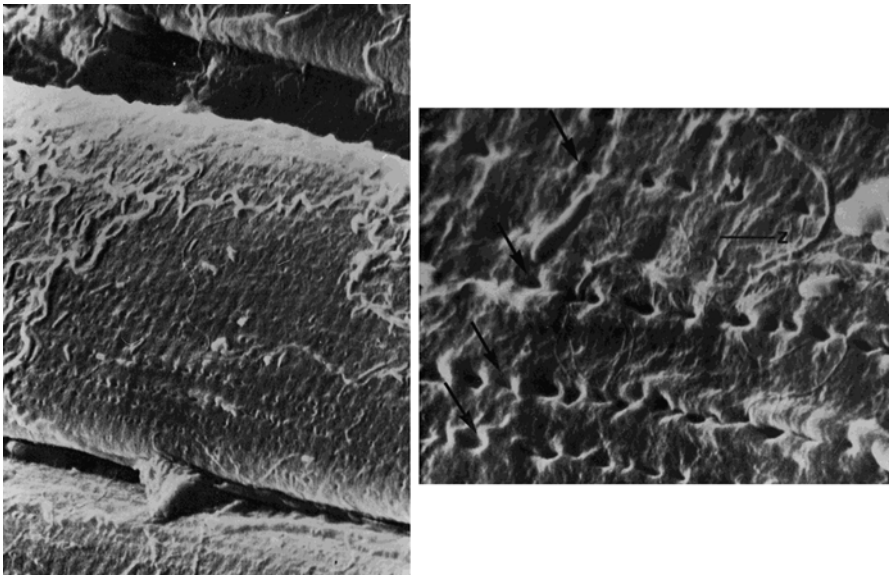


Fig. 4.7 Scanning electron micrographs of the surface of a frog muscle fiber. *Left*: lower magnification (×2,500) showing the striated pattern of the myofibrils that is evident at the fiber surface with the lighter ridges corresponding to the A bands and the dark furrows corresponding to the I bands. *Right*: higher magnification (×10,000) where *arrows* indicate the parallel rows of openings corresponding to the T system. The openings are found at the level of the I band in the vicinity of the Z line and are distributed circumferentially around the fiber (McCallister and Hadek 1970. With permission Elsevier)

after-potential. With repeated stimulation this after-potential became larger and returned to the resting level more slowly than after a single stimulus. They interpreted this observation as the accumulation of K^+ during the repeated stimulation "in an intermediary space" that was located between the major portion of the sarcoplasm and the external fluid. All of these experiments suggested that the T system is in electrical continuity with the surface membrane.

The conclusion that the unique electrical properties of skeletal muscle fibers could be attributed to the presence of the T system soon was confirmed in dramatic fashion. John N. Howell (Howell and Jenden 1967; Howell 1969), a graduate student at UCLA at the time, made the seminal observation. If a frog muscle is soaked in a solution made hypertonic by adding glycerol, the transverse tubules became disrupted when the muscle was returned to an isotonic salt solution. These muscles were no longer responsive to stimulation. Brenda R. Eisenberg (now Brenda Russell) and Robert (Bob) S. Eisenberg (1968) showed that this treatment destroyed 98 % of the transverse tubules. Bob Eisenberg and Australian physiologist Peter W. Gage (1967) made an extensive study of the electrical properties of these muscle fibers. They found that action potentials did not produce a mechanical response and that the negative after-potential was absent after a single stimulus or a train of stimuli.

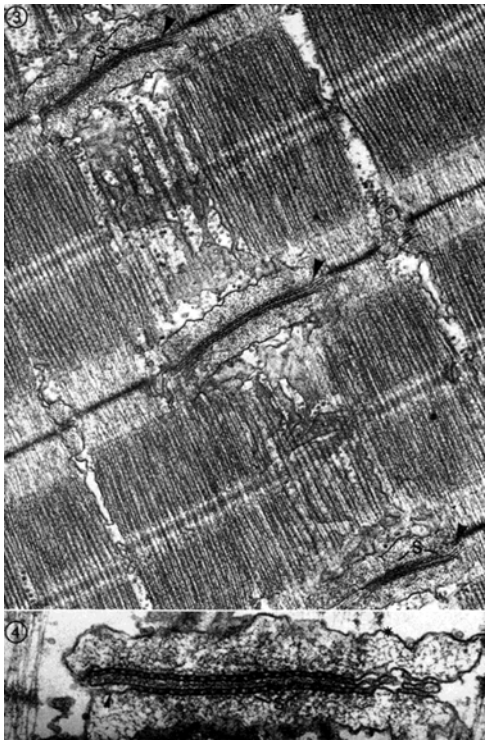


Fig. 4.8 Electron micrographs showing periodic structures connecting the T system to the sarcoplasmic reticulum in frog muscle fibers. These structures have been called "SR feet" by Franzini-Armstrong. Magnification ×45,000 above and ×100,000 below (Franzini-Armstrong 1970. With permission Rockefeller University Press)

Also the membrane capacitance was strikingly reduced. Thus the matter was settled: the T system was in electrical contact with the surface membrane, responsible for the characteristic properties of the muscle action potential and necessary for muscle activation.

4.5.2 Is the Spread of Activation into the T System Active or Passive?

Huxley and Taylor (1958) interpreted their results to indicate that inward spread of electrical activity into the muscle fiber was a passive process. They based this interpretation upon the observation that the distance to which the contraction spread inwards from the fiber surface was graded according to the strength of the pulse applied. Nonetheless, they still considered the mechanism of inward spread to be an open question. Hugo Gonzales-Serratos (d. 2011) came to Andrew Huxley's laboratory from Mexico in the early 1960s to work for a Ph.D. He devised a remarkable experiment to determine the velocity of inward spread of contraction in a single frog muscle fiber. Gonzalez-Serratos compressed a frog fiber in gelatin until all the

myofibrils became wavy. When the fiber was stimulated to produce a twitch, the active myofibrils straightened and this spread of inward contraction was recorded with high-speed cine microphotography at a remarkable 2,000–3,000 frames per second (Gonzalez-Serratos 1966, 1971). The contraction spread inward with a propagation velocity of 7 cm/s at 20 °C. Thus it would take less that 1 ms for the contraction to reach the middle myofibrils of a typical 100 μm frog fiber. As the temperature was decreased the propagation velocity decreased with a Q_{10} of about 2. Passive electronic spread would be expected to be almost temperature insensitive. Thus these results suggested for the first time that the propagation of the excitatory process along the tubules was probably a regenerative process like an action potential rather than a passive one. When Andrew Huxley gave his Croonian lecture in 1967 (Huxley 1971), he still wasn't completely convinced but did finally acquiesce when the results of LeRoy (Roy) L. Costantin (1970) clearly indicated that a Na^+ current in the T tubule membrane contributed to the tubule spread of the excitatory process, as in an action potential. The radial spread of contraction was less effective in fibers exposed to a bathing medium with greatly reduced Na^+ concentration or to tetrodotoxin which was known to block the increase in Na^+ permeability associated with an action potential. The results strongly supported the existence of a sodium-regenerative process in the T system. For one of the few times in his research career Andrew Huxley got it wrong. The inward spread of electrical activity leading to muscle contraction was active and not passive.

Thus the first steps of excitation-contraction coupling as defined by Alexander Sandow were elucidated both functionally and structurally.[4] What was missing in 1958 was a general understanding of what happened next. Huxley and Taylor (1958) emphasized that there was little evidence concerning the final step from the hypothetical transverse networks to the filaments themselves. It turned out that there was evidence that Ca^{2+} played a crucial role in muscle activation but much of this evidence was either forgotten or ignored.

4.6 Ca^{2+} and Contractility: The Early Studies on Muscles

The history of Ca^{2+} and skeletal muscle contraction is a curious one. It involved forgotten clues, feuds around the merits of doing experiments on "living" muscles versus "dead" muscle components and strong personalities. There were different "clubs", the cell physiologists and the biochemists, who spoke different "languages" and who weren't able or willing to consider the contributions of the other club. For the biochemist, there were many confounding issues (Franzini-Armstrong 1998).

[4] Jozef Zachar (1925–2000) produced in 1971 a remarkable monograph entitled: "Electrogenesis and Contractility in Skeletal Muscle Cells" which covers excitation-contraction coupling in great experimental detail, especially from a comparative muscle point of view. At the time of the publication, he was a Professor at the Institute of Normal and Pathological Physiology, Bratislava, Czechoslovakia (now the Slovak Republic).

Some of these issues included: variable and unknown Ca^{2+} contamination of reagents, especially ATP; contamination due to Ca^{2+} leaching from the glassware; variable purity of protein preparations; and the difficulty of detecting the low concentrations of Ca^{2+} that produced contraction of the model systems. Also there was the confusing issue that ATP could cause contraction of a muscle model system but also relaxation. Finally there was the problem that many biochemists had difficulty in believing that something as simple and common as the Ca^{2+} could regulate something as important as human movement. In the end, the Ca^{2+} hypothesis of contraction would become accepted but not without much hard work and some famous experiments.

The cell physiologists found the early clues which for reasons described below were set aside. They believed strongly that the best, maybe only, way to gain a full understanding of cellular function was to study living cells. Books with titles such as "The Dynamics of Living Protoplasm" by Heilbrunn (1956) and "Explorations into the Nature of the Living Cell" by Robert Chambers and his son Edward L. Chambers (1961) clearly expressed the authors' point of view. They took a general physiology approach and looked for commonality in cell function across the animal kingdom. In a study published in 1932, Robert Chambers and H. P. Hale examined the effects of freezing and thawing on different cells including segments cut from frog muscle fibers. They routinely used the response to Ca^{2+} as an indicator of a healthy fiber. The contractility of the fibers was tested by blowing a small quantity of 0.1 M $CaCl_2$ with a micropipette on the surface of the fiber. They noted that this procedure induced a pronounced contraction of a healthy fiber. (The Ca^{2+} likely entered the fiber segment through the cut ends.)

In a paper entitled "the action of calcium on muscle protoplasm" in 1940, Lewis Victor Heilbrunn[5] at the University of Pennsylvania described in detail his Ca^{2+} release theory of muscle contraction. He proposed that in a muscle fiber at rest, Ca^{2+} was stored in the cortex of the cell, a 10 μm thick region just under the cell membrane. This Ca^{2+} was then released into the more or less fluid portion of the cell interior to cause contraction upon muscle stimulation. In support of his hypothesis, he observed that when cut frog fibers were exposed to an isosmotic solution of $CaCl_2$ (90 mM) there was a strong contraction and the fiber segment lengths decreased by 70 % (Fig. 4.9). He believed that the Ca^{2+} diffused into the fiber segment through the cut ends.

[5] Lewis Victor Heilbrunn (1892–1959) spent the majority of his career in the department of zoology at the University of Pennsylvania where he trained more than 50 scientists for the Ph.D. He was a prophetic author who wrote his first book, The Colloid Chemistry of Protoplasm, in 1928. He was a true general physiologist whose research interests ranged from the study of protoplasm in cell division, ameboid movement, fertilization and muscle contraction. He was a strong personality, a decorated aviator in World War I, who held steadfastly to his scientific beliefs. He is best known for his advocacy for the universality of the role of Ca^{2+} in regulation of cell function. Unfortunately he did not live long enough to see his idea vindicated. He died in an automobile accident in 1959. He would have gotten great joy out of Anthony K. Campbell's book entitled: Intracellular Calcium—Its Universal Role as Regulator (1983). For more on Heilbrunn, see Steinbach (1960), Shreeve (1983) and Gross (1986).

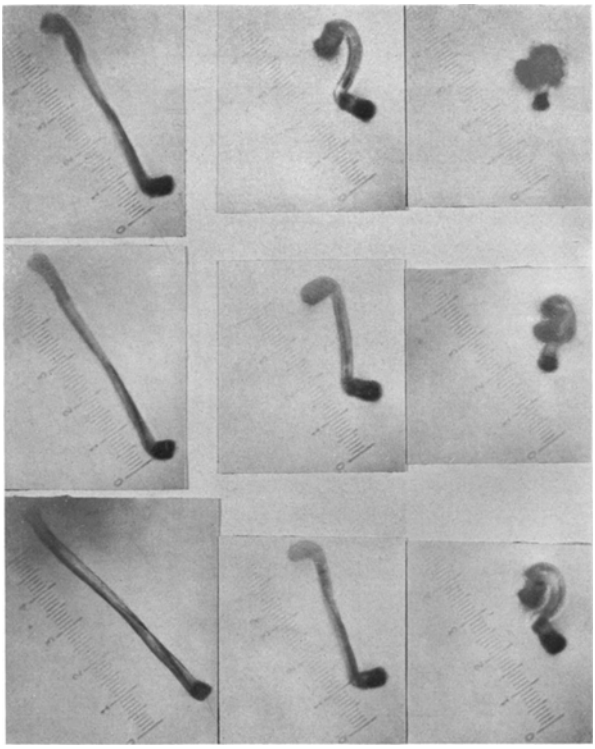


Fig. 4.9 Shortening of an isolated muscle fiber in isosmotic $CaCl_2$. The fiber was cut from a frog muscle. The succession of stages is from *left* to *right*. The first photograph was taken in Ringer's solution; the second, 10 s after addition of $CaCl_2$. Subsequent pictures were taken at 10 s intervals (Heilbrunn 1940. With permission The University of Chicago Press)

Kamada and Kinosita (1943) of the Tokyo Imperial University confirmed Heilbrunn's observations in a paper extraordinary for its time, its place and elegance of techniques. But the paper was virtually unknown in the west until well after World War II. They investigated the effects of various solutions on the contraction or in their words "coagulum formation" of isolated, intact or cut, frog muscle fibers. They injected solutions into the fibers through micro-pipettes with tip diameters of 2–5 μm. Most effective in producing contraction of the naturally occurring ions was a solution containing 83 mM $CaCl_2$. If the amount of injected solution was small, the local contraction was followed by relaxation.

Heilbrunn and Wiercinski (1947) conducted a study that was at first considered influential, then set aside and now considered classic. Their point of view was made clear when they emphasized that the phenomenon of muscular contraction occurs in living rather than in dead muscle. They also utilized single cut fibers from the frog. Ironically, many physiologists would consider these cut fibers to be "dead" or at least injured. These cut fiber segments were immersed in a Ca^{2+} free solution. Various solutions were injected into the fiber segments through a micropipette and the extent of segment shortening was measured. Of the physiologically occurring ions only Ca^{2+} resulted in a strong contraction with an average fiber segment shortening of 44 % in a few seconds. Solutions with a Ca^{2+} concentration as low as

200 μM produced a contraction. Considering that the amount of solution injected into the fiber segment was about 10 % of the segment volume, this suggests that the steady Ca^{2+} concentration might have been as low as 20 μM. The fiber segments did not relax. Heilbrunn believed that muscle contraction was a process analogous to blood clotting. In contrast, injection of solutions resulting in a final fiber segment concentration of about 8 mM $MgCl_2$ or 12.6 mM KCl or 12.3 mM NaCl produced only small changes of the fiber segment length (less than 10 %). The results were detailed and convincing. This paper and the accompanying hypothesis of Ca^{2+} regulation of contraction at first were influential but only for a very short time.

There was a problem with Heilbrunn's Ca^{2+} theory of muscle contraction, a fatal problem. In 1949 A. V. Hill showed that it was impossible to explain the rapidity of frog muscle contraction by diffusion of a substance from the cortex of the cell (see above). Hill was a Nobel Laureate and his word was "instant law" (Shreeve 1983). Thus Heilbrunn's hypothesis was set aside. In a sense the "baby (Ca^{2+}) was thrown out with the bath water" (location of Ca^{2+} in cell cortex at rest). Of course these events occurred before the recognition of the inward spread of electrical activity in a muscle fiber.

There was another reason that Heilbrunn's work was discredited and that was that Heilbrunn's myopic view of the work of others, especially the biochemists, made matters worse. He believed that Ca^{2+} was a universal regulator of cellular function in all cells. His perceived over insistence on the role of Ca^{2+} as a universal regulator led some of his colleagues to call him a "calcium maniac" (Shreeve 1983). That he was a first rate scholar there could be no doubt. Besides his original publications, he also published single authored books. Examples include his textbook entitled "An Outline of General Physiology" that went through three editions and his monograph the "Dynamics of Living Protoplasm". In an unusually candid assessment of Heilbrunn some years after his death, Gross (1986), then director of the Marine Biological Laboratory at Woods Hole where Heilbrunn spent many summers, characterized Heilbrunn as a remarkable scholar. But he went on to state that Heilbrunn was committed to an outmoded way of thinking about cell chemistry and structure. He didn't believe that the function of a cell could be understood by looking at its individual pieces. He was dogmatic and disdainful of the biochemists whose influence was ascending dramatically in the 1940s and 1950s. For example, concerning the then confusing dual role for ATP in muscle model systems, Heilbrunn (1956) had this to say (Heilbrunn 1956. With permission Elsevier):

> One thing is certain, adenosine triphosphate, or ATP, is a powerful word in all our modern thinking about muscle…words seem to have taken the place of the incantations used by magicians in the days before modern science. Whereas the old magicians were content with their abracadabra, we now have much more impressive words like adenosine triphosphate. Adenosine triphosphate—and the muscle contracts; adenosine triphosphate and it relaxes again.

And there was more, Heilbrunn believed that the effect of ATP on dead glycerinated muscle fibers could scarcely be the same as that on the living fibers. Ebashi (1980) noted that the fact that Heilbrunn did not seem to appreciate the role of ATP may have discredited the concept that Ca^{2+} itself was important at the molecular level. So it is not surprising that Heilbrunn's work garnered little respect from the biochemists. The fact that Heilbrunn spent his summers at the Marine Biological

Laboratory at Woods Hole, Massachusetts, where Albert Szent-Gyorgyi and his group had a year round residence must have led to some tense moments between these two great but dogmatic scholars. Nonetheless, Hill's (1949) criticism came and "abracadabra": Heilbrunn's Ca^{2+} release hypothesis was discarded.

In a somewhat sobering close to his assessment of Heilbrunn, Gross concluded with some advice for young investigators (Gross 1986. No objection from Marine Biological Laboratory. Author could not be located):

> Perhaps they will consider, however, these few generalizations, which *I know* are true: Being right isn't enough. What you say, however right, must be said in a currently acceptable language, must not violate too brutally current taste, and must somehow signal your membership in a respectable professional club. If you want to succeed you must play *all* of the game, not just the innings in laboratory or library.

That wasn't the end of the Ca^{2+} story from a physiological perspective. Niedergerke (1955), after finishing his contribution to the experiments leading to the sliding filament model of muscle contraction with Andrew Huxley, essentially confirmed the observations of Heilbrunn and Wiercinski. He injected Ca^{2+} from a micropipette by electrolytic transport into a living muscle fiber from the frog. The myofibrils near the pipette tip rapidly shortened and slowly lengthened, but not completely, once the current was turned off. Injection of a solution of KCl or $MgCl_2$ did not induce a contraction. Niedergerke concluded that the local contraction and partial relaxation suggested that Ca^{2+} activated a specific link of the contractile cycle. His observations were described in a brief publication in the proceedings of the Physiological Society. Niedergerke was a member of the right "professional club" but unfortunately he did not follow-up these initial observations with a full study. He moved on immediately to study the role of Ca^{2+} in cardiac muscle contraction.

4.7 Ca^{2+} and Contractility: Observations on Model Systems

The early physiological studies provided definite clues for an important role for Ca^{2+} in contraction but these studies were essentially descriptive. It was through detailed biochemical studies on muscle model systems that a molecular picture for the role of Ca^{2+} in contraction emerged. Despite these clues of the role of Ca^{2+} in muscle contraction, Ebashi and Endo (1968) have stated that "It is ironic that recognition of the essential role of Ca ion in contraction has resulted mainly from the investigation into the mechanism of relaxation." Various simplified model systems were developed to mimic contraction. Some of these model systems included (Ebashi and Endo 1968; also see Chap. 1): (1) reconstituted or "synthetic" actomyosin made from purified actin and myosin, (2) "natural" actomyosin which likely contained other structural proteins made by isolating actomyosin from muscle, (3) actomyosin threads made from synthetic or natural actomyosin, (4) myofibrils, and (5) glycerinated muscle fibers. These systems could be made to "contract" by addition of ATP and Mg^{2+} at an appropriate ionic strength. Contraction of model systems (1), (2) and (4) is represented by superprecipitation and increases in actomyosin ATPase activity. In the presence of Mg.ATP, the actomyosin threads shorten and the

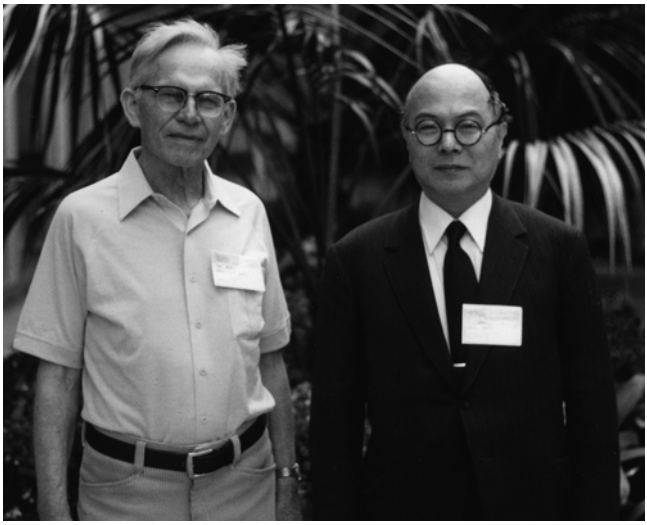


Fig. 4.10 Emil Bozler and Setsuro Ebashi in 1989 at Ohio State University during a symposium on smooth muscle in honor of Bozler. Ebashi's development of the calcium concept was stimulated by the observations of Bozler in 1954 (see Fig. 4.11 and associated text). They were long time friends (Photo: author's collection)

glycerinated fibers develop force and can do work by shortening. The glycerinated fiber preparation is most suitable for mechanical studies. In the view of the biochemists, all of these systems mimic muscle contraction but in none of them was relaxation observed before 1950.

The first important step was taken by Emil Bozler[6] (Fig. 4.10) at Ohio State University. He was interested in the mechanism of skeletal muscle relaxation. He utilized bundles of glycerol-extracted psoas muscle fibers from the rabbit to study relaxation. What he discovered was pathfinding. A bundle of fibers developing a steady force due to the addition of Mg.ATP completely relaxed when exposed to a solution containing the Ca^{2+} chelator ethylenediamine tetraacetate (EDTA) (Fig. 4.11) (Bozler 1954). In turn the bundle could be made to contract once again by addition of a 2 mM $CaCl_2$. Thus Ca^{2+} caused a contraction and Ca^{2+} binding led to relaxation. The interpretation seemed simple but Bozler interpreted his results in a complex way, and as it turned out, incorrect way. He proposed that Ca^{2+} was permanently bound to actomyosin and that the chelator blocked this Ca^{2+} and inhibited contraction. The excess added Ca^{2+} bound to the chelator and contraction ensued. Nonetheless these were

[6] Emil Bozler (1901–1995). born in Steingebronn, Germany, came to the department of physiology at Ohio State University in 1936 and spent his entire career there, becoming a professor emeritus in 1971 and thereafter continuing to work for another 20 years. He is best known for his contributions to smooth muscle physiology where he has been called the "father of smooth muscle physiology". He classified smooth muscles into two categories: unitary (visceral) and multiple-unit. He also discovered the pacemaker prepotential in cardiac muscle using extracellular electrodes in 1943. His publications span more than 60 years from 1925 to 1990. His work was deeply respected in Japan and he and Setusro Ebashi were friends. See Rall (1990).

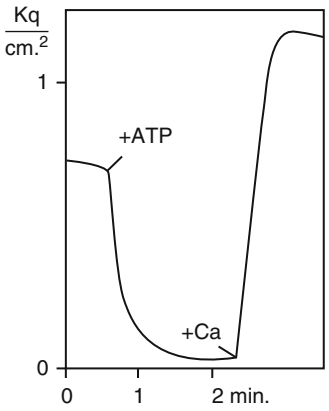


Fig. 4.11 Relaxation of muscle fibers in response to a Ca^{2+} chelator. Skeletal muscle fibers from the rabbit were extracted in a glycerol solution to remove membranes and soluble proteins. Contraction of the fiber bundle was induced by a solution containing ATP and $MgCl_2$. Once steady force was reached, the fiber bundle was exposed to a solution containing the Ca^{2+} chelator EDTA and relaxation ensued. The bundle contracted once again on exposure to the solution containing 1 mM $CaCl_2$ (Bozler 1954. With permission Rockefeller University Press)

Fig. 4.12 Annemarie Weber (1923–2012), M.D., in 1959 was the first to show that Ca^{2+} in the micromolar range regulated the ATPase activity of isolated myofibrils, a model system for skeletal muscle contraction (Franzini-Armstrong 1998. With permission Elsevier)

crucial experiments because for the first time they demonstrated the importance of Ca^{2+} in contraction of a muscle model system and also because they introduced the concept of controlling Ca^{2+} concentration with a Ca^{2+} chelator.

Annemarie Weber (Fig. 4.12) was stimulated by Bozler experiments and in 1959, using EDTA, she was the first to show that isolated myofibrils must be in equilibrium

with a very low concentration of Ca^{2+} in order to have a maximal rate of ATP hydrolysis (Weber 1959). It was difficult to know exactly what that Ca^{2+} concentration was but her data indicated that it was likely to be less than 10 μM free Ca^{2+} concentration. Thus the concentration of Ca^{2+} necessary for contraction in muscles or model systems seemed to be getting strikingly smaller as techniques advanced. It would get even smaller.

At about the same time Setsuro Ebashi[7] (Fig. 4.10) at the University of Tokyo was pursuing the mechanism of muscle relaxation also using model systems. Based on Bozler's results, he reasoned that the extent of relaxation of a glycerinated muscle preparation should vary in proportion to the affinity of the chelator for Ca^{2+}. He obtained six Ca^{2+} chelators with varying affinities for Ca^{2+} to test this idea (Ebashi et al. 1960). They measured the "relaxation activity" as the time that it took to initiate lengthening of a contracted glycerinated fiber bundle after the introduction of the chelator solution. As hoped the chelator with the highest Ca^{2+} affinity, GEDTA (now known as EGTA, ethylene glycol tetraacetic acid), exhibited the greatest relaxation activity, i.e., shortest time to start relaxation. But when all six chelators were considered, there was no correlation between Ca^{2+} affinity and relaxation activity. This result was a great disappointment to Ebashi because it did not support his belief in the role of Ca^{2+} in contraction and relaxation. In 1959 he went to the Rockefeller Institute in New York to work with Fitz Lipmann. Sometime after the June 1959 submission of the paper to the Journal of Biochemistry and before May 1960, Ebashi realized that he made a simple but major mistake. He did not make allowance for the fact that the various Ca^{2+} chelators also had various affinities for Mg^{2+} which he had in his solutions. When this correction was made, the correlation became excellent (Fig. 4.13). In May of 1960 he sent off a letter to the editor of the Journal of Biochemistry and the new result was published in July 1960 (Ebashi 1960). Ebashi (1994) always deeply regretted this error. In the letter to the Journal of Biochemistry (Ebashi 1960), he acknowledged Annemarie Weber's work when he indicated that her work and that of his laboratory indicated the role of Ca^{2+} in contraction of the actomyosin system. So Weber and Ebashi were both thinking along the same lines with regard to role of Ca^{2+} in muscle contraction and relaxation. But there was much more hard work to be done.

One of the main problems was that their fellow biochemists had a difficult time accepting the proposal that something as simple and common as Ca^{2+} could control muscle contraction. There was another problem. The experiments indicating the role of Ca^{2+} in contraction were done in the presence of synthetic calcium chelators

[7] Setsuro Ebashi (1922–2006) was born in Tokyo and received his M.D. degree in 1944 from the University of Tokyo and served as a naval surgeon and was detained in Shanghai after the war. After returning to Japan in 1946, he received his Ph.D. from the University of Tokyo under the mentorship of Professor Hiroshi Kumagai in 1954. He spent the majority of his career in the department of pharmacology at the University of Tokyo. Ebashi received many awards, nationally and internationally, including the Order of Cultural Merit, the highest scientific honor in Japan. He was a member of the Japan Academy and a Foreign Member of the Royal Society and Foreign Associate of the National Academy of Sciences in the United States. For further details see Endo (2006) and Otsuka (2007).

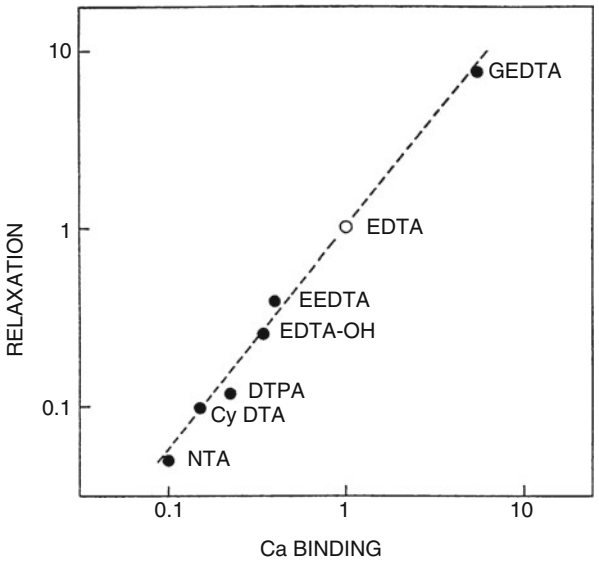


Fig. 4.13 Relationship between calcium binding by various chelating compounds and relaxation of glycerol extracted muscle fibers. Relaxation was measured as the time it took to initiate lengthening of a contracted fiber bundle after the introduction of the chelator solution. Activity of each compound was expressed as its value relative to that of EDTA. Calcium binding was adjusted for the variable binding of Mg^{2+} by the chelators. GEDTA is now referred to as EGTA. For full names of the chelators, see Ebashi et al. (1960) (Ebashi 1960. With permission Oxford University Press)

which, of course, did not occur naturally in muscles. It was important to show the effects of Ca^{2+} in the absence of Ca^{2+} chelators. Given the ease of contamination of reagents and actomyosin with Ca^{2+} in the micromolar range, this would be a heroic experiment. In 1961, Ebashi undertook what he has called the most difficult experiments of his career. He went to extreme lengths to minimize Ca^{2+} contamination in the glassware, reagents and actomyosin preparations before starting the experiment. He isolated "natural" actomyosin from rabbit muscle and measured contraction as the increase in turbidity that accompanied the superprecipitation of actomyosin upon addition of ATP. The time course of superprecipitation was markedly enhanced by Ca^{2+} in the submicromolar range (Fig. 4.14) (Ebashi 1961a). This result was crucial in that it indicated that the effects of Ca^{2+} were not an artifact of Ca^{2+} chelators.

At about the same time, Weber and Winicur (1961) examined the effect of Ca^{2+} on the superprecipitation of a "synthetic" or reconstituted actomyosin system, i.e., purified myosin and purified actin. This system, of course, was the most basic model of muscle contraction. The superprecipitation and accompanying ATPase activity was generally inhibited in the presence of EGTA, but not always. This was potentially a major problem. Some of their preparations were Ca^{2+} insensitive. In their summary, they stated that some actomyosin preparations were inhibited only to a small extent by EGTA and that this effect was a property of the actin moiety. Thus

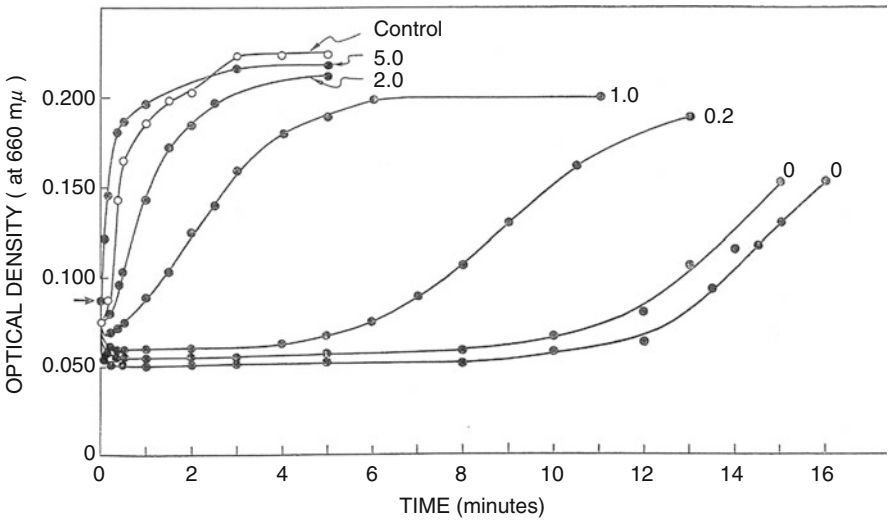


Fig. 4.14 Superprecipitation of natural actomyosin as a function of time in the presence of variable concentrations of free Ca^{2+}. Superprecipitation was measured by following the increase in turbidity. This increase in turbidity was taken as a measure of contraction. Ca^{2+} concentration ranged from 0 added to 5 **μ**M. One of the two tests containing no added Ca^{2+} was done at the beginning of the series of this experiment and the other, at the end. Note there is a substantial acceleration of superprecipitation of actomyosin with a Ca^{2+} concentration as low at 2 **μ**M (Ebashi 1961a. With permission Oxford University Press)

not all synthetic actomyosin preparations were the same. Some preparations were different and this difference may somehow be associated with actin itself. Earlier Perry and Grey (1956), in a short note, also observed that the ATPase activity of synthetic actomyosin was insensitive to EDTA addition. Weber and Herz (1963) went on to show that exchangeable Ca^{2+} bound to myofibrils in the submicromolar range and enhanced, in a complex relationship, superprecipitation and ATPase activity (Fig. 4.15). Thus the free Ca^{2+} concentration necessary to cause contraction in these model systems was remarkably low. Weber and Herz also noted that there were "nonrelaxing" actomyosins which superprecipitate even in the presence of a Ca^{2+} chelator. These experiments were especially complex with very low Ca^{2+} concentrations necessary, possible Ca^{2+} contamination from various sources, multiple agents that bind Ca^{2+} and Mg^{2+} and even uncertainly about the protein nature of the preparations.

Despite the barriers, Weber and Ebashi made much progress but still not all the pieces fit together and people were not convinced. Ebashi (1994) has stated that at the international muscle biochemistry symposium held in Massachusetts in May of 1962 Annemarie Weber and he were essentially the only two participants who believed in the role of Ca^{2+} in contraction and relaxation. One of the main criticisms directed against the Ca^{2+} concept was that Ca^{2+}, while effective on a crude system such as glycerinated fibers or natural actomyosin, was not effective on reconstituted

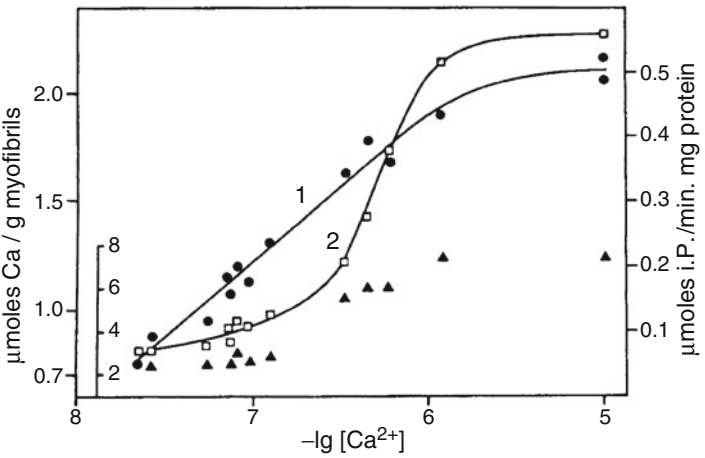


Fig. 4.15 Ca^{2+} binding, ATPase activity and superprecipitation of myofibrils as a function of ionized calcium concentration. Ordinates: *left*, Ca^{2+} binding (*curve 1*); *inset left*, superprecipitation (*triangles*); *right*, ATPase activity (*curve 2*). Abscissa, log of the free Ca^{2+} concentration. Note that Ca^{2+} binding to the myofibrils in the submicromolar range leads to acceleration of ATPase activity and superprecipitation (Weber and Herz 1963. With permission The American Society for Biochemistry and Molecular Biology)

actomyosin which was composed of separately prepared actin and myosin and was much purer than natural actomyosin. Basically it was asserted that a factor that was unable to act on a pure system could not be of vital importance (Ebashi 1980).

4.8 Discovery of the Ca^{2+} Receptor in Muscle Activation: Troponin

Ebashi and his colleagues took up this challenge in a series of remarkable papers, mostly "letters to the editor" from 1963 to 1968. The end result was the discovery and characterization of a new protein that Ebashi called troponin, the first intracellular Ca^{2+} binding protein. The first letter went to Nature and the next five letters were sent to the Journal of Biochemistry. The Journal of Biochemistry is an English language journal published in Tokyo on behalf of the Japanese Biochemical Society. Ebashi published most of his papers throughout the 1960s in this journal. One can suppose that there were at least two reasons for this approach. First, publishing significant papers in a Japanese journal with international distribution certainly promoted Japanese biochemistry and this was very important to Ebashi. Furthermore, letters to the editor were rapidly published. Ebashi could submit a letter in April and it would appear in print in June of July. Of course this was important in establishing priority in a rapidly moving field.

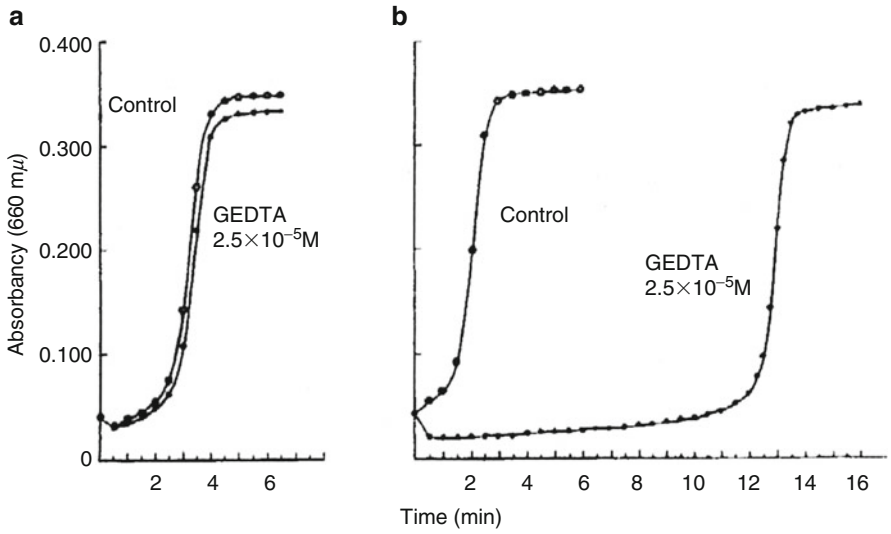


Fig. 4.16 Effect of tropomyosin-like protein on the superprecipitation of "natural actomyosin". Natural actomyosin (*control*) was made Ca^{2+} insensitive by treatment with trypsin. Superprecipitation was measured as an increase in turbidity versus time and taken as a measure of contraction. (**a**) Effect of GEDTA (EGTA) on the trypsin-treated natural actomyosin. (**b**) Effect of GEDTA on a mixture of trypsin-treated natural actomyosin plus purified tropomyosin-like protein. Note that the addition of tropomyosin-like protein changes the preparation from Ca^{2+} insensitive (**a**) to very Ca^{2+} sensitive (**b**) (Ebashi 1963. With permission Nature Publishing Group)

In a letter to Nature in 1963, Ebashi (1963) noted that a preparation of natural actomyosin isolated from rabbit skeletal muscle made Ca^{2+} insensitive by treatment with trypsin (Fig. 4.16a) could be made Ca^{2+} sensitive again, i.e., superprecipitate, by addition of a solution containing a "tropomyosin-like protein" to the mixture (Fig. 4.16b). This tropomyosin-like protein solution, also called native tropomyosin by Ebashi, exhibited all the properties of the tropomyosin protein discovered by Kenneth Bailey[8] at University of Cambridge in 1946. Tropomyosin is a very elongated fibrous protein associated with the myofibrils. While not derived from myosin itself, it has an amino acid composition similar to myosin. Bailey (1946) thought that tropomyosin might be a precursor or prototype of myosin, possibly even a subunit of myosin, and that is the reason for its name.

[8] Kenneth Bailey (1909–1963) spent most of his research career in the biochemistry department at the University of Cambridge. Beside his own discoveries relating to tropomyosin and invertebrate paramyosin (Bailey 1946), important discoveries were made by people training in his laboratory. Tien-chin Tsao (Tianqin Cao) discovered the subunit nature of the myosin molecule (see Chap. 3). B. B. Marsh discovered the 'relaxing factor' of muscle. Bailey also trained scientists who would become well known in the muscle field, including S. V. Perry (see Chap. 3) and J. C. Ruegg. Bailey was elected to a Fellowship at Trinity College in 1948 and to the Fellowship of the Royal Society in 1953. Despite his scientific success, he experienced and was treated for recurrent attacks of acute depression that worsened over the years. Tragically, he committed suicide in 1963 (Huxley 2007). Chibnall (1964) has written a biographical sketch of Bailey.

But native tropomyosin was somehow different from tropomyosin prepared according to the Bailey procedure because Bailey's tropomyosin did not restore Ca^{2+} sensitivity to the trypsin-treated natural actomyosin or synthetic actomyosin system. Some protein along with tropomyosin must be in the solution. In 1964, Ebashi and his wife Fumiko Ebashi described the properties and actions of this unnamed "new protein" in greater detail (Ebashi and Ebashi 1964). They simply referred to it as a "new protein component" that participated in the superprecipitation of synthetic actomyosin or trypsin-treated natural actomyosin. In a letter to the editor of the Journal of Biochemistry in 1965, Ebashi and Kodama (1965) for the first time reported the separation of the "tropomyosin-like protein" into two different components. In another brief letter to the editor of the same journal in 1966, Ebashi and Kodama (1966) gave this new protein the name "troponin" and showed that troponin interacted with F-actin but only in the presence of tropomyosin. Thus the tropomyosin-like protein or native tropomyosin consisted of tropomyosin and troponin and these proteins together bound to F-actin and restored Ca^{2+} sensitivity to synthetic actomyosin preparations.

Much more needed to be known about tropomyosin and troponin and their location and function in the myofibril. Endo et al. (1966) in yet another letter to the editor determined the location of native tropomyosin on myofibrils. Myofibrils isolated from rabbits were exposed to trypsin in order to digest tropomyosin and troponin. These myofibrils were then incubated with fluorescently labeled tropomyosin and troponin and observed by phase contrast and fluorescence microscopy. The results suggested strongly that the tropomyosin and troponin were located along the entire length of the thin filaments. These results were confirmed by fluorescent antibody staining of tropomyosin and troponin. In a fourth letter to the editor, Ohtsuki et al. (1967) showed in an immuno-electron microscopic study that antibodies to troponin were distributed at 400 Å intervals along the entire thin filament with 24 periodicities counted on either side of the Z line.

In the final letter to the editor in this series, Ebashi et al. (1967) proved that troponin was the calcium receptive protein in the model contractile systems in a very imaginative way. Strontium (Sr^{2+}) was known to substitute for Ca^{2+} in activating skeletal and cardiac natural actomyosin systems but with a lower potency than Ca^{2+}. The concentration of Sr^{2+} necessary for the activation of the skeletal muscle system was more than 20 times higher than that of Ca^{2+} but in cardiac muscle, it is only 3–4 times higher. All four proteins, myosin, actin, tropomyosin and troponin, were purified from both skeletal and cardiac muscle. All possible combinations were tested to determine the relative sensitivity to Sr^{2+} versus Ca^{2+}. Irrespective of the origin of the other three proteins, if troponin was prepared from skeletal muscle, the relative Sr^{2+} sensitivity of the reconstituted actomyosin system was low, but if prepared from cardiac muscle, it was high. These results clearly indicated that troponin was the site of the regulatory action of Ca^{2+} or Sr^{2+}. They concluded (Ebashi et al. 1967. With permission Oxford University Press):

> It is conceivable that binding and detaching of Ca^{++} to and from troponin might be of primary importance in regulation of muscle contraction, i.e., some conformational change of the troponin molecule induced by the removal of Ca^{++} might inhibit interaction of adjoining

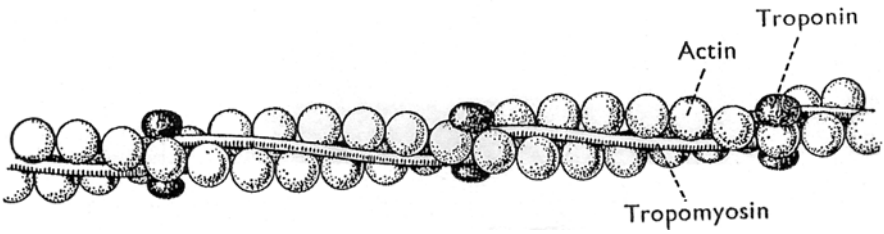


Fig. 4.17 A proposed model for the structure of the thin filament. In this model it is assumed that two molecules of tropomyosin and troponin exist in each period of the thin filament. The pitch of the double helix in the thin filament formed by the actin molecules is considered to be 360–370 Å, which is slightly shorter than the period due to troponin (Ebashi et al. 1969. With permission Cambridge University Press)

> actin molecule with myosin, and this inhibition might be cancelled by Ca^{++}. However, before accepting this explanation, we must answer the question as to how the troponin molecules, which are distributed along the thin filaments at a 400 Å periodicity (5), can exert their influence on those actin molecules which are located at some distance from adjacent two troponin molecules.

In 1968, Ebashi, Kodama and Ebashi published a full description of troponin, its preparation and physiological function, based mostly on the preliminary reports already described. They estimated that a troponin molecule bound about four Ca^{2+} with half of the binding of a high affinity in the submicromolar range and the other half with a 25-fold lower affinity. Tropomyosin did not bind Ca^{2+}. They emphasized that troponin alone could not restore Ca^{2+} sensitivity to Ca^{2+}-insensitive actomyosin preparations but rather also required tropomyosin. Furthermore, based on their relative molecular weights and content in the myofibrils, they speculated that it was plausible that two troponin molecules and two tropomyosin molecules exist per pitch of 400 Å of the thin filament. Ebashi et al. (1969) proposed a model for the structure of the thin filament (Fig. 4.17). In conclusion, Ebashi et al. (1968) stated that the results supported the hypothesis that troponin was the Ca^{2+}-receptive protein of the contractile system, i.e., the contraction-triggering action of Ca^{2+} became effective only when it bound to troponin. Thus the first intracellular Ca^{2+} receptor protein was discovered and its potential role in muscle contraction and relaxation proposed.

Hartshorne and Mueller (1968) at Carnegie-Mellon University went on to show that troponin could be fractionated into two distinct components. But Marion L. Greaser and John Gergely in 1971 at the Boston Biomedical Research Institute discovered that troponin was formed by three components in equimolar ratios (see Chap. 6). In 1972 Ebashi agreed with Greaser and Gergely that troponin is functionally composed of three components. Much more would be learned in the ensuing years about troponin and its components but the framework for the role of Ca^{2+} in muscle activation now was established.

4.9 Mechanism of Relaxation: The Mysterious "Relaxing Factor"

To many people, Setsuro Ebashi is best remembered for the discovery of troponin and characterization of its function. But Ebashi (1990) has said that the discovery of troponin was a natural consequence of the "calcium concept" and could have been done by any scientist, "perhaps even more elegantly." It was the development of the "calcium concept" and his publication of it in 1961 (Ebashi 1961a) that Ebashi considered to be his most important work. He has stated that this article was the culmination of nearly 10 years of inquiry into the mechanism of the relaxing factor and would remain "indelibly imprinted" in his memory" (Ebashi 1990).

The story of the relaxing factor began in 1951 with a discovery by a meat scientist working at the Low Temperature Station at Cambridge on a project designed to improve the quality of whale meat for human food (Marsh 1966). That scientist was B. B. Marsh from New Zealand who did the investigation in Kenneth Bailey's laboratory at the University of Cambridge. Whale meat wasn't available so Marsh (1951, 1952) examined, in the centrifuge, the effects of ATP on the time course of the decrease in volume of fragmented fibers in a homogenate of rabbit skeletal muscle. As a control for centrifugal packing of the fiber fragments, the time course of the volume change in the absence of ATP (rigor) was measured and subtracted from the time course of volume change in the presence of ATP (Fig. 4.18). When this correction was made, a striking and fundamentally important result was observed (Fig. 4.18, lower panel). The volume of the fiber fragments in the homogenate first increased, then became stable and finally slowly decreased as the ATP was exhausted. This effect was reversible and could be repeated by further addition of ATP. In the process of examining what was responsible for the volume increase or swelling of fiber fragments, Marsh also did the experiment by replacing the supernatant with isotonic KCl. Now a very different result appeared. ATP immediately caused a profound shrinkage or syneresis of the fragmented fibers, reducing the volume beyond what would be expected by centrifugal packing alone. This effect was irreversible. Marsh went on to examine the fiber fragments in the microscope under these two experimental conditions. When shrinkage occurred the fiber fragments shortened and when swelling occurred the fragments lengthened. Thus there was something in the supernatant that caused swelling or fiber fragment lengthening, i.e., relaxation. This relaxing effect in the presence of supernatant could also be overcome by addition of 1–2 mM $CaCl_2$. He concluded that the results indicated the existence of a substance, soluble in 0.16 KCl solution, which must be intimately concerned in volume increase effects. He referred to this substance simply as the "factor". This factor was normally present in the muscle. Over time this factor became known as the relaxing factor or sometimes also called the Marsh factor. Bendall (1952), also at the Low Temperature Station at Cambridge, extended Marsh's observations to muscle fiber model systems, including glycerinated muscle fibers. He observed that a fiber bundle that shortened in response to ATP could be made to relax by addition of a preparation containing the relaxing factor and

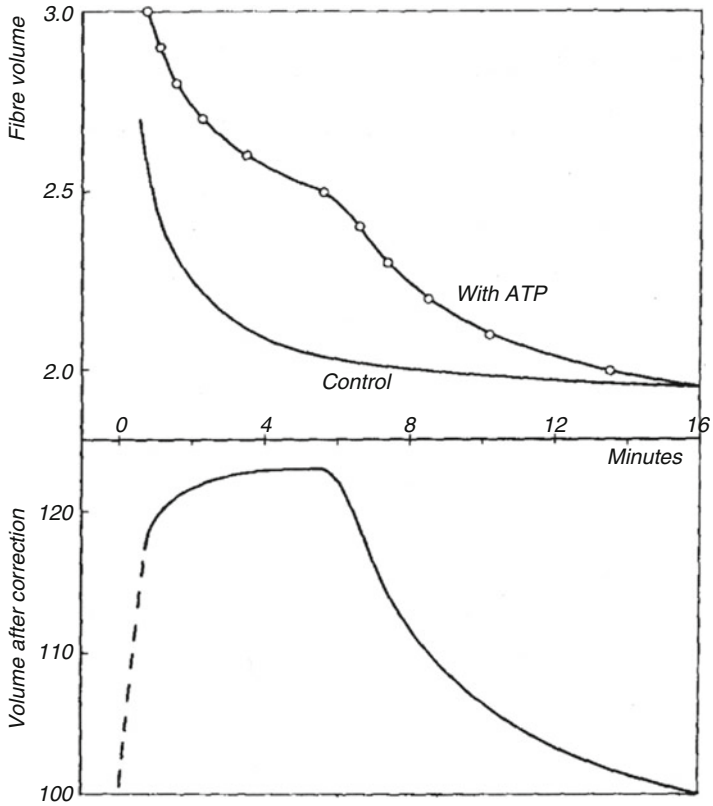


Fig. 4.18 The swelling effect of ATP on the volume of fiber fragments in a homogenate of rabbit skeletal muscle. *Upper panel*: time course of changes in fiber volume during centrifugation before (control) and after addition of ATP (at time zero) to a homogenate containing rigor fiber fragments. The decrease in volume of the rigor fragments (control) represents the effect of centrifugal packing. *Lower panel*: volume change of ATP containing fragments after subtracting the centrifugal packing curve. Note that the initial effect of ATP is to cause a swelling of the fiber fragments, i.e., a relaxing effect (Marsh 1952. With permission Elsevier)

ATP. Even though these observations were provocative, there would be missteps before the true nature of the muscle relaxing factor would be revealed almost 10 years later.

The next important step in the identification of the nature of the relaxing factor came in Hiroshi Kumagai's laboratory at the University of Tokyo. In 1955 Kumagai et al. (F. Takeda would later become Setsuro Ebashi's wife) showed that the relaxing factor was not myokinase or creatine phosphokinase as was previously proposed. Rather the active factor contained a considerable amount of lipid and exhibited a strong adenosine triphosphatase activity. It appeared to be similar to an ATPase bound to particulate matter that was discovered in 1948 by W. Wayne Kielley and

Otto Meyerhof at the University of Pennsylvania. In 1958 Ebashi concluded that the relaxation of glycerinated psoas muscle fibers required the presence of muscle granules and furthermore that the ATPase activity of the granules paralleled their effect on relaxation (Ebashi 1958).

As Ebashi was undertaking his studies of the relaxing factor during the 1950s, he was stimulated by Emil Bozler's observation in 1954 that the Ca^{2+} chelator, EDTA, imitated all the known effects of the relaxation factor. In extracted muscle fibers which have contracted in a solution containing ATP, the addition of EDTA caused relaxation, the subsequent addition of $CaCl_2$ contraction (Bozler 1954). As discussed above, Ebashi tested this Ca^{2+} concept by comparing the ability of chelators with varying affinities for Ca^{2+} to cause relaxation of glycerinated muscle fibers. The experimental results initially were disappointing as no correlation was observed (Ebashi et al. 1960). Somewhat discouraged, Ebashi went in late 1958 to spend a year in Fritz Lipmann's laboratory at the Rockefeller Institute in hopes of studying enzymology. Lipmann encouraged Ebashi to continue with his investigation of the relaxing factor. Just after his arrival, he realized his error in not considering the Mg^{2+} binding affinity of the Ca^{2+} chelators. Once corrected the correlation between relaxation and Ca^{2+} binding was excellent (Fig. 4.13). Also at this time Annemarie Weber (1959) discovered that small amounts of Ca^{2+} were necessary for contraction of myofibrils. The pieces were starting to come together.

Ebashi thought that the relaxing factor might be an ATP dependent Ca^{2+} chelator. He set out to test this hypothesis in Lipmann's laboratory. These experiments were greatly facilitated by the recent introduction of radio isotopes into biology in the form of Ca^{45}, C^{14}, P^{32}. His results were clear cut. The particulate fraction isolated from homogenized rabbit skeletal muscle that contained ATPase activity and produced muscle fiber relaxation was found to concentrate radioactive Ca^{2+} (Ca^{45}) up to 1,400-fold in an ATP dependent manner (Fig. 4.19) (Ebashi and Lipmann 1962). Ebashi (1985) has stated that this "cherished" experiment was the only experience in his life where the result completely lived up to his expectation. Furthermore George Palade[9] examined the particulate preparation in the electron microscope and concluded that the preparation contained resealed fragments or vesicles of the sarcoplasmic reticulum that he and Porter characterized in 1957 (Porter and Palade 1957). Muscatello et al. (1961) also showed that the microsomes utilized in relaxation experiments were fragments of the sarcoplasmic reticulum.

As a result of this work, Ebashi in 1961 put forward for the first time the "calcium concept" of muscle contraction and relaxation. Ebashi (1961a) described his evidence that Ca^{2+} activated the contractile proteins and that the fragmented vesicles of the sarcoplasmic reticulum induced relaxation by binding Ca^{2+} in the presence of

[9] In the Ebashi and Lipmann (1962) paper, George Palade performed the electron microscopy of the particulate preparation that accumulated Ca^{2+}, interpreted the electron micrographs, wrote the related methods and legends but yet was not a co-author on the paper. This is very unusual by modern day standards, especially since the identification of the particulate preparation as fragmented sarcoplasmic reticulum was an important finding.

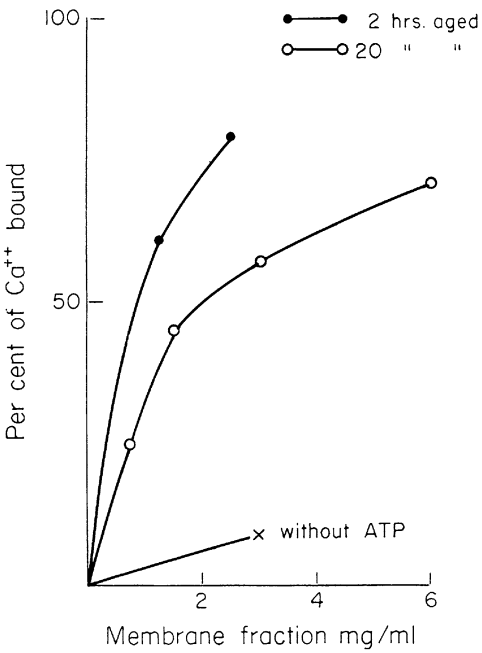


Fig. 4.19 Influence of membrane fraction (fragmented sarcoplasmic reticulum) on the ATP dependent Ca^{2+} concentrating reaction. An aged membrane fraction was less effective in accumulating calcium (Ebashi and Lipmann 1962. With permission Rockefeller University Press)

ATP. Ebashi was most proud of this paper because it was the result of 10 years of investigation and led to the development of the calcium concept. Ebashi concluded (Ebashi 1961a. With permission Oxford University Press):

> …we suggest the following picture of muscle contraction. In resting muscle calcium is highly concentrated in the endoplasmic reticulum; the concentration of calcium inside the myofibril is too low to cause the shrinking of contractile protein. When the muscle is excited, the concentrated calcium in some portion of the endoplasmic reticulum is released by the electrical influence due to the depolarization of the muscle membrane, and the calcium thus released causes in turn the shrinking of the actomyosin system. When the excitation is over, calcium release ceases and the liberated calcium is recaptured by the endoplasmic reticulum (the relaxing factor); consequently the shrunken actomyosin-system is restored to its relaxed state.

A second paper in 1961 (Ebashi 1961b) was a review of the results leading to the calcium concept. Thus the calcium concept of muscle contraction and relaxation was born. Much work would need to be done to prove the correctness of the hypothesis.

On the surface of it everything seemed fine but there was a problem for the respectful Ebashi who had the honor of working in the laboratory of a Nobel Laureate. Lipmann was slow to believe that anything as simple as Ca^{2+} could control muscle contraction and relaxation. Ebashi conducted the experiments in 1959 but the paper wasn't received by the Journal of Cell Biology until April of 1962. Thus there is an unusual statement in the introduction to the paper (Ebashi and

Lipmann 1962) indicating that the work was done about 3 years previously but, "due to various circumstances", publication was delayed. The reason for this statement was that Wilhelm Hasselbach[10] and Madoka Makinose (see footnote 10) at the Max Planck Institute for Medical Research in Heidelberg reported similar results a year earlier (Hasselbach and Makinose 1961).

Hasselbach started working on the identity of the relaxation factor with Hans H. Weber in 1953 (Hasselbach 1989). They found that the relaxing factor effectiveness was enhanced in the presence of the Ca^{2+} binding agent oxalate. Annemarie Weber (Hans Weber's daughter) suggested that he determine if removal of the microsomes from their preparation by centrifugation would affect relaxing activity. He confirmed Ebashi's findings and found that relaxing activity was present in the microsomes. In 1961; 1962 and 1963, Hasselbach and Makinose published a series of classic papers describing the relationship between Ca^{2+} uptake by the microsomes and ATP hydrolysis. Their results were very similar to those of Ebashi and Lipmann (1962). Hasselbach and Makinose discovered that oxalate which diffused into the microsomal vesicles could greatly increase Ca^{2+} uptake by the vesicles by forming a complex with Ca^{2+} in the vesicles and thus reducing the free Ca^{2+} concentration inside the vesicles. The ability of microsomes to remove Ca^{2+} from the medium depended on the presence of ATP and Mg^{2+} but Mg^{2+} was not transported into the vesicles. They calculated that the free Ca^{2+} concentration was higher in the mircosomes than in the medium. Thus the Ca^{2+} is transported against a concentration gradient. They boldly concluded that the microsomes function as a Ca^{2+} concentrating system, i.e., the calcium pump (Die Calciumpumpe), the energy for the pump being derived from ATP (Hasselbach and Makinose 1961).

Ebashi and Lipmann (1962) found that the hydrolysis of ATP by the vesicles was accompanied by an exchange of ATP and ADP. This ATP-ADP phosphate exchange reaction was interpreted as indicating a reversible phosphorylation of the membrane: ATP + membrane ↔ ADP + membrane-P. In 1962 Hasselbach and Makinose correlated for the first time the time course of Ca^{2+} uptake and ATP hydrolysis with the rapidly proceeding transfer of the terminal phosphate of ATP to ADP. The activation of this exchange activity of the transport system by Ca^{2+} was the first indication of the occurrence of a phosphorylated intermediate in a transport reaction cycle. Hasselbach and Makinose (1962) speculated that the unknown substance became a

[10] Wilhelm Hasselbach (b.1921) received his M.D. from the University of Marburg in 1949 after being released as a prisoner of war by the US Army, first Infantry Division early in 1945. He became a member of H. H. Weber's laboratory and published an influential paper on selective extraction of myosin form myofibrils in 1951. He then moved to Heidelberg from Tubingen with H. H. Weber in 1954. In 1964 he succeeded Weber as director of the Max Planck Institute for Medical Research in Heidelberg. He received the Feldberg Prize in 1963 for his work establishing the calcium pump of the sarcoplasmic reticulum. He retired from the Max Planck Institute in 1990. Madoka Makinose first joined Weber's research group in 1957 as a postdoctoral fellow from Sapporo Medical College in Japan. He returned to Germany permanently in 1960 and played a pivotal role with Hasselbach in the development of the evidence for the calcium pump. He continued to collaborate with Hasselbach and also worked independently on various aspects of calcium pump function with his last paper appearing in 1992.

"carrier by phosphorylation" and that the Ca^{2+} complex of the phosphorylated carrier diffused to the inner surface of the membrane of the vesicles and there the phosphate group was split off from the carrier leading to a large decrease in Ca^{2+} affinity of the carrier. These results were confirmed and extended by Yuji Tonomura (1923–1982) and Taibo Yamamoto (Yamamoto and Tonomura 1967) at Osaka University. From their study, especially of the phosphorylation kinetics, they developed the first reaction schemes for the calcium pump. The sarcoplasmic reticulum calcium pump is now classified as a P-type ion pump or P-ATPase since it functions via the formation of a phosphorylated intermediate. Some other P-ATPases include the Na,K-ATPase and the Ca-ATPase of the plasma membrane.

In 1963 Hasselbach and Makinose revised their earlier estimate of the stoichiometry of the calcium pump and now concluded that stoichiometry was two Ca^{2+} transported per one ATP hydrolyzed. Weber et al. (1966) verified this conclusion and extended it over a wide range of Ca^{2+} concentrations.

When Hasselbach visited the Rockefeller Institute in 1962, he "encountered fierce criticism" from Lipmann and his co-workers (Hasselbach 1989). They objected to the use of oxalate as a Ca^{2+} trapping agent. They did not believe that Ca^{2+} was accumulated by an ATP supported pump but preferred an ATP dependent Ca^{2+} binding process. Hasselbach was unaware of Ebashi's results "which were still in one of Lipmann's drawers". The findings stayed in "Lipmann's drawer" for nearly 2 years.

An important unaddressed question was could the calcium pump operate rapidly enough to explain the fast relaxation observed in muscles? Ebashi thought that the pump was not fast enough to explain relaxation and he thus was thinking in terms of an ATP dependent Ca^{2+} binding to the vesicles in analogy with the effects of calcium chelators. He did a time resolved spectrophotometric investigation of rate of Ca^{2+} binding to the vesicles (Ohnishi and Ebashi 1964) and found that in the presence of Mg^{2+} and ATP, Ca^{2+} was very rapidly bound to the relaxing vesicles. They concluded that "for the time-being" it was most plausible to suppose that the main part of Ca^{2+} was bound to the surface of sarcoplasmic reticulum (surface which is in contact with sarcoplasm) and only a minor part is accumulated inside the vesicles. Ebashi thought that the conclusions of Hasselbach and Makinose were dependent on the non-physiological action of oxalate. In the end, Hasselbach and Makinose would be shown to be correct as the results of Ohnishi and Ebashi were not confirmed by other investigators. Furthermore by comparing the rate of Ca^{2+} pumping by vesicles of the fragmented sarcoplasmic reticulum with the rate of frog muscle relaxation at low temperature, Weber et al. (1966) concluded that the speed of the Ca^{2+} uptake by reticular transport system in skeletal muscle was adequate to explain the rate of relaxation within a factor of 2.5. This agreement is reasonable given the fact that the relaxation of an intact muscle was compared to Ca^{2+} uptake by isolated vesicles of the sarcoplasmic reticulum. But in the long run more direct evidence would be needed.

Hasselbach and Makinose also made a mis-step in their interpretation. Because they knew that ATP induced superprecipitation of purified actomyosin was not affected by Ca^{2+}, they (Hasselbach 1989) developed the working hypothesis that in

presence of ATP the microsomes produced a "soluble calcium-sensitive relaxing factor". They tried to incorporate their view of the calcium pump into this hypothesis. In the early 1960s, other laboratories also were pursuing the hypothesis that the sarcoplasmic reticulum produced a "soluble relaxing factor". It was soon after the discovery of cyclic AMP by Earl W. Sutherland in 1958 (Kresge et al. 2005) and many investigators felt that the soluble relaxing factor would turn out to be a complex organic molecule. For example, Laszlo Lorand (1964) speculated that the membrane system of the sarcoplasmic reticulum might act as a "metabolic assembly synthesizing a diffusible relaxation substance" that would somehow prevent the contractile process on the myofibrils from occurring.

Hasselbach (1989) has stated that he abandoned this idea of a soluble relaxing factor after seeing the results of Weber et al. (1963). They determined that the relaxing factor could remove Ca^{2+} from actomyosin or myofibrils to an extent sufficient to explain the relaxing effect. Furthermore the vesicles of the relaxing factor were capable through the mechanism of Ca^{2+} accumulation of reducing the outside concentration of Ca^{2+} to such low levels that the equilibrium between Ca^{2+} and actomyosin was shifted toward dissociation. Finally the time course of Ca^{2+} removal paralleled the time course of inhibition of ATPase and reversal of syneresis. With regard to the effects of the relaxing factor, they concluded that it was not necessary to postulate any additional mechanism—such as a soluble relaxing substance—to explain its effect on actomyosin systems (Weber et al. 1963). The concept of the soluble relaxing factor finally became obsolete when Ebashi discovered the Ca^{2+} binding protein troponin.

The next important step was to couple the biochemical results regarding the role of Ca^{2+} in contraction and relaxation with muscle structure. The biochemical results showed that Ca^{2+} could be sequestered in the fragmented sarcoplasmic reticulum. Where was Ca^{2+} located in the resting muscle fiber? To answer this question, Costantin et al. (1965) utilized a frog muscle fiber preparation developed by Reiji Natori[11] (1954; 1986) in which the surface membrane was peeled off in paraffin oil leaving the intracellular components intact. This muscle fiber preparation is called a mechanically "skinned" muscle fiber. Following a recommendation by Andrew Huxley (Podolsky 1989), they perfused this resting "skinned" fiber with a Ca^{2+} solution containing oxalate in order to increase the participation of Ca^{2+} and to make it easier to locate calcium deposits in the electron microscope. They found that electron-opaque material, probably calcium oxalate, accumulated in the terminal sacs of the sarcoplasmic reticulum. They concluded that these regions of Ca^{2+} accu-

[11] Reiji Natori (d. 2006) graduated from the Jikeikai School of Medicine in 1936 and spent his entire career in the department of physiology at the Jikeikai School of Medicine where he became a professor emeritus in 1977. He developed the skinned muscle fiber preparation in 1949. Natori (1986) has given a chronological account of his studies utilizing skinned muscle fibers. In the mechanically skinned muscle fiber, the surface membrane has been removed allowing access to the fiber interior which still contains the structural and biochemical components of the intact fiber. In contrast, in the glycerinated muscle fiber preparation developed by Albert Szent-Gyorgyi not only the surface membrane but also the intracellular membranes have been destroyed and soluble proteins removed.

mulation represented the intracellular Ca^{2+} sink that controlled the relaxation phase of the contraction-relaxation cycle. Furthermore they noted that their proximity to the transverse tubules which were implicated in intracellular stimulus conduction suggested that they might also be regions from which Ca^{2+} was released to trigger contraction. Thus the biochemical results and muscle structure were brought together in a most satisfying way. By the mid- to late nineteen sixties, the "calcium concept" had become well established. Ebashi and Endo (1968) and Weber (1966) have written excellent reviews covering this important, exciting and sometimes confusing period of research.

Thus during the development of the calcium concept of muscle contraction and relaxation three "giants" dominated the stage. Setsuro Ebashi was the first to articulate the calcium concept which guided research during this period. Annemarie Weber was the first to show that Ca^{2+} was required for contraction of model muscle systems. Wilhelm Hasselbach (and Makinose) was the first to prove that a calcium pump caused the effects of the relaxing factor. Finally, Ebashi was the first to discover the myofibrillar calcium receptor troponin.

4.10 The Ca^{2+} Transient: Contraction and Relaxation in Muscles

Despite the spectacular advances made by the biochemists, it was still crucial to prove in muscles that the proposed changes in free Ca^{2+} concentration during contraction and relaxation actually conformed to the calcium concept. Some of the important research in this regard in the 1960s was performed on unconventional muscle preparations, namely giant muscle fibers isolated from invertebrates. Peter Caldwell (1927–1979), of Bristol University, regularly worked at the Laboratory of the Marine Biological Association in Plymouth, England and had collaborated with Alan Hodgkin and Richard Keynes. Thus he was well aware of the experiments where substances where injected into the interior of squid axons through cannulation of the axon. He reasoned that that same procedure would be possible with the giant muscle fibers from the spider crab, *Maia squinado*. These fibers could reach diameters of 1–2 mm. He and Walster (1963) developed just such a muscle preparation. They were able to microinject various substances through a cannula into the muscle fiber and then insert an electrode longitudinally into the fiber for transmembrane potential measurements. Their results were reminiscent of those of Heilbrunn and Wiercinski in 1947 in that of the ions injected only Ca^{2+} gave a substantial contraction of the fiber. Hildegard Portzehl from the University of Bern and Johann Caspar Ruegg from the Max Planck in Heidelberg joined Caldwell at the Plymouth laboratory in 1963. They injected solutions containing low concentrations of Ca^{2+} stabilized by EGTA into the giant fibers (Portzehl et al. 1964). The goal was to determine the threshold concentration of free Ca^{2+} that was needed to cause contraction in an intact muscle fiber. The Ca^{2+}-EGTA buffers which just caused contraction

contained 0.3–0.7 μM free Ca^{2+}. Thus the free Ca^{2+} concentration in resting muscle fibers must be lower than these values. These results were in good agreement with those of Annemarie Weber (Weber and Herz 1963) on isolated myofibrils and purified actomyosin. These experiments were an important step in linking the biochemistry with the physiology of muscle contraction. In 1965 Christopher C. Ashley,[12] who did his doctoral research with Peter Caldwell, showed that intracellular injections of EGTA into single cannulated muscle fibers were able to suppress, almost completely, the contractions induced by various contractile agents such as caffeine, high K^+ or electrical stimulation (Ashley et al. 1965). Richard J. Podolsky (1923–2001) had just finished postdoctoral training at University College London and was now at the NIH Naval Medical Research Institute. He applied Ca^{2+} to the Natori skinned muscle fiber preparation in oil through a micropipette with a tip diameter of 1 μm (Podolsky 1964). He found that this local application of Ca^{2+} resulted in a local contraction but only if the sarcomere length was less than 3.7 μm. Since this critical sarcomere length for contraction in response to added Ca^{2+} was very close to the length found by Huxley and Peachey (1961) for activation by electrical stimulation of an intact muscle fiber, it was reasonable to conclude that that electrical stimulation was followed by Ca^{2+} release. Thus the evidence for the calcium concept was moving from the biochemistry of actomyosin and isolated myofibrils into the realm of intact muscle fibers.

But what was really needed was a direct measure of the expected time course of changes in intracellular free Ca^{2+} concentration with muscle contraction. The first investigators to attempt to measure the so-called calcium transient in intact muscle were Frans F. Jobsis (1929–2006) and medical student Michael J. O'Connor at Duke University (1966). They introduced the Ca^{2+} sensitive dye murexide into toads by repeated intraperitoneal injection and observed changes in murexide absorbance in the isolated sartorius muscle in response to electrical stimulation. Because murexide is a dye that undergoes absorbance changes upon binding Ca^{2+}, they attributed the optical signals to calcium transients. They did not follow up these preliminary observations with a complete study at least in part because of the difficulty in getting murexide reproducibly into the muscle.

The first comprehensive investigation of intracellular calcium transients in intact muscle was performed by Ashley and Ellis Ridgway (see footnote 12) at the University of Oregon. These interesting and imaginative studies represent literally

[12] Christopher C. Ashley (1941–) received his Ph.D. from Bristol University under the guidance of Peter Caldwell. After 3 years as a Fulbright Scholar in the United States, he returned to the department of zoology at the University of Bristol. He eventually moved to the university laboratory of physiology in Oxford (now the department of physiology, anatomy and genetics, University Oxford). Throughout his career he has investigated various aspects of the regulation of muscle contraction by Ca^{2+} with publications that span more than 40 years. Ellis B. Ridgway received his Ph.D. in biology (1968) from the University of Oregon under Graham Hoyle. Following postdoctoral fellowships in London with Andrew Huxley and in Cambridge with Alan Hodgkin, Dr. Ridgway joined the department of physiology and biophysics at the Medical College of Virginia (now the Virginia Commonwealth University) in 1972. His research has revolved around assessing the role of Ca^{2+} ion in a number of biological phenomena.

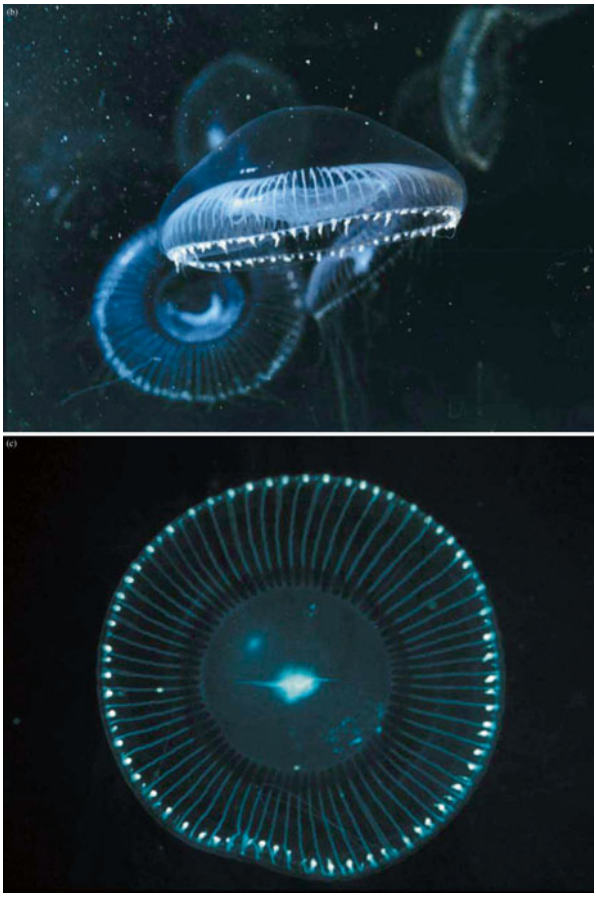


Fig. 4.20 The jellyfish, *Aequorea forskalea*, and its bioluminescence. *Top*: The light emitting cells of the jellyfish are located in the edge of the umbrella at the base of the tentacles. *Bottom*: *Aequorea* forskalea emits a greenish light upon agitation. During the light emission, blue light of the aequorin molecules is converted to green light by a cofactor, the green fluorescent protein (GFP) (Shimomura 2005. With permission John Wiley & Sons Inc)

one of the more "colorful" chapters in the history of muscle research. The story begins not with muscle at all but with the discovery of a photoprotein by Osamu Shimomura, Frank H. Johnson and Yo Saiga in 1962 at Princeton University. Shimomura, on leave from the University of Nagasaki, came to the biology department at Princeton University which was at the time the "mecca" for the study of bioluminescence. The world's leading authorities were there including E. Newton Harvey and Frank Johnson. They investigated the mechanism of the luminescence of the jellyfish, *Aequorea aequorea* (also referred to as *Aequorea victoria* or *Aequorea forskalea* after its discoverer Peter Forskal in 1775 [Harvey 1957]) which could be collected in abundance at Friday Harbor in the state of Washington. The jellyfish are nearly transparent and will only flash when stimulated or agitated (they don't sting) and then they emit a greenish luminescence. The jellyfish are shaped like a hemispherical umbrella and the light emitting organs are located along the edge of the umbrella at the base of the tentacles (Fig. 4.20). From these organs they isolated a protein that emitted a bluish light in response to very low levels of

Ca^{2+}. About a thousand jellyfish had to be collected with hand nets to yield, upon purification, 0.5 mg of protein. They called this photoprotein, aequorin.[13] Before aequorin was discovered bioluminescent systems were thought to be all of the luciferin (the substrate)/luciferase (the enzyme) type. In contrast aequorin emitted light in response to Ca^{2+} only and did not require an enzyme. Thus aequorin was a new type of bioluminescent system, a photoprotein. In 1963 Shimomura, Johnson and Saiga suggested that aequorin might be utilized as a Ca^{2+} indicator for biological research.

Ashley and Ridgway took up this suggestion with the idea of measuring the calcium transient in a muscle fiber by measuring the time course of aequorin light emission. At about the time of the discovery of aequorin, Graham Hoyle and T. Smyth (1963) at the University of Oregon discovered giant muscle fibers (up to 2 mm in diameter) in the barnacle *B. nubilus*. Ashley was already familiar with cannulation of giant fibers and he and Ridgway applied this technique to the barnacle fibers while working with Hoyle's laboratory group. They injected aequorin into the fiber and then inserted an electrode in the fiber to measure transmembrane potential and then stimulated the fiber to contract. The barnacle muscle fibers do not normally produce an all-or-none action potential when stimulated. They produce graded responses upon electrical stimulation. What Ashley and Ridgway saw when the fiber was stimulated must have been tremendously exciting. The fiber produced a blue flash of light. This light was actually visible to the dark adapted eye! They measured the time course of light emission with a photomultiplier (Ridgway and Ashley 1967; Ashley and Ridgway 1968, 1970). In what has become a classic figure (Fig. 4.21), they measured simultaneously the graded electrical response, the calcium transient as the light emitted by aequorin and the isometric force developed by the muscle fiber. The onset of the calcium transient occurred just after the onset of membrane depolarization but before the onset of detectable force. The calcium transient peaked early in the mechanical response and was virtually complete at peak force and in somewhat of a surprise mechanical relaxation occurred at essentially resting calcium levels. They (Ashley and Ridgway 1970) concluded that the simplest explanation of this latter observation was that, while Ca^{2+} was required for the generation of tension, it was not required for the maintenance of tension since at peak tension Ca^{2+} was already re-accumulated by the SR during the exponential fall of the calcium transient. This simple interpretation, of course, leads to the conclusion that relaxation involves some process other than Ca^{2+} removal from the contractile machinery. This interpretation would turn out to be incorrect but the apparent result of relaxation occurring at resting levels of free Ca^{2+} concentration would

[13] Shimomura et al. (1962) also isolated another protein which they called "green protein", later known as "green fluorescent protein" or GFP from the same organism. GFP is not required for luminescence but serves as a wavelength converter, accepting energy that would otherwise be emitted as blue light and emitting green light in its place. It was the discovery of GFP by Shimomura, the subsequent utilization of its light emission to monitor gene expression by Martin Chalfie at Columbia University and the modification of the color of light emission of GFP that allowed the monitoring of several biological processes at the same time by Roger Y. Tsien at the University of California at San Diego that led the three to share the Noble Prize in chemistry in 2008.

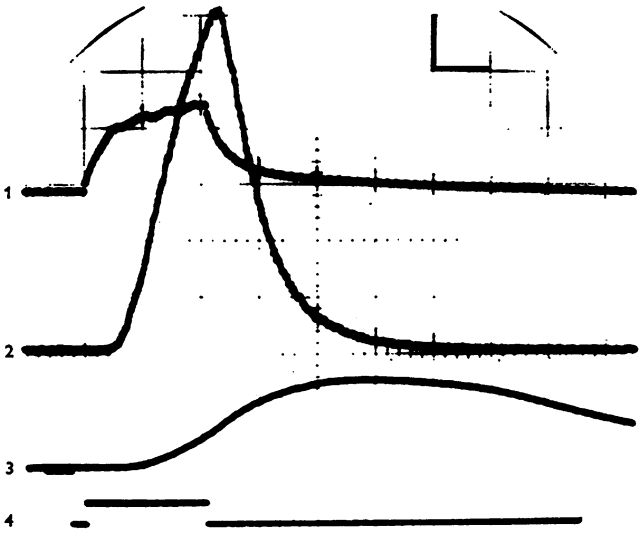


Fig. 4.21 Simultaneous recordings of membrane potential, calcium transient and isometric force in a single barnacle muscle fiber. Trace 1 is the graded membrane potential monitored by an intracellular recording electrode in response to a single depolarizing pulse of 200 ms duration. Trace 2 is the Ca^{2+}-mediated light emission from aequorin recorded by a photomultiplier. Trace 3 is isometric force. Trace 4 is a stimulus mark which indicates the onset and cessation of the stimulus pulse. Note the temporal sequence of events is from initiation of electrical activity to the increase in free Ca^{2+} concentration leading to force development (Ashley and Ridgway 1970. With permission John Wiley & Sons Inc)

require further investigation. They went on to perform a thorough study of the effects of stimulus strength, stimulus duration, repeated stimulation on the calcium transient and isometric tension. This was a powerful study in that it made it feasible to measure in muscle the temporal relationships of membrane potential, calcium transients and force production.

Since this study was done on an unconventional muscle preparation, there was interest in measuring calcium transients in the more usual preparations utilized for electrical and mechanical studies, such as frog and toad muscle fibers. In about 1966 John Blinks (1931–2012) at the Mayo Foundation took up this challenge (Blinks et al. 1976). It wouldn't be until 1973 that Blinks was able to report the results of his collaborators, postdoctoral fellows Reinhardt Rudel from Germany and Stuart Taylor from Alexander Sandow's laboratory at New York University (Blinks 1973). They recorded the time course of aequorin light emission in response to electrical stimuli of various frequencies, from single twitches to a fused tetanus in toad muscle fibers. These experiments were particularly difficult. Aequorin, a large molecule of molecular weight about 21,000 Da had to be pressure injected through a microelectrode into the fiber without damaging the fiber. Also it turned out that aequorin tracked the time course of the Ca^{2+} changes with a lag which was more of a problem the faster the calcium transient. Finally the aequorin response was non linear with

changes in free Ca^{2+}. [For a review of the technical issues relating to aequorin as a biological Ca^{2+} indicator, see Blinks et al. 1976.] There would be other interesting and important experiments conducted measuring calcium transients with aequorin in skeletal and cardiac muscle fibers. But eventually the use of aequorin as an indicator of changes in free Ca^{2+} concentration during muscle contraction would be replaced by indicators that were easier to obtain, easier to get into fibers, faster in response to changes of free Ca^{2+} and linear in response to changes in free Ca^{2+}. Measurement of changes in free Ca^{2+} concentration in muscle fibers has become a routine procedure today (see Chap. 8). Nonetheless the early experiments by Ashley and Ridgway and Blinks, Taylor and Rudel were seminal in opening up the field of calcium transient measurement in muscle fibers.

If the generalizations inferred from studies on subcellular systems are applicable to the intact muscle, exchangeable Ca^{2+} should be bound by troponin during contraction and thus one would expect to find exchangeable Ca^{2+} absent from the region of the thin filaments in resting muscle and present in significant amounts in contacting muscle. In the mid to late 1960s, Saul Winegrad in the department of physiology at the University of Pennsylvania built upon his postdoctoral work in the laboratory of Andrew Huxley. He was the first to attempt to measure the location of Ca^{2+} in a muscle at rest and during and after tetanic contraction. He developed autoradiographic techniques to measure the intracellular location of ^{45}Ca in frog skeletal muscles. He demonstrated that Ca^{2+} was located primarily in the region of the terminal cisternae in the resting muscle (Winegrad 1970). Sections of muscles rapidly frozen during contraction and then fixed were characterized by a decline in ^{45}Ca radioactivity in the region of the terminal cisternae and a rise in activity in the region of the sarcomere occupied by the thin filaments. Winegrad (1970) concluded that during a tetanus, most of the Ca^{2+} in the terminal cisternae shifted to the region of the thin filaments. Even though this technique did not have great spatial resolution and would be superseded later by elegant experiments using electron probe microanalysis (Somlyo et al. 1981), the results were consistent with the calcium concept.

4.11 The Later Years: Setsuro Ebashi

The 1960s were incredibly productive years for Ebashi and his collaborators. In 1965 Ebashi, his wife Fumiko Ebashi and Koscak Maruyama (Ebashi and Ebashi 1965; Maruyama and Ebashi 1965) announced the discovery of a new structural protein in muscle which they named α-actinin. Their laboratory subsequently localized this protein to the z-band (Masaki et al. 1967). Then Ebashi and collaborators discovered M-protein (Masaki et al. 1968) which was located at the M-line of the sarcomere. In 1967 Eijiro Ozawa, Keizo Hosoi and Ebashi discovered that Ca^{2+} in a concentration that activates muscle contraction also activated glycogenolysis by reversibly converting phosphorylase b to phosphorylase a.

Weber (1994) has summed up what she called Ebashi "great achievements". She emphasized that Ebashi had introduced all the major features of an intracellular

signaling system into biology: first, a target protein, troponin, for the intracellular messenger, Ca^{2+}; second, the concept that a messenger should coordinate cellular activities around a major task: in this case, switching on the energy supply for mechanical work, i.e., activation of phosphorylase kinase by the same Ca^{2+} concentration necessary for Ca^{2+} binding to troponin and third, a mechanism to terminate the message, ATP driven Ca^{2+} uptake by the sarcoplasmic reticulum. These were indeed great achievements.

Ebashi went on to investigate the regulatory proteins in cardiac and smooth muscle. He and his collaborators proposed that smooth muscle activation was initiated by Ca^{2+} binding to a protein that they called leiotonin, an analogue of troponin, which along with tropomyosin was associated with the actin filaments of smooth muscle (Ebashi et al. 1977). They proposed that the leiotonin-tropomyosin system was the regulatory factor common to all kinds of vertebrate smooth muscle. This idea never became accepted and it is now thought that smooth muscle is activated via a Ca^{2+} dependent myosin light chain kinase system as originally proposed by David J. Hartshorne and his colleagues (Aksoy et al. 1976). Even as late as 1995 Ebashi was still clinging to the idea of leiotonin's role in smooth muscle activation but it was not to be and leiotonin seems to have drifted quietly into the past. This apparent mis-step in no way lessens the great scientific achievements of Ebashi or his monumental role in promoting Japanese muscle biochemistry and the careers of Japanese scientists.

By the end of the 1960s much had been discovered about the role of Ca^{2+} in muscle contraction and relaxation. These discoveries included the development of the calcium concept, elucidation of the pathway of spread of the surface electrical activity into the fiber interior, location of Ca^{2+} at rest, measurement of the time course of changes in free Ca^{2+} concentration during contraction and relaxation, determination of the sensitivity of the contractile apparatus to Ca^{2+}, discovery of the role of troponin in activation of contraction, and the role of the sarcoplasmic reticulum calcium pump in sequestering Ca^{2+} and causing relaxation. But in an important sense this understanding wasn't truly molecular in nature. It was really a broad outline that would require elucidation of detailed molecular mechanisms to have a complete understanding of the role of Ca^{2+} in muscle contraction and relaxation. Many of these molecular mechanisms would be elucidated throughout the rest of the twentieth century.

References

Andersson-Cedergren E (1959) Ultrastructure of motor end plate and sarcoplasmic components of mouse skeletal muscle fiber as revealed by three-dimensional reconstructions from serial sections. J Ultrastr Res 2(Suppl 1):5–191

Ashley CC, Caldwell PC, Lowe AG (1965) The amount of EGTA needed to suppress the contractile responses of single Maia muscle fibres and its relation to the amount of calcium released during contraction. J Physiol 179:32–33P

Ashley CC, Ridgway EB (1968) Simultaneous recording of membrane potential, calcium transient and tension in single muscle fibres. Nature 219:1168–1169
Ashley CC, Ridgway EB (1970) On the relationships between membrane potential, calcium transient and tension in single barnacle muscle fibres. J Physiol 209:105–130
Aksoy MO, Williams D, Sharkey EM, Hartshorne DJ (1976) A relationship between Ca^{2+} sensitivity and phosphorylation of gizzard actomyosin. Biochem Biophys Res Commun 69:35–41
Bailey K (1946) Tropomyosin: a new asymmetric protein component of muscle. Nature 157:368–369
Bendall JR (1952) Effect of the 'Marsh factor' on the shortening of muscle fibre models in the presence of adenosine triphosphate. Nature 170:1058–1060
Bennett HS (1955) Modern concepts of structure of striated muscle. Am J Phys Med 34:46–67
Bennett HS (1956) The sarcoplasmic reticulum of striped muscle. J Biophys Biochem Cytol 2:171–174
Bennett HS (1960) The structure of striated muscle as seen by the electron microscope. In: Bourne GH (ed) The structure and function of muscle, vol 1, Structure. Academic, New York, pp 137–181
Bennett HS, Porter KR (1953) An electron microscope study of sectioned breast muscle of the domestic fowl. Am J Anat 93:61–105
Bezanilla F, White MM, Taylor RE (1982) Gating currents associated with potassium channel activation. Nature 296:657–659
Blinks JR (1973) Calcium transients in striated muscle cells. Eur J Cardiol 1:135–142
Blinks JR, Prendergast FG, Allen DG (1976) Photoproteins as biological calcium indicators. Pharmacol Rev 28:1–93
Bozler E (1954) Relaxation in extracted muscle fibers. J Gen Physiol 38:149–159
Buchthal F, Sten-Knudsen O (1959) Impulse propagation in striated muscle fibers and the role of the internal currents in activation. Ann N Y Acad Sci 81:422–445
Caldwell PC, Walster G (1963) Studies on the micro-injection of various substances into crab muscle fibres. J Physiol 169:353–372
Campbell AK (1983) Intracellular calcium: its universal role as regulatory. Wiley, New York
Chambers R, Chambers EL (1961) Explorations into the nature of the living cell. Harvard University Press, Cambridge
Chambers R, Hale HP (1932) The formation of ice in protoplasm. Proc R Soc Lond B B110:336–352
Chibnall AC (1964) Kenneth Bailey (1909–1963). Biogr Mems Fell Roy Soc Lond 10:1–13
Costantin LL (1970) The role of sodium current in the radial spread of contraction in frog muscle fibers. J Gen Physiol 55:703–715
Costantin LL, Franzini-Armstrong C, Podolsky RJ (1965) Localization of calcium-accumulating structures in striated muscle fibers. Science 147:158–160
Ebashi S (1958) A granule-bound relaxation factor in skeletal muscle. Arch Biochem Biophys 76:410–423
Ebashi S (1960) Calcium binding and relaxation in the actomyosin system. J Biochem 48:150–151
Ebashi S (1961a) Calcium binding activity of vesicular relaxing factor. J Biochem 50:236–244
Ebashi S (1961b) The role of "relaxing factor" in contraction-relaxation cycle of muscle. Prog Theor Phys 17:35–40
Ebashi S (1963) Third component participating in the superprecipitation of "natural actomyosin". Nature 200:1010
Ebashi S (1972) Separation of troponin into its three components. J Biochem 72:787–790
Ebashi S (1980) Regulation of muscle contraction. Proc R Soc Lond B 267:259–286
Ebashi S (1985) Relaxing factor, sarcoplasmic reticulum and troponin—a historical survey. In: Fleischer S, Tonomura Y (eds) Structure and function of sarcoplasmic reticulum. Academic, New York, pp 1–18
Ebashi S (1990) The first correct prediction. Citation classic. Current Contents, October 29

Ebashi S (1994) Calcium ion and muscle contraction. In: Maruyama K, Nonomura Y, Kohama K (eds) Calcium as cell signal. Igaku-Shoin, Tokyo, pp 1–11
Ebashi S, Ebashi F (1964) A new protein component participating in the superprecipitation of myosin B. J Biochem 55:604–613
Ebashi S, Ebashi F (1965) α-actinin, a new structural protein from striated muscle. I. Preparation and action on actomyosin-ATP interaction. J Biochem 58:7–12
Ebashi S, Ebashi F, Fujie Y (1960) The effect of EDTA and its analogues on glycerinated muscle fibers and myosin adenosinetriphosphatase. J Biochem 47:5–59
Ebashi S, Ebashi F, Kodama A (1967) Troponin as the Ca++-receptive protein in the contractile system. J Biochem 62:137–138
Ebashi S, Endo M (1968) Calcium and muscle contraction. Prog Biophys Mol Biol 18:123–183
Ebashi S, Endo M, Ohtsuki I (1969) Control of muscle contraction. Quart Rev Biophys 2:351–384
Ebashi S, Kodama A (1965) A new protein factor promoting aggregation of tropomyosin. J Biochem 58:107–108
Ebashi S, Kodama A (1966) Interaction of troponin with F-actin in the presence of tropomyosin. J Biochem 59:425–426
Ebashi S, Kodama A, Ebashi F (1968) Troponin. I. Preparation and physiological function. J Biochem 64:465–477
Ebashi S, Lipmann F (1962) Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. J Cell Biol 14:389–400
Ebashi S, Mikawa T, Hirata M, Toyo-oka T, Nonomura Y (1977) Regulatory proteins in smooth muscle. In: Casteels R, Godfraind T, Ruegg JC (eds) Excitation-contraction coupling in smooth muscle. Elsevier/North-Holland, Amsterdam, pp 325–334
Eisenberg B, Eisenberg RS (1968) Selective disruption of the sarcotubular system in frog Sartorius muscle. J Cell Biol 39:451–467
Eisenberg RS, Gage PW (1967) Frog skeletal muscle fibers: changes in electrical properties after disruption of transverse tubular system. Science 158:1700–1701
Endo M (1964) Entry of a dye into the sarcotubular system of muscle. Nature 202:1115–1116
Endo M (2006) Setsuro Ebashi (1922–2006). Nature 442:996
Endo M, Nonomura Y, Masaki T, Ohtsuki I, Ebashi S (1966) Localization of native tropomyosin in relation to striation patterns. J Biochem 60:605–608
Endo M, Tanaka M, Ogawa Y (1970) Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. Nature 228:34–36
Franzini-Armstrong C (1970) Studies of the triad. I. Structures of the junction in frog twitch fibers. J Cell Biol 47:488–499
Franzini-Armstrong C (1998) Annemarie Weber: Ca^{2+} and the regulation of muscle contraction. Trends Cell Biol 8:251–254
Franzini-Armstrong C (2002) Veratti and beyond: structural contributions to the study of muscle activation. Rend Fis Acc Lincei 13:289–323
Franzini-Armstrong C, Porter KR (1964) Sarcolemmal invaginations constituting the T system in fish muscle fibers. J Cell Biol 22:675–696
Freygang WH, Goldstein DA, Hellam DC (1964) The after-potential that follows trains of impulses in frog muscle fibers. J Gen Physiol 47:929–952
Gonzalez-Serratos H (1966) Inward spread of contraction during a twitch. J Physiol 185:20–21P
Gonzalez-Serratos H (1971) Inward spread of activation in vertebrate muscle fibres. J Physiol 212:777–799
Graham J, Gerard RW (1946) Membrane potentials and excitation of impaled single muscle fibers. J Cell Com Physiol 28:99–117
Greaser ML, Gergely J (1971) Reconstitution of troponin activity from three protein components. J Biol Chem 246:4226–4233
Gross PR (1986) The truth about calcium. MBL Newsletter 8

Hartshorne DJ, Mueller H (1968) Fractionation of troponin into two distinct proteins. Biochem Biophys Res Commun 31:647–653
Harvey EN (1957) A history of luminescence from the earliest times until 1900. Volume 44 of Memoirs of the American Philosophical Society, Philadelphia
Hasselbach W (1989) From frog lung to calcium pump. In: Tosteson DC (ed) Membrane transport: people and ideas. American Physiological Society, Bethesda, pp 187–201
Hasselbach W, Makinose M (1961) Die calciumpumpe der "erschlaffungsgrana" des muskels und ihre abhangigkeit von der ATP-spaltung. Biochem Z 333:518–528
Hasselbach W, Makinose M (1962) ATP and active transport. Biochem Biophys Res Commun 7:132–136
Hasselbach W, Makinose M (1963) Uber den mechanismus des calcium-transportes durch die membranen des sakoplasmatischen reticulums. Biochem Z 339:94–111
Heilbrunn LV (1940) The action of calcium on muscle protoplasm. Physiol Zool 13:88–94
Heilbrunn LV (1956) The dynamics of living protoplasm. Academic, New York
Heilbrunn LV, Wiercinski FJ (1947) The action of various cations on muscle protoplasm. J Cell Comp Physiol 29:15–32
Hill AV (1948) On the time required for diffusion and its relation to processes in muscle. Proc R Soc Lond B 136:446–453
Hill AV (1949) The abrupt transition from rest to activity in muscle. Proc R Soc Lond B 136:399–420
Hodgkin A (1992) Chance & design: reminiscences of science in peace and war. Cambridge University Press, Cambridge
Hodgkin AL, Horowicz P (1960) The effect of sudden changes in ionic concentration on the membrane potential of single muscle fibres. J Physiol 153:370–385
Howell JN, Jenden DJ (1967) T-tubules of skeletal muscle: morphological alterations which interrupt excitation-contraction coupling. Fed Proc 26:553
Howell JN (1969) A lesion of the transverse tubules of skeletal muscle. J Physiol 201:515–533
Hoyle G, Smyth T (1963) Giant muscle fibers in a barnacle, Balanus nubilus Darwin. Science 39:49–50
Huxley AF (1957a) Muscle structure and theories of contraction. In: Butler JAV, Katz B (eds) Progress in biophysics and biophysical chemistry, vol 7. Pergamon Press, London, pp 255–318
Huxley AF (1957b) Local activation of striated muscle from the frog and crab. J Physiol 135:17–18
Huxley AF (1971) The activation of striated muscle and its mechanical response. Proc R Soc Lond B 178:1–27
Huxley AF (1995) E-C coupling: the pre-Ebashi era. In: Maryama K, Nonomura Y, Kohama K (eds) Calcium as cell signal. Igaku-Shoin, Tokyo, pp 23–29
Huxley AF (2004) Andrew F. Huxley. In: Squire LR (ed) The history of neuroscience in autobiography, vol 4. Elsevier Academic Press, Amsterdam, pp 282–319
Huxley AF (2007) Biological actions of calcium. In: Ebashi S, Ohtsuki I (eds) Regulatory mechanisms of striated muscle contraction. Springer, New York, pp 3–6
Huxley AF, Niedergerke R (1958) Measurement of the striations of isolated muscle fibres with the interference microscope. J Physiol 144:403–425
Huxley AF, Peachey LD (1961) The maximum length for contraction in vertebrate striated muscle. J Physiol 156:150–165
Huxley AF, Straub RW (1958) Local activation and interfibrillar structures in striated muscle. J Physiol 143:40–41
Huxley AF, Taylor RE (1955a) Function of Krause's membrane. Nature 176:1068
Huxley AF, Taylor RE (1955b) Activation of a single sarcomere. J Physiol 130:49–50
Huxley AF, Taylor RE (1958) Local activation of striated muscle fibers. J Physiol 144:426–441
Huxley HE (1964) Evidence for continuity between the central elements of the triads and extracellular space in frog Sartorius muscle. Nature 202:1067–1071

Jobsis FF, O'Connor MJ (1966) Calcium release and reabsorption in the Sartorius muscle of the toad. Biochem Biophys Res Commun 25:246–252
Kamada T, Kinosita H (1943) Disturbances initiated from naked surface of muscle protoplasm. Jpn J Zool 10:469–493
Katz B (1948) The electrical properties of the muscle fibre membrane. Proc R Soc Lond B 135:506–534
Kielley WW, Meyerhof O (1948) Studies on adenosinetriphosphatase of muscle. II. A new magnesium-activated adenosinetriphosphatase. J Biol Chem 176:591–601
Kresge N, Simoni RD, Hill RL (2005) Earl W. Sutherland's discovery of cyclic adenine monophosphate and the second messenger system. J Biol Chem 280:e39
Kuffler SW (1946) The relation of electric potential changes to contracture in skeletal muscle. J Neurophysiol 9:367–377
Kumagai H, Ebashi S, Takeda F (1955) Essential relaxing factor in muscle other than myokinase and creatine phosphokinase. Nature 176:166
Ling G, Gerard RW (1949) The normal membrane potential of frog sartorius fibers. J Cell Comp Physiol 34:383–396
Lorand L (1964) General discussion of chapter 24. In: Gergely J (ed) Biochemistry of muscle contraction. Little Brown and Co., Boston, p 252
Marsh BB (1951) A factor modifying muscle fibre synaeresis. Nature 167:1065–1066
Marsh BB (1952) The effects of adenosine triphosphate on the fibre volume of a muscle homogenate. Biochim Biophys Acta 9:247–260
Marsh BB (1966) Relaxing factor in muscle. In: Briskey EJ, Cassens RG, Trautman J (eds) The physiology and biochemistry of muscle as a food. University of Wisconsin Press, Madison, pp 225–236
Maruyama K, Ebashi S (1965) α-actinin, a new structural protein from striated muscle. II. Action on actin. J Biochem 58:13–19
Masaki T, Endo M, Ebashi S (1967) Localization of 6S component of α-actinin at the Z-band. J Biochem 62:630–632
Masaki T, Takaiti O, Ebashi S (1968) "M-substance", a new protein constituting the M-line of myofibrils. J Biochem 64:909–910
Mazzarello P (2002) Emilio Veratti and the first application of the "black reaction" to muscle research: a historical account. Rend Fis Acc Lincei 13:273–288
McCallister LP, Hadek R (1970) Transmission electron microscopy and stereo ultrastructure of the T system in frog skeletal muscle. J Ultrastr Res 33:360–368
Muscatello U, Andersson-Cedergren E, Azzone GF, von der Decken A (1961) The sarcotubular system of frog skeletal muscle. A morphological and biochemical study. J Biophys Biochem Cytol 10:201–218
Nastuk WL, Hodgkin AL (1950) The electrical activity of single muscle fibers. J Cell Comp Physiol 35:39–74
Natori R (1954) The property and contraction process of isolated myofibrils. Jikeikai Med J 1:119–126
Natori R (1986) Skinned fibres of skeletal muscle and the mechanism of muscle contraction: a chronological account of the author's investigations into muscle physiology. Jikeikai Med J 33(Suppl 1):1–74
Niedergerke R (1955) Local muscular shortening by intracellularly applied calcium. J Physiol 128:12–13P
Ohnishi T, Ebashi S (1964) The velocity of calcium binding of isolated sarcoplasmic reticulum. J Biochem 55:599–603
Ohtsuki I, Masaki T, Nonomura Y, Ebashi S (1967) Periodic distribution of troponin along the thin filament. J Biochem 61:817–819
Otsuka M (2007) Setsuro Ebashi (1922–2006). Proc Jpn Acad Ser B 83:179–180
Ozawa E, Hosoi K, Ebashi S (1967) Reversible stimulation of muscle phosphorylase b kinase by low concentration of calcium ions. J Biochem 61:531–533

Page S (1964) The organization of the sarcoplasmic reticulum in frog muscle. J Physiol 175:10–11P
Peachey LD (1959) Doctoral dissertation. The Rockefeller Institute, New York
Peachey LD (1965) The sarcoplasmic reticulum and transverse tubules of the frog's Sartorius. J Cell Biol 25:209–231
Peachey LD, Huxley AF (1964) Transverse tubules in crab muscle. J Cell Biol 23:70–71A
Perry SV, Grey TC (1956) Ethylenediaminetetra-acetate and the adenosinetriphosphatase activity of actomyosin systems. Biochem J 64:5P
Podolsky RJ (1964) The maximum sarcomere length for contraction of isolated myofibrils. J Physiol 170:110–123
Podolsky RJ (1989) Membrane transport in excitation-contraction coupling. In: Tosteson DC (ed) Membrane transport: people and ideas. American Physiological Society, Bethesda, pp 291–302
Porter KR, Palade GE (1957) Studies on the endoplasmic reticulum. III. Its form and distribution in striated muscle cells. J Biophys Biochem Cytol 3:269–300
Portzehl H, Caldwell PC, Ruegg JC (1964) The dependence of contraction and relaxation of muscle fibres from the crab maia squinado on the internal concentration of free calcium ions. Biochim Biophys Acta 79:581–591
Rall JA (1990) Emil Bozler: sixty years of investigation into the fundamental nature of muscle contraction. In: Sperelakis N, Wood JD (eds) Frontiers in smooth muscle research. Wiley-Liss, New York, pp 1–15
Ridgway E, Ashley CC (1967) Calcium transients in single muscle fibres. Biochem Biophys Res Comm 29:229–234
Robertson JD (1956) Some features of the ultrastructure of reptilian skeletal muscle. J Biophys Biochem Cytol 2:369–380
Robertson JD (1958) The cell membrane concept. J Physiol 140:58–59P
Robertson JD (1987) The early days of electron microscopy of nerve tissue and membranes. Int Rev Cytol 100:129–196
Sabatini DD, Bensch K, Barrnett RJ (1963) Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J Cell Biol 17:19–58
Sandow A (1952) Excitation-contraction coupling in muscular response. Yale J Biol Med 25:176–201
Shimomura O (2005) Discovery of aequorin and green fluorescent protein. J Microsc 217:3–15
Shimomura O, Johnson FH, Saiga Y (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. J Cell Comp Physiol 59:223–239
Shimomura O, Johnson FH, Saiga Y (1963) Microdetermination of calcium by aequorin luminescence. Science 140:1339–1340
Shreeve J (1983) Calcium and the man. MBL Newsletter 8
Singer SJ, Nicholson GL (1972) The fluid mosaic model of the structure of cell membranes. Science 175:720–731
Somlyo AV, Gonzalez-Serratos H, Shuman H, McClellan G, Somlyo AP (1981) Calcium release and ionic changes in the sarcoplasmic reticulum of tetanized muscle: an electron-probe study. J Cell Biol 90:577–594
Steinbach HB (1960) L. V. Heilbrunn, general physiologist. Science 131:397–399
Tiegs OW (1924) On the mechanism of muscular action. Aust J Exp Biol Med Sci 1:11–29
Veratti E (1902) Ricerche sulla fine struttura della fibra muscolare striata. Memorie Inst Lomb Sci Lett (Cl Sci Math Nat) 19:87–133
Veratti E (1961) Investigations on the fine structure of striated muscle. J Biophys Biochem Cytol 10(Suppl):1–59
Watanabe A (1958) Initiation of contraction by transverse and longitudinal current flow in single muscle fibers. Jpn J Physiol 8:123–137
Weber A (1959) On the role of calcium in the activity of adenosine 5'-triphosphate hydrolysis by actomyosin. J Biol Chem 234:2764–2769

Weber A (1966) Energized calcium transport and relaxing factors. In: Sanadi DR (ed) Current topics in bioenergetics, vol 1. Academic, New York, pp 203–254
Weber A (1994) Biological roles of some actin-binding proteins. In: Maruyama K, Nonomura Y, Kohama K (eds) Calcium as cell signal. Igaku-Shoin, Tokyo, pp 221–230
Weber A, Herz R (1963) The binding of calcium to actomyosin systems in relation to their biological activity. J Biol Chem 238:599–605
Weber A, Herz R, Reiss I (1963) On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum. J Gen Physiol 46:679–702
Weber A, Herz R, Reiss I (1966) Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. Biochem Z 345:329–369
Weber A, Winicur S (1961) The role of calcium in the superprecipitation of actomyosin. J Biol Chem 236:3198–3202
Winegrad S (1970) The intracellular site of calcium activation of contraction in frog skeletal muscle. J Gen Physiol 55:77–88
Yamamoto T, Tonomura Y (1967) Reaction mechanism of the Ca^{++}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. I. Kinetic studies. J Biochem 62:558–575
Zachar J (1971) Electrogenesis and contractility in skeletal muscle cells. University Park Press, Baltimore

Chapter 5
Mechanics and Energetics of Muscular Contraction: Before Sliding Filaments and into the Modern Era

The experimental results "have led to important modifications of some of the fundamental and accepted principles of muscle physiology." (Fenn 1923. With permission John Wiley & Sons Inc).

W. O. Fenn (1923)

The idea was that if the length was suddenly changed by a small amount during a tetanus, the kinetics of the cross-bridge cycle would be revealed in the tension record, just as the kinetics of the ion channels had been revealed in the time course of the current after a sudden change of voltage. (Simmons 1992. With permission Cambridge University Press)

R. M. Simmons (1992)

Until this heat is accounted for it is hard to feel much confidence in theories of contraction that neglect its existence. (Gilbert et al. 1973. With permission Cold Spring Harbor Laboratory Press)

C. Gilbert et al. (1973)

The goal of the kinetic studies of ATP hydrolysis by actomyosin was: "to arrive at a simple model, one that could be correlated with the steps in the contraction cycle." (Taylor 2001. With permission pending American Society for Cell Biology)

E. W. Taylor (2001)

5.1 Introduction

Muscle is first and foremost a machine that utilizes fuel to generate force and do work. This fact was emphasized in a very colorful way by Douglas (Doug) R. Wilkie, a professor in the department of physiology at University College London. In preparation for a lecture on muscle mechanics and energetics to engineers, Wilkie generated a notice of the lecture in terms that would be familiar to engineers. The notice of the lecture appeared in the publicity section of Nature in 1969 and is reproduced in Fig. 5.1. Muscle is indeed a motor with special properties. The topic of the mechanical and energetic properties of muscle is a venerable one dating back to the nineteenth century. The modern era began when the results of mechanical and

J.A. Rall, *Mechanism of Muscular Contraction*, Perspectives in Physiology,
DOI 10.1007/978-1-4939-2007-5_5

The Institution of Electrical Engineers has distributed the following notice of a lecture to be given to the joint IEE/IERE medical and biological electronics group on February 11, 1969:

Available now: LINEAR MOTOR. Rugged and dependable: design optimized by world-wide field testing over an extended period. All models offer the economy of 'fuel-cell' type energy conversion and will run on a wide range of commonly available fuels. Low stand-by power, but can be switched within msecs to as much as 1 kW mech/Kg (peak, dry) Modular construction, and wide range of available subunits, permit tailor-made solutions to otherwise intractable mechanical problems.

Choice of two control systems:

(1) ***Externally triggered mode.*** **Versatile, general-purpose units. Digitally controlled by picojoule pulses. Despite low input energy level, very high signal-to-noise ratio. Energy amplification 10^6 approx. Mechanical characteristics: (1 cm modules) max. speed; optional between 0.1 and 100 mm s^{-1}. Stress generated: 2 to 5 X 10^5 N m^{-2}.**

(2) ***Autonomous mode with integral oscillators.*** **Especially suitable for pumping applications. Modules available with frequency and mechanical impedance appropriate for**

(a) Solids and slurries (0.01 – 1.0 Hz)

(b) Liquids (0.5 – 5 Hz); lifetime 2.6 X 10^9 operations (typ.) 3.6 X 10^9 (max)- independent of frequency

(c) Gases (50 – 1,000 Hz)

Many optional extras **e.g. built-in servo (length and velocity) where fine control is required. Direct piping of oxygen. Thermal generation. Etc.**

Good to eat.

The lecture is by Professor D. R. Wilkie. The subject is muscle.

Fig. 5.1 An advertisement in Nature for a lecture to be given to engineers by physiologist Doug Wilkie in 1969. Reprinted with minor editorial correction (Wilkie 1969. With permission Nature Publishing Group)

energetic investigations started to be interpreted in terms of the sliding filament attached cross-bridge model of muscle contraction in the 1960s and early 1970s. Along the way empirical models of muscle contraction were developed only to be superseded by new results and new models. Muscle energetics evolved into a specialized field away from muscle chemistry and then came back when the energy

liberation during muscle contraction was compared to the observed chemical changes. These so-called energy balance experiments led to surprising and sometimes confusing results. Throughout the first half of the twentieth century the mechanics and energetics field was dominated by one scientist, a scientist of great intellectual ability and strength of personality, Archibald Vivian (A. V.) Hill. Hill made many important discoveries but also he sometimes got it wrong.

5.2 Mechanics of Muscular Contraction: The Classical Approach

5.2.1 The Visco-Elastic Model of Muscle Contraction

Gasser and Hill (1924) conducted an important study of muscle mechanics that led to the development of the visco-elastic (viscous-elastic) model of muscle contraction. Hill (1922) had previously shown in studies on humans that the force exerted by a muscle decreased as the speed of shortening increased. This result was unexpected based on the prevailing elastic model of muscle contraction. According to the elastic theory of contraction, upon stimulation the muscle effectively became a stretched elastic body. But the force exerted by a purely elastic body when stretched is determined by its length, and is independent of the velocity of shortening. The observation that, during isotonic shortening, force is determined by velocity indicated that the elastic theory could not be correct. But there was a problem. Since Hill's experiments were done on humans, it was possible that what he observed might be a property of the nervous control of contraction and not a property of the muscle itself. Thus Gasser and Hill set out to determine if this observation was an intrinsic property of the muscle or a function of the nervous system. They verified with isolated frog sartorius muscles that the force-shortening relationship was a fundamental property of the muscle itself. The active muscle behaved as though it possessed a much greater viscosity than an inactive one. The effect of speed of shortening on the force exerted by a muscle was proposed to be due to the fact that muscle consisted of an elastic network containing a viscous fluid. The idea was that some of the force in the elasticity was damped or used up internally in working against the viscosity. More force would be absorbed at high velocities, leaving less to appear at the ends of the muscle. They also did experiments where they rapidly released or stretched a muscle during an isometric contraction. During a so called "quick release" they observed that force fell below the isometric value of the shorter length and then rose again, approaching it asymptotically. They were able to develop an artificial elastic-viscous system that mimicked all of the mechanical phenomena that they observed in muscles. From these experiments Gasser and Hill concluded that the dependence of tension on the speed of shortening could be explained by the physical properties of a viscous-elastic model. Thus the visco-elastic model of muscle contraction was borne. Despite the fact that this model was able to explain many mechanical observations, it never should have been conceived as a mechanism of muscle contraction because it was wrong and in 1924 A. V. Hill already knew it.

5.2.2 *The Fenn Effect*

Before the Gasser and Hill paper was published, Wallace Osgood Fenn[1], working under Hill's guidance, already had performed a mechanical and energetic investigation with isolated muscles from the frog that would prove beyond doubt that muscle could not be working via a visco-elastic mechanism. These two papers published in 1923 and 1924 in the *Journal of Physiology* were destined to become classics in the muscle field. Fenn set out to determine the maximum efficiency of muscle contraction by measuring the work done and the energy liberated (as heat plus work) during a contraction-relaxation cycle. In his introduction Fenn (1923) stated that the experimental results "have led to important modifications of some of the fundamental and accepted principles of muscle physiology." What did Fenn find? How did his results alter fundamental principles of muscle physiology? Fenn measured the energy liberation as heat production using a sensitive system developed in Hill's laboratory. [For more about measurement of muscle heat production see the energetics section below.] This system was so sensitive to electrical and mechanical disturbances in the laboratory at Manchester University that Fenn actually did many of the experiments in the cellar of A. V. Hill's house where room temperature was usually 6–8 °C! He even graciously thanked Mrs. A. V. Hill for allowing him to utilize that basement for the experiments. Fenn examined muscle contractions where work was performed in a variety of different ways and came to the same conclusion each time (Fenn 1923, 1924). Whenever a muscle shortened upon stimulation and did work in lifting a weight, an extra amount of energy was mobilized which did not appear in an isometric contraction. Hence less energy was liberated in an isometric contraction than in any contraction in which the muscle was allowed to shorten. This fundamental result is clearly seen in the example in Fig. 5.2. This figure displays the energy liberated (as heat + work) above the value recorded for the isometric contraction. The muscle performing maximum work liberated greater than 30 % more energy than observed in the isometric contraction.

The visco-elastic model of muscle contraction supposed that the stimulated muscle was a "new elastic body" which possessed potential energy that could be converted to work or heat upon contraction. The main point was that the amount of energy liberated was predicted to be constant and thus independent of the whether work was done or not. In the isometric contraction all the potential energy would be converted to heat and in the working contraction some of it would be converted to

[1] Wallace Osgood Fenn (1893–1971), received a Ph.D. from Harvard University in 1919. From 1922 to 1924 he did research in the laboratories of A. V. Hill and H. H. Dale. He returned to the United States in 1924 to become the chair of the department of physiology at The University of Rochester School of Medicine and Dentistry for 36 years before becoming the Director of the Space Science Center at The University of Rochester. Fenn made major research contributions in muscle physiology, the mechanics of movement, electrolyte physiology and respiratory physiology. He was instrumental in promoting physiology worldwide. He was elected President of numerous societies, including the American Physiological Society (1945–1948) and International Union of Physiological Sciences (1968–1971). Fenn was elected to membership in the National Academy of Sciences in 1943. He was the recipient of five honorary degrees (Rahn 1979).

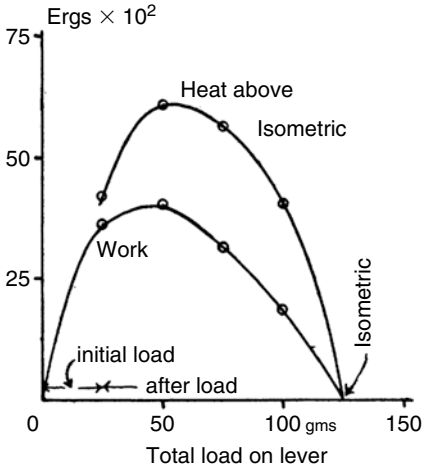


Fig. 5.2 Variation of work and heat production in isotonic contractions of isolated frog sartorius muscle as a function of increasing load. The heat curve represents only the heat in excess of the isometric heat production. Thus the isometric heat is made to coincide arbitrarily with the line of zero work. Since the heat measurements were made over a contraction relaxation cycle, they represent the energy liberated as heat + work during the contraction and relaxation. The muscle performing maximum work liberated 30 % more energy as heat + work than observed in the isometric contraction (Fenn 1923. With permission John Wiley & Sons Inc)

work and the rest to heat. Either way the total energy liberated should be constant. This prediction was clearly contrary to Fenn's results. Fenn concluded (Fenn 1923. With permission John Wiley & Sons Inc):

> …the existence of this excess energy liberation appears to be incompatible with the assumption that a stimulated muscle is a new elastic body possessing elastic potential energy. The shortening of a muscle appears to be an active process and not merely analogous to the release of a spring previously stretched. The energy used in the performance of work is developed at the time when the work is done and does not represent potential energy already developed before shortening begins.

Thus the energy that a muscle liberates during contraction is not pre-determined but can be modified after the stimulus by the nature of the contraction. This fundamental observation has become known as the Fenn Effect[2]. A. V. Hill coined the term "Fenn Effect" but Wallace Fenn never referred to the phenomenon as the Fenn Effect (Bouman 1972). Nevertheless Fenn's observations must be explained by any credible theory of muscle contraction. So even though the visco-elastic model can predict some of the mechanical features of a contraction, it cannot explain the mechanism of contraction.

[2] The Fenn Effect should not be confused with the Feng Effect. T. P. Feng (1907–1995), a Chinese scientist from Tsing Hua University working under A. V. Hill's guidance found in 1932 that the metabolism of a resting skeletal muscle was increased when the muscle was stretched.

Fenn's experiments like those of Einar Lunsgaard in the 1930 (see Chap. 1) are unique in the history of the muscle field in that they both unequivocally defeated an existing hypothesis. For Lunsgaard it was the lactic acid theory of contraction and for Fenn the visco-elastic mechanism of contraction. Yet unlike Lunsgaard's results, Fenn's results took time to "sink in".

Of course Hill was well aware of Fenn's experiments and results before the paper with Gasser was published in 1924. He knew that these results were the death knell for the visco-elastic mechanism of muscle contraction. Yet Hill went on thinking in terms of the visco-elastic mechanism for some time thereafter. Many years later, Hill reflected on his thinking at that time (Hill 1970. With permission Cambridge University Press):

> What seemed to me at the time to explain our findings (and many that had preceded them), namely the visco-elastic theory of muscle contraction, was wholly wrong; and the experiments on the visco-element model are now irrelevant. But the theory took…an unconscionable time dying…I knew Fenn's conclusions very well and they were obviously the death warrant of the visco-elastic theory; yet I went on thinking in terms of it for a long time after. It is odd how one's brain fails to work properly when pet theories are involved.

A. V. Hill is not the only scientist to fall into the trap of believing in a "pet" theory long after it has been shown to be wrong. It happened to Albert Szent-Gyorgyi with the mechanism of muscle contraction and even Setsuro Ebashi with leiotonin in smooth muscle.

5.2.3 Hill's Two Component Model of Muscle Contraction

Hill's classic experiments in 1938 finally convinced him to think differently about muscle. He measured the velocity of muscle shortening under different loads and also the time course of heat production before, during and after shortening of isolated frog sartorius muscles. This paper is a cornerstone in the history of muscle mechanics and energetics. Hill's measurements of the time course of heat production during a contraction were a major technical advance and the results agreed with Fenn's conclusions. These experiments will be considered in the energetics section below.

Hill (1938) also carefully measured muscle shortening velocity under various loads. The resulting relationship that he found is shown in Fig. 5.3. Hill fit the data empirically with an equation of the form of a displaced hyperbole:

$$\left(\mathbf{P}+\mathbf{a}\right)\left(\mathbf{v}+\mathbf{b}\right)=\mathbf{constant}$$

where P = the load during shortening, v = velocity of shortening and a and b constants given values to fit the data. During an isometric contraction, P_o, v is zero and the constant becomes equal to $(P_o + a)b$. Hill called this equation the "characteristic equation" and related it to the heat production during shortening (see below). It would turn out that all muscles exhibit the same general form for the force versus velocity relation though its scale varies very widely with muscle, animal and temperature, etc.

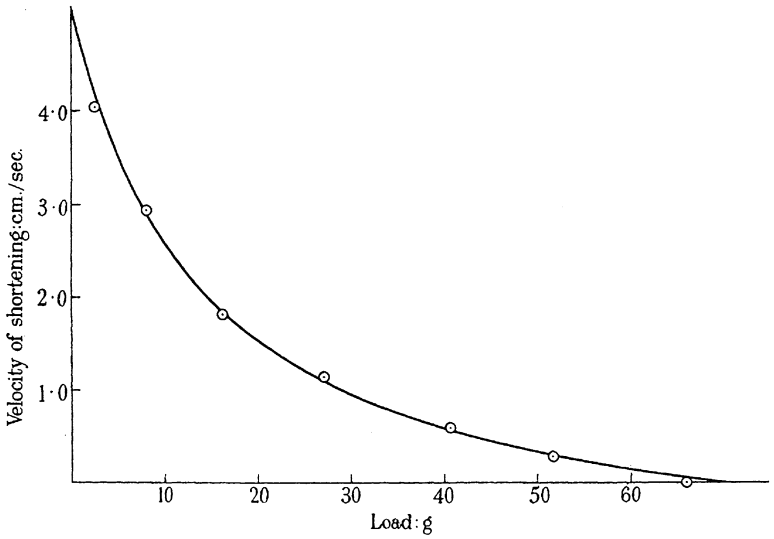


Fig. 5.3 Relationship between load and velocity of shortening in isotonic contractions of isolated frog sartorius muscle at 0 °C (Hill 1938. With permission The Royal Society)

The results of the mechanical and energetic experiments led Hill to propose a new two component model of muscle contraction. The first component, the contractile component (CC) was described by the relationship of the velocity of muscle shortening to the load. He assumed that this relationship was obeyed instantaneously upon stimulation. The second component of the model was an undamped elasticity that was proposed to be in series with the contractile component, i.e., the series elastic component (SEC). Its properties were determined by a force-extension curve and it was assumed to be unaffected by the state of activation of the muscle. Presumably the series elastic component represented in large part the muscle tendons and connections to the measuring apparatus but the possibility was not excluded that some of the series elasticity resided in the muscle fibers themselves. This model could explain at least qualitatively, though Hill (1938) said "exactly similar", the time course of force development during an isometric contraction and force recovery during quick release experiments.

The two-component model simulated the behavior of a muscle over the range of lengths where no resting tension was present, but it was not appropriate at longer lengths because the CC in the resting state offered no permanent resistance of stretch. In order to simulate the mechanical properties of muscles at lengths where resting tension is present, a third element capable of supporting the resting tension must be present in parallel with the CC. Originally the parallel elastic component (PEC) was thought to be due exclusively to the connective tissue sheaths surrounding the fibers but it would turn out that components within the muscle fibers were also important, specifically the protein titin (Horowits et al. 1986) (see Chap. 7).

Test of the two component model. Brain Jewell, a graduate student working with Doug Wilkie in the department of physiology at University College London, put Hill's two component model of contraction to a quantitative test (Jewell and Wilkie 1958). They asked the following question: could the two component model accurately predict the time course of force development in an isometric contraction and the time course of force recovery after a quick release? To answer this question they measured the force-velocity relationship of the CC and the force-extension relationship of the SEC and utilized these relationships to predict the changes in the time course of force development during an isometric contraction and after a release. Their results did not agree with the two component model. The model predicted a faster rise of tension than was actually observed experimentally. Something was wrong with the model. Jewell and Wilkie concluded that the basic premises of the two component model were incorrect. The force-velocity relationship was not established instantaneously and the SEC was not unaffected by the degree of muscle activation. These results did not mean that the two component model of muscle contraction wasn't useful to explain empirically many aspects of contraction (see below).

5.3 Mechanics of Muscular Contraction: The Contemporary Approach

With the introduction of the sliding filament model of contraction in 1954 and the kinetic model of contraction developed by A. F. Huxley in 1957, attention turned to the study of muscle mechanics from the perspective of cross-bridge behavior. Advances in this regard would come from the investigation of mechanical transients, i.e., the immediate responses of a muscle to a rapid change of load or length.

5.3.1 Velocity Transients

Richard Podolsky (1960) at the NIH was the first to critically explore this brief period after a change in load. With careful attention to reduce oscillations due to the system, he found oscillations that could be attributed to the muscle itself. Shortening velocity did not reach a steady value until more that 15 ms after the release in frog sartorius muscles at 0 °C. These results implied that the force-velocity relation is not instantaneously obeyed when the tension changes, i.e., the properties of the contractile component are not completely described by the steady-state force-velocity relation. Civan and Podolsky (1966) extended these results to fiber bundles with the same conclusions. Podolsky (1960) concluded that the transients directly reflected the working of the contractile element and thus that the kinetics provided a quantitative basis for evaluating models of the contractile process. Thus the modern era of the study of muscle mechanics began with the investigation of mechanical transients.

5.3.2 *Tension Transients*

Andrew Huxley also was interested in mechanical transients but he and his collaborators approached the subject by inducing a rapid change in muscle length, rather than load, and then recording the resulting transient change in force, i.e., tension transients rather than velocity transients. He took this approach because changes in length could be made more rapidly than changes in load. Thus the temporal resolution would be improved. Nonetheless the velocity and tension transients exhibited essentially the same oscillatory behavior (Armstrong et al. 1966).

The tension transients were intensely investigated in the early 1970s by Andrew Huxley and his collaborator Robert Malcolm Simmons and later also with Lincoln E. Ford. Bob Simmons[3] came to the Huxley laboratory in the department of physiology at University College London in early 1967. His background was in physics and protein X-ray crystallography and muscle mechanics was a strange world to him at first. Besides contributing to all the experimental results, Simmons built a force transducer with a 10 kHz frequency response to follow the rapid changes in force expected after a rapid length change. Lincoln Ford, M.D., joined the laboratory in 1971 for 2 years after postdoctoral training with Richard Podolsky at the NIH. Ford built a motor that allowed changes in muscle fiber length to be complete in as short a time as 0.2 ms. Along with the spot-follower and servo system designed by Al Gordon and Fred Julian (see Chap. 3) the stage was set for some of the most demanding experiments in the history of muscle mechanics.

In analogy with the voltage clamp experiments of Hodgkin and Huxley many years earlier, "the idea was that if the length was suddenly changed by a small amount during a tetanus, the kinetics of the cross-bridge cycle would be revealed in the tension record, just as the kinetics of the ion channels had been revealed in the time course of the current after a sudden change of voltage" (Simmons 1992).

The experiments that led to a new hypothesis of cross-bridge action put forward by Huxley and Simmons (1971b) were completed by 1971 (Huxley and Simmons, 1971a). These results were later refined and extended in 1973–1974 after improvements in the recording equipment, primarily developing a faster motor for length changes. In the typical idiosyncratic way of Andrew Huxley the results were not published in full until 1977, 1981, 1985 and 1986 by Ford, Huxley and Simmons. The theory described in the Nature paper (Huxley and Simmons 1971b) and these four papers represent the foundation for the modern view of transient mechanics of cross-bridge behavior.

[3] Robert Malcolm Simmons (b. 1938) collaborated with Andrew Huxley for 12 years at University College London resulting in the Huxley-Simmons model of cross-bridge action. Simmons then moved to the biophysics unit at King's College London where he remained for the rest of his research career. During this time he performed remarkable mechanical studies at the single molecule level. These studies included measurement of the force generation and step size of single myosin molecule interactions with actin in collaboration with James Spudich while on sabbatical at Stanford University (Finer et al. 1994) and measurements of the elasticity and unfolding of single molecules of the muscle protein titin (Tskhovrebova et al. 1997).

The idea of the basic experiment was to stimulate a frog muscle fiber to produce an isometric tetanus and then rapidly change its length, usually by less than 0.4 %, by either stretching or releasing the fiber as fast as possible, often in a step of 0.2 ms duration. Furthermore the experiment was done under sarcomere length control so that any effect of the elasticity of the tendons and connections to the apparatus would be eliminated. Resolution was enhanced by doing the experiments at low temperature, typically 0–3 °C. Initial experiments were done at a resting sarcomere length of 2.0–2.2 μm. The force response to a length step could be divided into four sequential phases (Ford et al. 1977):

Phase 1: initial elastic change during the step, complete in 0.2 ms.
Phase 2: after the step was completed, rapid partial recovery of force towards the original value, lasting 2–5 ms
Phase 3: slowing or reversal of recovery, lasting 10–50 ms
Phase 4: much slower return of force to its original value

These phases are shown in Fig. 5.4, on a slow time base (upper pair of traces) revealing the whole contraction with release and recovery and on a fast time base (lower pair of traces) showing phases 1 and 2 only.

Ford et al. (1977) concentrated their analysis on phases 1 and 2 because it was in these phases where they believed they could obtain insight into the behavior of attached cross-bridges. They thought that the cross-bridges remained attached throughout phases 1 and 2 for the following reasons. First, the step length change was very small, less than 6 nm per half sarcomere, and thus not likely to be large enough to detach the cross-bridges. Second, the recovery of force in step 2 was 50 times faster than the maximum velocity of muscle shortening and thus not likely to involve detachment and reattachment of cross-bridges. The analysis was arduous and deep. The 1977 paper in the Journal of Physiology is 77 pages long, certainly one of the longest ever published in the 100 plus year history of that journal.

Phase 1 of the tension transient. The initial change in force occurred synchronously with the applied length change which indicates that the fibers possess an elasticity which is almost linear and almost undamped. Its stiffness ($\Delta F/\Delta L$) is such that an instantaneous shortening of about 6 nm per half-sarcomere brought the force to near zero from its isometric value. This result is shown in the example in Fig. 5.5 where relative force is plotted versus step amplitude. The instantaneous elasticity is represented as T_1, the force at the end of the first phase (T_o is the maximum isometric force). The instantaneous force-extension relationship is nearly a straight line that approaches the length axis at a sharp angle and a negative tension appeared at the force transducer when a very large step was applied. These observations suggest that the structures responsible for the stiffness of the fiber remain rigid when they are not under tension. Thus this elasticity acts more like a spring than a rubber band.

Where in the fiber is the instantaneous elasticity located? Huxley and Simmons (1970) originally thought that this elasticity might be located in the filaments themselves or perhaps in the Z lines. To test this hypothesis, they stretched the resting fiber to decrease the overlap of thick and thin filaments and thus to decrease the isometric force production. If the elasticity existed in the Z lines which are in series

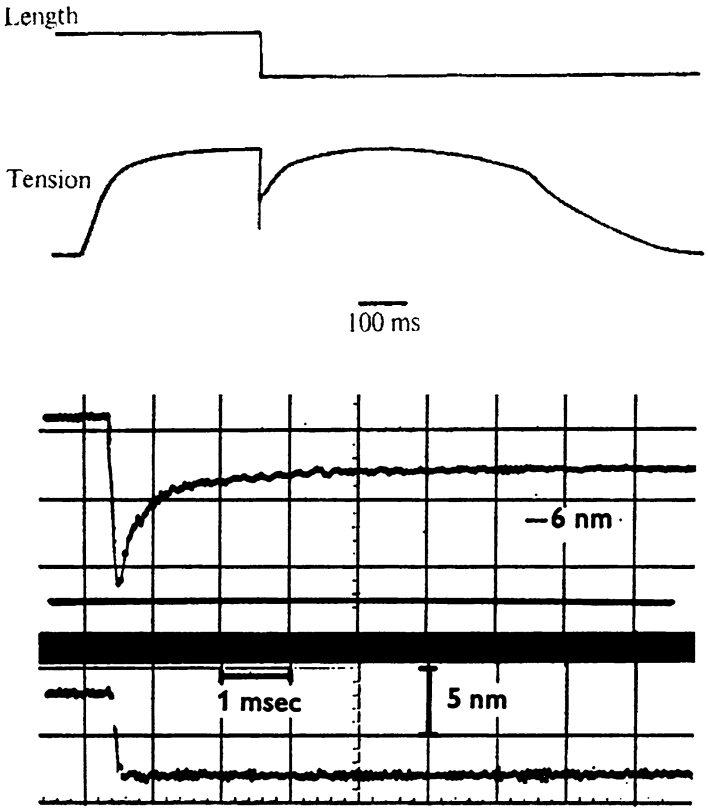


Fig. 5.4 The "tension transient": the time course of change of tension in response to a sudden change of muscle length. Upper pair of traces: length and tension on a slow time scale so as to show the whole tetanic contraction (Huxley and Simmons, 1971b. With permission Nature Publishing Group). The initial phase of tension recovery after the step is so fast that the down stroke and upstroke appear as a single line. Lower records: a part of a similar contraction which includes the step, on a time scale approximately 100 times faster, to show the early recovery phase. Frog muscle fiber, 0 °C (Huxley et al. 1994. With permission John Wiley & Sons Inc)

with the cross-bridges, then the stiffness of the elasticity would not change with decreased force, i.e., ΔF would decrease but so would ΔL, and the stiffness which is the ratio of $\Delta F/\Delta L$ represented by the slope of the T_1 curve would remain constant. The T_1 curve of Fig. 5.5 would simply be shifted to the right and remain parallel to the result at full overlap. When they did the experiment (Huxley and Simmons 1971a; Ford et al. 1981), the results were clear cut and their hypothesis was wrong. Figure 5.6 shows the slope and thus the stiffness of the T_1 decreased in proportion to the decrease in force. Thus the elasticity did not exist in the Z-lines and also not likely in the filaments. They concluded (Ford et al. 1981) that "probably well over 90 %" of the elasticity existed in the cross-bridges themselves.

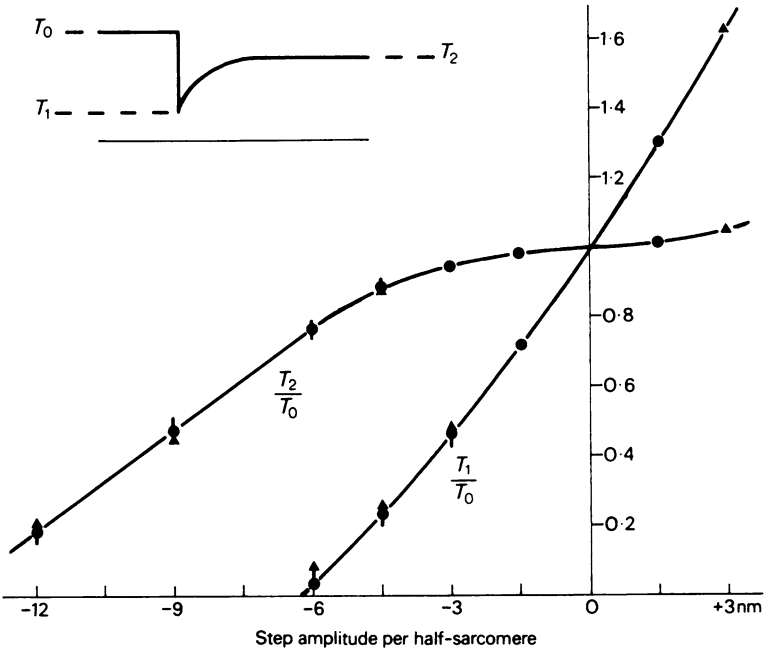


Fig. 5.5 Phase 1 and phase 2 components of the tension transient in a frog muscle fiber as functions of step amplitude and direction. Phase 1: extreme tension change during step (labeled T_1) and phase 2: tension approached during early recovery (labeled T_2). Both phases expressed as fractions of T_o, the isometric tension immediately before the step. Sarcomere length 2.2 μm at a temperature of 2.5 °C (Ford et al. 1977. With permission John Wiley & Sons Inc)

There was further evidence that the instantaneous elasticity did not reside in the Z-line. Because the series elasticity is conceived of as a spring, when the initial isometric force is markedly reduced (ΔF), the amplitude of the step length change (ΔL) necessary to bring the force instantaneously to zero should also be reduced. Instead the step length change necessary to drop the force to zero (Fig. 5.6) remained the same at both fiber lengths. This result also is consistent with the idea that the rapid elasticity resides in the cross-bridges which are not in series with each other but add up in parallel as force increases. Nonetheless they could not exclude the possibility that some elasticity existed in the filaments. Much later, structural evidence would be presented that indicates that the filaments do in fact contribute to the fiber elasticity (Huxley et al. 1994).

Besides providing information about the nature of cross-bridges, these results had practical consequences. Since the fiber stiffness was attributed to the cross-bridges and since force increases as the cross-bridges add up in parallel, this meant that fiber stiffness could be taken as an indicator of total number of attached cross-bridges. Fiber stiffness became a useful parameter to measure in a variety of experiments. For example, the stiffness of a muscle fiber decreased as the velocity of shortening increased with a limiting value of about 40 % of the maximum

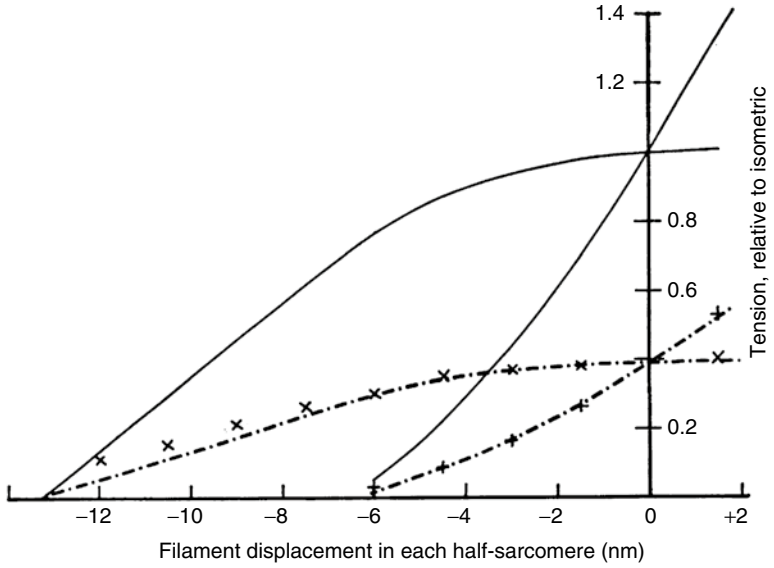


Fig. 5.6 Effect of fiber length on the values of phases 1 (T_1) and 2 (T_2) of the tension transient. Solid lines are curves fit to results from steps where sarcomere length was 2.2 μm, i.e., all cross-bridges are overlapped by thin filaments. T_1 (+) and T_2 (x) from the same fiber stretched to a sarcomere length of 3.1 μm, i.e., overlap reduced to approximately 40 %. Interrupted curves have been scaled down from the continuous curves by the factor of 0.4. Note that phases 1 and 2 scale with force and that the step change in length necessary to bring force to zero is the same at each sarcomere length. These results are consistent with the hypothesis that fiber stiffness resides in the cross-bridges (Huxley 1974. With permission Johns Wiley & Sons Inc)

isometric stiffness near the maximum shortening velocity (Ford et al. 1985). The decline of stiffness was interpreted as due largely to a reduced number of attached cross-bridges during shortening.

Phase 2 of the tension transient. During the first few milliseconds after the step the force at the end of the second phase, T_2, recovered part of the way toward the level which existed before the step (see Fig. 5.4 lower pair of traces). With small stretches and releases this early recovery was almost complete such that the plot of peak recovered force (T_2) versus step amplitude was nearly horizontal as shown in Fig. 5.5. With larger releases the line curved downwards, reaching zero in a release of about 14 nm per half-sarcomere. The time course of phase 2 is much more rapid for large releases than for small ones, and even slower for stretches. Since the T_2 curve, like the T_1 curve, scaled with force in the stretched fiber (Fig. 5.6), it too was considered to be a property of the cross-bridge. Ford et al. (1974) showed by inducing a small stretch at the peak of phase 2 that the stiffness of the fiber was the same as the stiffness of the fiber before the release. This result suggested that during phase 1 and 2 the cross-bridges remain attached.

Visco-elastic behavior of cross-bridges. Huxley and Simmons (1971b) explained these properties in terms of visco-elastic behavior of the cross-bridge. They envisioned that the cross-bridge functioned like a Voigt element. This hypothesis is

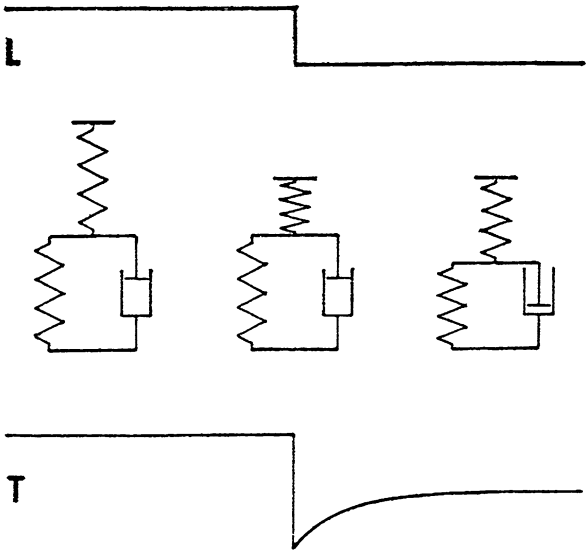


Fig. 5.7 Mechanical properties of the cross-bridge as a visco-elastic system. Diagram of a Voigt element to show how it responds to a sudden decrease in length. Synchronous with the length change, the undamped spring shortens and tension decreases. Later the spring damped by the dashpot shortens and tension recovers (Simmons and Jewell 1974. With permission Elsevier)

shown in Fig. 5.7 where an undamped elastic element is in series with a damped elastic element, i.e., an element possessing both elasticity and viscosity (the viscosity represented by a dashpot). In this model the T_1 curve would represent the force-extension properties of the undamped elastic element and the T_2 curve would represent the result of the combination of the undamped and damped elasticity. In Fig. 5.7, a rapid decrease in length leads first to a decrease in force in the undamped elastic element and then the damped elastic element shortens stretching the undamped elastic element and force rises. Thus, according to this view, the damped elastic element would represent the force-generator in the cross-bridge.

This model leads to a prediction concerning the distance over which the cross-bridge remains attached to a thin filament during the cross-bridge cycle. Consider a step shortening of 10 nm. After this step, the fiber redevelops 40 % of the isometric force in phase 2 (see T_2 of Fig. 5.5) and during this time the fiber stretches the undamped elasticity by 4 nm (T_1 of Fig. 5.5). Since the undamped and damped elasticity are in series in this model, their length changes are additive. Thus the damped elasticity has underdone 6 nm of shortening (10 nm total length change minus 4 nm undamped elasticity length change). From this result Huxley (1974) concluded that some "active" element in the cross-bridge was capable of taking up approximately 6 nm of shortening while maintaining a tension not much less than what it exerts in an isometric contraction. Furthermore the cross-bridge has to generate a movement of the order of about 5 nm to stretch the undamped elastic element during a maximum isometric contraction before the release. Thus the total range over which the cross-bridge actively generates movement is probably 10–12 nm. Huxley (1974) noted that this estimate of the range of action agreed "as well as can be expected" with the value of 10 nm calculated by Huxley (1960). This range of

cross-bridge action would become known as the power stroke and its magnitude would be a subject of intense research interest in the coming years.

In evaluating this model as representative of cross-bridge behavior two unexpected non-linearities had to be taken into consideration. The Voigt model in its simplest form, i.e. with linear springs, predicts that that the effect of step size on force recovery would be linear, i.e. T_2 should have constant slope. Contrary to prediction, the T_2 curve deviates grossly from a straight line (Fig. 5.5): for moderate-sized shortening steps it is almost horizontal, and then curves downwards, approaching a slope somewhat less than that of the T_1 curve. This result immediately suggested to Huxley and Simmons that the structures responsible for the force recovery were not just passive visco-elastic elements but active elements, the force generators themselves, i.e., the cross-bridges. Furthermore the passive Voigt model predicts that the time course of force redevelopment would be independent of the step size (and step direction). This prediction was also contrary to fact. Simmons and Jewell (1974) have stated that Andrew Huxley developed the model to explain these non-linear properties.

5.3.3 Huxley-Simmons Model of Cross-Bridge Action

The model with its mathematical underpinning is described in a Nature article by Huxley and Simmons (1971b) and later described qualitatively by Huxley and Simmons in 1973. They made the following assumptions (Huxley and Simmons 1971b, 1973):

1. The movement of an attached cross-bridge performing work takes place in a small number of steps going from one step to the next of a series of stable positions with progressively lower potential energy.
2. There is instantaneous elasticity within each cross-bridge allowing it to shift from one of these stable positions to the next without a simultaneous displacement of the whole thick and thin filaments relative to one another.

In order to explain the dependency of the rate of force redevelopment on step size (and direction), Huxley and Simmons (1971b) put forth the following argument: the greater the shortening step, the less the tension in each elastic element of the cross-bridge and therefore the less the energy necessary for the forward stepping movement of the cross-bridge. This motion therefore occurs more rapidly, and shows itself as a recovery phase 2 with a shorter half-time.

In order to visualize their arguments, they proposed that the undamped elasticity and damped elasticity existed in the cross-bridge as shown in Fig. 5.8 (Huxley and Simmons 1971b). This representation was in the context of the Andrew Huxley's 1957 model and Hugh Huxley's 1969 swinging-titling cross-bridge model. The undamped elasticity was assumed to be located in the S-2 subfragment of the myosin molecule and the damped elasticity in the S-1 subfragment of the myosin. Figure 5.9 (Huxley 1980) shows how the cross-bridge might operate during force

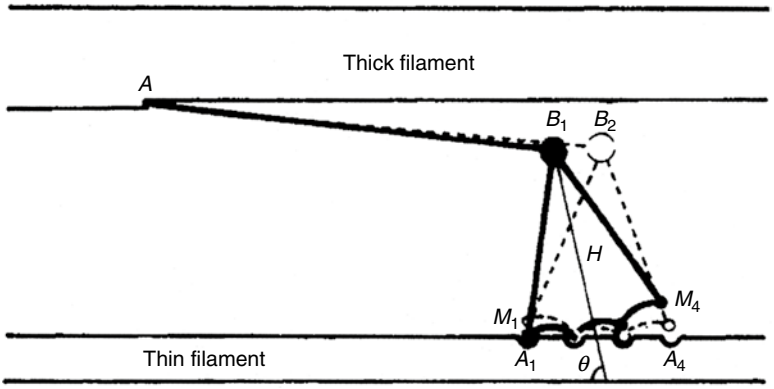


Fig. 5.8 Diagram showing assumed cross-bridge properties in the Huxley-Simmons model. The myosin head H is connected to the thick filament by a link AB containing the undamped elasticity which shows up as the T_1 curve in the whole fiber. Full line shows head in position where M_1A_1 and M_2A_2 attachments are made; broken lines show position where M_2A_2 and M_3A_3 attachments are made. The movement of the cross-bridge between stable states of varying potential energy results in the T_2 curve in the fiber (Huxley and Simmons 1971b. With permission Nature Publishing Group)

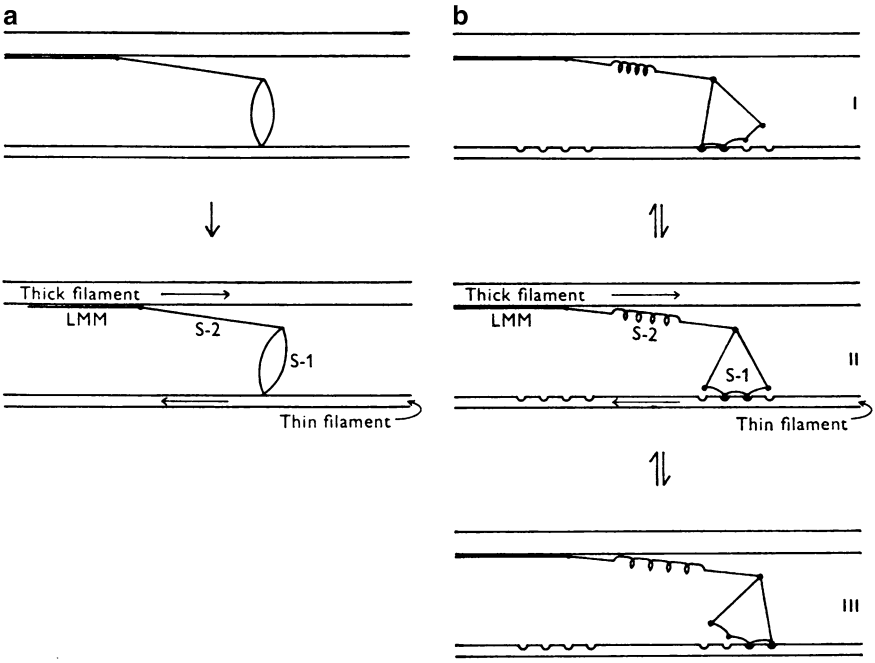


Fig. 5.9 Comparison of the swinging-titling cross-bridge model of the H. E. Huxley (1969) to the proposal of Huxley and Simmons (1971b). (**a**) Diagram of active cross-bridge adapted from Huxley (1969). In this model force originates in a tendency for the myosin head (S-1) to rotate relative to the thin filament, and is transmitted to the thick filament by the S-2 portion of the myosin molecule acting as an inextensible link. Flexible points at each end of S-2 permit S-1 to rotate, and allow for variations in the separation between filaments. (**b**) Modification of (**a**) by Huxley and Simmons (1971b) to incorporate the elastic and stepwise-shortening elements deduced from the tension transients (Huxley 1974. With permission John Wiley & Sons Inc)

development in comparison to Hugh Huxley's proposal of 1969. According to the Huxley-Simmons proposal (Huxley 1980), the strength of binding of the attached sites is higher in position II than position I and in position III than position II. During isometric contraction the myosin head oscillates rapidly between the three stable positions. The myosin head can be detached from position III with the utilization of a molecule of ATP and this is the predominant process during shortening. During stretch the myosin head can dissociate from position I without utilization of ATP.

It is important to understand that this model was not designed to replace Huxley's 1957 model but rather to become a part of it, applicable during the time the cross-bridge was attached in an isometric contraction at constant activation. It is also important to note that the kinetic experiments of Huxley, Simmons and Ford could not prove the location within the cross-bridge of the elastic structures. What was proposed was simply one possibility. Huxley and Simmons (1973) emphasized that within the context of the Hugh Huxley swinging-titling cross-bridge model there are several ways to arrange the elastic structures. Later, Huxley (1974) stated that he used the Huxley and Simmons (1973) proposal in his own thinking about the contractile mechanism but acknowledged that there was no specific evidence supporting this mechanism and that there were many other possibilities. Huxley and Simmons felt that other techniques would have to be employed to locate these elastic structures in the cross-bridges.

What about phase 3 and phase 4 of the tension transient? These phases were not extensively investigated by Ford et al. (1977) but they noted that the latter part of phase 4 exhibited a time course indistinguishable from that of the rise of tension over the same range at the start of the tetanus. With regard to phase 3 they merely noted that its physical basis was not yet definitely known. Julian et al. (1974) were able to generate a mathematical model that could explain the whole of the tension transient by incorporating the results of the Huxley and Simmons (1971b) into the Huxley 1957 model. Thus phases 3 and 4 can be accounted for by detachment and re-attachment of cross-bridges with kinetics not unlike those postulated in the 1957 theory.

As influential as the Huxley-Simmons model of cross-bridge action has been, Andrew Huxley was nonetheless somewhat disappointed. In the 1971 Huxley and Simmons (1971b) promised "a more complete treatment" of the theoretical aspects of the model and in 1977 Ford, Huxley and Simmons promised a theoretical paper that would attempt an explanation of the experimental results on the basis of the theory outline by Huxley and Simmons (1971b, 1973). Those promises were not realized, at least in part because the investigators moved on to other projects. They did not achieve what Andrew Huxley had hoped for, namely a synthesis of the results into a comprehensive theory as he and Hodgkin had generated for nerve conduction in 1952 (Huxley 2004).

It should be pointed out that these results do not imply that Hill's two component model of contraction is wrong. After all, many muscles have tendons with sufficient compliance to dominate the measured elasticity of the muscle and these tendons fit Hill's definition of the SEC. It is just that the two component model of Hill does not give insight into the mechanism of muscle contraction and cross-bridge action.

Simmons and Jewell (1974) and Huxley (1980) have written excellent reviews of the development of thought relating to models of muscle mechanics and McMahon (1984) has provided a lucid description of the mathematical aspects of the mechanical properties of muscle, including a description of the ideal Voigt (Kelvin) element, in light of the experiments of Ford, Huxley and Simmons.

Throughout the process of developing more and more complex mechanical models of muscle contraction, none of the investigators could possibly have imagined that one day the mechanical properties of force-generators would be investigated at the level of a single myosin molecule (Finer et al. 1994) (see Chap. 9).

5.4 Energetics of Muscular Contraction

5.4.1 Archibald Vivian Hill: Scientist, Humanitarian, Public Servant

No aspect of the study of muscle contraction was dominated to a greater extent in the first half of the twentieth century than the way that the muscle energetics field was dominated by Archibald Vivian Hill (1886–1977), known to all as A. V. Hill (Fig. 5.10). His publications on muscle energetics started in 1910 and ended in 1964. He shared the 1922 Nobel Prize in Physiology or Medicine with Otto F. Meyerhof (the prize was actually awarded in 1923). Hill was only 39 years old when he won the prize for his discoveries relating to the production of heat in the muscle. Hill pioneered a biophysical approach to physiology. He stated his philosophy as it related to physiology clearly in 1924 when he said: "…there is little evidence- indeed, I would be bold and say there is no evidence- that such living creatures can, in any manner or degree, evade the ordinary laws of mechanics, chemistry and physics, the principles of the conservation of energy and mass." (Hill 1924. With permission Nature Publishing Group) Hill believed that precise measurement and precise definition were needed in physiology. He never wavered from these beliefs. Furthermore with regard to the investigation of energetics, Hill (1965) believed that the accurate measurement of energy liberation during contraction, while admittedly not pointing unequivocally to the chemical processes that occur, nonetheless provided a framework into which these processes must be fitted. It was improving the "sensitivity and speed of the methods" and the establishment of an energetics "framework" in as precise a way as possible that occupied much of Hill's research effort over fifty years. This goal caused Hill in his later years to do something that on the surface seemed strange: re-doing earlier experiments with greater accuracy using continually improving equipment of his design. He called this process "operation re-examine".

That Hill saw as his legacy precise measurement and precise definition is clear from his book "Trails and Trials in Physiology" published in 1965 when he was 79 years old. There is a chapter on design of thermopiles for measurement of heat production, chapters on amplification of the signal using galvanometers, a chapter on

Fig. 5.10 A. V. Hill in his laboratory conducting an experiment on muscle energetics. The portrait was painted by H. Andrew Freeth (1912–1986) in 1957 when Hill was 71 years old. The portrait is in the possession of King's College, Cambridge (Hill 1959. With permission Annual Reviews)

analysis of heat records and one on "units, definitions, physical and chemical constants, useful quantities and relations" among others. His research was not in general hypothesis driven but rather precision driven. When he did have a hypothesis it would sometimes turn out to be wrong. A hypothesis that was subsequently proven wrong never bothered Hill. In this book he described his belief regarding hypotheses (Hill 1965. With permission Taylor & Francis Group):

> Now I do not regret these hypotheses, or even the titles of the papers; because they have set people (including myself) thinking and devising new experiments. That indeed is the chief purpose of hypotheses. I have long believed, and am still inclined to believe, that all theories of muscular contraction are wrong…But they have been very useful in stimulating new research. In fact *many of the best theories are self-destructive*, by provoking fresh inquiry and leading to new facts which they cannot explain. *The only useless theories are those that cannot be tested and can "explain" everything.*

Of course this statement was made in the 1960s after the discovery of the sliding filament hypothesis of muscle contraction in 1954. Apparently Hill wasn't convinced. What is especially informative and entertaining about "Trails and Trials" is Hill's commentary on the over 300 publications by him and those scientists who came to work with him.

Many of the important results generated in Hill's laboratory were produced by scientists who came to work with him. There were over 100 scientists from 20 different countries. Hill would provide these "young friends" as he called them with the equipment to use for their experiments and give guidance on use of the equipment (Hill 1965). Often he would suggest a topic for investigation as he did for Wallace O. Fenn. But Hill did not include his name on their papers unless he had a direct hand in the investigation. The classic example is the work of Fenn (1923, 1924) described earlier in this chapter.

Hill was also interested in the measurement of heat production in nerves. Hill (1965) believed that if it could be shown that heat was produced all along a nerve during transmission, then the purely physical theory of conduction would be untenable. A distributed relay system would be required with energy derived from chemical change. It took many years to show convincingly that the nerve action potential was associated with a measurable heat production (Abbott et al. 1958).

To measure the heat production associated with a single muscle twitch or with the generation of a single action potential was technically extremely demanding. In a single muscle twitch the temperature change was about 3×10^{-3} °C and the temperature change associated with the passage of a single action potential was remarkably small, about 10^{-5} °C (Hill 1965). The development of equipment to measure these small changes in temperature with accuracy and speed was a major component of Hill's research effort. This work was greatly facilitated by an outstanding instrument maker, A. C. Downing, who worked with Hill for over thirty years. The instrument for measuring muscle temperature change was, and for the most part still is, the thermopile. A thermopile is a series of thermocouples. The thermoelectric method depends on the fact that in a circuit made up of two appropriately dissimilar metals (a 'thermocouple') a voltage is generated when the temperature of one of the junctions is altered with respect to the other junction. This voltage can be used to measure the change of temperature. A large number of thermocouples can be joined in series to make a 'thermopile', the alternate junctions being 'hot' and 'cold'. The voltage is then multiplied in proportion. In those days, the small current associated with this electrical change was then amplified by a galvanometer. The galvanometer was extremely sensitive to mechanical and electrical disturbances and thus many of the experiments were done in a cellar, sometimes the cellar of A. V. Hill's house (see description of Fenn's work above). From 1920 to 1950 Hill and Downing increased the temporal resolution of the heat recording system by a thousand fold. The evolution of the thermopile and recording system was one Hill's major contributions to muscle research.

Hill was also a pioneer in the study of what is now called exercise physiology. In the 1920s he wanted to extend his work on isolated muscle mechanics and energetics to the study of exercising humans. The ideas behind the investigations on humans were derived partly from Hill's interest in athletics and hard muscular work, he was an avid runner, and partly from the recent work on fatigue and oxidative recovery in isolated muscles (Hill, 1965). His hope was that experiments on humans and on isolated muscle would throw light on one another. Bassett (2002) has described Hill's contributions as an exercise physiologist.

Hill's scientific expertise led him into positions of national and international prominence and no description of him would be complete without mentioning his service to his profession and his endeavors as a humanitarian and public servant (Katz 1978). For over 60 years he was active in scientific organizations including the Physiology Society, the Royal Society of London and the International Union of Physiological Sciences (IUPS), among others. He occupied leadership positions in all of these societies.

One of the issues that Hill believed in deeply was the promotion of international cooperation and collaboration in research. It was mentioned in Chap. 1 that Hill arranged for the transport by sea of 400 scientists from 22 countries to the United States for an IUPS meeting in Boston in 1929. Hill saw this international cooperation in serious jeopardy when the Nazis came to power in Germany in 1933. When he was invited to give the Thomas Henry Huxley Memorial lecture in Birmingham in November of 1933, Hill concentrated his remarks on the Nazi persecution of Jewish scientists. The lecture was published in an abbreviated form in December in Nature (Hill 1933). His remarks drew a strong reply in the February issue of Nature, 1934, from Professor Johannes Stark (1934a), a German Nobel Laureate in physics, who defended the actions of the Nazi government. Stark's comments led to another letter from Hill (1934a) and a second reply from Stark (1934b) in Nature in April, 1934. Hill (1934b) finally ended the exchange with an appeal for funds to support the Academic Assistance Council which helped exiled Jewish scientists find a location in England or the United States.[4] This exchange of letters in Nature had a profound impact on Bernard Katz, a young German medical student who was a Jew of Polish descent studying at the University of Leipzig. After reading the correspondence, Katz was determined that nothing would prevent him from going to England to work with A. V. Hill. Katz has called Hill his "his great friend and teacher" (Katz 1996). Katz (1978) has provided insight into A. V. Hill's personality and his admiration for Hill (Katz 1978. With permission The Royal Society):

> In fact, committed though he was throughout his life to work in the laboratory, it was his concern for others, the encouragement he gave to young colleagues, his upright defence not only of the cause of science, but of scientific men who had been driven from their places of work and needed help, in short it was his devotion to such wider issues, outside the boundaries of his own research, through which he exerted his most important influence on other people's lives and on the course of events.

Katz spent 5 years with Hill working on the mechanics of muscle contraction. He returned later to University College London, at Hill's invitation, to become the assistant director of research in the biophysics unit of the department of physiology. He subsequently became the first chair of the newly formed biophysics department. Katz won the Nobel Prize in Physiology or Medicine in 1970 for his investigations into the mechanism of transmitter release at the neuromuscular junction. He shared

[4] The Huxley Memorial Lecture in its unabridged form and subsequent exchanges between A. V. Hill and Johannes Stark in Nature are gathered together, with comments, by Hill in a book entitled "The Ethical Dilemma of Science and Other Writings". The book also includes other writings by Hill over a 40 year period (Hill 1962).

the prize with Julius Axelrod and Ulf von Euler. Von Euler also worked in Hill's laboratory in the 1930s (von Euler 1935). They were not the only Nobel laureates to work with A. V. Hill as Herbert Gasser also received the Nobel Prize in 1944.

From 1940 to 1945 A. V. Hill served as an elected Member of Parliament for Cambridge University. At times it must have been a difficult situation for a person of "precision" to be in the political world of "compromise". Nonetheless Hill seemed to appreciate the political acumen of many of his fellow politicians. Bernard Katz (1978) has written a detailed and affectionate biographical memoir of Hill and Katz (1996) also has commented extensively on Hill in his own autographical chapter.

Possibly the best way to summarize A. V. Hill's personality and attitude toward science is to relay a story told by Katz (1996). After giving a public lecture on "the mechanism of muscle contraction" during his first visit to the United States in 1924, a disapproving person in the audience asked Hill what was the practical use of his research. Hill's thinking and his response was (Katz 1996. With permission Elsevier):

> To prove to an indignant questioner on the spur of the moment that the work I do was useful seemed a thankless task and I gave it up. I turned to him with a smile and finished. "To tell you the truth we don't do it because it is useful but because it's amusing." The answer was thought of and given in a moment: it came from deep down in my soul…And if that is not the best reason why a scientist should do his work, I want to know what is.

And that in a "nutshell" summarizes A. V. Hill. Hill died in 1977 at the age of 90. In 2009 the University of Manchester, where Hill was the chair of physiology from 1920 to 1923, opened the A. V. Hill building which houses members of the Faculty of Life Sciences and Faculty of Medicine and Human Sciences. Hill's papers are located in Churchill College, Cambridge (Frank 1978)[5].

5.4.2 *Energetics of Muscular Contraction: Establishing the Basic Framework*

A. V. Hill's first paper on muscle heat production appeared in 1910 and he soon became interested in establishing an energetic "balance sheet" by relating heat production to the chemical change in lactic acid observed by Fletcher and Hopkins (1907) and Meyerhof (1920) (see Chap. 1). This work led to Hill and Meyerhof sharing the Nobel Prize for 1922. But Hill then diverted his attention from the concept of the balance sheet to the establishment of the energetic framework of muscular contraction with the greatest possible precision.

[5] A. V. Hill's papers at the Archives Centre at Churchill College, Cambridge, England include reprints of publications, correspondence with almost 900 individuals and institutions (numbering 10,000–15,000 items), 57 pocket diaries (an unbroken run from 1924 to 1977), five large scrapbooks of photographs and clippings about Hill and his work and three volumes of "Memories and Reflections" written in the late 1960s and 1970s (privately circulated) (Frank 1978).

Energetics of Isometric Contractions. Hill (1913a) showed in 1913 that a substantial amount of heat production associated with an isometric contraction of isolated frog muscle actually appeared after the contraction was complete. This "delayed heat" production, or recovery heat as Hill called it, depended on oxygen. It was later found that about 20 % of the delayed heat remains after oxygen is removed. The history of characterization of this anaerobic delayed heat has been described by Hill (1965). In 1914 a young German scientist Viktor Weizsacker (1886–1957) working in Hill's laboratory made the significant observation that the heat production during muscle contraction and relaxation, the so-called "initial heat production", was unaltered by the absence of oxygen in contrast to the recovery heat production which was oxygen dependent (Weizsacker 1914). Thus the chemical reactions that occur during contraction must be non-oxidative in nature. The next important step was made by Hill and Hartree (1920). This paper began a very fruitful fourteen year collaboration between Hill and Hartree which resulted in considerable improvements in the recording system and data analysis. They introduced a numerical method for analyzing the records to correct for slowness of the instrumental response. By this means the time course of heat production could be deduced, not only in the slow processes of recovery but also in the more rapid processes immediately following excitation. During an isometric contraction and relaxation of a of frog skeletal muscle at 0 °C there were three distinguishable phases of heat production: (a) an initial rapid production, diminishing gradually in rate as the stimulus proceeds; (b) a smaller constant heat-production maintained so long as the stimulus is maintained, and ending shortly after the stimulus ceases; and c) a relatively large evolution of heat, occurring rather suddenly during the later stages of relaxation. These three phases were associated (a) with the development, (b) with the maintenance and (c) with the disappearance of the mechanical response. A fourth phase, associated with the recovery processes, was measured after contractile relaxation at 20 °C. Hill and Hartree acknowledged that an investigation of heat production would enable the generation of a framework but that it would be incapable of filling in the details of the picture since the laws of thermodynamics "deal with events, not with the mechanism of events". Nonetheless the framework was starting to take shape, albeit in a qualitative way.

Energetics of Isotonic and Eccentric Contractions. The experimental results of Fenn (1923, 1924) that indicted that whenever a muscle shortens and does work an extra amount of energy is liberated are the cornerstone of the muscle energetics framework as it relates to isotonic contractions (see Fig. 5.2). In these experiments, described earlier, Fenn recorded the total amount of energy (as heat plus work) liberated in a contraction-relaxation cycle but not the time course of energy liberation. With major improvements in thermopile and galvanometer design due to A. C. Downing, Hill in 1938 was able to measure the time course of heat production during shortening of a contraction that was initially isometric. The thermopile/galvanometer system responded sixty fold more rapidly than the system used by Hill and Hartree in 1920. It was now possible to observe the time course of heat production with high temporal resolution. Hill (1938) observed that when a muscle shortened, in a maintained tetanic contraction, it liberated extra energy in two forms, (1) as

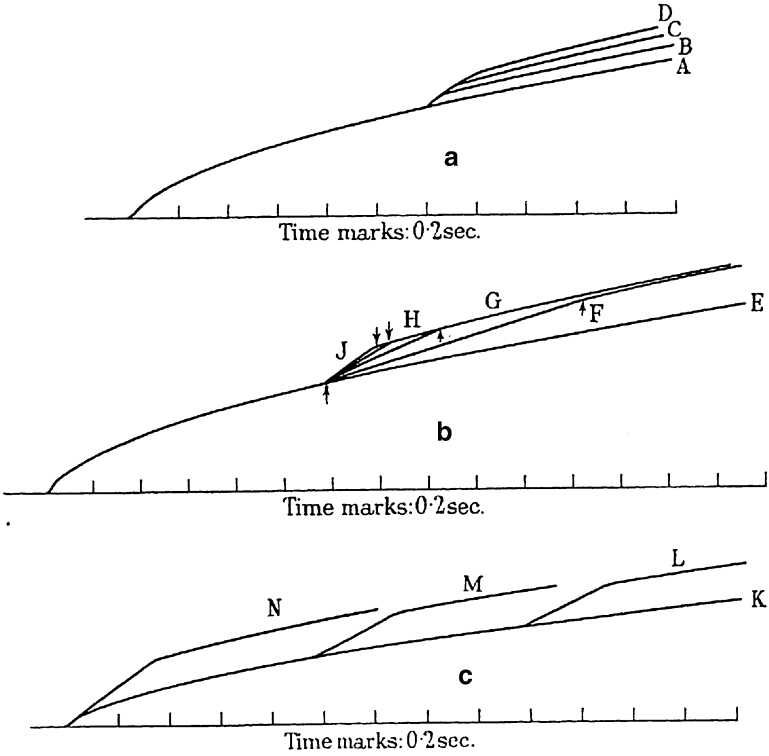


Fig. 5.11 Shortening heat production during isotonic shortening, initiated during an isometric tetanus. Frog sartorius muscles contracting at 0 °C. *Upper panel*, shortening different distances (D greatest distance, A no shortening) under constant load. *Middle panel*, shortening a constant distance under different loads (F heaviest load, J lightest load). *Lower panel*, muscle released at different times during the isometric tetanus but shortening a constant distance against a constant load (Hill 1938. With permission The Royal Society)

"shortening heat", in amount proportional to the shortening, and (2) as external mechanical work. The shortening heat was independent of the load, and therefore of the work done and the speed of shortening. The rate of energy liberation at any moment, i.e., of work + heat, was a linear function of the load, increasing as the load diminished. These characteristics of the shortening heat are shown in Fig. 5.11. The baseline for measuring shortening heat was the time course of heat production in an isometric tetanus. This isometric heat production displays the same features as observed by Hill and Hartree in 1920. The heat is produced initially at a high rate, which Hill (1949a) called the activation heat, and later at a lower steady rate called the maintenance heat production. The shortening heat data extended Fenn's observations into the time domain and were consistent with Fenn's conclusions. Hill also found that when a contracting muscle was slowly stretched the rate of energy liberation was decreased compared to the rate in an isometric contraction. Hill now finally gave up his support for the visco-elastic model of the muscle contraction.

Hill performed a number of studies to determine the relationship between heat production and the onset of force development. He clearly showed that heat production preceded the onset of contraction Hill (1953).

The results of Hill's mechanical and energetic investigations, especially those in 1938, characterizing the force-velocity relation and energy liberation during isometric and shortening contractions were extremely influential. Huxley (1957) in developing his kinetic model of "side-piece" (cross-bridge) action designed the model to fit the data in Hill's 1938 paper. Later Hill (1964a), as part of "operation re-examine", discovered using still again improved methods that the shortening heat was actually load dependent. This observation required Huxley (1973) to revise his kinetic model of contraction to fit the new data. But there were more serious challenges ahead. Huxley had assumed that the time course of energy liberation measured by Hill reflected the time course of ATP hydrolysis by the muscle. This assumption came under serious attack when Francis D. Carlson of Johns Hopkins University declared that the shortening heat was an artifact due to muscles shortening on a thermopile. Hill was able to counter the criticism (Hill 1965, pp. 328–329). Nonetheless this criticism emphasized the important point that heat production is non-specific and that one could not simply assume that the heat production represented the hydrolysis of high energy phosphate. Clearly the energy liberation had to be related to the chemical changes that occurred during contraction and relaxation. The energetics framework was essentially complete before 1960 but yet at that time there was no direct evidence that ATP or phosphocreatine was actually broken down during muscle contraction. Before a balance sheet could be drawn up there had to be improvements in the sensitivity and time resolution of the chemical analysis. This would turn out to be arduous work and would lead to numerous surprises and uncertainties.

5.4.3 Chemistry of Muscular Contraction

Since the discovery by Engelhardt and Ljubimova in 1939 that myosin exhibited ATPase activity and the development of muscle model systems (actomyosin threads and glycerinated muscle fibers) by Albert Szent-Gyorgyi in the 1940s (Szent-Gyorgyi 1945, 1949) that required ATP to contract, it was generally assumed that ATP was hydrolyzed during contraction of living muscle (see Chap. 1 for details). Yet there was no direct evidence of ATP breakdown during a contraction. In 1949 and again in 1950 A. V. Hill (1949) issued "a challenge to biochemists" to measure ATP hydrolysis, not in muscle extracts which cannot contract, but rather in contracting muscles.

Carlson and Siger (1960) did confirm the results of Lundsgaard (1930 and see Chap. 1) in elegant experiments that showed a linear decrease in the phosphocreatine content of frog sartorius muscles in which recovery reactions were inhibited by iodoacetate and nitrogen versus the number of isometric twitches. But they found no change in the ATP content of the muscles (Fig. 5.12). The problem with a series of contractions was that one didn't know when the energy was provided and not even whether it was provided during contraction or relaxation.

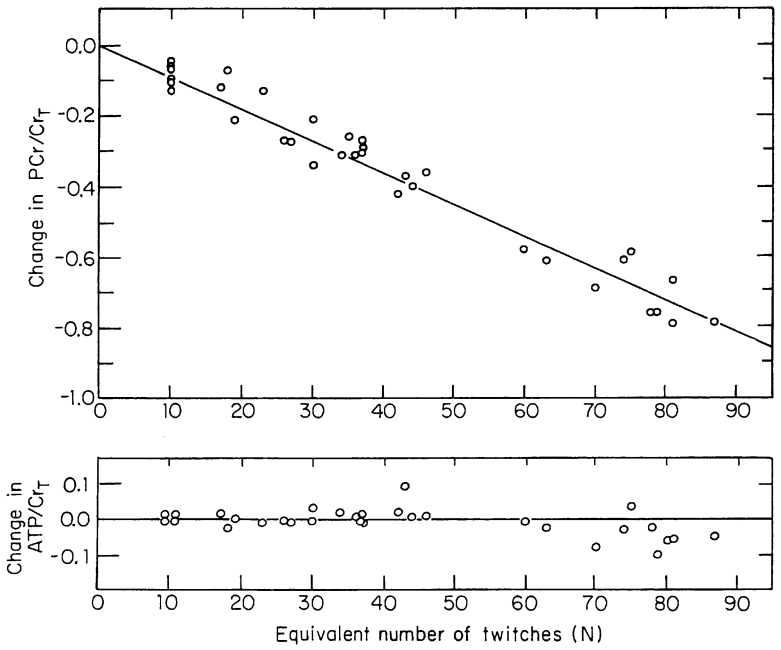


Fig. 5.12 Changes in phosphocreatine (PCr) and ATP in a frog sartorius muscles stimulated to produce thirty isometric twitches at 0 °C. Muscles were incubated in iodoacetate to block glycolysis and contracted in nitrogen to block oxidative phosphorylation. Hence PCr supplied the only source of energy to replenish ATP. PCr content decreased linearly with the number of twitches but ATP concentration stayed almost constant until the PCr was nearly gone. Cr_T represents the total creatine content (Cr+PCr) (Carlson and Siger 1960. With permission Rockefeller University Press)

There were a number of technical problems that had to be overcome in order to measure the changes in high energy phosphate during a single contraction. First the muscle had to be rapidly frozen within a fraction of a second. Rapid freezing techniques using organic fluids such as the freons, isopentane or petroleum ethers cooled to low temperatures such as −80 or −170 °C in order to ensure rapid cooling were utilized. Then there was the problem that a muscle could not be utilized as its own control. Also the amount of high energy phosphate hydrolysis expected in a single contraction was extremely small compared to the resting levels. With improved techniques Wilfried F. H. M. Mommaerts and colleagues at UCLA were able to demonstrate the breakdown of phosphocreatine in a single brief isotonic tetanus of frog muscle (Mommaerts et al. 1962).

In contrast to measurement of changes in phosphocreatine content during contraction, the measurement of changes in ATP hydrolysis during contraction faced an apparently insurmountable problem: the rapid regeneration of ATP from phosphocreatine catalyzed by the enzyme creatine phosphoryltransferase (ATP: creatine phosphokinase). At a scientific meeting in 1959 an astute observation by Robert

E. Davies[6] from the department of animal biology at the University of Pennsylvania led to the ultimate solution to the dilemma. Kuby and Mahowald (1959) from the Institute for Enzyme Research at the University of Wisconsin made a presentation in which they described the selective inhibition of isolated ATP-creatine phosphokinase and myokinase enzymes by the reagent 1-fluoro-2,4-dinitrobenzene (FDNB). They were hoping to shed some light on the mechanism of action of the ATP transphorylase reaction with these studies. Davies made note of their observation but didn't try the reagent on muscle for 2 years primarily because it was difficult to believe that a reagent normally considered to be nonspecific because of its general reactivity with primary amino, phenolic, imidazole and sulfhydryl groups could have a specific effect on only the enzymes of interest (Kushmerick 2001). But when Dennis Cain, a graduate student, in the Davies laboratory tried the experiment, it worked (Cain and Davies 1962)! They utilized pairs of the slow contracting rectus abdominus muscles of the frog at 0 °C frozen either at once, the control, or at the peak of a 1.3 s isotonic tetanus. The muscle was capable of only about three normal contractions but that didn't matter because they only wanted results from one contraction. They found a significant 35 % decrease in ATP content of the stimulated muscles. They concluded that these experiments demonstrated "directly and clearly" that the energy comes directly from the breakdown of ATP. Infante and Davis (1962) went on to extend these observations to the frog sartorius muscle. They found a breakdown of ATP during the rising phase of the isotonic twitch and a further breakdown during relaxation. Finally, there was direct proof in living muscle that ATP breakdown occurs during a single contraction. It was now time to turn attention once again to the balance sheet.

5.4.4 Energy Balance Experiments and Unexplained Energy

By the early 1960s the energetics framework was well established and it was assured that high energy phosphate was split during contraction. Now it came time to ask if the extent of hydrolysis of high energy phosphate could account for all of the energy observed: (1) in complete contraction-relaxation cycles and (2) during the time

[6] Robert Ernest Davies (pronounced "Davis") (1919–1993), born in England, received a Ph.D. from the University of Sheffield in 1949. He worked there as part of Sir Hans Krebs research unit studying the energy supply for solute and ion transport. He moved to the University of Pennsylvania in 1955 where he undertook the challenge of measuring ATP breakdown during muscle contraction. Davies was the chairman of the department of animal biology in the school of veterinary medicine from 1961 to 1973. Davies trained students who would go on to become prominent in the muscle energetics field, including Martin Kushmerick, Nancy Curtin and Thomas Butler. Davies was a true university citizen who was actively involved in all aspects of campus life, especially issues of personnel procedures and faculty rights. He strongly advocated for the rightful place of women in academic ranks. He was an avid outdoors enthusiast and he died suddenly while rock climbing in Scotland. Davies was a member of the Royal Society (1966). See the biographical sketch by Kushmerick (2001) for further details.

course of a single isometric or isotonic contraction. If the answers were yes, then it was highly likely that all of the chemical reactions were identified. If the answers were no, then there must be unknown reactions occurring. These questions would turn out to be vexing and would require nearly two decades before satisfactory answers could be provided.

Energy Balance in Repeated Contraction-Relaxation Cycles. In an energy balance experiment the energy liberation in a contraction-relaxation cycle is compared to the expected energy liberation based on the extent of the known chemical reactions. Formally this relationship, based on the first law of thermodynamics, is:

$$\mathbf{h} + \mathbf{w} = \Sigma \xi_{\mathbf{i}} \cdot \Delta \mathbf{H}_{\mathbf{i}}$$

where h + w is the heat (h) and work (w) or energy[7] liberated by the muscle (J/g), $\xi_{\mathbf{i}}$ is the extent of the ith chemical reaction (mol/g) and $\Delta \mathbf{H}_{\mathbf{i}}$ is the molar enthalpy change for the ith reaction (J/mol). Initially energy balance experiments were performed under conditions where recovery reactions were blocked with iodoacetate (to block glycolysis) and nitrogen (to block oxidative phosphorylation). The experiments were also performed with frog skeletal muscles at 0 °C. Frog muscles were utilized because of the extensive data already collected on the mechanical and energetic properties of these muscles. The muscles were studied at low temperatures to slow down contraction and the onset of recovery. Typically, pairs of sartorii were utilized in which one muscle of the pair was stimulated and the energy liberation measured with the other muscle serving as the unstimulated control. Both muscles were frozen after the last contraction and chemical change measured. Under these experimental conditions it was suspected that that net reaction occurring was the hydrolysis of phosphocreatine (PCr). In the initial energy balance experiments, repeated contraction-relaxation cycles were examined in order to build up a change in PCr that could be readily measured.

Wilkie[8] (1968) in the department of physiology at University College London performed an extensive energy balance study. He found that in a variety of different types of contraction-relaxation cycles, including a series of isometric twitches, isomet-

[7] Technically the heat + work liberated when a muscle contracts at a constant pressure is called the enthalpy liberation. But since the volume of a muscle changes very little during contraction, there is essentially no difference between the enthalpy liberation and the energy liberation.

[8] Douglas Robert Wilkie (1922–1998), was an English physiologist who received his M.D. degree from Yale University. He joined the department of physiology at University College London in 1945 where he remained throughout his research career before retiring in 1988. Wilkie made important contributions to the understanding of muscle mechanics and energetics. He pioneered the application of nuclear magnetic resonance (NMR) spectroscopy to the energetics of contraction in isolated muscles and muscles in humans. His 1960 paper on "Thermodynamics and the interpretation of biological heat measurements" is a masterpiece of insight and clarity of writing. He championed the need for energy balance experiments in understanding the energetics of muscle contraction. Wilkie also was interested in human powered flight and in 1959 he became a member of the Man-Powered Aircraft Group Committee of the Royal Aeronautical Society whose goal was to promote human powered flight. Wilkie became a fellow of the Royal Society in 1971. See Woledge (2001) for a biographical sketch of Douglas Wilkie.

ric tetani, contractions with positive or with negative work, the heat + work was produced in direct proportion to the change in PCr. The proportionality constant was −11 kcal/mol (−46 kJ/mol). Wilkie called this value the "in vivo" ΔH for PCr splitting.

Earl Homsher in Mommaerts' laboratory at UCLA also did experiments where the heat + work and PCr splitting were measured in repeated isometric twitches (Homsher et al. 1972). These experiments specifically examined the effect of muscle length on the mechanics and energetics of contraction. They took advantage of the insight gained from the elucidation of the sliding filament model of muscle contraction. Thus a frog semitendinosus muscle at rest was stretched to a sarcomere length where the overlap of the thick and thin filaments was vanishingly small. When the muscle was stimulated at this length to produce an isometric twitch, repeated twitches or an isometric tetanus, the heat produced was taken as a measure of the energetics of the cycling of Ca^{2+}. They found that the amount of energy liberated in an isometric contraction declined in a linear manner as the overlap of thick and thin filaments was reduced (Fig. 5.13). Furthermore about 25–30 % of the energy liberated at maximum filament overlap remained when isometric force was reduced to nearly zero at the long muscle length. Similar results were obtained by I. Christopher H. Smith in Roger C. Woledge's laboratory at University College London (Smith 1972). Homsher et al. and Smith called this remaining energy the "activation heat" (AH) and Homsher et al. attributed it to (Homsher et al. 1972. With permission John Wiley & Sons Inc):

> the sum of the thermal accompaniments of the liberation of calcium into the sarcoplasm, its movement to and from the myofibrillar binding sites, and its return to its storage site by an ATP-dependent transport process in the sarcoplasmic reticulum. In a completely reversed cycle, all other processes should thermally cancel, so that the net AH would derive from the hydrolysis of ATP associated with the cycling of calcium.

Homsher et al. (1972) went on to determine the amount of PCr splitting resulting from repeated isometric twitches under the same conditions. The deduced molar enthalpy change for PCr splitting at long sarcomere lengths was −10 kcal/mol (−42 kJ/mol) and at optimum sarcomere length the value was −11 kcal/mol (−46 kJ/mol). These experiments were important because they gave the first clear estimate of the relative metabolic cost of cycling Ca^{2+} during a contraction-relaxation cycle, i.e., approximately one in every three ATP molecules split during an isometric contraction was utilized to cycle Ca^{2+} with the remaining ATP utilized by the cross-bridges to develop force. Another important result from these experiments was that the fraction of total heat production attributed to the activation heat was about the same in a twitch and in a tetanus even though much more heat was produced in the tetanus. This result implied that the activation heat was produced continually and that Ca^{2+} continued to cycle from the sarcoplasmic reticulum to the thin filaments and back again to the sarcoplasmic reticulum during the tetanus.

With regard to energy balance, there was agreement in that both the Wilkie and Mommaerts/Homsher laboratories concluded that the energy liberated during repeated contraction-reaction cycles was explained by the extent of PCr splitting with an "in vivo" molar enthalpy change for PCr splitting of −42 to −46 mJ/mol.

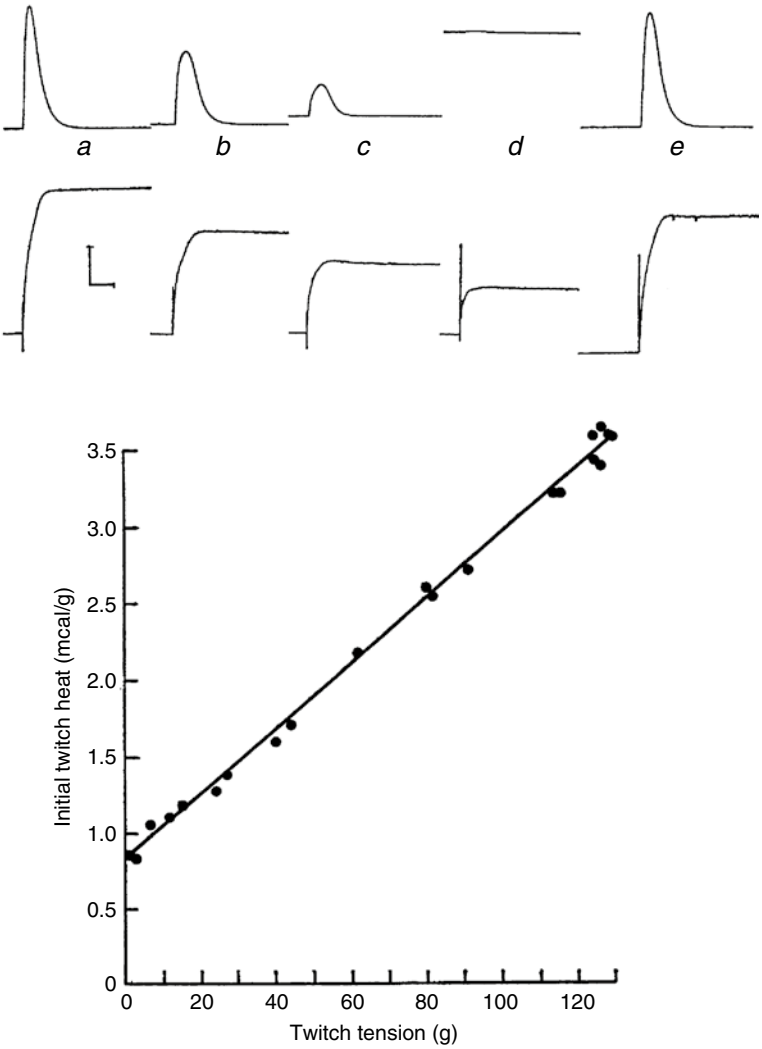


Fig. 5.13 Relationship between isometric twitch force and heat production in frog semitendinosus muscles at 0 °C stretched to reduce the overlap of thick and thin filaments. Upper records, force; middle records, heat. The length of the muscles in successive panels goes from lo (*a*) to 1.5 lo (*d*) and back to lo (*e*). The base line displacement of force records indicates the resting force exerted by the muscle. *Lower panel*: plot of isometric twitch heat versus force. The intercept on the ordinate, the heat attributed to Ca^{2+} cycling, is 25 % of the maximum heat liberated at lo (Homsher et al. 1972. With permission John Wiley & Sons Inc)

Thus the issue seemed settled until Roger Craven Woledge, A. V. Hill's last student, pointed out a major error in reasoning. The molar enthalpy change for a particular reaction must be determined not in vivo but rather in vitro using calorimetry to measure heat production. When Woledge (1973) carried out this experiment for PCr

splitting, he derived a value of −34 kJ/mol[9], a value significantly lower than the so called "in vivo" value for ΔH_{PCr}. This result meant that there was significantly more energy liberated in these repeated contraction-relaxation cycles than could be explained by PCr splitting.

Energy Balance Experiments in a Single Isometric Tetanus. What reaction(s) produced this "unexplained" energy? Was it produced during or after contraction? Clearly what needed to be done was to measure the time course of unexplained energy production during a single contraction. Technically this was extremely difficult since all reactions had to be rapidly stopped for chemical analysis. The problem of rapid freezing was solved by K. Melvin Kretzschmar who was doing his thesis work in Doug Wilkie's laboratory (Kretzschmar and Wilkie 1969). The idea was to "smash" the muscles with hammers cooled in liquid nitrogen at a predetermined time during a contraction. These flattened muscles could be frozen in less than 100 msec. Energy liberation was measured during muscle contraction in parallel experiments. Using this apparatus, Gilbert et al. (1971) did the first energy balance experiment during a single isometric tetanus. To avoid possible complications of enzyme inhibitors, they utilized frog sartorius muscles uninhibited in oxygen. Their results were clear: more energy was liberated than could be explained by amount of PCr splitting (Fig. 5.14). During the first 2 s of contraction the majority of the energy liberated could not be attributed to PCr splitting. Gilbert et al. concluded (1973): "Until this heat is accounted for it is hard to feel much confidence in theories of contraction that neglect its existence. The explanation may well turn out to be something remote from the contractile system…but equally well, it may not."

Thus the "age of innocence" ended for muscle energetics and an age of uncertainty began. It could no longer be assumed that the time course of energy liberation during a muscle contraction was a direct measure of the time course of high energy phosphate splitting. Something else was going on and it would take over 10 years to develop plausible hypotheses to explain what might be occurring. There were many difficulties in sorting out this problem. A primary difficulty was associated with the experimental preparation, frog skeletal muscles. Results seemed to vary from batch to batch of frogs, from season to season and between frog species. European frogs produced more unexplained energy than North American frogs. Slowly and painfully problems were solved and hypotheses took shape. The majority of this important work was done by Nancy A. Curtin (Fig. 5.15) and Roger C. Woledge (Fig. 5.15) in London and Earl Homsher (Fig. 5.16) and colleagues at UCLA.

Throughout the next 10 years the properties of the unexplained energy in isometric contractions were elucidated. Unexplained energy was produced at an exponentially decaying rate with a time constant of about 1 s and thus went to completion

[9] The splitting of PCr can be written as: $PCr^{2-} \rightarrow Cr + HPO_4^{2-}$ When considering the molar enthalpy change for the splitting of PCr in a muscle, the heat associated with the intracellular buffering reactions must be taken into account because protons are transferred from the buffers to $HPO4^{2-}$. Furthermore Mg^{2+} is required for activity. Thus a series of linked equilibria are involved. After taking into account the likely intracellular buffer reactions in frog skeletal muscle, Woledge (1973) derived a value of −34 kJ/mol for the molar enthalpy change for PCr splitting at 0 °C at pH 7 and pMg 2.5.

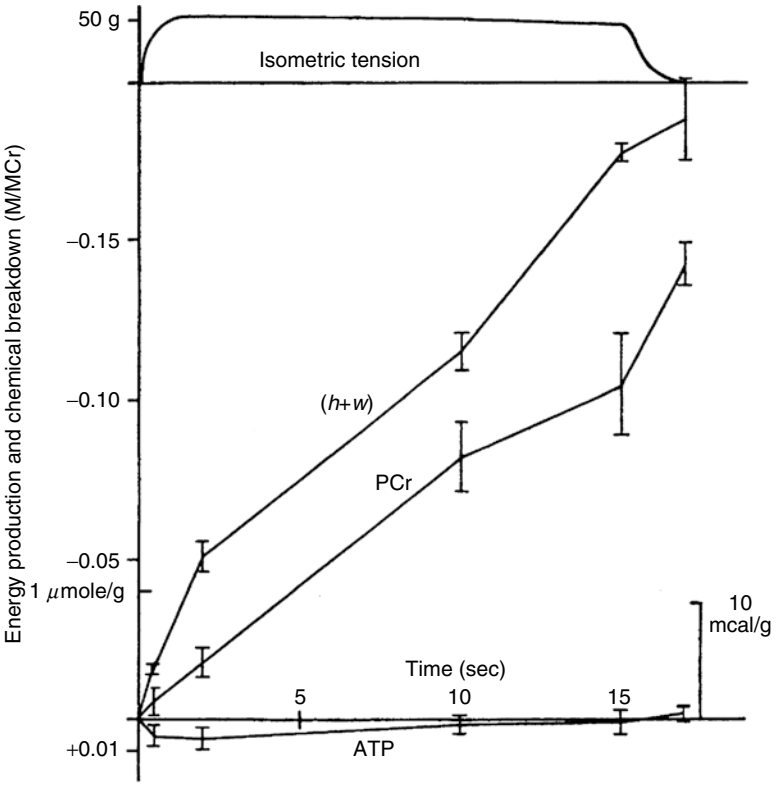


Fig. 5.14 Unexplained energy evolution during a single isometric tetanus. Physical and chemical changes during and after a 15 s isometric tetanus. Top trace: force during a the tetanus. The *lower graph* shows the physical and chemical changes, measured in mole/mol creatine (M/M Cr). The heat + work (h + w), measured in mcal/mmol Cr, is plotted in terms of equivalent chemical units (M/M Cr) using a conversion factor of 11 kcal/mol (46 kJ/mol) (Had the value of 34 kJ/mol been used for the conversion, the unexplained energy would have been even greater). The majority of the energy liberated during the first few seconds of stimulation is unexplained (Gilbert et al. 1971. With permission John Wiley & Sons Inc)

within about 5 s of stimulation (Curtin and Woledge 1979 and Homsher et al. 1979) (Fig. 5.17). Thereafter all of the energy liberation could be attributed to PCr splitting. Thus the stable maintenance heat production was a direct measure of the rate of PCr splitting. If a second tetanus was elicited soon after a first tetanus, the unexplained energy was dramatically reduced in the second tetanus (Curtin and Woledge 1977). Furthermore the full value of unexplained energy appeared again in a second tetanus after about 30 s of rest (Homsher et al. 1987). During the 30 s rest period PCr was split with little energy liberation, i.e., there was a negative energy balance. The magnitude of the negative energy balance suggested that the processes producing unexplained energy during contraction were reversed during the 30 s rest period after contraction.

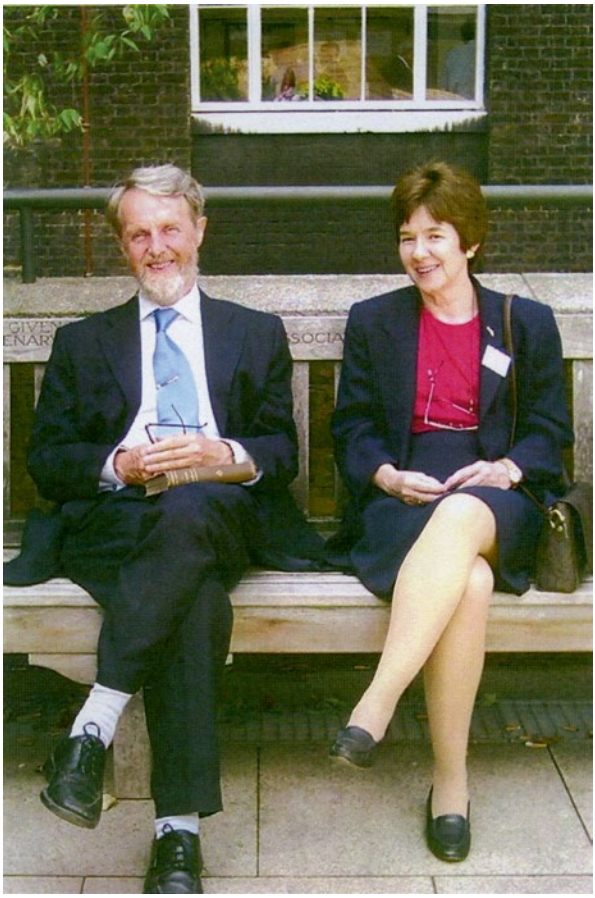


Fig. 5.15 Nancy A. Curtin and Roger C. Woledge. Curtin did doctoral studies with Robert E. Davies at the University of Pennsylvania and received a Ph.D. in 1972. After postdoctoral study in the department of physiology at University College London, she joined the faculty in the department of physiology at Charing Cross Hospital Medical School which eventually merged with Imperial College London. She has remained in London throughout her research career and became an emeritus professor in 2011. Roger Craven Woledge received a Ph.D. working with A. V. Hill at University College London. He remained there to join the faculty of the department of physiology and he received emeritus professor status in 2003. Woledge has carried on A. V. Hill's legacy by investigating the mechanical and energetic properties of isolated muscles and intact human muscles. They are best known for their work in the muscle energetics field. The names "Curtin and Woledge" are famous in muscle energetics (Photo: courtesy of N. A. Curtin)

An important clue resulted from the observation that the unexplained energy was not greatly decreased when a muscle was stimulated at a long sarcomere length (Homsher and Kean 1981 and Curtin and Woledge 1981). This result strongly suggested that the unexplained energy was not associated with cross-bridge cycling but rather with Ca^{2+} cycling. Taken together the results suggested that the isometric

Fig. 5.16 Earl Homsher (on *left*) talking to Setsuro Ebashi at the International Conference on Muscle Energetics, Yufuin, Japan, 1988. Homsher received a Ph.D. from the University of Pittsburgh. He then joined Wilfried F. H. M. Mommaerts' laboratory at UCLA as a postdoctoral fellow and stayed to become a faculty member in the department of physiology becoming an emeritus professor in 2010. He, possibly as much as anyone in the muscle field, has mastered whatever technique that is necessary to pursue basic questions about muscle contraction. He has been a true renaissance man in the muscle field (Photo: author's collection)

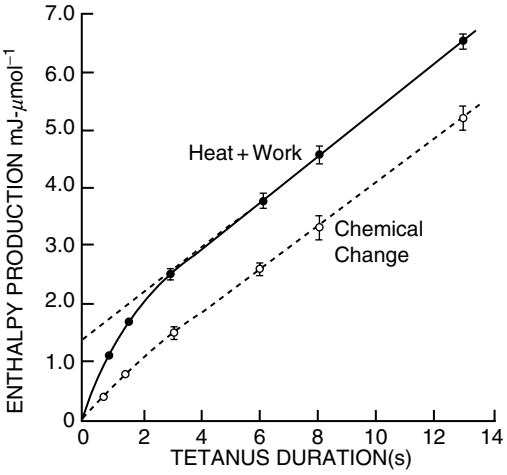


Fig. 5.17 Relationship between total energy liberation (heat + work) and explained energy liberation during an isometric tetanus of frog skeletal muscle. The solid curve shows the observed energy produced plotted as mJ/**μ**mol of total creatine (ordinate) against tetanus duration in seconds. The broken curve shows the net chemical change (PCr split) at various time intervals during the tetanus. The chemical change has been multiplied by the molar enthalpy change (34 kJ/mol) for PCr splitting to yield the energy liberation that can be explained by PCr splitting. Note that after 5 s of stimulation at 0 °C the unexplained energy has completely evolved and the steady rate of energy liberation can be explained by PCr splitting (Homsher et al. 1979. With permission Rockefeller University Press)

unexplained energy had more to do with the events of muscle activation and early stages of contraction than with development and maintenance of force caused by actomyosin interaction.

The general hypothesis developed over these years was that the isometric unexplained energy was due to an incomplete thermodynamic cycle(s) (Curtin and Woledge 1978). The essence of such a cycle is that the initial state is restored when the cycle has been completed. If one part of the cycle is spontaneous, then free energy must be supplied, by coupling to a reaction such as ATP splitting, at another step to complete the cycle. The total heat for the completed cycle will then be equal to the heat of ATP splitting. However, if the cycle is not completed during the time considered, one would observe the heat from initial step of the cycle. If this incomplete thermodynamic cycle is not been taken in account in the energy-balance calculation, an unexplained energy would result.

The specific hypothesis developed for isometric unexplained energy was that it was due to an incomplete cycle associated with Ca^{2+} movements during contraction that was not reversed until after contraction. It was proposed (Woledge et al. 1985) that the isometric unexplained energy was due to Ca^{2+} binding to the intracellular Ca^{2+} binding protein parvalbumin, a protein known to be in high concentration in frog skeletal muscles (Gosselin-Rey and Gerday 1977). Smith and Woledge (1985) showed that Ca^{2+} binding to parvalbumin liberated heat as does Ca^{2+} binding to troponin (Potter et al. 1976). The basic idea was that Ca^{2+} binding to troponin would occur extremely rapidly and that parvalbumin would bind Ca^{2+} during contraction at a rate limited by rate of Mg^{2+} dissociation from parvalbumin and that the Ca^{2+} would be returned slowly to the sarcoplasmic reticulum at the expense of ATP splitting after contraction limited by the rate of Ca^{2+} dissociation from parvalbumin. This hypothesis gave a plausible quantitative explanation for the isometric unexplained energy production during contraction and its reversal after contraction at the expense of ATP splitting. For further discussion, see Woledge et al. (1985). [For more information on the role of parvalbumin in muscle relaxation, see Chap. 8] According to this hypothesis, early in an isometric tetanus the time course of energy liberation is dominated by heat production associated with Ca^{2+} binding to troponin and parvalbumin accompanied by PCr splitting for force development. Later in the tetanus the heat production is completely explained by PCr splitting for force maintenance and for the continuous return of Ca^{2+} to the sarcoplasmic reticulum. After contraction PCr is split to slowly return Ca^{2+} bound to parvalbumin to the sarcoplasmic reticulum and thus complete this thermodynamic cycle.

Energy Balance Experiments in Contractions with Muscle Shortening. The early investigations of unexplained energy were conducted with isometric contractions. But it was entirely possible that muscles that shorten and do work could also produce unexplained energy and it could well be due to reactions that were different from those producing isometric unexplained energy. A. V. Hill thought that it would be "very odd" if this were true and he (Hill 1966) issued "a further challenge to biochemists" to determine the relationship of the heat of shortening during contraction to the chemical change during shortening. Hill suggested comparing an isometric contraction to one shortening under a light load for the same duration. Since it

was known from energetic studies that the shortening contraction would liberate energy at substantially greater rate than in the isometric contraction (Hill 1964a), the prediction was that the shortening contraction should also split high energy phosphate at a greater rate. The results as it turned out were "very odd" indeed.

Martin J. Kushmerick and Roy Larson in Bob Davies' laboratory took up Hill's further challenge. They did not measure the rate of energy liberation but they did measure the rate of ATP splitting and found it to be greater in the isometric contraction than in the isotonic contraction. These results (Kushmerick et al. 1969) strongly suggested that there was likely more energy liberated during rapid muscle shortening than could be explained by ATP splitting. Homsher and postdoctoral fellow Jack A. Rall did measure the energy liberation in an isometric contraction and in a contraction where the muscle shortened rapidly under a light load (Rall et al. 1976). The experiments were designed so that the muscles under the two conditions would liberate the same amount of energy. When this happened they found that the shortening muscle split only 25 % as much PCr as the isometric muscle. Thus they confirmed the conclusion of Kushmerick et al. (1969). Clearly more energy was liberated during rapid muscle shortening then could be explained by PCr splitting.

Was this unexplained energy during rapid shortening due to the same reactions that produced unexplained energy during the isometric contraction or was another process involved? To address this question it was necessary to design an experiment where the reactions associated with isometric unexplained energy would be nearly complete before rapid shortening occurred. Homsher et al. (1981) stimulated muscles to produce a 3 s tetanus. During the first 2 s the muscle contracted isometrically, then the muscle was released to shorten near maximum velocity for 300 ms and then the muscle redeveloped force for another 700 ms (see Fig. 5.18, left). Since isometric unexplained energy was known to be produced at a decreasing rate with a time constant of about 1 s, at 2 s of isometric stimulation the isometric unexplained energy would be about 90 % evolved. They found that less than half of the energy liberated during the rapid shortening period could be accounted for by simultaneous chemical reactions. Furthermore in the post-shortening period the observed energy liberation was less than that expected from the simultaneous chemical reactions. But over the whole cycle of shortening and force recovery there was an energy balance (Fig. 5.18, right). They concluded that there was an exothermic shift in the population of cross-bridge states during rapid shortening such that a relatively slow subsequent step prevented many of these cross-bridges from completing the cycle and splitting ATP until after the end of shortening. This is another example of an incomplete thermodynamic cycle. This cycle involves cross-bridge transitions during rapid shortening and not Ca^{2+} movements. To complete the picture, Homsher et al. (1984) did energy balance experiments with a similar design but in this instance the muscle shortened at about one-half of the maximum velocity and thus did a substantial amount of work. Under these conditions they found that the time course of energy liberation during shortening and recovery could be completely explained by the PCr split. Thus there was an energy balance throughout the shortening and force recovery periods.

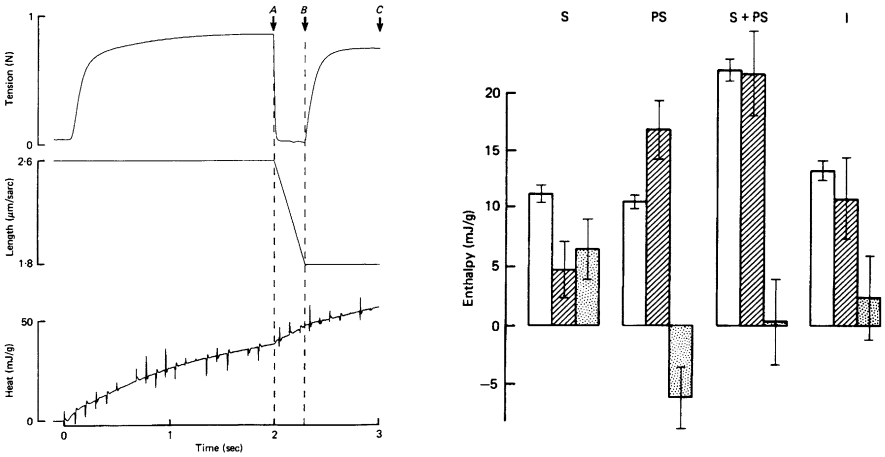


Fig. 5.18 Energy balance in rapidly muscle shortening. *Left Panel*: Time course of force and heat production during an isometric tetanus in which rapid shortening was induced followed by isometric recovery of force. Records of muscle force (top), length (*middle*) and heat production (*bottom*) in a 3 s tetanus. After 2 s of contraction the muscle was released at constant velocity for 0.3 s. In the chemical experiments on a separate group of muscles, the changes during shortening were estimated by freezing one muscle of a pair at A and its contralateral muscle at B; changes in the post-shortening period were determined from a comparison of paired muscles frozen at B and C. *Right Panel: Unshaded bars*, observed enthalpy (heat plus work); diagonal shading, explained enthalpy; *dots*, unexplained enthalpy. *Columns* from *left* to *right* show results for the rapid shortening period (S), post-shortening period (PS), the sum of these two values (S + PS) and the period (I) (of duration equal to that of S plus PS) of an isometric tetanus. See text for details (Homsher et al. 1981. With permission John Wiley & Sons Inc)

The muscle rapidly shortening under a light load was unique in producing reversible unexplained energy. What could explain this observation? Results from calorimetric determinations of reaction heats showed that large heat changes accompanied various changes in the state of the actomyosin system (reviewed by Curtin and Woledge 1978) and these changes likely contributed to the unexplained energy produced during rapid shortening.

By the mid 1980s the era of energy balance studies came to a close. The following general conclusions were reached based on research with frog skeletal muscles at 0 °C. One, at about 30 s after a contraction relaxation cycle, all of the energy liberated can be explained by high energy phosphate splitting. Two, during the first 5 s of an isometric tetanus, more energy is liberated than can be explained by the extent of high energy phosphate splitting. This isometric unexplained energy is associated with Ca^{2+} movements during contraction, most likely Ca^{2+} binding to parvalbumin. Three, the steady rate of energy liberation during an isometric tetanus is totally attributed to high energy phosphate splitting. About 30 % of this steady energy liberation is due to ATP hydrolysis by the calcium pump of the sarcoplasmic reticulum and about 70 % due to ATP hydrolysis during cross-bridge cycling. Four, when a muscle is allowed to shorten and do work after isometric unexplained energy

liberation has evolved in a tetanus, all of the energy liberation can be explained by high energy phosphate splitting. Five, if the muscle shortens from the isometric tetanus at a velocity near the maximum velocity, unexplained energy is produced during shortening and an excess of high energy phosphate splitting occurs during force redevelopment after shortening. Over the shortening and force redevelopment cycle all the energy liberated can be explained by high energy phosphate splitting. Thus there is a phase shift between energy liberation and high energy phosphate splitting. This phase shift is unique to rapidly shortening muscles and is likely attributed to thermal changes during transitions in the cross-bridge cycle. In general the unexplained energy liberation in isometric and isotonic contractions can be attributed to incomplete thermodynamic cycles involving calcium movements and cross-bridge cycling.

The final, and is some ways, most important conclusion is that one cannot assume that the time course of energy liberation from moment to moment in a contraction is a direct measure of high energy phosphate splitting. This conclusion has important implications for interpreting models of muscle contraction. What one would really like is a non destructive, high temporal resolution, measure of the time course of ATP hydrolysis. Michael A. Ferenczi and colleagues at the National Institute for Medical Research, Mill Hill, London employed such a technique for studying muscle contraction by using a fluorescently labeled phosphate binding protein (Ferenczi et al. 1995). In this case a fluorescence change is generated when inorganic phosphate binds to the phosphate binding protein (see Chap. 9, Fig. 9.5). This imaginative technique is limited to skinned muscle fibers and limited to contractions of relatively short duration. Thus it is still a reality that there is no simple direct way to monitor the time course of ATP splitting during contraction.

Important reviews covering the energy balance era have been written by Curtin and Woledge, 1978; Kushmerick 1983 and Homsher 1987. The monograph by Woledge et al. (1985) is a milestone in the muscle energetics field. They have put an extensive repository of mechanical and energetic data into perspective with information on techniques and speculation on theories of muscle contraction.

5.4.5 Efficiency and Economy of Muscular Contraction

Since the nineteenth century there has been an interest in the determinations of the efficiency of muscular contraction. Efficiency is of interest intrinsically and in developing quantitative models of cross-bridge function. A. V. Hill started to address this issue in 1913(b) and followed up with detailed studies of efficiency in 1939 and 1964 (Hill 1964b). Somewhat surprisingly there has been confusion over the proper definition of efficiency. Woledge et al. (1985) found it hard to understand how w/(h + w) and thermodynamic efficiency got "muddled up" and why A. V. Hill was so unrepentant about it. What got "muddled up"? Why was A. V. Hill "unrepentant"? What is the proper definition of efficiency? And finally, how does efficiency differ from economy of muscular contraction?

In 1960 Doug Wilkie produced an influential review on thermodynamics as it relates to interpretation of biological heat measurements, especially those derived

from muscle studies. He stated that the only valid measure of efficiency is: (free energy transformed)/(total free energy made available). The free energy transformed is the work (w) done during the contraction. An estimate of the free energy (w_{max}) made available requires identification of the chemical reaction(s) yielding energy and knowledge of the thermodynamic parameters of these reactions. The w_{max} obtainable from a reaction is equal to the free energy change (ΔG) for that reaction times the extent (ξ) of the reaction. The free energy change is related to the enthalpy (ΔH, heat + work) of the reaction by:

$$\Delta \mathbf{G} = \Delta \mathbf{H} - \mathbf{T}\Delta \mathbf{S}$$

where T is absolute temperature ΔS is the entropy change for the reaction. The entropy change may be positive, negative or zero. Thus the thermodynamic efficiency (ε_{therm}) is defined as:

$$\varepsilon_{\mathbf{therm}} = \mathbf{w} / \mathbf{w}_{max} = \mathbf{w} / \xi \cdot \Delta \mathbf{G}$$

The experimental difficulty with $\varepsilon_{\mathbf{therm}}$ is the difficulty in measurement of the ΔGs for the reactions occurring in muscle. ΔG for a given reaction depends upon the cellular environment, including concentrations of reactants and products, and in general can only be estimated from known reactions occurring during muscular contraction. Hill (1913b) knew the thermodynamic definition of efficiency. But since the pertinent reactions and their ΔGs were not known when he did his studies, he opted for a definition of efficiency that could be determined experimentally. This efficiency he called "mechanical efficiency" ($\varepsilon_{\mathbf{mech}}$) and it is expressed as:

$$\varepsilon_{\mathbf{mech}} = \mathbf{w} / \mathbf{w} + \mathbf{h} = \mathbf{w} / \xi \cdot \Delta \mathbf{H}$$

Hill (1965) agreed with Wilkie but added that since the change of free energy was not usually known, the "thermodynamic efficiency" was not of practical utility.

Hill (1939, 1964b) characterized the "mechanical efficiency" as a function of load and speed of shortening in frog skeletal muscle. The efficiency had a broad "bell shaped" dependency on load with a maximum value of 0.45 at a load of 50 % of the isometric value.

How does the mechanical efficiency relate to the thermodynamic efficiency? The energy balance experiments described above suggest that the relationship can be complicated depending on conditions of the experiments. In order to derive the thermodynamic efficiency from measurements of mechanical efficiency, the experiment must be done under conditions where all the energy liberation can be ascribed to PCr splitting. This requirement is met when a frog muscle: (a) is stimulated to produce an isometric contraction for a few seconds in order to avoid the initial isometric unexplained energy and (b) then released to do work at a velocity around 0.5 Vmax in order to avoid the unexplained energy that appears at higher velocities of shortening. Under these conditions:

$$\varepsilon_{\text{therm}} = \varepsilon_{\text{mech}} \mathbf{X} \Delta \mathbf{H}_{\mathbf{PCr}} / \Delta \mathbf{G}_{\mathbf{ATP}}$$

Since $\Delta \mathbf{H}_{\mathbf{PCr}}/\Delta \mathbf{G}_{\mathbf{ATP}}$ has a value of about 0.68 [ΔH_{PCr} of 34 kJ/mol (Woledge and Reilly 1988) and ΔG_{ATP} of ~50 kJ/mol (Kushmerick and Davies 1969)], the maximum thermodynamic efficiency of frog muscle contraction is about 30 %. What one would really like to know is the efficiency of chemomechanical conversion by the cross-bridges. To estimate this value, it is necessary to correct for non-cross-bridge utilization of ATP during contraction, i.e., the ATP utilized for Ca^{2+} pumping. This correction increases the maximum thermodynamic efficiency of the cross-bridges by about 10–15 % to a value of about 35 %[10]. Thus in general the thermodynamic efficiency will be less than mechanical efficiency.

Does the efficiency of muscle contraction vary among animal species? The most suggestive data that it does vary comes from the mechanical efficiency measurements on tortoise skeletal muscle by Roger Woledge (1968). He found a maximum mechanical efficiency for tortoise muscle of 0.77, clearly much higher than that observed by Hill for frog muscle (0.45). A comparison of mechanical efficiency in mammalian fast- and slow-twitch muscle suggests that the slow-twitch soleus muscle is more efficient than the fast-twitch extensor digitorum muscle (Barclay 1994). It has been suggested that slowness of contraction might be related to the higher muscle efficiency but this is still considered highly speculative. Chris Barclay (1999) has written a brief and lucid review of efficiency of isolated muscle and its relationship to animal locomotion. For detailed recent reviews on muscle efficiency and cross-bridge models, see Smith et al. (2005), Barclay et al. (2010).

During a maintained isometric contraction a muscle liberates energy due to high energy phosphate splitting but the muscle does not perform external work. Thus it is inappropriate to consider the efficiency of an isometric contraction. Instead another term, the economy of contraction, is defined as the ratio of the maintained isometric force to the steady rate of energy liberation or high energy phosphate splitting. This term was first utilized by Emil Bozler (1930) to characterize the extremely low rate of energy utilization to maintain a given force during smooth muscle contraction. The economy of muscle contraction varies widely across the animal kingdom and exhibits an inverse relationship to the power output of a muscle (Rall 1985). There seems to be a trade off of economy and power output, i.e., a muscle cannot be highly economical in maintaining isometric force and also be powerful in doing work.

[10] When a muscle shortens under a load where the mechanical efficiency is maximum, the rate of energy (heat + work) liberation is about threefold greater than the isometric rate of heat production. About 30 % of the isometric heat production is attributable to ATP utilization for Ca^{2+} pumping (Homsher et al. 1972). Assuming that this rate is unchanged during the shortening, the non-cross-bridge energy liberation (activation metabolism) would be about 10–15 % of the rate of energy liberation during shortening.

5.4.6 *Energy Liberation and ATP Cleavage During Stretch of Active Muscle*

In his classic experiments, Fenn (1923, 1924) showed that a muscle shortening and doing work liberated more energy than during an isometric contraction. He also found that when work was done on the muscle by stretching during contraction more heat was liberated than during an isometric contraction (Fenn 1924). The work done on the muscle might be expected to appear quantitatively as heat. In that case the *net* energy liberated by the muscle would be the total heat output minus the work done on the muscle in the contraction with stretch. After subtraction, Fenn's results showed that the muscle liberated less net energy when stretched despite producing a greater force during the stretch.

This issue was taken up by A. V. Hill and his collaborators some years later. Bernard (Bud) C. Abbott et al. (1951) found that when a frog or toad skeletal muscle was stretched by about 10 % of its initial length during a twitch or short tetanus the muscle resisted strongly and work was done stretching the muscle (Fig. 5.19). The total heat produced up to the end of relaxation was greater than it would have been had no work been performed on the muscle. All the work done on the muscle was assumed to be converted into heat but the excess heat was too small to account for all the work done. Compared to the isometric heat production, the net energy liberation during the contraction-relaxation cycle with stretch was decreased by 30–70 %. In contrast if the stretch is applied entirely during the relaxation phase, when activity is over but force is till present, the whole of the work performed reappears as heat. The "missing" work amounted to about 50 % of the total work done on the muscle. There was no apparent damage to the muscles. Where did the work go? Abbott et al. (1951) suggested three possibilities:

(a) that the work was absorbed in driving backwards chemical processes which had occurred during contraction;
(b) that the work was absorbed in some other unknown chemical or physical process;
(c) that the work was wholly degraded into heat, but that chemical processes normally occurring in contraction were prevented by the stretch.

They clearly favored the possibility (a) that the work of stretch caused the reversal of the chemical processes occurring during contraction. They appeared to favor this conclusion, in part, because it seemed "natural" to suppose that if a muscle converts chemical energy into mechanical energy while doing work that doing work on the muscle would convert mechanical energy back into chemical energy. But they were clear to point out that without specific evidence that thermodynamics could not provide it was impossible to decide between these hypotheses.

Hill and Howarth (1959) studied the effect of stretch on muscle energy liberation in further detail and produced a paper with the provocative title: "The reversal of chemical reactions in contracting muscle during an applied stretch". They found that with a contraction-relaxation cycle the net energy liberation by toad skeletal

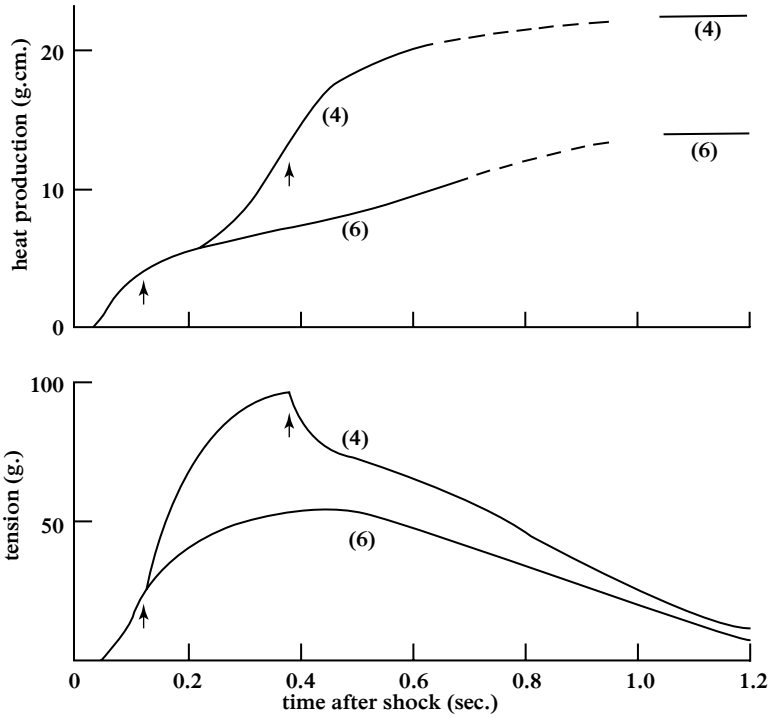


Fig. 5.19 Heat production and force with stretch of a toad skeletal muscle during a twitch. The stretch started 120 msec after the stimulus and lasted 260 msec resulting in a total stretch of 11 % of the muscle length. *Upper panel*: heat production during the isometric contraction (labeled 6) and during the contraction with stretch (4). *Lower panel*: force response for the isometric twitch (6) and the contraction with stretch (4). Beginning and end of stretch shown by *arrows*. The isometric heat traces at the short and long lengths were nearly identical (trace at short length is shown). For this muscle about 50 % of the work appeared as heat by the end of the contraction relaxation cycle and net energy liberation was suppressed by about 70 % (Abbott et al. 1951. With permission The Royal Society)

muscle that was stretched could be reduced to zero! This was a remarkable result. Apparently the muscle itself had produced no heat at all although it had resisted the stretch very strongly. They also examined the time course of heat production, work and elastic energy throughout a contraction with and without stretch. During the stretch the rate of net energy liberation wasn't just suppressed but actually exhibited a negative slope and then the rate increased again after the stretch to return the total net heat production to about zero by the end of relaxation. Thus during the stretch the muscle was gaining internal energy. Hill and Howarth interpreted these results as clear evidence of reversal of chemical processes appearing during contraction. Hill (1960) brought these results to a wide audience after his lecture at the University of Rochester was published in Science. He didn't speculate about mechanism because attention might be drawn away from the facts and Hill believed that the facts had to be explained by any theory of muscle contraction. But he did go on to

conclude that a biochemical process in a living muscle cell could be reversed by the application of external mechanical work. Hill was as provocative as ever but was he correct? These results were in effect another challenge to the biochemists to actually measure ATP splitting during muscle contractions with stretch.

In 1963 Xavier Aubert and Georges Marechal at Louvain examined the phosphocreatine metabolism of stimulated IAA-treated frog sartorii during stretch. They found that with a slow stretch there was about a 30 % decrease in PCr breakdown when compared with the unstretched control isometric muscle. This result was in reasonable agreement with the heat measurements of Abbott et al. (1951). Also Infante et al. (1964) found about a 50 % reduction in ATP splitting with stretch. Both groups concluded that stretch inhibited ATP splitting but did not reverse the chemical reactions. Wilkie reviewed the situation in a 1966 (Wilkie 1966). He emphasized that it was important to distinguish between reversal of a chemical reaction and suppression of the reaction. He believed that the chemical results along with the results of Abbott et al. (1951) showed suppression and not evidence for reversal of chemical reactions. With regard to the results of Hill and Howarth (1959), Wilkie was skeptical because their analysis involved measuring a small difference between two large quantities and it put a large strain on the myothermic technique because it demanded uniformity of temperature in the muscle and a high level of accuracy in the absolute calibration. In short he doubted the validity of Hill and Howarth's results. Hill (1965) commented that he and Howarth were convinced that the experimental results are correct. With hindsight there is another reason to question the results of Hill and Howarth. Since about 30 % of the energy and ATP splitting in a contraction is associated with the re-accumulation of Ca^{2+} by the sarcoplasmic reticulum calcium pump (Homsher et al. 1972), it is difficult to see how there could be a 100 % suppression of the energy liberated during a contraction with stretch. After all, the muscle relaxed and thus Ca^{2+} must have been sequestered. The mystery has remained to this day.

There is a loophole in the ATP suppression argument. During the stretch some partial reversal of ATP splitting could have occurred which resulted in a net suppression of ATP splitting by 30–50 %. This idea was tested by Jean-Marie Gillis and Marechal (1974). They found a small incorporation of ^{32}P into ATP during contraction but no difference in incorporation during isometric, shortening or lengthening contractions. They concluded that there was no evidence to support the hypothesis that mechanical work done on a muscle could be used to synthesize ATP.

Is it to be concluded that the negative slope of the energy liberation during stretch observed by Hill and Howarth (1959) was an artifact? Recent results of Linari et al. (2003) suggest that it wasn't an artifact. When single frog fibers were stretched during contraction, they too observed a negative slope of the energy liberation versus time that suggested that the fiber was gaining energy during the stretch. These results were qualitatively similar to those of Hill and Howarth. But they interpreted the results not as a reversal of ATP splitting but rather as energy storage in structures in the muscle with stretch. This transiently stored energy was later dissipated as heat after the stretch was complete. The sum of the energy change during stretch and the heat production after stretch was always positive which suggested that some ATP was split during stretch at least accounting for the ATP split by the calcium pump.

Thus there are multiple events occurring with stretch: suppression of ATP splitting by the cross-bridges and transient storage of energy within the muscle. Loiselle et al. (2010) recently have reviewed the history of this interesting problem.

5.5 Mechanism of Actomyosin ATPase: Relationship to Cross-Bridge Cycle

It had been known since the work of Szent-Gyorgyi (1951) that ATP acted both as a substrate for myosin and as a regulator of the interaction between actin and myosin. But it was a mystery as to how these effects might relate to muscular contraction. A major break through came from work in Edwin W. Taylor's[11] laboratory in the department of biophysics at the University of Chicago. In the mid-1960s Taylor (2001) was interested in the mechanism of mitosis and he and graduate students Gary Borisy and Richard Weisenberg discovered the basic protein subunit of the microtubules which was later named tubulin. The microtubules were known to be constituents of the mitotic spindle fibers. The next important question in Taylor's view was: what causes the chromosomes to move? He proceeded to study the actomyosin ATPase system (Taylor 2001) because in 1965 little was understood about how any motile system worked and Taylor felt that it was necessary to first concentrate on muscle actomyosin to understand the principles of mechano-chemical coupling. He concluded that to determine the steps in the actomyosin ATPase cycle it was necessary to apply the method of transient kinetics. Even though the Taylor laboratory had no experience in the transient kinetics field and the necessary equipment was not commercially available, they did have access to an excellent machine shop. They designed and had built both a stopped-flow and quenched flow apparatus for the transient kinetic studies. The goal from the beginning was (Taylor 2001) "to arrive at a simple model, one that could be correlated with the steps in the contraction cycle".

Taylor and graduate student Richard W. Lymn[12] started with measurement of the pre-steady state phosphate liberation during the hydrolysis of ATP (substrate is

[11] Edwin William Taylor is often acknowledged as the "father of cytoskeletal research." His pioneering investigations using biochemical approaches revealed the structure of cytoskeletal systems in cells. In addition, his biophysical studies elucidated the mechanism by which molecular motors convert chemical energy into mechanical force. He received his Ph.D. in biophysics from the University of Chicago in 1957 and has spent the majority of his research career there. In 1999, Taylor received the E.B. Wilson Medal, the highest honor awarded by the American Society for Cell Biology. He was elected as a fellow of the Royal Society of London in 1978 and as a member of the National Academy of Sciences in 2001.

[12] Richard W. Lymn did his pioneering work establishing the Lymn-Taylor model of actomyosin ATPase while working toward his Ph.D. in 1970 in Taylor's Laboratory at the University of Chicago. After postdoctoral study at the Laboratory of Molecular Biology in Cambridge and at the NIH, Lymn became a health science administrator at the NIH in 1978 where he launched the Muscle Biology Program within the NIH. He and his family also have established the Lymn Foundation promoting improvements in muscle biology by fostering the research careers of young investigators.

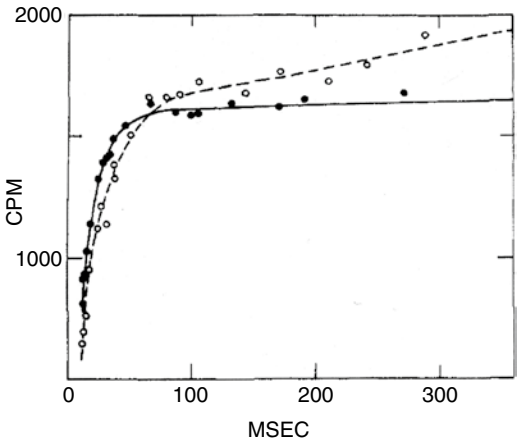


Fig. 5.20 Early phase of the hydrolysis of ATP by HMM and acto-HMM. (*filled circle*) Phosphate liberation by HMM. (*unfilled circle*) phosphate liberation by acto-HMM. The rate of phosphate liberation is similar for HMM and acto-HMM at about 50^{-1} but the steady state rate of phosphate liberation (the linear portion of the curve) is ten times higher for acto-HMM (0.9 s^{-1}) than for HMM. (On this short time scale the steady state rate of phosphate liberation for HMM is almost a *flat line*.) Thus the phosphate burst is much faster than the steady state rate of phosphate liberation for both HMM and acto-HMM. The rate of the phosphate burst depends on ATP concentration and reaches a maximum of about 100 s^{-1} (Lymn and Taylor 1971. With permission American Chemical Society)

actually MgATP) by myosin using a chemical quenched flow technique (Lymn and Taylor 1970). ATP is rapidly mixed with myosin and the reaction stopped (or quenched) at various times with a strong acid solution. They then measured the ^{32}P liberated from the labeled γ phosphate of ATP. The phosphate liberated during ATP cleavage included that which was released from myosin during cleavage and that which was displaced from the complex of myosin and ADP and Pi (M.ADP.Pi) when the acid denatured the protein. The products in the M.ADP.Pi complex were not covalently bound, in contrast to the ion pump ATPases where a phosphorylated intermediate was isolated. With this technique they were able to follow the exponential time course of phosphate liberation. The rate of this "phosphate burst" increased as a function of ATP concentration and the rate greatly exceeded the steady state rate of ATP hydrolysis by myosin. The initial experiments with whole myosin were performed at a very high ionic strength of greater than 0.5 M in order to solubilize the myosin. In order to study the system at a more nearly physiological ionic strength (in the 100–200 mM range), phosphate liberation was measured using the heavy meromyosin subfragment of myosin (HMM) which is soluble at all ionic strengths. Similar results were found with a maximum rate of Pi liberation during cleavage of ATP by HMM of about 100 s^{-1} which was considerably greater than the steady state rate of ATP hydrolysis of less than 0.1 s^{-1} (Fig. 5.20). They concluded that the rate-limiting step which determined the steady-state rate must be the dissociation of the enzyme-product complex or some other slow step which followed

bond hydrolysis. If this prediction is valid, then one would expect that the myosin in a resting muscle would be predominantly in the form of M.ADP.Pi. This prediction was verified by Marston (1973) who found that decomposition of the M.ADP.Pi complex formed in relaxed glycerol-extracted muscle fibers was the rate limiting process in the myosin ATPase.

Lymn and Taylor (1971) expanded their study to consider ATP hydrolysis by actomyosin, actually acto-HMM. The actin was in the form of F-actin since only F-actin activates the myosin ATPase. When they mixed ATP with actomyosin and stopped the reaction at various times in the quenched flow apparatus, they found that the size of the phosphate burst and its time course were similar to that observed for HMM alone but that the steady state rate of ATP hydrolysis was stimulated by actin (Fig. 5.20). A crucial question was whether the acto-HMM-ATP complex dissociated before or after the ATP was hydrolyzed. In order to answer this question they rapidly mixed ATP with acto-HMM and followed the change in turbidity as an indicator of dissociation in a stopped-flow apparatus. They found that the rate of the turbidity decrease was too fast to measure and thus concluded that the dissociation rate was likely greater than 1000 s^{-1} at 20 °C. This result indicated that dissociation of actin and myosin occurred before hydrolysis of the bound ATP and thus that the hydrolysis of ATP by myosin occurred after dissociation of actin. The simplest mechanism to explain these results was that actin recombined with the myosin-product complex and then rapidly displaced the products. They were able to demonstrate the ability of actin to increase the rate of product dissociation from myosin by use of a rapid column separation procedure. By means of this procedure it was shown that actin combined with the M.ADP.Pi complex and displaced the products of the hydrolysis reaction. Actin accelerated product dissociation five to tenfold.

Lymn and Taylor summarized their results in the following way (Lymn and Taylor 1971. With permission American Chemical Society):

This series of experiments establishes some general features of the mechanism of ATP hydrolysis by acto-HMM. The main results are:

(a) an early burst of ATP hydrolysis of approximately the same magnitude and with similar values for the rate constants as for HMM alone;
(b) dissociation of acto-HMM as measured by turbidity change occurs by a very fast step following ATP binding. The actual step…is at least 10 times larger than the rate of splitting of ATP, consequently the system largely dissociates into A + M.ATP;
(c) the step in which ATP is hydrolyzed must occur predominantly on the free myosin, M.ATP → M.ADP.Pi and
(d) actin can react with the myosin-product complex causing the products to be released at a much faster rate than from myosin alone. Actin activation of myosin ATPase therefore occurs at the product release step and not at the ATP hydrolysis step.

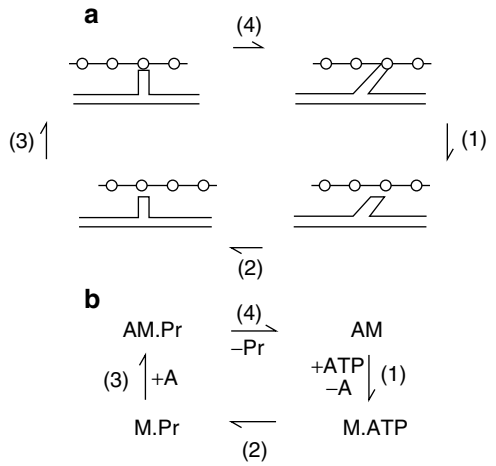


Fig. 5.21 Proposed relationship of actomyosin ATPase to the cross-bridge cycle in muscle. (**a**) Contractile cycle for a single cross-bridge as proposed by Huxley (1969). Actin sites which are suitably oriented to interact with a cross-bridge are indicated by *circles*. (**b**) Proposed steps in the chemical mechanism for ATP hydrolysis by actomyosin. Binding of ATP and dissociation of actin are shown as a single step because actin dissociation is very fast following substrate binding. Pr stands for reaction products, ADP and phosphate (Lymn and Taylor 1971. With permission American Chemical Society)

They presented the following scheme with forward rate constants for the various steps as the simplest explanation of their results:

$$\begin{array}{ccccc} & & \overset{4}{} & & \overset{7}{} \\ \mathbf{AM+ATP} & \overset{}{\leftrightarrow} & \mathbf{AM.ATP} & \quad \mathbf{AM.ADP.P} & \overset{}{\leftrightarrow}\ \mathbf{AM+ADP+P} \\ & & \mathbf{5}\uparrow\downarrow(-\mathbf{A}) & \quad \mathbf{6}\downarrow\uparrow(+\mathbf{A}) & \\ & \overset{1}{} & & \overset{2}{} & \overset{3}{} \\ \mathbf{M+ATP} & \overset{}{\leftrightarrow} & \mathbf{M.ATP} & \overset{}{\leftrightarrow} & \mathbf{M.ADP.P} \overset{}{\leftrightarrow}\ \mathbf{M+ADP+P} \end{array}$$

They then went on to speculate as to how this scheme might relate to the "swinging-titling" cross-bridge model proposed by Hugh Huxley (1969) (see Fig. 3.23). Their figure is shown as Fig. 5.21. The four steps in the cross-bridge cycle are shown in Fig. 5.21a: (1) the dissociation of the actin-cross-bridge complex, (2) movement of the free myosin cross-bridge, (3) recombination of the cross-bridge with actin, and (4) the power stroke. The four main steps in the enzyme mechanism are shown in Fig. 5.21b. These steps are: (1) the binding of ATP and very rapid dissociation of actomyosin, (2) the splitting of ATP on the free myosin, (3) recombination of actin with the myosin-products complex, and (4) the displacement of products. Thus the proposed chemical mechanism provided a very satisfying explanation of how a cycle involving dissociation and recombination of myosin with actin could be coupled to ATP hydrolysis and to the proposed cross-bridge mechanism of muscle contraction.

These experiments were a major break through for various reasons. First, they provided an explanation for the dual role of ATP as a substrate for myosin and as a regulator of the interaction of actin and myosin. Second, the results could be related to the proposed cross-bridge cycle in a very pleasing way. But possibly most important, these experiments initiated a dialogue between the biochemists and the physiologists and structural biologists that would lead to major advances in the identification of chemical and mechanical steps in the contractile cycle. The paper by Lymn and Taylor (1971) appeared 2 years after Hugh Huxley proposed "swinging-titling cross-bridge" theory of contraction in 1969 and just 2 months after the seminal paper on transient kinetics of muscle contraction by Huxley and Simmons (1971b). Various lines of evidence were starting to come together.

Lymn and Taylor (1971) were careful to point out that their proposed mechanism for actomyosin ATPase was "a provisional and no doubt oversimplified mechanism". In particular they noted that there may be other intermediate states in the hydrolytic process (step 2) and that nothing could be said about the important question of configuration changes accompanying the binding or splitting of substrate or the dissociation of products. Indeed their goal was to establish the predominant states in the actomyosin ATPase cycle and to relate them to the cross-bridge cycle. Many refinements would subsequently be made to the Lymn-Taylor model with many more intermediates states added but nonetheless this model established the basic framework for further experiments.

Lymn and Taylor didn't consider the reversibility of the various steps in the cycle. It is important to evaluate the reverse rate constants because they have bearing on the steady-state concentration of each intermediate during the ATPase cycle. For example, this point is important as it relates to the cleavage of ATP. One might have guessed that the cleavage step of ATP would dissipate the chemical free energy of the ATP molecule resulting in a low energy M.ADP.Pi complex unable to fuel muscle contraction. It turns out that this isn't true as was shown by David Trentham and Clive Bagshaw then at the University of Bristol because the cleavage step (step 2 in the Lymn-Taylor scheme) is readily reversible (Bagshaw and Trentham 1973). This fact means that there is only a small negative free-energy change for the cleavage step which is advantageous for efficient chemical to mechanical energy exchange during muscle contraction.

Furthermore Lymn and Taylor (1971) utilized relatively low actin concentrations to avoid high viscosity of the resulting solutions. Under these conditions the actomyosin ATPase in not strongly activated by actin. Evan Eisenberg and his collaborators Leonard A. Stein, Richard P. Schwarz and P. Boon Chock at the NIH also performed experiments elucidating the actomyosin ATPase mechanism but they did so under conditions considerably different from those of Lymn and Taylor (Stein et al. 1979, 1981). They investigated the steps in the actomyosin ATPase cycle that determined the maximum steady state ATPase rate *(Vmax)* and the binding between myosin subfragment one (S-1) and actin which occurred when the ATPase activity was close to *Vmax.* The affinity of actin for myosin increases as ionic strength decreases. If the ionic strength of the solution is lowered to a non-physiological value of about 10 mM, the same low actin concentration now gave near maximal

actomyosin ATPase activity. Under these conditions, they found two main differences from the Lymn and Taylor scheme. First, the rate of the phosphate burst increased markedly with increasing actin concentration whereas the Lymn-Taylor model predicted that the rate should remain nearly constant. They concluded (Stein et al. 1981) that it was difficult to see how the rate constant of the initial Pi burst could nearly double at high actin concentrations unless the initial Pi burst occurred "without dissociation of the acto-S-1 complex." They found no evidence for an obligatory dissociation step when ATP bound to actomyosin but rather suggested a direct conversion of AM.ATP to AM.ADP.Pi. Second, they found that the intermediates M.ATP and M.ADP.Pi had equal weak binding affinity for actin to form AM.ATP and AM.ADP.Pi respectively (steps 5 and 6 in the Lymn and Taylor scheme above). They concluded that these complexes were in a state of rapid equilibrium. Under the rapid equilibrium approximation, it is assumed that the noncovalent binding of substrates to the enzyme and the dissociation of newly formed products from the intermediate complexes is infinitely faster than any chemical steps. Thus the association-dissociation steps are essentially in equilibrium on the time scale of the ATPase cycle. These *weak binding* states were distinguished from the *strong binding*, high affinity, states of AM and AM.ADP.

In the kinetic scheme proposed by Stein and Eisenberg, actin did not dissociate from myosin during the hydrolysis step. As applied to muscle, they envisioned that the power stroke was generated when the cross-bridge went from the weakly bound AM.ADP.Pi state to the strongly bound AM.ADP state. The role of ATP was to return the cross-bridge to the weakly bound states rather than to detach it. The power stroke was therefore reversed while the cross-bridge was still attached to actin. On the surface this seemed to be an unacceptable model to explain cross-bridge activity in muscle where detachment of the cross-bridge from actin was proposed to be obligatory. Stein et al. (1981) explained this counter intuitive observation in the following way. They speculated that a cross-bridge may cycle between states weakly attached to actin with an angle near 90° and states strongly attached to actin with an angle near 45°. Because there was no mandatory detachment step, a cross-bridge may detach only when, during isotonic contraction, it was elastically distorted and thus in rapid equilibrium with an unattached state. They concluded that the absence of a mandatory detachment step in vitro suggested that cross-bridge detachment in vivo may be a mechanical event rather than strictly a biochemical process. The concept that a cross-bridge generates force during the transition from a weakly to strongly bound state originated from the results of the Eisenberg group. With regard to the weak binding states, their affinities are increased at low ionic strength. At physiological ionic strengths the weakly attached intermediates are largely in the dissociated state and hence would not contribute significantly to the force developed by the muscle.

There was no disagreement about the results between Lymn and Taylor and Eisenberg and Stein. The experiments were done under different conditions and gave different results. The differences between the Lymn-Taylor and Stein-Eisenberg schemes also point out that it is not possible to duplicate in solution the conditions inside a muscle fiber, i.e., high ionic strength and high effective actin concentration.

Furthermore the solution experiments cannot duplicate the mechanical events that exist in a muscle where force is generated and work is performed. For example, if a step in the cycle is coupled to a structural change that produces force or motion, the rate and equilibrium constants must be affected by the strain energy that is stored in the transition. Plus these experiments did not take into account any effects on the actomyosin ATPase that might relate to the presence of Ca^{2+} and troponin and tropomyosin in the filaments of muscle. Nevertheless, one would expect that the basic framework observed in the solution studies would be applicable to the muscle fiber with the caveat that rates of transition between intermediates and even pathways of predominant intermediates might be dependent on mechanical conditions. Clearly what was needed were measurements of the kinetics of actomyosin ATPase in systems that retained the three-dimensional geometry of the proteins in the muscle fiber and also allowed for variation in mechanical constraints on the intermediate states, e.g., myofibrils or skinned muscle fibers. This information then could be related directly to mechanical and structural parameters of muscle fibers.

New techniques would be developed throughout the 1980s to investigate the biochemical kinetics in structured systems including nucleotide analogs, isotopically labeled compounds, probe molecules, and especially photochemical compounds ("caged" compounds) that readily diffuse into the filament lattice (see Chap. 9). Also an increasing number of chemically identifiable (mainly by intrinsic fluorescence changes) intermediates or isomers in the actomyosin ATPase cycle would be discovered in solution studies. For example, it soon was determined that there are at least seven steps associated with the hydrolysis of ATP by S-1 in solution (compared to the three steps in the Lymn-Taylor scheme above), including the cleavage step and various isomerizations and rapid equilibrium steps (Bagshaw et al. 1974). Each of the intermediates in the hydrolysis of ATP by myosin may have a counterpart in which actin is bound to myosin. Furthermore there may be exchange between the members of each pair of states via binding of actin. Thus the solution studies would result in an exceedingly complex mechanism of actomyosin ATPase. The challenge would be to determine which of these various intermediates could be related to the cross-bridge cycle. Earl Homsher (Woledge et al. 1985) has written a useful primer on enzymes kinetics and described the experimental results leading to the Lymn-Taylor and Stein-Eisenberg models.

References

Abbott BC, Aubert XM, Hill AV (1951) The absorption of work by a muscle stretched during a single twitch or a short tetanus. Proc Roy Soc Lond B 139:86–104

Abbott BC, Hill AV, Howarth JV (1958) The positive and negative heat production associated with a nerve impulse. Proc Roy Soc Lond B 148:149–187

Armstrong CF, Huxley AF, Julian FJ (1966) Oscillatory responses in frog skeletal muscle fibres. J Physiol 186:26–27P

Aubert X, Marechal G (1963) Le bilan energetique des contractions musculaires avec travail positif ou negtif. J Physiol Paris 55:186–187

Bagshaw CR, Trentham DR (1973) The reversibility of adenosine triphosphate cleavage by myosin. Biochem J 133:323–328
Bagshaw CR, Eccleston JF, Eckstein F, Goody RS, Gutfreund H, Trentham DR (1974) The magnesium ion-dependent adenosine triphosphate of myosin: two-step processes of adenosine triphosphate association and adenosine diphosphate dissociation. Biochem J 141:351–364
Barclay CJ (1994) Efficiency of fast- and slow-twitch muscles of the mouse performing cyclic contractions. J Exp Biol 193:45–78
Barclay CJ (1999) Efficiency of energy conversion by muscles and its relation to efficiency during locomotion. In: Hargreaves M, Thompson M (eds) Biochemistry of exercise. Human Kinetics, Champaign, pp 115–123
Barclay CJ, Woledge RC, Curtin NA (2010) Inferring crossbridge properties from skeletal muscle energetics. Prog Biophys Mol Biol 102:53–71
Bassett DR (2002) Scientific contributions of A. V Hill: exercise physiology pioneer J Appl Physiol 93:1567–1582
Bouman HD (1972) In memoriam: Wallace O. Fenn, Ph.D., 1893–1971. Am J Phys Med 51:57–61
Bozler E (1930) The heat production of smooth muscle. J Physiol 69:442–462
Cain DF, Davies RE (1962) Breakdown of adenosine triphosphate during a single contraction of working. Biochem biophys Res Comm 8:361–366
Carlson FD, Siger A (1960) The mechanochemistry of muscular contraction. I. The isometric twitch. J Gen Physiol 44:33–60
Civan MM, Podolsky RJ (1966) Contraction kinetics of striated muscle fibres following quick changes in load. J Physiol 184:511–534
Curtin NA, Woledge RC (1977) A comparison of the energy balance in two successive isometric tetani of frog muscle. J Physiol 270:455–471
Curtin NA, Woledge RC (1978) Energy changes and muscular contraction. Physiol Rev 58:690–761
Curtin NA, Woledge RC (1979) Chemical change and energy production during contraction of frog muscle: how are their time course related? J Physiol 288:353–366
Curtin NA, Woledge RC (1981) Effect of muscle length on energy balance in frog skeletal muscle. J Physiol 316:453–468
Engelhardt WA, Ljubimova MN (1939) Myosine and adenosinetriphosphate. Nature 144:668–669
Feng TP (1932) The effect of length on the resting metabolism of muscle. J Physiol 74:441–454
Fenn WO (1923) A quantitative comparison between the energy liberated and the work performed by the isolated Sartorius muscle of the frog. J Physiol 58:175–203
Fenn WO (1924) The relation between the work performed and the energy liberated in muscular contraction. J Physiol 58:373–395
Ferenczi MA, He Z-H, Chillingworth RK, Brune M, Corrie JET, Trentham DR, Webb MR (1995) A new method for the time-resolved measurement of phosphate release in permeabilized muscle fibers. Biophys J 68:191s–193s
Finer JT, Simmons RM, Spudich JA (1994) Single myosin molecule mechanics: piconewton forces and nanometer steps. Nature 368:113–119
Fletcher WM, Hopkins FG (1907) Lactic acid in amphibian muscle. J Physiol 35:247–309
Ford LE, Huxley AF, Simmons RM (1974) Mechanism of early tension recovery after a quick release in tetanized muscle fibres. J Physiol 240:42–43P
Ford LE, Huxley AF, Simmons RM (1977) Tension responses to sudden length change in stimulated frog muscle fibres near slack length. J Physiol 269:441–515
Ford LE, Huxley AF, Simmons RM (1981) The relation between stiffness and filament overlap in stimulated frog muscle fibres. J Physiol 311:219–249
Ford LE, Huxley AF, Simmons RM (1985) Tension transients during steady shortening of frog muscle fibres. J Physiol 361:131–150
Ford LE, Huxley AF, Simmons RM (1986) Tension transients during the rise of titanic tension in frog muscle fibres. J Physiol 372:595–609

Frank RG (1978) The J. H. B. archive report. The A. V. Hill papers at Churchill College, Cambridge. J Hist Biol 11:211–214
Gasser HS, Hill AV (1924) The dynamics of muscular contraction. Proc Roy Soc Lond B 96:398–437
Gilbert C, Kretzschmar KM, Wilkie DR, Woledge RC (1971) Chemical change and energy output during muscular contraction. J Physiol 218:163–193
Gilbert C, Kretzschmar KM, Wilkie DR (1973) Heat, work, and phosphocreatine splitting during muscular contraction. Cold Spring Harb Symp Quant Biol 37:613–618
Gillis JM, Marechal G (1974) The incorporation of radioactive phosphate into ATP in glycerinated fibres stretched or released during contraction. J Mechanochem Cell Mot 3:55–68
Gosselin-Rey C, Gerday C (1977) Parvalbumin from frog skeletal muscle: isolation and characterization. Structural alterations associated with calcium binding. Biochim Biophys Acta 492:53–63
Hill AV (1913a) The energy degraded in the recovery processes of stimulated muscles. J Physiol 46:28–80
Hill AV (1913b) The absolute mechanical efficiency of the contraction of an isolated muscle. J Physiol 46:435–469
Hill AV (1922) The maximum work and mechanical efficiency of human muscles, and their economical speed. J Physiol 56:19–41
Hill AV (1924) Thermodynamics in physiology. Nature 113:859–862
Hill AV (1933) International status and obligations of science. Nature 132:952–954
Hill AV (1934a) International status and obligations of science—reply to Stark. Nature 133:290
Hill AV (1934b) International status and obligations of science—reply to Stark. Nature 133:615
Hill AV (1938) The heat of shortening and the dynamic constants of muscle. Proc Roy Soc Lond B 126:136–195
Hill AV (1939) The mechanical efficiency of frog's muscle. Proc Roy Soc Lond B 127:434–451
Hill AV (1949a) The heat of activation and the heat of shortening in a muscle twitch. Proc Roy Soc Lond B 136:195–211
Hill AV (1949b) Adenosine triphosphate and muscular contraction. Nature 163:320
Hill AV (1950) A challenge to biochemists. Biochim Biophys Acta 4:4–11
Hill AV (1953) Chemical change and mechanical response in stimulated muscle. Proc Roy Soc Lond B 141:314–320
Hill AV (1959) The heat production of muscle and nerve, 1848–1914. Annu Rev Physiol 21:1–19
Hill AV (1960) Production and absorption of work by muscle. Science 131:897–903
Hill AV (1962) The ethical dilemma of science and other writings. Chapter Four: Refugees, The Scientific Book Guild, London, p. 205-268
Hill AV (1964a) The effect of load on the heat of shortening of muscle. Proc Roy Soc Lond B 159:297–318
Hill AV (1964b) The efficiency of mechanical power development during muscular shortening and its relation to load. Proc Roy Soc Lond B 159:319–324
Hill AV (1965) Trails and trials in physiology. Williams & Wilkins, Baltimore
Hill AV (1966) A further challenge to biochemists. Biochem Z 345:1–8
Hill AV (1970) First and last experiments in muscle mechanics. Cambridge University Press, Cambridge
Hill AV, Hartree W (1920) The four phases of heat-production of muscle. J Physiol 54:8–128
Hill AV, Howarth JV (1959) The reversal of chemical reactions in contracting muscle during an applied stretch. Proc Roy Soc Lond B 151:169–193
Homsher E (1987) Muscle enthalpy production and it relationship to actomyosin ATPase. Annu Rev Physiol 49:673–690
Homsher E, Kean CJC (1981) Unexplained enthalpy production in isometric contractions and its relation to intracellular calcium movements. In: Grinnell AD, Brazier MB (eds) The regulation of muscle contraction: excitation-contraction coupling. Academic, New York, pp 337–347
Homsher E, Mommaerts WFHM, Ricchiuti NV, Wallner A (1972) Activation heat, activation metabolism and tension-related heat in frog semitendinosus muscles. J Physiol 220:601–621

Homsher E, Kean CJ, Wallner A, Garibian-Sarian V (1979) The time-course of energy balance in an isometric tetanus. J Gen Physiol 73:553–567
Homsher E, Irving M, Wallner A (1981) High-energy phosphate metabolism and energy liberation associated with rapid shortening in frog skeletal muscle. J Physiol 321:423–436
Homsher E, Yamada T, Wallner A, Tsai J (1984) Energy balance studies in frog skeletal muscles shortening at one-half maximal velocity. J Gen Physiol 84:347–360
Homsher E, Lacktis J, Yamada T, Zohman G (1987) Repriming and reversal of the isometric unexplained enthalpy in frog skeletal muscle. J Physiol 393:157–170
Horowits R, Kempner ES, Bisher ME, Podolsky RJ (1986) A physiological role for titin and nebulin in skeletal muscle. Nature 323:160–164
Huxley AF (1957) Muscle structure and theories of contraction. In: Butler JAV, Katz B (eds) Progress in biophysics and biophysical chemistry, vol 7. Pergamon, London, pp 255–318
Huxley AF (1973) A note suggesting that the cross-bridge attachment during muscle contraction may take place in two stages. Proc Roy Soc Lond B 183:83–86
Huxley AF (1974) Review lecture: muscular contraction. J Physiol 243:1–43
Huxley AF (1980) Reflections on muscle. Liverpool University Press, Liverpool
Huxley AF (2004) Andrew F. Huxley. In: Squire LR (ed) The history of neuroscience in autobiography, vol 4. Elsevier, Amsterdam, pp 284–318
Huxley AF, Simmons RM (1970) A quick phase in the series-elastic component of striated muscle, demonstrated in isolated fibres from the frog. J Physiol 208:52–53P
Huxley AF, Simmons RM (1971a) Mechanical properties of the cross-bridges of frog striated muscle. J Physiol 218:59–60P
Huxley AF, Simmons RM (1971b) Proposed mechanism of force generation in striated muscle. Nature 233:533–538
Huxley AF, Simmons RM (1973) Mechanical transients and the origin of muscular force. Cold Spring Harb Symp Quant Biol 37:669–680
Huxley HE (1960) Muscle cells. In: Brachet J, Mirsky AE (eds) The cell, vol 4. Academic, New York, pp 365–481
Huxley HE (1969) The mechanism of muscular contraction. Science 164:1356–1366
Huxley HE, Stewart A, Sosa H, Irving T (1994) X-ray diffraction measurements of the extensibility of actin and myosin filaments in contracting muscle. Biophys J 67:2411–2421
Infante AA, Davis RE (1962) ATP breakdown during a single isotonic twitch of frog Sartorius muscle. Biochem biophys Res Comm 9:410
Infante AA, Klaupiks D, Davies RE (1964) Adenosine triphosphate: changes in muscles doing negative work. Science 144:1577–1578
Jewell BR, Wilkie DR (1958) An analysis of the mechanical components in frog's striated muscle. J Physiol 143:515–540
Julian FJ, Sollins KR, Sollins MR (1974) A model for the transient and steady-state mechanical behavior of contracting muscle. Biophys J 14:546–562
Katz B (1978) Archibald Vivian Hill, 26 September 1886–3 June 1977. Biogr Mems Fell R Soc Lond 24:71–149
Katz B (1996) Sir Bernard Katz. In: Squire LR (ed) The history of neuroscience in autobiography, vol 1. Society for Neuroscience, Washington, DC, pp 348–381
Kretzschmar KM, Wilkie DR (1969) A new approach to freezing tissues rapidly. J Physiol 202:66–67P
Kuby SA, Mahowald TA (1959) Studies on the ATP-transphosphorylases. Fed Proc 18:267
Kushmerick MJ (1983) Energetics of muscle contraction. In: Peachey LD, Adrian RH, Geiger SR (eds) Handbook of physiology: section 10, skeletal muscle. American Physiological Society, Bethesda, pp 189–236
Kushmerick MJ (2001) Robert Ernest Davies. 17 August 1919–7 March 1993. Biogr Mems Fell R Soc Lond 47:141–157
Kushmerick MJ, Davies RE (1969) The chemical energetics of muscle contraction. II. The chemistry, efficiency and power of maximally working Sartorius muscles. Proc Roy Soc B 174:315–353

Kushmerick MJ, Larson RE, Davies RE (1969) The chemical energetics of muscle contraction. I. Activation heat, heat of shortening and ATP utilization for activation-relaxation processes. Proc Roy Soc B 174:293–313

Linari M, Woledge RC, Curtin NA (2003) Energy storage during stretch of active single fibres from frog skeletal muscle. J Physiol 548:461–474

Loiselle DS, Tran K, Crampin EJ, Curtin NA (2010) Why has reversal of the actin-myosin cross-bridge cycle not been observed experimentally? J Appl Physiol 108:1465–1471

Lundsgaard E (1930) Weitere untersuchungen uber muskelkontraktionen ohne milchsaurebildung. Biochem Z 227:51–83

Lymn RW, Taylor EW (1970) Transient state phosphate production in the hydrolysis of nucleoside triphosphates by myosin. Biochem 9:2975–2983

Lymn RW, Taylor EW (1971) Mechanism of adenosine triphosphate hydrolysis by actomyosin. Biochem 10:4617–4624

Marston S (1973) The nucleotide complexes of myosin in glycerol-extracted muscle fibres. Biochim Biophys Acta 305:397–412

McMahon TA (1984) Muscle, reflexes, and locomotion. Princeton University Press, Princeton

Meyerhof O (1920) Ueber die beziehung der milchsaure zur warmebildung and arbeitsleistung des muskels in der anaerobiose. Arch f ges Physiol 182:232–283

Mommaerts WFHM, Seraydarian K, Wallner A (1962) Demonstration of phosphocreatine splitting as an early reaction in contraction of frog Sartorius muscle. Biochim Biophys Acta 63:75–81

Podolsky RJ (1960) Kinetics of muscular contraction: the approach to the steady state. Nature 188:666–668

Potter JD, Hsu F, Pownall HJ (1976) Thermodynamics of Ca^{+2} binding to troponin C. J Biol Chem 252:2452–2454

Rahn H (1979) Wallace Osgood Fenn, 1893–1971. National Academy of Sciences. pp 141–173

Rall JA (1985) Energetic aspects of skeletal muscle contraction: implications of fiber types. In: Terjung RL (ed) Exercise and sport sciences reviews, vol 13. MacMillan, New York, pp 33–74

Rall JA, Homsher E, Wallner A, Mommaerts WFHM (1976) A temporal dissociation of energy liberation and high energy phosphate splitting during shortening in frog skeletal muscles. J Gen Physiol 68:13–27

Simmons RM (1992) A. F. Huxley's research on muscle. In: Simmons RM (ed) Muscular contraction, Cambridge University Press, p 19-42

Simmons RM, Jewell BR (1974) Mechanics and models of muscular contraction. In: Linden RJ (ed) Recent advances in physiology. Churchill Livingstone, Edinburgh, pp 87–147

Smith ICH (1972) Energetics of activation in frog and toad muscle. J Physiol 220:583–599

Smith SJ, Woledge RC (1985) Thermodynamic analysis of calcium binding to frog parvalbumin. J Muscle Res Cell Motil 6:757–768

Smith NP, Barclay CJ, Loiselle DS (2005) The efficiency of muscle contraction. Prog Biophys Mol Biol 88:1–58

Stark J (1934a) International status and obligations of science- reply to Hill. Nature 133:290

Stark J (1934b) The attitude of the German government towards science- reply to Hill. Nature 133:614

Stein LA, Schwarz RP, Chock PB, Eisenberg E (1979) Mechanism of actomyosin adenosine triphosphatase. Evidence that adenosine 5′-triphosphate hydrolysis can occur without dissociation of the actomyosin complex. Biochem 18:3895–3909

Stein LA, Chock PB, Eisenberg E (1981) Mechanism of the actomyosin ATPase: Effect of actin on the ATP hydrolysis step. Proc Natl Acad Sci 78:1346–1350

Szent-Gyorgyi A (1945) Studies on muscle. Acta Physiol Scand 9(Suppl 25):1–115

Szent-Gyorgyi A (1949) Free-energy relations and contraction of actomyosin. Biol Bull 96:140–161

Szent-Gyorgyi A (1951) Chemistry of Muscular Contraction, 2nd edn. Academic, New York

Taylor EW (2001) 1999 E. B. Wilson lecture: the cell as molecular machine. Mol Biol Cell 12:251–254

Tskhovrebova L, Trinick J, Sleep JA, Simmons RM (1997) Elasticity and unfolding of single molecules of the giant muscle protein titin. Nature 387:308–312
von Euler US (1935) Some factors influencing the heat production of muscle after stretching. J Physiol 84:1–14
Weizsacker V (1914) Myothermic experiments in salt solutions in relation to the various stages of a muscular contraction. J Physiol 48:396–427
Wilkie DR (1960) Thermodynamics and the interpretation of biological heat measurements. Prog Biophys Biophys Chem 7:259–298
Wilkie DR (1966) Muscle. Annu Rev Physiol 28:17–38
Wilkie DR (1968) Heat work and phosphorylcreatine break-down in muscle. J Physiol 195:157–83
Wilkie DR (1969) Soft Sell? Nature 221:306
Woledge RC (1968) The energetics of tortoise muscle. J Physiol 197:685–707
Woledge RC (1973) In vitro calorimetric studies relating to the interpretation of muscle heat experiments. Cold Spring Harb Symp Quant Biol 37:629–634
Woledge RC (2001) Douglas Robert Wilkie. 2 October 1922–21 May 1998. Biogr Mems Fell R Soc Lond 47:483–495
Woledge RC, Reilly PJ (1988) Molar enthalpy change for hydrolysis of phosphorylcreatine under conditions in muscle cells. Biophys J 54:97–104
Woledge RC, Curtin NA, Homsher E (1985) Energetic Aspects of Muscle Contraction. Academic, London

Chapter 6
1972 Cold Spring Harbor Symposia on Quantitative Biology: The Mechanism of Muscle Contraction—Problem Solved?

Our symposium each year gives us the opportunity to seek an exploding phase of biology and to bring together most of the key practitioners. (Watson 1973. With permission Cold Spring Harbor Laboratory Press) J. D. Watson, Director, Cold Spring Harbor Laboratory 1973

J. D. Watson, Director, Cold Spring Harbor Laboratory 1973

By the early 1970s, the time of the Cold Spring Harbor Symposium on muscle contraction, it was generally accepted that the sliding filament moving crossbridge model was correct. (Huxley 1996. With permission Annual Reviews)

H. E. Huxley 1996

On the one hand was the view that the attachment-detachment cycle of A. F. Huxley (1957), plus the structural picture of H. E. Huxley (1969), plus the biochemical cycle of Lymn and Taylor (1971), plus the force-generating step of A. F. Huxley and Simmons (19171b) equaled the muscle problem solved. We found this astonishing, as we knew we were just scratching the surface of the problem. (Simmons 1992. With permission Cambridge University Press)

R. M. Simmons 1992

6.1 Introduction

In 1972 a "watershed" meeting took place in idyllic Cold Spring Harbor, New York. In some ways the meeting represented the end of the "classical" period of muscle investigation during which time the basic principles of muscle activation and contraction were established. The meeting also represented the opening of the contemporary era of muscle study before the explosion of molecular biology and structural biology would "super charge" the field. It also represented the beginning of many muscle workshop style meetings throughout the twentieth century and beyond. The meeting was held at the Cold Spring Harbor Laboratory and organized by Noble Laureate James D. Watson, the laboratory director, with the advice of Carolyn Cohen, John Gergely, Andrew Huxley, Hugh Huxley and Andrew Szent-Gyorgyi. In the forward to the published volume, Watson explained the purpose of the Cold

J.A. Rall, *Mechanism of Muscular Contraction*, Perspectives in Physiology,
DOI 10.1007/978-1-4939-2007-5_6

Spring Harbors Symposia on Quantitative Biology and why muscle was "ripe" for selection as the 37th symposium:

> Our symposium each year gives us the opportunity to seek an exploding phase of biology and to bring together most of the key practitioners…With the emergence of the sliding filament model the field of muscle contraction has become in itself a major intellectual discipline, with its own well-defined objectives. On no occasion, however, since the sliding filament model was first presented has it been possible to bring together all the many people whose research directly bears on the molecular event underlying the contraction process.

The meeting itself ran from the evening of June 6 through noon of June 13, 1972. There were 78 presentations and approximately 200 participants at the meeting. Formal sessions occurred in the mornings with informal discussion in the afternoons. The meeting was opened with a, no doubt, colorful address by Albert Szent-Gyorgyi. Albert Szent-Gyorgyi had not worked in the muscle field for 20 years but his classic work leading to the discovery of actin and true myosin during World War II opened up the classical era of muscle investigation (see Chap. 1).

In his summary at the end of the meeting, Huxley (1973b) commented on the "rather gratifying" fact that virtually everyone at the meeting was conducting their research assuming the validity of the sliding filament, attached crossbridge mechanism of contraction. "Rather gratifying" indeed. The meeting must have been a great triumph for Hugh Huxley. He and his ideas had come a long way since the days back in Cambridge in the early 1960s when he had those uncomfortable lunches being challenged by Francis Crick to provide a mechanism to explain his X-ray diffraction results on muscle (see Chap. 2). Hugh Huxley also made another pertinent observation. The participants, while using different approaches and techniques, were beginning to relate their results to the results of other investigators. There was an evolving sense that they were "part of a community". Scientists were listening to the presentations outside of their own sub-discipline and trying to understand how the results of others could help the development of their own ideas. This could be a painful, but necessary, process as the sub-disciplines became more specialized and were often based on esoteric technology. Nonetheless the interaction of multi-disciplines became the hallmark of this and future muscle meetings.

Of course the major contributors to the development of the classic period of muscle research were at the meeting. But a noteworthy aspect of the participant list was the large number of junior investigators who would go on to develop distinguished careers by further advancing the muscle field. With regard to the presentations at the meeting, some were updates of well known on-going projects but others introduced entirely new topics and ideas to a wider audience. A partial list of the some of the novel topics presented at the meeting include: the steric blocking model of muscle activation, myosin binding protein C, thick filament regulation of contraction in invertebrate muscle, a new sarcoplasmic reticulum protein named calsequestrin, nomenclature for the three components of troponin, and a peculiar single headed myosin molecule that would open up a whole new field of investigation. These, and other selected topics, will be considered below. Some of the topics will be followed throughout the 1970s and into the 1980s and then returned to in later chapters. Other topics will be considered into the twenty-first century. Also some

topics not discussed at the meeting will be considered. In a sense the meeting will be utilized as a starting point to trace the development of ideas about muscle activation and contraction throughout the 1970s and beyond.

6.2 Sequence, Subunits and Assembly of Muscle Proteins

6.2.1 Amino Acid Sequence of Actin

The first scientific session dealt with actin and myosin structure, roles of the myosin light chains and assembly of myosin into filaments. Some of these presentations were a general introduction to what would become entirely new sub-fields of muscle study. Elzinga and Collins (1973) from the department of muscle research at the Boston Biomedical Research Institute described the substantial completion of the first step in establishing the structure of rabbit skeletal muscle actin by determining the amino acid sequence. They commented on the need to establish the tertiary structure of actin. Actin had not yet been crystallized because of its tendency to form polymers. To get around this problem, there was a need to develop a derivative of actin that will not polymerize. They were exploring various chemical procedures. It would not be until 1990 that this problem was solved and the crystal structure of actin elucidated in Kenneth C. Holmes' laboratory at the Max-Planck-Institut fur medizinische Forschung in Heidelberg by Kabsch et al. (1990). To prevent the polymerization of actin, it was combined with bovine pancreatic deoxyribonuclease I (DNase I) whose structure was known and the structure of the complex determined at about 3 Å by X-ray diffraction. Holmes et al. (1990) then produced an atomic model of the actin filament. (For more on the atomic structure of actin and the actin filament, see Chap. 9.)

6.2.2 Myosin Light Chains: Structure and Function

By the time of the meeting, it was generally accepted that the light chain fraction of myosin from rabbit skeletal muscle migrated as three bands on gel electrophoresis in the presence of sodium dodecyl sulfate (SDS)[1]. It had been proposed that there were two classes of myosin light chains. The alkali light chains (A1 and A2) which were essential for enzymatic activity of myosin and the DTNB light chains. Each

[1] Leonard Ornstein (1964) described the theoretical aspects of the gel electrophoresis technique and Baruch Joel (B.J.) Davis (1964) described the practical application for separating serum proteins. Shapiro et al. (1967) modified the technique by adding the anionic detergent sodium dodecyl sulfate (SDS) as a denaturing substance. Weber and Osborn (1969) at Harvard University showed that SDS gel electrophoresis could separate proteins according to molecular weight in the range of 11–220 kDa.

heavy meromyosin subfragment-1 (S-1) contained one alkali light chain and one DTNB light chain. Thus the myosin molecule was a hexamer with two heavy chains and four light chains (see Chap. 3 for a description of the research leading to these conclusions). There still were many uncertainties and unanswered questions. Was A2, molecular weight 17 kDa, a genuine light chain or a proteolytic breakdown product of A1, molecular weight 21 kDa? Was the DTNB light chain really a component of myosin or an unwanted contaminant of myosin purification? After all, removal of the DTNB light chain had no effect on the myosin ATPase activity. Did the two heads of a myosin molecule have an identical light chain composition or could the two heads be non-identical? And of course, what was the function of the light chains? These questions would not all be answered at the meeting but new and exciting possibilities would be proposed.

Weeds and Frank (1973) from the Laboratory of Molecular Biology at Cambridge described a structural study of the light chains of rabbit skeletal myosin. They determined the amino acid composition of A1 and A2. The major part of both proteins was identical with two important differences: (a) A1 contained an additional 41 residues at its N-terminal end and (b) the N-terminal eight residues of A2 contained five amino-acid replacements. Thus they were highly homologous but clearly different proteins. Weeds and Frank suggested that the two light chains may reflect the asymmetry of the two 'heads' of myosin or that they may arise from different isoenzymes of myosin present in the muscles. They noted that Locker and Hagyard (1967) from the Meat Industry Research Institute of New Zealand had described heterogeneity of myosin light chains in skeletal and cardiac muscles from different animal species. Weeds and Frank favored the possibility that at least two populations of myosin were present in rabbit skeletal muscle but clearly more evidence was required. This work subsequently was published in full (Frank and Weeds 1974).

Lowey and Holt (1973) described an immunochemical approach to the isolation of the myosin light chains. In order to avoid introducing any chemical modification into myosin by thiol reagents or denaturing solvents, they prepared antibodies against both classes of light chains with the idea of using the antigen-antibody reaction to dissociate the light chains from myosin. The results were somewhat disappointing. The DTNB light chain could be dissociated by the appropriate antibody but antibodies against A1 light chain showed only a limited reaction with heavy meromyosin S-1 (HMM S-1) and did not dissociate the alkali light chains. But it was not completely a false trail. They commented that although the existence of the alkali light chains was not disputed, the DTNB light chain still was dismissed as a contaminant. They were able to minimize the possibility that the DTNB light chain was a contaminant of myosin by showing that a fluorescently labeled antibody to the DTNB light chain localized entirely to the A band of isolated myofibrils with no detectable staining of the I band. They concluded that the absence of any fluorescence in the I-band and the lack of immunological reactivity of anti-DTNB with proteins of the relaxing system made it highly unlikely that DTNB light chains were related to the regulatory proteins of the thin filament. Thus the DTNB light chain was in all probability a genuine myosin light chain.

In the 1950s Edwin G. Krebs and Edmond H. Fischer at the University of Washington described the first enzymatic reaction regulated by a phosphorylation/dephoshorylation mechanism. The enzyme glycogen phosphorylase exists in two forms (*b* and *a*). This enzyme catalyzes the rate-limiting step of glycogenolysis. Krebs and Fischer discovered that the reversible interconversion of the inactive phosphorylase *b* to the physiologically active phosphorylase *a* involved a phosphorylation/dephoshorylation mechanism. The phosphorylation was mediated by an enzyme that they termed phosphorylase kinase. This work would eventually lead to them sharing the Noble Prize in Physiology or Medicine in 1992 (Krebs 1992). By the end of the 1960s, 15 years after phosphorylase kinase had been discovered, phosphorylation was still thought of as a rather specialized control mechanism largely confined to the regulation of glycogen metabolism (Krebs 1992; Fischer 1992; Cohen 2002). But this conclusion would change dramatically in the 1970s and by the end of the century several hundred protein kinases would be discovered.

Krebs and Beavo (1979) developed criteria for establishing that an enzyme undergoes physiologically significant phosphorylation and dephosphorylation. These criteria included:

1. Demonstration in vitro that the enzyme can be phosphorylated stoichiometrically at a significant rate in a reaction(s) catalyzed by an appropriate protein kinase(s) and dephosphorylated by a phosphoprotein phosphatase(s).
2. Demonstration that functional properties of the enzyme undergo meaningful changes that correlate with the degree of phosphorylation.
3. Demonstration that the enzyme can be phosphorylated and dephosphorylated in vivo or in an intact cell system with accompanying functional changes.
4. Correlation of cellular levels of protein kinase and/or phosphoprotein phosphatase effectors and the extent of phosphorylation of the enzyme.

Samuel Victor Perry and his colleagues at the University of Birmingham asked whether or not myosin could be regulated by phosphorylation. They presented intriguing preliminary results at the meeting that suggested that the DTNB light chain might play a regulatory role in myosin function. Perrie et al. (1973a) showed that a serine residue in the DTNB light chain component of skeletal myosin could be phosphorylated by a protein kinase system present in muscle. But the phosphorylation did not have a large effect on the ability of myosin to hydrolyze ATP. They were exploring the possibility that the system may be involved in the regulation of myofibrillar ATPase either in addition to or in association with the troponin and the tropomyosin complex. These results subsequently were published in detail (Perrie et al. 1973b). In a note added in proof in that paper E. M. V. Pires, M. A. W. Thomas, H. A. Cole and S. V. Perry reported the discovery of a protein kinase that phosphorylated the myosin light chain. This kinase required Ca^{2+} for full activity. They provisionally named it "myosin light chain kinase". While far from satisfying the criteria of Krebs and Beavo (1979), nonetheless this investigation opened a chapter in the regulation of skeletal muscle contraction by phosphorylation that would continue to be pursued into the twenty-first century (see Chap. 8 for further details and for a recent review see Stull et al. 2011).

Later Perry and collaborators (Frearson et al. 1976) also demonstrated that a light chain component of myosin from smooth muscle could be phosphorylated by an enzyme present in smooth muscle. Apolinary Sobieszek from the Institute of Molecular Biology, Aarhus University, Denmark at a meeting on the biochemistry of smooth muscle in 1975, described a Ca^{2+} mediated phosphorylation of a myosin light chain isolated from chicken or turkey gizzard smooth muscle (Sobieszek 1977). This phosphorylation resulted in the activation of actomyosin ATPase activity of smooth muscle. Furthermore the myosin light chain of about 20 kDa became phosphorylated at the same Ca^{2+} concentrations required to activate the actin-activated ATPase activity of myosin. He concluded that these results suggested that in vivo the actin-myosin interaction was regulated via a phosphorylation of the myosin molecule. This evidence coupled with the growing awareness that smooth muscle did not contain troponin suggested that smooth muscle activation by Ca^{2+} may occur via a thick filament mechanism involving myosin phosphorylation. This area of research became very exciting throughout the latter part of the twentieth and into the twenty-first century (for a review see Kamm and Stull 1985).

Thus the picture that was just beginning to emerge at the Cold Spring Harbor meeting was that along with Ca^{2+} regulation of muscle contraction via thin filaments there might also be Ca^{2+} regulation via thick filaments. Of course a possible role for phosphorylation in muscle activation was by no means proven at this time.

There was yet another way in which muscle could be activated by Ca^{2+} and that was by a direct stimulation of actomyosin ATPase by Ca^{2+} binding to myosin. Andrew Szent-Gyorgyi's laboratory at Brandeis University presented evidence to support this possibility (see Fig. 3.9). John Kendrick-Jones, Eva M. Szentkiralyi (Andrew's wife) and Szent-Gyorgyi (1973) described a recent study that they had undertaken with molluscan muscle (Kendrick-Jones et al. 1970; Szent-Gyorgyi et al. 1973). Scallops swim via jet-propulsion mediated by a large fast contracting striated muscle. The muscle contains fibers with A and I bands and sarcomeres with a resting sarcomere length similar to that observed in vertebrate striated muscle. But scallop muscle did not contain appreciable amounts of troponin (though the thin filaments did contain tropomyosin) and isolated actin containing filaments did not bind Ca^{2+}. These results suggested that muscle activation was not via a thin filament mechanism. Furthermore there was no evidence for myosin phosphorylation. They found that the actin activated ATPase of myosin from scallop muscle required Ca^{2+} binding to myosin. Tropomyosin was not required for regulation. They further determined that the removal of a light chain with EDTA from myosin caused the Ca^{2+} control to be lost and the actomyosin ATPase occurred at a maximum rate. Calcium control could be regained by recombination of the EDTA light chain with myosin. The light chain seemed to inhibit the actin-activated ATPase activity of scallop myosin in the absence of Ca^{2+} by blocking those sites which combine with actin. Strangely the EDTA light chain did not itself bind Ca^{2+} but its removal decreased Ca^{2+} binding to myosin. Its presence on myosin was necessary for Ca^{2+} binding to another myosin light chain, the essential light chain. Furthermore only two-headed myosin molecules were regulated. Simmons and Szent-Gyorgyi (1985) went on to show that what was observed with isolated myosin was also observed in

skinned scallop muscle fibers. Removal of the EDTA light chain caused that fiber bundle to generate maximum force that was independent of Ca^{2+} and Ca^{2+} control of contraction could be restored completely by re-addition of the EDTA light chain to the scallop muscle.

Szent-Gyorgyi naturally wondered to what extent this thick filament regulation by Ca^{2+} existed in the animal kingdom. At the meeting Lehman et al. (1973) presented results from a comparative investigation of thick and thin filament regulation of actomyosin ATPase by Ca^{2+}. Numerous invertebrate species exhibited thick filament regulation and some even showed dual thick and thin filament regulation. Tropomyosin was present in all muscles. From this limited data they hypothesized that animals from phyla which evolved early would exhibit myosin-linked regulation and that regulation utilizing troponin would be the result of a more recent evolutionary development. Subsequently Lehman and Szent-Gyorgyi (1975) examined nearly 100 different species from different phyla and concluded that their hypothesis was wrong and that there was no evidence that actin control via troponin represented a relatively recent evolutionary development. Nonetheless they clearly established that Ca^{2+} could activate muscle contraction by either a thin filament, thick filament or dually regulated mechanism. Szent-Gyorgyi (2007) continued to explore the subtleties of thick filament activation of molluscan muscle down to the level of the three dimensional structure and function of myosin.

It was not possible to know exactly how the light chains were arranged in HMM S-1 until 1993 when Ivan Rayment and Hazel M. Holden and colleagues at the University of Wisconsin determined the three dimensional structure of vertebrate myosin S-1 at a resolution of 2.9 Å (Raymentet al. 1993b). The essential and regulatory light chains were clearly seen to bind to the long neck or so called lever arm of S-1. This structure suggested that the light chains also might play a mechanical role in stabilizing S-1 (See Chap. 8 for more on the function of myosin light chains and Chap. 9 for the crystal structure of myosin.).

At the time of the Cold Spring Harbor meeting there were uncertainties about the role(s) of the myosin light chains but also excitement about the emerging diversity of the regulation of muscle contraction by Ca^{2+}.

6.2.3 C-protein (Myosin Binding Protein C, MyBP-C): Discovery, Characterization and Localization

It had been known since 1949 that fine striations separated by about 40 nm could be seen in electron micrographs in the I and A bands of the sarcomere (Draper and Hodge 1949). By the time of the Cold Spring Harbor meeting, tropomyosin and troponin were well established as thin filament proteins interacting with F-actin. Using immunoelectron microscopy Ohtsuki et al. (1967) showed that the striations in the I bands were due to troponin molecules distributed at about 38 nm intervals along the whole thin filament (see Fig. 4.17). There was considerably more uncertainty with regard to possible accessory proteins related to the thick filaments.

In 1968 Ebashi's laboratory (Masaki et al. 1968) had discovered a protein they called "M-substance" located at the M-line of myofibrils. Huxley (1963) reasoned that there had to be something else, some substance that determined the length of the thick filaments. In his classic paper of 1963, Huxley showed that isolated myosin spontaneously formed thick filaments whose length varied considerably (see Fig. 3.14). But in the intact fiber, the thick filaments were always of a precise length of 1.6 μm. Therefore something was missing from the isolated myosin experiments, something that determined thick filament length. Huxley (1967) further observed in the electron microscope that there were 11 fine striations visible in each half of the A band in unlabelled sections of skeletal muscle at a spacing of about 44 nm. Could these spacings contain the thick filament length determining proteins?

At the meeting, Gerald Offer from Jean Hanson's MRC biophysics laboratory at King's College London summarized work that suggested that at least one other protein was bound to myosin in the thick filaments (Offer 1973). This work was done in collaboration with Roger Starr, Elizabeth Rome and Roger Craig at King's College and with visiting scientist Carl Moos and with Frank A. Pepe at the University of Pennsylvania. The approach that Starr and Offer (1971) took was to look for impurities in preparations of myosin since any protein of the thick filaments might be expected to bind to myosin during conventional purification procedures and thus be a persistent impurity. They utilized the new technique of sodium dodecyl sulfate (SDS) gel electrophoresis to separate proteins and protein subunits according to molecular weight (see Footnote 1). A myosin preparation isolated from rabbit skeletal muscle run on a SDS gel exhibited the characteristic heavy chain at 200 kDa and the three light chains in the 14–27 kDa range. But also there were numerous bands in between the heavy chain and light chain bands. Starr and Offer (1971) labeled them alphabetically according to their mobility starting with the heavy chain band as "A". A prominent band was observed at the "C" position.[2] They chose to study the C-protein in detail. A purified preparation of C-protein exhibited a molecular weight of about 140 kDa, a length of about 40 nm with essentially zero alpha helical content in a single polypeptide chain. A further study of C-protein indicated that it did not bind Ca^{2+} in the presence of Mg^{2+}, it inhibited actomyosin ATPase by half and constituted about 1–2% of the myofibrillar protein (Offer et al. 1973). Carl Moos made a brief presentation during the discussion that indicated that C-protein bound strongly to the light meromyosin and the myosin rod (Moos 1973). Offer considered C-protein to be a newly characterized myofibrillar protein, not a proteolytic breakdown product, since its physical characteristics did not resemble myosin, actin, troponin, tropomyosin or M-line proteins.

The next step was to determine the location of C-protein in the myofibril. Offer collaborated with Frank A. Pepe to develop an antibody against C-protein. Roger Craig, a graduate student working with Jean Hanson at King's College, took the first

[2] Other prominent bands in the gel of the myosin preparation that were found to exist in the myofibril included bands labeled B, F and H (Starr and Offer 1971). The B band was likely a M-line protein and the F band was attributed to the enzyme phosphofructokinase (Starr and Offer 1982). The H band with a molecular weight of 74 kDa was homologous to C-protein and found to exist in stripe 3 of the A band (see Footnote 3 below).

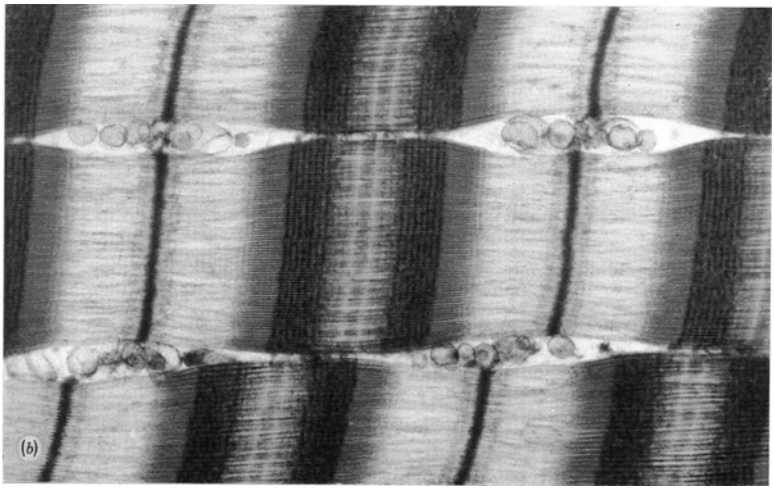


Fig. 6.1 Labeling of glycerinated psoas muscle fibers from the rabbit with a highly purified antibody to C-protein. The labeling patterns observed in the electron microscope show that C-protein is located on seven stripes, spaced about 43 nm apart in each half of the A-band (Craig and Offer 1976. With permission The Royal Society)

electron micrographs showing that the antibody to C-protein appeared as nine stripes separated by about 43 nm in each half of the A band of glycerinated rabbit skeletal muscle. There was also an increase in the density at the M-line which turned out to be due to the fact that the C-protein preparation was not pure (Craig and Offer 1976). When antibodies were made against a C-protein preparation of higher purity, Craig and Offer found no increased density at the M-line and now seven stripes in each half of the A band (Fig. 6.1). Similar conclusions were reached by Pepe and Drucker (1975). These results implied that there must be other protein material located in the A band. Thus C-protein could account for seven to nine of these striations. Some of the stripes closest to the M-line of the thick filament do not appear to be made up of C-protein.[3]

Because the C-protein stripes were narrow and the C-protein molecule long, Offer (1973) speculated that the C-protein was arranged circumferentially relative to the thick filament axis rather than axially. He calculated that there are about two C-protein molecules per stripe. Offer went on to speculate about the function of the C-protein. He rejected the possibility that C-protein acted as a thick filament length determining protein because of its restricted location in the A band and because it seemed to have the same periodicity as the myosin helix repeat. Rather it seemed that the structure of the thick filament determined the location of C-protein binding.

[3] The precise number of C-protein stripes in the A band is dependent on muscle fiber type (Bennett et al. 1986). Three isoforms of C-protein are known to exist in adult muscle: fast skeletal, slow skeletal (originally described as X-protein) and cardiac. Separate genes encode each isoform. A related protein, H-protein, smaller than C-protein in skeletal muscle is localized to the third stripe from the M-line of the A band. It is interesting to note that C-protein does not occur in stripes 1 and 2. See Flashman et al. (2004) for a review.

Offer (1973) opted for a more mundane possibility for the role of C-protein. He suggested that if the C-protein molecule was elongated, it could wrap around the circumference of the backbone of the thick filament. This arrangement would allow two C-protein molecules to make contact and form "a collar" around the filament.

That there could be more to the story was shown in 1978 by Moos and collaborators (1978). They found that C-protein, besides binding strongly to light meromyosin and the myosin rod, could also bind to actin. The binding to actin was considerably weaker than the binding to myosin but C-protein did inhibit the actin actomyosin ATPase. They commented that while there was no direct evidence that interaction of C-protein with actin was physiologically significant, the length of the C-protein molecule was sufficient to make contact with the thin filaments in muscle while remaining attached to the thick filament. Furthermore Starr and Offer (1978) showed that C-protein also bound to myosin subfragment-2. They concluded with the interesting speculation (Starr and Offer 1978. With permission Portland Press Limited):

> If, in resting muscle, the subfragment-2 regions of the myosin molecules making up the thick filaments were attached to the light-meromyosin part of the filament shaft by C-protein, the outward movement of the cross-bridges of heavy meromyosin thought to occur during contraction (Huxley 1969) would be prevented. It is an intriguing possibility that, if the binding of C-protein to the subfragment-2 region or to light meromyosin were regulated, this could form the basis of a mechanism to regulate the interaction of myosin heads with actin.

The interest in C-protein languished somewhat until it was discovered that the cardiac muscle isoform of C-protein was phosphorylated in response to β-adrenergic stimulation (Jeacocke and England 1980). This phosphorylation suggested a possible functional role for C-protein in the heart. Weisberg and Winegrad (1996) showed that phosphorylation of C-protein (now called Myosin Binding Protein-C, MyBP-C) extended the cross-bridges from the backbone of the native myosin thick filaments. Reminiscent of the earlier speculation by Starr and Offer (1978), they suggested that this effect could alter the rate constants for attachment to and detachment from the thin filament and thereby modify force production in activated cardiac muscle. The interest in cardiac MyBP-C really accelerated in the mid 1990s when two groups showed that mutations in MyBP-C were some of the most frequent causes of hypertrophic cardiomyopathy (for a comprehensive review see Flashman et al. 2004). A recent structural study, using electron tomography[4], in intact skeletal muscle (Luther et al. 2011) showed that MyBP-C does not form a collar around the thick filament, as originally suggested by Offer, but rather reaches out to the thin filament (Fig. 6.2). All of this information has made the possibility that MyBP-C plays an important functional role in contraction a very intriguing and active area of research in the cardiac muscle field.

[4] Electron Tomography is a technique for obtaining detailed 3D structures of subcellular macromolecular objects. It is an extension of traditional transmission electron microscopy and uses a transmission electron microscope to collect data. In the process, a beam of electrons is passed through the sample at incremental degrees of rotation around the center of the target sample. This information is collected and used to assemble a three dimensional image of the target. Current resolutions of electron tomography systems are in the 5–20 nm range.

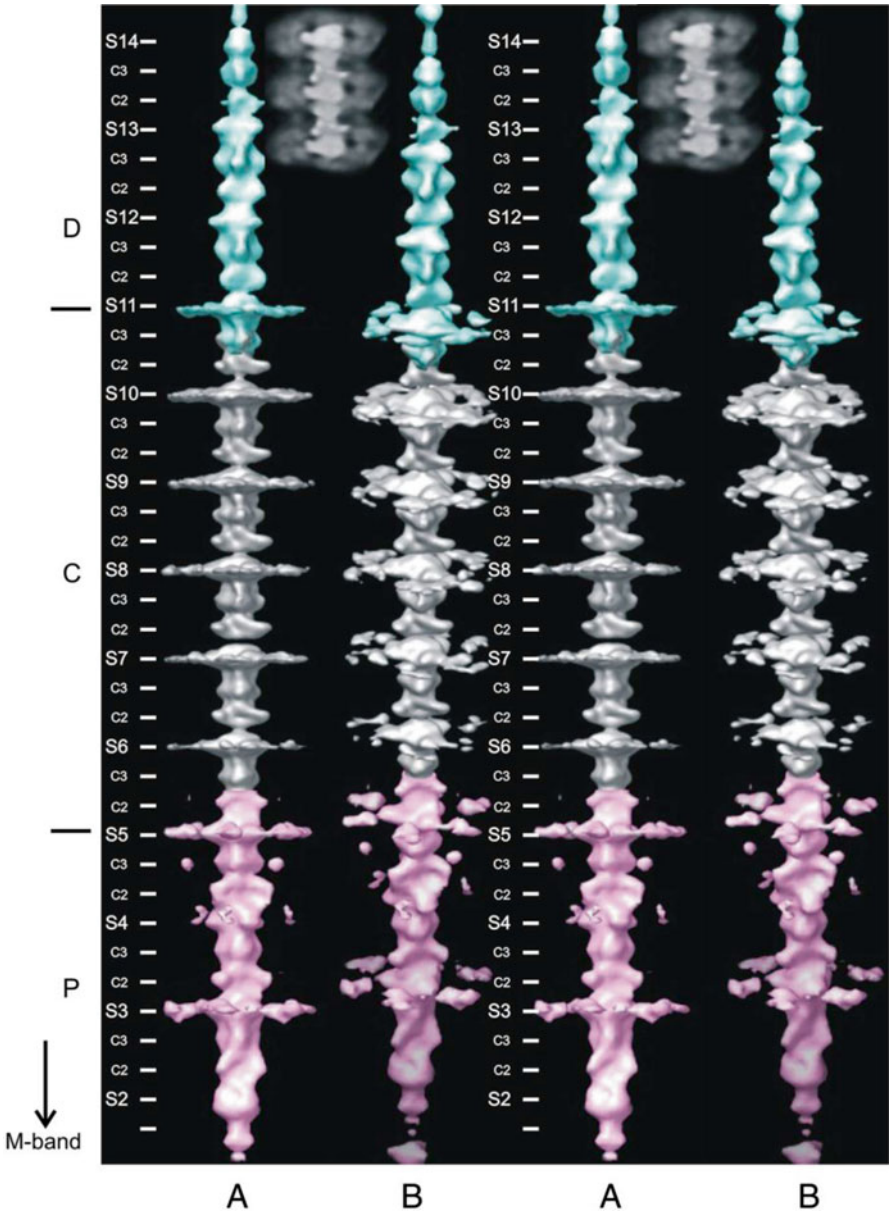


Fig. 6.2 Tomographic reconstruction of a thick filament. (**A**, **B**) Interleaved stereo images of averaged frog muscle thick filament tomograms; (**A**) face view, (**B**) tilted 20°. MyBP-C is present at stripes S5–S11. Between these stripe levels are two layers of density due to crowns of myosin heads with a periodicity of about 14.3 nm (labeled c2 and c3). (Luther et al. 2011. With permission National Academy of Sciences)

Thus the discovery of C-protein did not solve the mystery of the constancy of thick filament length in muscle. With regard to other proteins bound to the thick filaments, a big surprise was in store in the late 1970s when Koscak Maruyama and colleagues (Maruyama et al. 1977) in Japan discovered the largest protein known. Koscak called it connectin. Independently Kuan Wang and collaborators at the University of Texas at Austin found a similar protein, actually a doublet (Wang et al. 1979). Wang called it titin. Connectin/titin was found to be a myosin binding protein that also bound to MyBP-C. What role did it play? Possibly it was the long sought filament length determining protein. See Chap. 7 for the fascinating story.

6.2.4 Structure and Assembly of Thick Filaments

Myosin is a remarkable protein. It is an enzyme that catalyzes the hydrolysis of ATP, a proposed force generator and also a structural protein that forms the backbone of the thick filaments. It is important to understand the structural role of myosin in developing theories of muscle contraction. Based upon the pioneering structural studies of H. E. Huxley in 1963 (see Chap. 3) on isolated native myosin filaments from vertebrate muscle and synthetic thick filaments, the following properties were established by the time of the Cold Spring Harbor meeting. The thick filaments were bipolar in structure with a central bare zone which did not exhibit cross-bridges. The myosin molecules exhibited anti-parallel packing in the bare zone and then parallel packing in the rest of the filament. Both ends of the filament were tapered at the outer extremities. The total length of a native thick filament was 1.6 μm but synthetic thick filaments exhibited varying lengths. Also the thick filaments were about 10–15 nm in diameter. The task then became one of describing the structure of the thick filaments in more detail. There were three main questions to address (Squire 1986). What was the arrangement (lattice) of myosin cross-bridges on the filament surface? In what way were the myosin molecules packed together to both produce this surface lattice and give the observed bipolar structure? What was the configuration (or orientation in space) of the myosin cross-bridges on the observed surface lattice? This configuration clearly could alter according to the physiological state of the muscle and would have direct relevance to the force-producing mechanism. These would turn out to be very difficult questions that would require numerous technical advances before real progress could be made.

6.2.5 Arrangement of Cross-bridges on the Thick Filament Surface

The first question related to the arrangement of cross-bridges on the thick filament surface. In 1957 H. E. Huxley produced classic electron micrographs of vertebrate muscle where cross-bridges were observed for the first time at intervals of about 40 nm along the thick filament (see Fig. 3.5). Because each myosin filament was

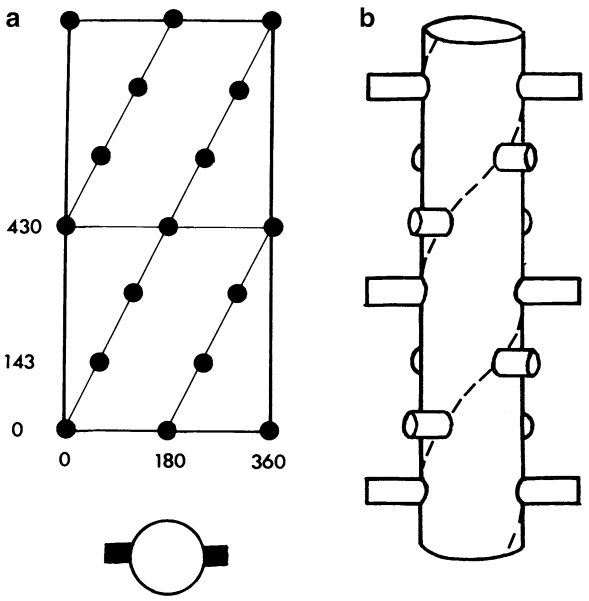


Fig. 6.3 Cross-bridges arranged on a two-stranded helix in a thick filament of striated muscle as proposed by Huxley and Brown (1967). (**a**) Radial projection (*above*) and cross-section (*below*). In this projection the back side of the thick filament has been sliced axially and the filament then laid flat. (**b**) A schematic three-dimensional view of thick filament with cross-bridges arranged on a two-stranded helix. The dashed line follows one of the helical strands. In this model there are six cross-bridges every 42.9 nm with a pair of cross-bridges every 14.3 nm (Squire 1981. With permission Springer)

surrounded by six adjacent actin filaments, it was suggested that there were six cross-bridges pointing to the six actin filaments in each 40 nm repeat.

The axial X-ray diffraction evidence from living muscle of Huxley and Brown (1967) showed that the cross-bridges were very likely arranged in an approximately helical manner with a 42.9 nm repeat (taken as equivalent to the 40 nm repeat observed in electron micrographs) and with a 14.3 nm axial translation or separation of cross-bridges. Thus there would be expected to be three cross-bridges per every 42.9 nm. The observation from electron micrographs of six cross-bridges to actin in a repeat similar to 42.9 nm suggested that perhaps there should be two such helices twisting around the same axis (see Fig. 3.17). Huxley and Brown derived a model for the cross-bridge array consisting of two helical strands of cross-bridges. Each strand is a helix with six cross-bridges per turn and having a pitch (axial distance between one turn of the helix and the next) of 2×42.9 nm and axial subunit translation of 14.3 nm. This model is illustrated in Fig. 6.3 where its radial projection and perspective view are shown. This arrangement is commonly termed a two-stranded, six units per turn helix. Even though each helix has a pitch of 2×42.9 nm, since there are two helical strands, the repeat of the whole structure is only 42.9 nm.

From the electron micrographs, Huxley (1963) was able to estimate that a single thick filament would contain about 200 myosin molecules, assuming one myosin molecule per observed cross-bridge. But there was what Huxley called a "stubborn" discrepancy. Huxley and Brown (1967) summarized the problem. The number of myosin molecules per thick filament expected on the basis of large-scale biochemical analyzes of muscle was about two times larger than the number of cross-ridges observed, first by electron microscopy and later confirmed by X-ray diffraction. Huxley and Brown (1967) concluded that either there were much bigger errors than

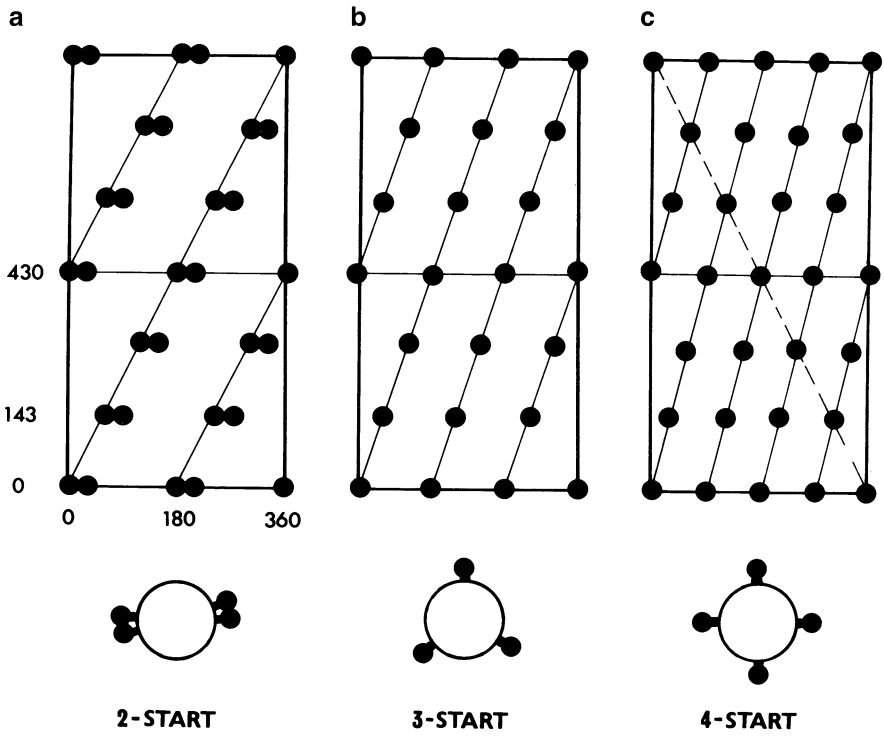


Fig. 6.4 Radial projections (*above*) and cross-sectional views (*below*) of thick filaments that exhibit the three alternative helical distributions of cross-bridges that account for the X-ray pattern observed in resting frog sartorius muscle. These alternatives also satisfy the requirement that at each 14.3 nm spaced axial level there are the heads of either three or four myosin molecules. Each projection in the lower figures corresponds to one myosin molecule. The three stranded model (**b**) turned out to be the correct description of the frog thick filament (Kensler and Stewart 1983). See text for details and see Fig. 6.5 (Squire 1981. With permission Springer)

had been realized in some of the generally accepted figures used in the calculations or that there was more than one, probably two, myosin molecules per cross-bridge. The radial projection and cross-sectional view of this speculated arrangement is shown in Fig. 6.4a. Earlier Huxley (1963) commented that perhaps it was pushing the data too far to expect perfect numerical agreement but nonetheless he believed that at least one of the values being used must be wrong. But which value was in error? It would take nearly 20 years to solve the mystery.

In 1972 John M. Squire, who was not at the Cold Spring Harbor meeting, pointed out that there were other possibilities for the cross-bridge surface lattice that were more compatible with the biochemical estimate of myosin in a muscle. The thick filament might exhibit a three stranded (300 myosin molecules per thick filament) or four stranded (400 myosin molecules per thick filament) helical arrangement of cross-bridges (Fig. 6.4b, c). The X-ray diffraction results could not distinguish

among these three possibilities since each would exhibit a 42.9 nm repeat with a 14.3 nm subunit axial translation.

The controversy continued throughout the 1970s without enough information to distinguish with certainty among the two-, three- or four-stranded models of the surface lattice of the vertebrate thick filament. The break through came in the early 1980s when Robert W. Kensler and Rhea J. C. Levine at what was then known as the Medical College of Pennsylvania developed new techniques to rapidly isolate native thick filaments from fresh invertebrate muscle in a well preserved natural state. The thick filaments of invertebrates exhibited cross-bridge arrangements that were more stable than those from vertebrate muscle and it was with invertebrates that a detailed description of the arrangement was first possible. Kensler and Levine (1982) examined the thick filaments isolated from *Limulus* telson muscle by electron microscopy and optical diffraction. The optical diffraction patterns of negatively stained thick filaments were similar to the X-ray diffraction patterns from living muscle. Their analysis of the electron micrographs and optical diffraction patterns supported a model for the thick filament in which the cross-bridges were arranged on a four-stranded helix with four cross-bridges at each 14.3 nm level, sometimes referred to as a "crown".

This was the result for an invertebrate muscle. What was the lattice arrangement of cross-bridges in a vertebrate thick filament? In 1983 Kensler and Murray Stewart applied the newly developed thick filament isolation procedures to frog skeletal muscle. The arrangement of cross-bridges was well preserved in the negatively stained thick filaments. They concluded the computer image analysis of negatively stained images of these filaments enabled the number of strands to be established "unequivocally". The data was consistent only with a three-stranded structure and could not be reconciled with either two- or four-stranded models. Figure 6.5, left shows the results of the computer image analysis that indicated that cross-bridges were arranged along three helical strands in the frog thick filament. Thus there were three cross-bridges per crown (Fig. 6.5, right) and finally a 20 year mystery was solved.

Therefore the "stubborn" discrepancy could be traced to electron microscopy. Apparently electron microscopy failed to establish the three-stranded nature of the vertebrate thick filament because of the flexibility of the cross-bridge attachments which led to disorder of the cross-bridges (Craig 1986). Also if only a fraction (2/3) of the total number of cross-bridges could attach to actin in rigor muscle and if only those cross-bridges that were attached to actin were sufficiently stabilized be preserved and visualized in the electron microscope, H. E. Huxley's observations could be explained (Squire 1981). Thus vertebrate thick filaments exhibit a three-stranded approximately helical arrangement of cross-bridges on the filament surface.

6.2.6 Packing of Myosin Molecules in a Thick Filament

This result does not give any direct information regarding the packing of the myosin tails into the filament shaft. To conclude that the tails were arranged in an approximately helical manner similar to that of the heads would not be justified without

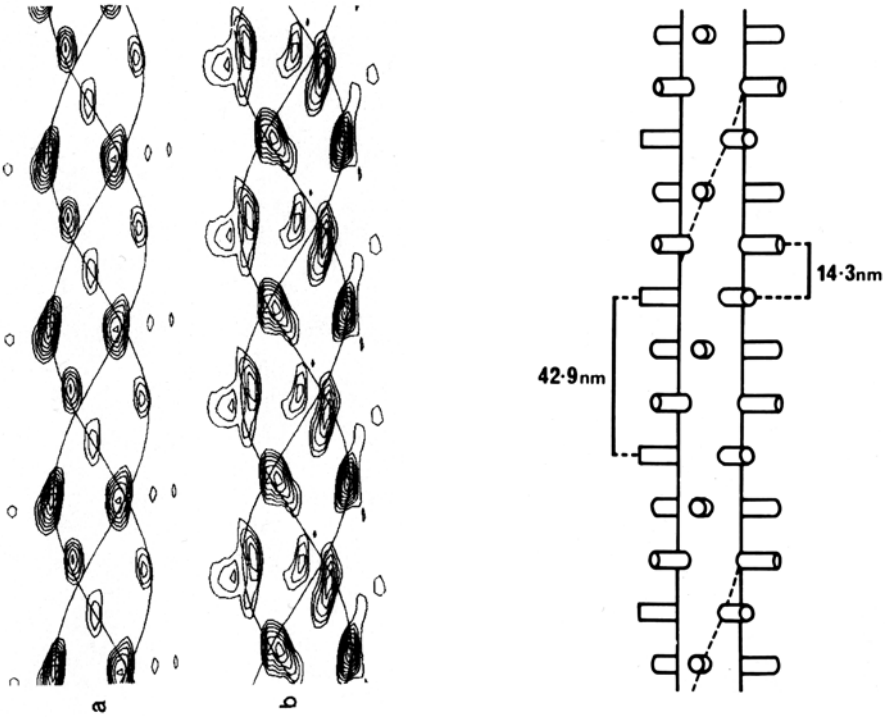


Fig. 6.5 Thick filament structure. *Left*: Computer filtered images of negatively stained thick filaments from frog striated muscle showing myosin cross-bridges every 14.3 nm along three helical strands. The density associated with the filament backbone was omitted in these computer filtered images to allow easier identification of the individual cross-bridges (Kensler and Stewart 1983. With permission Rockefeller University Press) *Right*: Schematic diagram showing the now accepted arrangement of cross-bridges on the myosin filament in vertebrate striated muscle. The cross-bridges are arranged on the thick filament surface as a three-stranded helix with pitch 3×42.9 nm and three cross-bridges every 14.3 nm. The dotted line traces the path of one of the helical strands. (Offer 1974)

further information. The next questions to consider were: in what way was the myosin molecules packed together to produce the observed surface lattice and bipolar structure and what was the mechanism of assembly? These would be particularly vexing questions and research is still on-going in this area. The major difficulty is that the myosin tail which is an α-helical coiled coil is only 2 nm in diameter. This dimension is at the limit of resolution of the electron microscope. Further perspective on the difficulty is gleaned from the following consideration. The portion of the myosin molecule held in the filament backbone (the tail) is about 150 nm long, there are three myosin cross-bridges at each level, and the levels are separated by 14.3 nm. Thus a cross-section of the thick filament could contain up to 30 myosin tails (150/14.3×3), 3 from each of 10 different, neighboring axial levels of cross-bridges-from molecules that are just "starting" to those that are about to give off their cross-bridges

(Craig 1986). These thirty individual myosin tails would be closely packed in the backbone and thus very difficult to discern with the electron microscope.

At the Cold Spring Harbor meeting, Pepe (1973), from the University of Pennsylvania, who developed the first comprehensive model of vertebrate thick filament structure in 1967 described his model. He would continue to work on the thick filament structure for 30 years. The most important facet of his model as it evolved over the years was the proposal that the thick filament backbone was built from parallel structural units (or subfilaments), about 4 nm in diameter, containing more than one myosin molecule (Ashton et al. 1992). But the units would be packed together such that the myosin molecules find themselves in a large number of non-equivalent environments which is generally considered to be an unlikely situation in macromolecular organelles. In 1971 John M. Squire, then at Aarhus University, proposed what he called the "general model" for the structure of myosin containing filaments. He noted that across the animal kingdom actin filaments had basically the same structure. In contrast myosin filaments could be cylindrical with widely varying diameters or even ribbon-like in shape. Nonetheless the myosin extracted from the various muscles was similar in structure and all of these myosin containing filaments exhibited an axial repeat of cross-bridges of 14.3 nm. Squire (1971) considered the possibility that the packing of myosin molecules was basically the same in all types of myosin-containing filaments. He made two assumptions. First, the myosin molecules in a particular thick filament would tend to be in equivalent environments and second, the molecular packing arrangements in different myosin filaments would be closely related. Thus he considered the various thick filament structures as variations on a theme. The proposal had considerable intellectual appeal. Based on this proposal, he rejected that two-stranded model of the thick filament and suggested that either a three- or four-stranded model would satisfy the "equivalency" criterion for myosin packing (Squire 1972). He also suggested that the vertebrate thick filament would contain a core protein similar to the paramyosin[5] core protein observed in invertebrate thick filaments. In this regard he was incorrect as a core protein has not been observed in vertebrate thick filaments. In this model the myosin tails were envisioned as assembling individually to form the backbone. The model has been called the molecular crystal model and is different than the subfilament model proposed by Pepe. Nonetheless there is strong experimental evidence for the existence of myosin subfilaments (Woodhead et al. 2005). Squire and Pepe never agreed on the structure of the thick filaments. After 40 years of study of the thick

[5] Ten years after Kenneth Bailey discovered tropomyosin in vertebrates, Bailey (1957) reported the characterization of what he considered a new form of tropomyosin in invertebrates. This "invertebrate tropomyosin" is now known as paramyosin. The name paramyosin was coined by Hall et al. (1946) earlier based on electron microscopic studies of invertebrate muscle fibrils since they had some properties similar to myosin but were yet also different. Paramyosin was found to be a two chain **α** helical coiled coil approximately 130 nm in length, 2 nm in diameter with a molecular weight of 220 kDa (Lowey et al. 1963). Paramyosin was first determined to be in the core of invertebrate thick filaments by Szent-Gyorgyi et al. (1971). It has similarities to the myosin rod. A paramyosin core allows invertebrate thick filaments to attain large diameters containing up to a seven stranded cross-bridge helix arrangement in striated scallop muscle (Squire 1986).

filament packing there is still some uncertainty about the precise arrangement of myosin molecules in the thick filament. Squire has written two outstanding monographs on the structural basis of muscular contraction (Squire 1981, 1986).

6.2.7 *Mechanism of Assembly of a Thick Filament*

The assembly mechanism of thick filaments was addressed at the Cold Spring Harbor meeting by William F. Harrington and his colleagues Morris Burt and Janice S. Barton from the department of biology at Johns Hopkins University (Harrington et al. 1973). They suggested that a dimeric species of myosin could be the initiating and possibly the building unit used in assembly of the thick filaments with the rate limiting step being the formation of the nucleating species from the initiating dimer units. Furthermore if the dimer had parallel geometry, then the bare central zone could be constructed by antiparallel association of the dimer species.

Since myosin molecules aggregate spontaneously when the ionic strength of a solution in reduced into the physiological range, it is highly probable that ionic or electrostatic interactions play a crucial role in thick filament formation. Thus one approach to investigating thick filament assembly is to examine the amino acid sequence of the myosin molecule in hopes that it will yield information about the axial arrangement of molecules in the thick filament. The myosin rod [2/3 light meromyosin (LMM) + 1/3 heavy meromyosin subfragment 2 (HMM S-2)] has two α-helices wrapping around one another to form an extended coiled coil approximately 150 nm long. This structure exhibits a seven amino acid helical repeat where the hydrophobic residues stabilize the structure by interacting with each other in the core of the coiled coil, while the hydrophilic residues lie at the surface. David A. D. Parry, then at Massey University in New Zealand, noted (1981) that in the amino structure of the rod of rabbit skeletal myosin there was strong evidence for another repeat of 28 amino acids resulting in an alternation of positively and negatively charged zones along the rod. He suggested that this repeat was compatible with myosin molecules in the thick filament being axially staggered with respect to one another by 14.3 nm.

McLachlan and Karn (1982) at the Laboratory of Molecular Biology in Cambridge examined the periodic charge distributions in the myosin rod amino acid sequence and concluded that these distributions matched the cross-bridge spacings observed in muscle. Sidney Brenner had established the soil nematode worm, *Caenorhabditis elegans* (*C. elegans*), as a model organism for the investigation of molecular development and molecular genetics. (His investigations resulted in him receiving the Noble Prize in 2002.) McLachlan and Karn deduced the complete amino acid sequence of the myosin rod of *C. elegans* from the DNA sequence of the cloned myosin (unc-54) gene. The myosin rod contained over 1,000 amino acids and exhibited strong homology with the myosin rods in mammalian striated muscle. They too noted the 28 residue repeat (zone) that contained a strong band of negative charges followed by a band of positive charges 14 residues away from it.

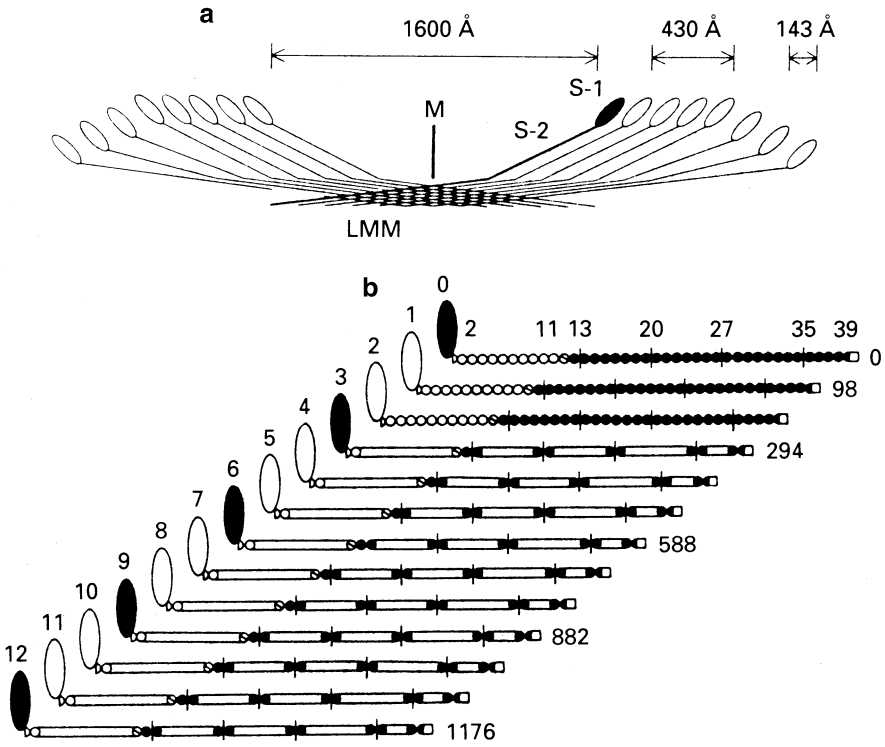


Fig. 6.6 Longitudinal packing of myosin molecules in the thick filament as suggested by the amino acid sequence of the rod section of the molecule. (**a**) The bipolar arrangement in the middle of the filament is shown with the central bare zone free of cross-bridges. (**b**) The parallel array of staggered myosin rods is shown. 28-residue repeats are represented as circles in rods 0–2, with the first 12 repeats (*white circles*) being in the S2 section of the rod. Successive rods are staggered by 98 residues resulting in cross-bridges being separated axially by 14.3 nm (McLachlan and Karn 1982. With permission Nature Publishing Group)

Their calculations indicated that there would be strong electrostatic attractions if two 28-residue zones were displaced axially by 14 residues or if two complete rods are shifted by any odd multiple of 14. The strongest electrostatic attractions would occur at 98 (7×14) and 294 (21×14) residue repeats. The 14.3 nm axial cross-bridge repeat in a thick filament corresponded to the 98 residue repeat and the 43 nm repeat to the 294 residue repeat (Fig. 6.6). They concluded that their one-dimensional model calculations suggested that direct interactions between complementary charges on parallel myosin rods staggered by 98 or 294 residues could account for the 14.3 and 43.0 axial spacings found in muscle.

With emerging genetic techniques, this plausible model for thick filament formation could be directly tested. Atkinson and Stewart (1991), also members of the Laboratory of Molecular Biology at Cambridge, concluded that although the 28-residue charge periodicity is important in myosin molecular interactions, it is

probably not the major driving force for myosin assembly but instead influences the detailed axial stagger of the interacting molecules. They expressed in *Escherichia coli* a cDNA clone corresponding to rabbit light meromyosin together with a number of modified polypeptides generated by directed site-specific mutagenesis. They found that a deletion of 92 residues at the C terminus of LMM disrupted myosin assembly whereas deletion of 92 residues from the N terminus did not radically disrupt myosin assembly. This was not what would be expected if the complementation of the zones of alternating charge were providing the driving force for myosin assembly because the same degree of complementation should be found with deletions from either terminus. Therefore, they concluded that the 28-residue charge repeat does not appear to be primarily responsible for myosin assembly. Leslie Leinwand and her colleagues Regina L. Sohn, Karen L. Vikstrom, Michael Strauss at the University of Colorado collaborated with Carolyn Cohen and Andrew Szent-Gyorgyi at Brandeis University to investigate further the importance of the C terminal portion of the myosin molecule in thick filament assembly (Sohn et al. 1997). They expressed segments of the myosin rod of human fast skeletal in *E. coli* and examined the formation of ordered paracrystals[6] of myosin rods. They took ordered paracrystal formation as a stringent test of proper assembly of myosin molecules. They found that proper assembly required a 29 residue sequence (residues 1874–1902) near the C terminus of the rod region. Thus, there appeared to be a specific sequence in the C terminal region of the myosin heavy chain rod which was necessary for ordered paracrystal formation and was sufficient to confer assembly properties to an assembly-incompetent rod fragment. They called this residue sequence the "assembly competence domain". They further concluded that the 28 residue zones of charge along the molecule were necessary but not sufficient to insure correct assembly.

This area of research is on-going. Despite the significant process, the precise mechanism of assembly and packing of myosin molecules in three dimensions into the bipolar filaments of vertebrate striated muscle is still not completely understood. This discussion does not consider other important topics. For example, what determines thick filament length? (see Chap. 7) What is the configuration (or orientation in space) of the myosin cross-bridges on the observed surface lattice in the relaxed and contracted state (see Chap. 9)? How is thick filament assembly regulated in vivo? And, of course, how is a sarcomere assembled? For a recent review discussing these latter two questions, see Kachur and Pilgrim (2008).

[6] In solutions of low ionic strength LMM precipitates to form ordered aggregates called paracrystals. The paracrystals can assume different forms. Spindle shaped paracrystals were first studied in the electron microscope by Philpott and Szent-Gyorgyi (1954) who observed an axial repeat of about 40 nm. Other spindle-shaped paracrystals (tactoids) have been observed but with periodicities different from 43 nm. However, almost without exception the repeats correspond to an integral multiple of 14.3 nm. Paracrystal formation is an indicator of the ability of LMM or myosin rods to assemble properly. See Bennett (1981) for examples of paracrystals with periodicities of 43 or 14.3 nm.

6.3 Muscle Regulatory Systems

6.3.1 Troponin

By the time of the Cold Spring Harbor meeting, Setsuro Ebashi's concept of Ca^{2+} as regulator of muscle contraction and relaxation was considered to be dogma. But there was still much to be learned. Ebashi and Kodama (1966) discovered and named the intracellular Ca^{2+} receptor troponin (see Chap. 4). Soon thereafter Hartshorne and Mueller (1968) showed that troponin consisted of two components. Troponin A bound Ca^{2+} and troponin B inhibited the actomyosin ATPase. But the real modern era of troponin investigation began in John Gergely's laboratory at the Boston Biomedical Research Institute. This ground breaking work involved two outstanding postdoctoral fellows who would go on to distinguished research careers in the muscle regulation field: Marion L. Greaser and John D. Potter. Greaser and Gergely (1971), using the technique of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, discovered that troponin consisted of three, not two, components. Reconstitution experiments indicated that all three components, in an approximate molar ratio of 1:1:1, were necessary to restore the Ca^{2+} requirement for the ATPase activity of actomyosin in the presence of tropomyosin.

There were five presentations on troponin at the meeting with general agreement that the troponin complex consisted of three proteins. Marion Greaser, now at the University of Wisconsin, and John Gergely described their recent results together (Greaser et al. 1973). It was at this meeting that they introduced the modern terminology for the troponin subunits. The names were based on the observed function of the proteins: TN-C bound Ca^{2+}, TN-I inhibited actomyosin ATPase activity in the presence or absence of $Ca^{2+}$and TN-T bound to tropomyosin. The estimated molecular weights of the proteins were 20,000, 24,000, 37,000 Da respectively. They also commented briefly about cardiac troponin. They stated that cardiac troponin contained subunits which differed markedly in molecular weight from those obtained from skeletal troponin. Elucidating these differences and their functional consequences would turn out to be a major field of study in the regulation of cardiac muscle regulation.

Greaser and Gergely subsequently introduced the terminology for the troponin components into the peer reviewed literature in 1973 along with evidence that TN-T interacted with tropomyosin (Greaser and Gergely 1973). They also observed in 1973 that the fragmented sarcoplasmic reticulum could remove Ca^{2+} from troponin. This result was consistent with the view that the sarcoplasmic reticulum controlled Ca^{2+} binding to troponin in vivo. In what would turn out to be a classic understatement, Greaser and Gergely in 1973 summed up their results by concluding that if it is assumed that Ca^{2+} binds to TN-C during contraction, there appears to be "a rather complicated series of protein-protein interactions". They went on to speculate that the Ca^{2+}-induced change in TN-C would be transferred either to TN-I and then to TN-T or in conjunction with TN-I to TN-T. The information would then go from TN-T to tropomyosin where it in turn would be conveyed to actin.

Thus the challenge became to understand how the proteins interacted with each other and with tropomyosin and actin. This effort began in earnest in John Gergely's

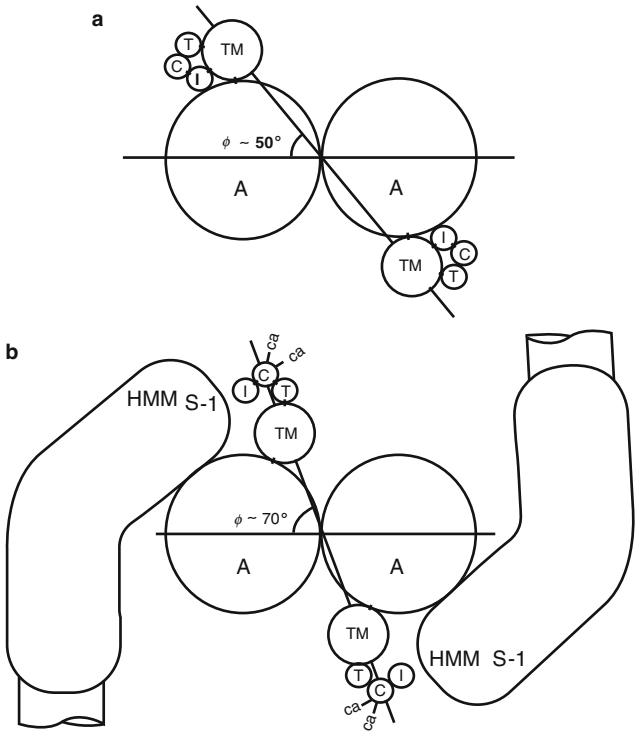


Fig. 6.7 Scheme of regulation of muscle contraction by troponin and Ca^{2+}. The relative positions of actin, tropomyosin and the head of the myosin molecule (HMM S-I) in the model are essentially as proposed by Spudich et al. (1972), Haselgrove (1973), Huxley (1973a, b), and Parry and Squire (1973). Key: *A* actin, *TM* tropomyosin, *T* TN-T, *I* TN-I, *C* TN-C; (**a**) relaxation in the absence of Ca^{2+}, (**b**) activation. Suggested interactions between proteins are indicated by *short connecting lines* (Potter and Gergely 1974. With permission American Chemical Society)

laboratory by postdoctoral fellow James D. Potter. They (Potter and Gergely 1974) determined the effect of Ca^{2+}on the interaction of troponin and its components with F-actin, tropomyosin, and the F-actin-tropomyosin complex. They utilized ultracentrifugal analysis to determine the interaction of the various components. The components were mixed and centrifuged and the pellets and supernatant checked with SDS gel electrophoresis. On the basis of the binding studies, they proposed the first model to explain how troponin and Ca^{2+} might regulate muscle contraction (Fig. 6.7). This model was based on the proposed positions of tropomyosin on the thin filaments during contraction and relaxation (see below the steric blocking model of activation described at the Cold Spring Harbor meeting).

Potter and Gergely (1975) then investigated the Ca^{2+} binding sites on troponin and troponin C. They found that there were two high affinity Ca^{2+} binding sites on troponin C that also bound Mg^{2+} competitively (Ca^{2+}–Mg^{2+} sites, 2×10^7 M^{-1}) and two sites with lower affinity for Ca^{2+} that did not bind Mg^{2+} (Ca^{2+}-specific sites, 2×10^5 M^{-1}). Studies on myofibrillar ATPase activity as a function of free Ca^{2+} con-

centration at two different free Mg^{2+} concentrations showed that the full activation by Ca^{2+} occurred only upon binding of Ca^{2+} to the two Ca^{2+}-specific binding sites in troponin but did not require binding of Ca^{2+} to the Ca^{2+}–Mg^{2+} sites. They suggested that the Ca^{2+}–Mg^{2+} sites may be involved in maintaining the protein in a conformation that was a prerequisite for activation by Ca^{2+}. These studies moved into the time domain when J. David Johnson, a postdoctoral fellow in the Potter laboratory, determined the rates of Ca^{2+} exchange with the Ca^{2+}-specific and Ca^{2+}–Mg^{2+} binding sites of troponin C (Johnson et al. 1979). They utilized a fluorescent probe attached to troponin C to monitor the structural changes associated with Ca^{2+} binding and removal from the high and low affinity sites and stopped flow kinetic analysis to monitor the rates of Ca^{2+} exchange. Calcium binding to both sites occurred very rapidly, within the few milliseconds mixing time of the stopped flow instrument. In contrast the rate of Ca^{2+} dissociation from the Ca^{2+}–Mg^{2+} sites of troponin C was greater than 200-fold slower than from the Ca^{2+}-specific sites. They concluded that the Ca^{2+} exchange and the structural changes involved with Ca^{2+} exchange with the Ca^{2+}–Mg^{2+} sites were too slow to be directly involved in muscle regulation. In contrast they concluded that Ca^{2+} exchange and the structural changes that occurred with Ca^{2+} exchange with the Ca^{2+}-specific sites were rapid enough to be involved in the regulatory process. This study, therefore, offered further support that the Ca^{2+}-specific sites were the regulatory sites for skeletal muscle contraction. Thus by the end of the 1970s it was well established that the troponin C had four Ca^{2+} binding sites and that the two Ca^{2+}-specific sites were likely the trigger sties for muscle contraction and the two Ca^{2+}–Mg^{2+} sites were likely structural sites. Much more would be learned about the molecular aspects of troponin and it interactions with tropomyosin and actin in the 1980s and beyond with the advent of powerful new molecular biology, genetic, structural and physiological techniques (see Chap. 8).

6.3.2 Parvalbumin: Structure of a Calcium Binding Protein

Around the time of the Cold Spring Harbor meeting another story was unfolding. This research seemed on the surface to be remote from the topics of the meeting and thus was not included in the program. But this research would lead to the discovery of the molecular structure of the Ca^{2+} binding site in hundreds of proteins, including troponin. The story started somewhat earlier in the laboratory of general biology directed by Marcel Dubuisson[7] at the University of Liege in Belgium. Dubuisson had a strong interest in skeletal muscle contraction and muscle proteins (Dubuisson

[7] Marcel Dubuisson (1903–1974) was the director of the laboratory of general biology at the University of Liege from 1949 to 1970 during which time he studied muscle proteins by electrophoresis methods. He wrote a monograph on muscle contraction in 1954. He had a long standing interest in marine biology and founded an aquarium at the university that opened in 1962 as the "Aquarium M. Dubuisson". His most important influence was as the rector (president) of the University of Liege from 1953 to 1971 during which time he was a strong visionary for modernization of the university. He created the oceanographic research station (STARESO) in Corsica in 1970.

1954). He founded an aquarium at the University of Liege and the research of the laboratory extended to the study of fish muscle proteins. Gabriel Hamoir, a member of the laboratory, was one of the pioneers in the study of fish muscle proteins with investigations that covered a period of four decades, starting in the early 1950s. In 1955 J. G. Henrotte in the Hamoir research group described the isolation and crystallization of a protein from carp myogen (soluble protein extract). 1965, Hamoir, Shoji Konosu and Jean-Francois Pechere (Hamoir and Konosu 1965; Konosu et al. 1965) published an extensive study of the myogen component of carp white and red muscle using electrophoresis and ultracentrifugation techniques. They found proteins of low molecular weight (9,000–13,000 Da) in high abundance in white but not in red muscle of the carp. There were three main highly water soluble components, closely related in molecular weight and amino acid composition, that were isolated from carp white muscle. These proteins could form crystals, as first described by Henrotte (1955). The proteins exhibited no enzymatic activity and their function was unknown. Similar proteins were found in frog but not mammalian muscle. They concluded that this group of proteins appeared to be specific for the white muscle of fishes and amphibians. It would be some years later before it was realized that these proteins also existed in mammalian muscle (see below) and in mammalian non-muscle cells.

Based on their low molecular weight and high solubility in water, Pechere (1968), who moved on to the département de biochimie macromoléculaire du C.N.R.S., Montpellier, France, suggested the general name "parvalbumin" for this group of proteins. He and his colleagues performed extensive studies on the parvalbumins throughout the 1970s. They found that parvalbumin bound two moles of Ca^{2+} tightly and made a quantitative determination of Ca^{2+} binding (Benzonana et al. 1972). Pechere noted the similarities between parvalbumin and troponin and proposed that they may both have similar functions in muscle (Pechere et al. 1971).

The crystal structure of parvalbumin: Robert H. Kretsinger had joined the department of biology at the University of Virginia in 1967 after postdoctoral training with John Kendrew in Cambridge where he worked on the crystal structure of metmyogloblin. He was interested in the parvalbumins because they were easily extractable, small, proteins that formed crystals that could be amendable to X-ray crystallographic investigations of structure. He hoped that solving the tertiary structure of these atypical proteins would contribute to the general understanding of the relationship of primary to tertiary protein structure. Plus the knowledge of their structure might also suggest their function (Kretsinger et al. 1971). Kretsinger undertook an investigation of the crystal structure of parvalbumin with postdoctoral fellow Clive Nockolds who would go on to spend his research career in the electron microscopy unit at the University of Sydney, Australia. When they determined the structure of parvalbumin, neither he nor Nockolds were aware that the protein bound Ca^{2+}. In their classic paper (Kretsinger and Nockolds 1973) entitled "carp muscle calcium-binding protein", they stated that they took no special precautions either to add or to remove Ca^{2+}. Only after the data collection was completed did they discover that the protein bound Ca^{2+}. Thus they didn't set out to determine the structure

of a Ca^{2+} binding protein. It happened by chance and, as it would turn out, good fortune because they made a fundamental discovery. One of the parvalbumins (the one isolated and crystallized by Henrotte) formed beautiful crystals that allowed its structure to be determined at a resolution of 1.85 Å. Once they knew that the protein bound Ca^{2+} they looked for the Ca^{2+} binding motif. The molecule had the approximate shape of a prolate ellipsoid of revolution. It was a smooth protein, free of clefts and grooves often characteristic of enzymes. The course of the main chain was best visualized in terms of the six helices A, B, C, D, E, and F. The helix C, the CD loop, and helix D were related to the EF region by an approximate twofold axis. Thus parvalbumin had two Ca^{2+} binding regions. With regard to the EF Ca^{2+} binding region, they observed that the over-all configuration of the EF region was remarkably similar to a right hand with thumb and forefinger extended at approximate right angle and the remaining three fingers clenched (Fig. 6.8). The thumb points toward the COOH terminus of helix F. The forefinger points along helix E in the NH_2-terminal direction. The clenched fingers trace the course of the EF loop about the Ca^{2+}. Thus EF hand Ca^{2+} binding proteins were inaugurated into the literature in 1973. Today there are known to be more than 1,000 EF hand Ca^{2+} binding proteins. Calcium is coordinated by six oxygen atoms in an octahedral arrangement. The ligands of the EF loop can be visualized as occupying the corners of an octahedron (Fig. 6.8).

Based on the amino acid sequence determination by John Collins and colleagues (Collins et al. 1973), Kretsinger and Barry (1975) predicted correctly that troponin C contained four EF hand Ca^{2+} binding motifs (see Chap. 8 for troponin C crystal structure). It was soon realized that parvalbumin was not limited to fish and amphibians as it was shown that mammalian parvalbumin isolated from rabbit skeletal muscle was homologous to fish parvalbumin (Enfield et al. 1975).

What was the function in muscle of this Ca^{2+} binding protein? Kretsinger and Nockolds (1973) stated that it seemed reasonable then to explore the idea that the protein functioned by mediating the concentration of Ca^{2+} in muscle. Indeed Jean-Marie Gillis, at the University of Louvain, and Charles Gerday, at the University of Liege (Gerday and Gillis 1976), and Pechere and colleagues (1977) put forward the idea that parvalbumin acted to promote relaxation in fast contracting skeletal muscle (see Chap. 8 for details). Jean-Francois Pechere died prematurely in 1978 and didn't live to realize how right he was concerning the role of parvalbumin in muscle relaxation. It is ironic that an obscure fish muscle protein turned out to be the prototype for defining Ca^{2+} binding sites in troponin C and Ca^{2+} binding in many other proteins. It is also ironic that the structure of parvalbumin was known at the atomic level well before there was any idea what its function might be. Eugene A. Permyakov, from the Russian Academy of Sciences in Moscow, and Kretsinger have completed an extensive analysis of the evolution of more than 1,000 EF-hand homolog Ca^{2+} binding proteins and identified 78 distinct subfamilies. This analysis along with the history of investigation of the role of Ca^{2+} in physiological processes, techniques used to study Ca^{2+} binding proteins and chemistry of Ca^{2+} are described in a book written by Permyakov and Kretsinger (2011).

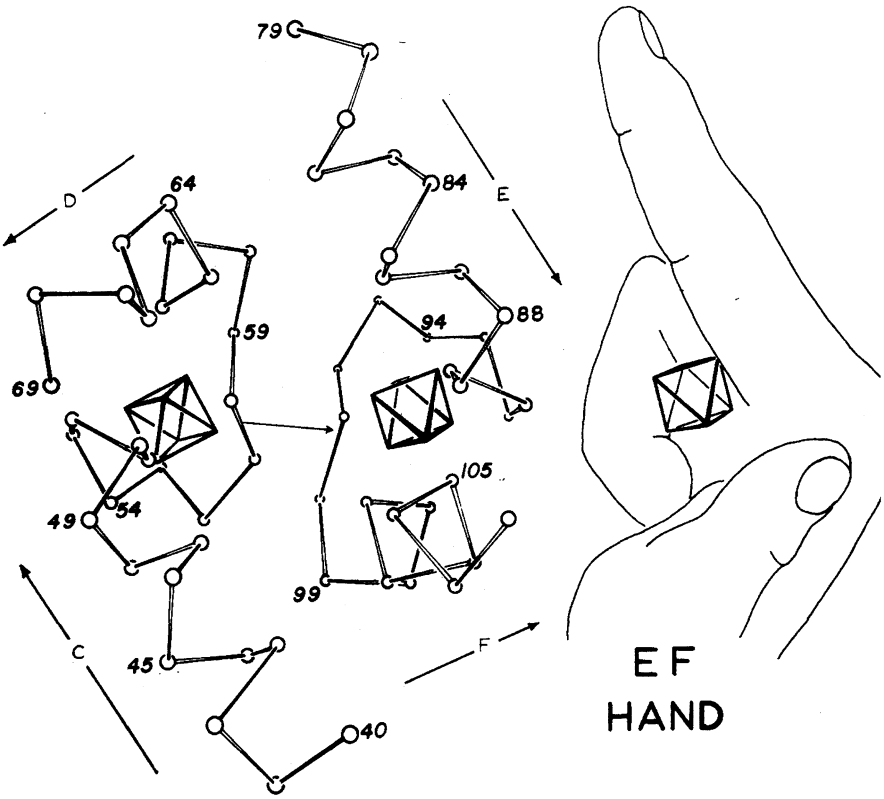


Fig. 6.8 Calcium binding regions of parvalbumin from carp muscle. Crystal structure of the CD and EF regions. These Ca^{2+} binding regions are thirty amino acids long. Helix E (and helix C) runs from the tip to the base of the forefinger. The flexed middle finger corresponds to the EF (and the CD) Ca^{2+} binding loop. Helix F (and helix D) runs to the end of the thumb. The EF region has been chosen as the evolutionary prototype, or "EF hand," as drawn to the *right* (Kretsinger and Barry 1975. With permission Elsevier)

6.3.3 Tropomyosin

By 1972 it was agreed that tropomyosin was critical in transferring the Ca^{2+} signal from troponin to seven actin monomers of the thin filament. Also the basic features of the tropomyosin molecule and its location in the myofibrils were reasonably well understood by that time. Tropomyosin, discovered and characterized by Kenneth Bailey (1946), is an elongated fibrous protein associated with the myofibrils. Bailey named the protein tropomyosin because of its similarity to myosin. He thought incorrectly that it might be a precursor of the myosin molecule. In 1950s Carolyn Cohen began a 50 exploration of fibrous proteins, particularly tropomyosin, and a 50 plus year collaboration with Andrew G. Szent-Gyorgyi. She was attracted to

tropomyosin because it was the smallest and simplest fibrous protein. Cohen and Szent-Gyorgyi (1957) found that tropomyosin exhibited a high alpha-helical content like the light meromyosin fragment of the myosin molecule. In his classic studies Pauling et al. (1951) discovered the alpha helix and reasoned that in complex fibrous proteins the alpha helices would tend to twist around one another and form a stable coiled coil. Crick (1953) pointed out that a coiled coil would be formed by the regular interlocking of side chains of the alpha helices, his "knobs-into-holes" analogy. He proposed that alpha helical proteins must have two-chain or three-chain structures. In 1963 Cohen and postdoctoral fellow Kenneth Holmes examined the X-ray diffraction pattern of an invertebrate muscle which was known to contain a large concentration of the fibrous protein paramyosin (Cohen and Holmes 1963). The X-ray pattern exhibited the predicted two-chain alpha-helical coiled coil structure. They then correctly deduced that tropomyosin, like paramyosin, also would have a two chain coiled-coiled structure. Hanson and Lowy (1963) made the provocative suggestion that two strands of tropomyosin followed the actin filament helix and were located on the outside of the actin filament in the two grooves between the strands of actin monomers. Ebashi and Kodama (1966) discovered that "native tropomyosin" contained tropomyosin and the new protein troponin. Ebashi and colleagues (Endo et al. 1966; Ohtsuki et al. 1967; also see Chap. 4) then utilized fluorescent and anti-body techniques to label tropomyosin and troponin. They determined that troponin and tropomyosin were located along the entire length of the thin filaments with troponin distributed at 400 Å intervals. They proposed that tropomyosin molecules were oriented end to end in the two grooves of the thin filament positioning the troponin molecules every 400 Å along the thin filament. Ebashi et al. (1969) put all of this information together and described the classic representation of the thin filament which outlined the basic functional scheme of muscle regulation (see Fig. 4.17).

Nonetheless the regulatory system was still understood only in a general way. What was needed was detailed information about the structure and interaction of the components. With regard to tropomyosin, Cohen and Longley (1966) constructed and examined tropomyosin paracrystals, the so-called "Cohen-Longley paracrystals". The paracrystals were initially made serendipitously when a technician mistakenly added a thousand times more Ca^{2+} than planned (Cohen 1975). These were exotic structures with stripes and bands exhibiting a striking 400 Å periodicity (Fig. 6.9). Caspar et al. (1969) examined the paracrystals with X-ray diffraction and electron microscopy. They concluded that the paracrystals were built up of rod shaped tropomyosin molecules, about 20 Å in diameter, associated head-to-tail in polar filaments with a 400 Å period. This conclusion meant that the tropomyosin molecule must be at least 400 Å long. At the Cold Spring Harbor meeting Cohen and colleagues described their recent investigations of the interaction of tropomyosin with troponin in paracrystals and nets (Cohen et al. 1973). Cohen (1975) summarized this work in an article in Scientific American.

In order to understand the structure of the tropomyosin molecule, a important starting point was the determination of the amino acid sequence of the molecule. Tropomyosin contained two chains of molecular weight about 34,000 Da each.

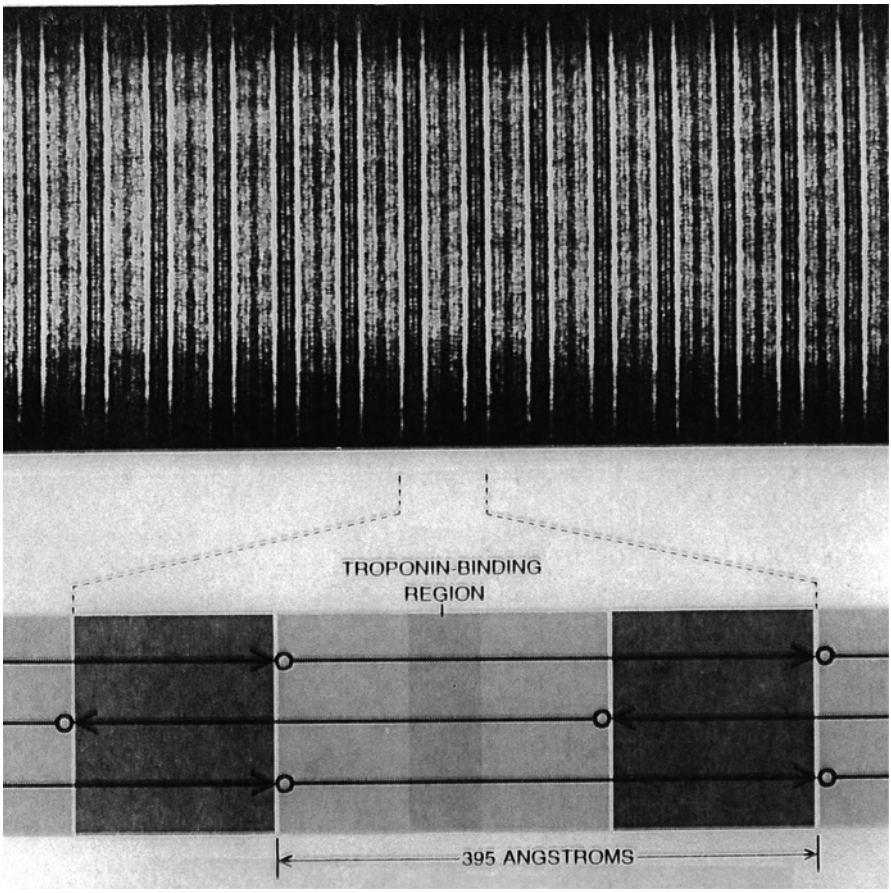


Fig. 6.9 Paracrystal of tropomyosin. *Top*: An electron micrograph of a paracrystal, negatively stained, shows a pattern of *light* and *dark regions* bounded by *thin white bands. Bottom*: The pattern is interpreted in the drawing. The *thin white bands* mark the ends of molecules, indicating a small overlap. The symmetrical, nonpolar pattern derived from polar tropomyosin molecules means there must be sets of molecules (*black arrows*) bonded head to tail to form filaments that are oppositely directed in the paracrystal. The filaments are shown as being straight with a period of about 400 Å (Cohen 1975) the molecule

Lawrence B. Smillie and colleagues (Hodges et al. 1973) at the University of Alberta described this sequence at the Cold Spring Harbor meeting. But there was a complication. Cummins and Perry (1973) showed that vertebrate tropomyosins were heterogeneous. They could be separated into two populations of slightly different chains of amino acids. The two subunits present in rabbit skeletal tropomyosin were named the α- and β-chains. They were shown to be virtually identical in amino acid composition except for their cysteine content. In skeletal muscle the α and β components of tropomyosin were present in the proportion of about 4:1. Subsequently Smillie and David Stone (Stone and Smillie 1978) published the com-

plete amino acid sequence of the α-chain of tropomyosin which consisted of 284 amino acids. The elucidation of the amino acid sequence of rabbit skeletal α-tropomyosin led to rational explanations for numerous aspects of: (a) the tropomyosin molecule, (b) tropomyosin-tropomyosin interaction and (c) tropomyosin interaction with other proteins. The stabilization of the coiled-coil structure was as predicted by Crick (1953). Nonpolar amino acids were spaced regularly along the chain, separated by an average of 3.5 subunits. The two chains could therefore be meshed by an interlocking of the nonpolar subunits. This meshing resulted in the "knobs-into-holes" packing arrangement which Crick predicted. Based on statistical analysis and model building, McLachlan and Stewart (1976a) concluded that the two chains of tropomyosin likely existed in a parallel, non-staggered, arrangement. The calculated molecular length from the amino acid sequence was 423 Å. Since the periodicity observed in paracrystals was about 400 Å, these results implied that there was a head-to-tail overlap of molecular ends of 8–9 residues. Tropomyosin which spans almost exactly seven actins, may be attached in a similar way to each actin because there appeared to be a 14-fold repeat in the amino acid sequence along the tropomyosin molecule. No pattern of identical repeating regions existed along the actin molecule but there appeared to be seven pairs of "quasi-equivalent" regions at which the tropomyosin may be linked to actin (McLachlan and Stewart 1976a). McLachlan and Stewart (1976b) also pointed out that the region of the amino acid sequence between residues 197 and 217 is atypical in being relatively devoid of acidic residues, in having a significantly high concentration of uncharged polar groups and a large hydrophobic surface on the broad face of the coiled-coil. They suggested that this region represented the main binding site for troponin-T. Thus much was learned from the amino acid sequence of tropomyosin but what one really wanted was an atomic structure of tropomyosin. Achievement of this goal would take Carolyn Cohen and her colleagues nearly 30 more years (Brown et al. 2001).

6.4 Muscle Structure

6.4.1 The Steric Blocking Model of Muscle Activation

During the muscle structure session at the Cold Spring Harbor meeting, a very exciting hypothesis was put forward concerning the regulation of muscle contraction. This hypothesis was called the "steric blocking model" of muscle activation. This model "seemed so neat" when it was put forward that it was quickly accepted (Squire 1994). Over the next 30 years the hypothesis would be discredited more than once only to rise again as technology advanced. The investigation of the hypothesis would draw upon major advances in three dimensional reconstructions of biological structures from electron micrographs, low-angle X-ray diffraction of living muscles using synchrotron radiation, biochemical kinetics, and atomic resolution of contractile proteins. There would be disagreements and confusion and even now all issues may not be settled.

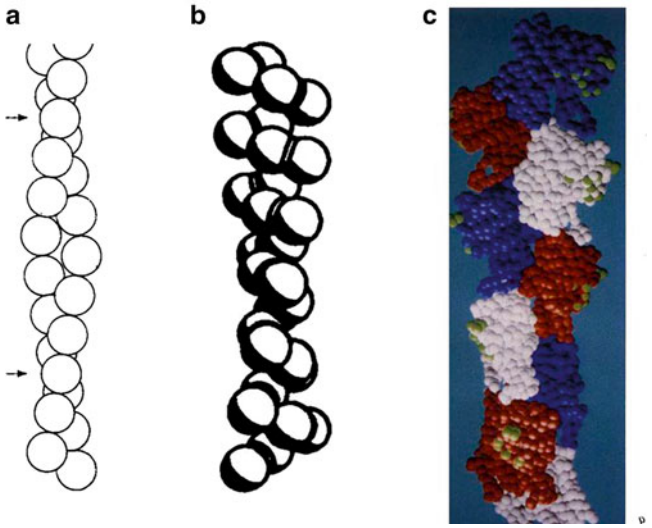


Fig. 6.10 Evolution of models of the actin filament. (**a**) Actin filament subunits drawn as spheres of diameter 5.5 nm. The centers of the subunits are helically arranged on the circumference of a cylinder. There are two twisted strands of subunits (Hanson and Lowy 1963. With permission Elsevier). (**b**) Each actin monomer consists of a dumbbell shape with its axis at about 75° to the helix axis (Egelman and DeRosier 1983. With permission Elsevier). (**c**) An atomic model of the actin filament based on the crystal structure of the actin monomer and X-ray diagram of oriented F-actin gels (Holmes et al. 1990. With permission Nature Publishing Group)

The story started well before the Cold Spring Harbor meeting. In their classic study of actin filament structure, Hanson and Lowy (1963) proposed that the actin filament consisted of two strands which were helically-wound around each other. Each strand was composed of subunits which were alike and approximately spherical (Fig. 6.10a and see Fig. 3.13). They went on to suggest a model in which there are two strands of tropomyosin which follow the actin helix and lie on the *outside* of the actin filament in the two grooves between the strands of actin monomers. In discussion of their results at a meeting of the Royal Society in 1963, Hanson and Lowy (1964) further speculated that "if tropomyosin B forms some sort of complex with actin in the intact muscle, as seems very probable, then it might exert some control over the contractile function of actin, say by masking certain monomers in the filament and thereby selecting the sites for interaction with myosin." (Hanson and Lowy 1964. With permission The Royal Society) This perceptive speculation would become a core concept in the steric blocking model. Soon thereafter Setsuro Ebashi and colleagues would discover troponin and establish the Ca^{2+} concept of muscle contraction and relaxation (see Chap. 4). The stoichiometry of the proteins of the thin filament was determined to be one troponin to one tropomyosin to seven actins (see Fig. 4.17).

The question then became how does Ca^{2+} binding to one troponin allow myosin binding to seven actin monomers. To begin to approach this question one had to

know the relationship of the contractile proteins, actin, tropomyosin, troponin and myosin to each other in three dimensions. A start in this direction was the pioneering study by Moore et al. (1970) when they determined the first, but not the last, three dimensional structure of thin filaments decorated with myosin S-1. They utilized the formalism developed by DeRosier and Klug (1968)[8] for the three dimensional (3D) reconstruction of biological structures from electron micrographs. DeRosier and Klug had demonstrated that a single electron micrograph of a helical object contained all the information necessary to reconstruct its structure in three dimensions. F-Actin, thin filaments and the complexes they form with myosin are all helical. Moore, Huxley and DeRosier determined separately the 3D maps of F-actin, thin filaments and decorated thin filaments. The electron micrographs were examined by optical diffraction and the structures reconstructed from the diffraction patterns using the programs developed by DeRosier and Klug. They then put these maps together to deduce the orientation of the myosin S-1 on the thin filament. The myosin S-1 molecules, which appeared somewhat elongated and curved, were attached to the actin filaments in a configuration in which they were both tilted and slewed with respect to the filament axis (Fig. 6.11). This was a heroic study. But there were limitations that the authors carefully discussed. The resolution was low, 2 nm at best. More serious there were concerns about the possible distortion of the structures by flattening in the stain. These possible artifacts would be a concern until the development of cryo-electron microscopy in the 1980s (see below). Nonetheless this study provided the first look at the relationship of myosin S-1 to the thin filament and was important in developing the steric blocking model.

The next important clue came from the laboratory of Jean Hanson. O'Brien et al. (1971) examined the optical diffraction patterns of electron micrographs of actin filaments in planar paracrystals. The side-by-side packing of F-actin in paracrystals provided straight filaments that allowed for better resolution of the optical diffraction patterns than observed from isolated filaments. They compared the diffraction pattern from purified actin filaments to that from "impure" actin filaments (containing troponin and tropomyosin) and related their results to the pattern attributed to the thin filaments in muscle. They found that when tropomyosin and troponin were added to actin filaments that the optical diffraction pattern changed. The first layer-line was now weak where before it was strong, and the second layer-line was strong where before it was weak. This change of relative intensities of the first and second layer-lines would occur if the additional material in the impure paracrystals were situated in the long-pitch helical grooves of the actin filament. They along with Valerie Lednev described the paracrystals of actin filaments and summarized their results at the Cold Spring Harbor meeting (Hanson et al. 1973). Thus the presence

[8] Aaron Klug (1926–) won the Nobel Prize in chemistry in 1982 for development of crystallographic electron microscopy and his structural elucidation of biologically important nucleic acid-protein complexes. Klug used methods of X-ray diffraction, electron microscopy and structural modeling to develop crystallographic electron microscopy in which a sequence of two-dimensional images of crystals taken from different angles are combined to produce three-dimensional (3D) images of the target.

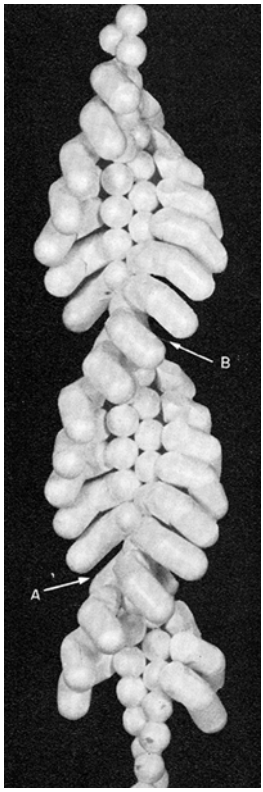


Fig. 6.11 The first model of actin filaments decorated with myosin S-1 based on three-dimensional (3D) reconstruction. The double helical filament of actin subunits has been decorated with myosin subfragment 1 (S-1). The curved myosin S-1 molecules are titled with respect to the long axis of the F-actin filament resulting in the characteristic *arrowhead* appearance of decorated actin filaments (see Chap. 3 Fig. 3.15). (Moore et al. 1970. With permission Elsevier)

of tropomyosin could be "seen" in the diffraction pattern of the actin filaments. With regard to the regulatory mechanism, they raised two possibilities. Tropomyosin might block the myosin-combining sites on the actin subunits while the muscle is relaxed and then move out of the way on activation. Alternatively tropomyosin might switch the actin subunits off by changing their conformation or their relationship to one another in the polymer during activation. The change in intensity of the second layer line attributed to the presence of tropomyosin on the actin filament would become a key element in defining the steric blocking model.

But where was tropomyosin in the 3D structure of the decorated thin filaments? James A. Spudich, Hugh Huxley and John T. Finch (1972) determined the structure of the actin-tropomyosin-troponin complex by optical diffraction and three-dimensional reconstruction from electron micrographs. They concluded that the tropomyosin strands lie off the center line in each of the two long-pitch grooves of the actin helix, in such a way that each tropomyosin molecule apparently contacts only one of the two actin helical chains which define those grooves. They then speculated that "…each long pitch chain of the two-start helix may be individually controlled by its own TM filament, perhaps by direct steric blocking of myosin attachment." (Spudich et al. 1972. With permission Elsevier) Thus the idea of steric blocking was taking shape.

The first reference to the steric blocking model was made by Hugh Huxley in abstract form in 1970 and 1971. Huxley stated (Huxley 1971. With permission Portland Press Limited):

> …recent X-ray diffraction studies on contracting muscles that show evidence for a substantial structural rearrangement in the thin filaments on activation. The latter may represent a change in position of tropomyosin, making possible the attachment of a myosin head carrying ATP (or its split products), in the configuration corresponding to the beginning of the working stroke, an attachment that is apparently blocked by tropomyosin plus troponin in the relaxed muscle.

What was the X-ray evidence that supported this hypothesis? The low angle X-ray pattern of living muscle is dominated by two helical structures, the helical arrangement of cross-bridges on the thick filaments and the helical structure of the thin filaments. Huxley found that a reflection corresponding to the second actin layer line was present in the patterns from muscles contracting at rest length, although this reflection was absent from the patterns from relaxed muscles. This was not a straight forward experiment. Given the low power of the then available X-ray sources, it required about 600–1,200 tetanic contractions over 20 h or more to accumulate enough resolution to observe a faint and diffuse change in the second actin layer line. Nonetheless this result could be explained if mass on the thin filament moved from the periphery of the thin filament toward the central axis of the filament. In light of the studies of O'Brien et al. (1971), it was suggested that this mass might represent the movement of tropomyosin.

The X-ray diffraction evidence and the quantitative model on which the steric blocking hypothesis was based was described at the Cold Spring Harbor meeting in two presentations. The first presentation was by John Haselgove (1973) who had just finished his Ph.D. thesis in the Laboratory of Molecular Biology at Cambridge. Haselgrove noted that the great disadvantage of X-ray diffraction studies on muscle was that they did not give information directly about the muscle structure. The structure must first be assumed from other studies and the structure then could be accepted if it gave a diffraction pattern like the observed pattern. He developed the computer programs to deduce the positions and intensities of the X-ray reflections from the assumed structure of the thin filament at rest and during contraction or rigor. The calculated patterns then were compared with actual observations obtained from live and rigor muscles. Haselgrove concluded that it was possible to simulate qualitatively the changes seen in the X-ray pattern by changing the position of the tropomyosin in the actin groove. What was unexpected was that the changes in diffracted intensity could be accomplished by a very small change in position of tropomyosin of about 15 Å. Thus he showed that the large change in the intensity of the second layer line that occurred when muscles contracted or passed into rigor could satisfactorily be explained by a movement of the tropomyosin molecule in the groove of the filament. The second presentation on the steric blocking model was given by Huxley (1973a). His own modeling using the Haselgrove programs led to the same conclusion. He stated that the most likely interpretation of the constancy of the actin structure was a virtually complete constancy in the internal structure of the actin monomers themselves when they are switched on or off. In his view this

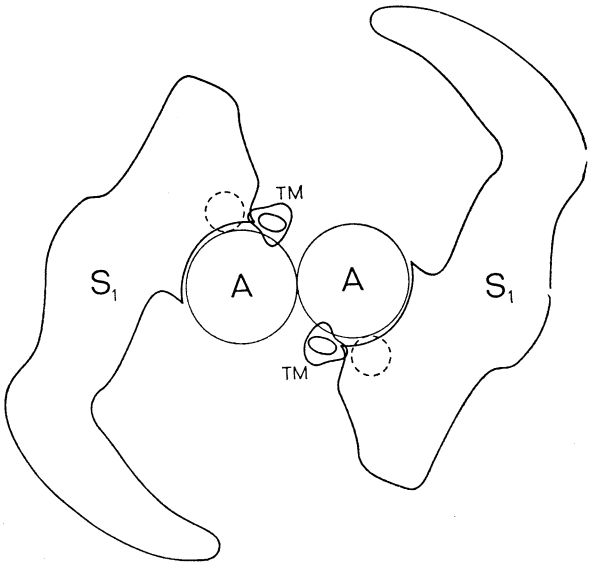


Fig. 6.12 Proposed structure of steric blocking model of muscle activation. Composite end-on view of actin-tropomyosin-myosin S-1 structure. The two tropomyosin (TM) positions correspond to the activated state (dotted shapes) and the relaxed state (solid shapes). This model assumes that the "sense" of the azimuthal position of tropomyosin with respect to the attachment site of myosin S-1 has been chosen correctly (see Fig. 6.13). The possible way in which tropomyosin could block the attachment of a cross-bridge is evident (Huxley 1973a. With permission Cold Spring Harbor Laboratory Press)

interpretation strongly favored the possibility that regulation was affected by a steric blocking mechanism involving tropomyosin. He put this conclusion in the context of the earlier structural studies of Moore et al. (1970) and Spudich et al. (1972) in a figure (Fig. 6.12). At rest tropomyosin physically or sterically blocked attachment of myosin to actin. During contraction tropomyosin moved into the groove of the thin filament allowing myosin to bind to actin and generate force. Thus Huxley and Haselgrove envisioned muscle activation as a simple on or off switch.

Both Haselgrove and Huxley acknowledged in their presentations that Parry and Squire (1973), who were not at the meeting, had simultaneously and independently performed similar calculations and came to similar conclusions. Their paper was submitted to the *Journal of Molecular Biology* 1 month before the June Cold Spring Harbor meeting. Thus all authors are considered to be the discovers of the steric blocking model though it is seems clear that the idea originated with Hugh Huxley.

Even though these authors envisioned the trigger for tropomyosin movement to be the binding of Ca^{2+} to troponin, there already were hints that the mechanism may be more complicated. Robert D. Bremel working on his Ph.D. thesis with Weber (1972) observed that in the presence of low concentrations of ATP, myosin could bind to actin in the absence of Ca^{2+} and that this binding then turned on the rest of the actin filament allowing myosin binding and ATP hydrolysis. They concluded

that rigor complexes elicited a cooperative response from actin filaments which caused all actin molecules of the filament to be turned on in the absence of Ca^{2+}. Thus the blocking of myosin attachment to actin in the absence of Ca^{2+} was not absolute at least in the presence of low ATP concentrations. Bremel et al. (1973) expanded on these results at the meeting. They showed in the presence of saturating ATP concentrations and Ca^{2+}, the acto-S-1 activity which was partially inhibited at a low S-l to actin concentration ratio became activated at a high S1 concentration. Thus there seemed to be some modulation of the on state by myosin itself. Furthermore Lowy and Vibert (1973), in a presentation at the meeting, noted a similar change in the second actin layer line when the invertebrate anterior byssus retractor muscle (ABRM) contracted. But this muscle has no troponin and thus the activation mechanism must somehow reside in the myosin molecule itself. Even though their results were consistent with tropomyosin movement during contraction, they pointed out that the extra electron density could also be introduced into the long grooves of the actin helix by some change in shape and/or orientation of the G-actin monomers.

6.4.2 Demise of the Steric Blocking Model?

Whereas there may have been some nuances to consider, in all, this discovery was very exciting and visually appealing and was soon accepted. But there would be trouble ahead and by the early 1980s it would be said (Chantler 1982) that the steric blocking model, as originally envisioned, had reached its "demise." The first problem was a structural one and it was a real surprise. Two possibilities exist for the placement of the tropomyosin strands in the actin filament grooves as shown in Fig. 6.13. In the development of the steric blocking model, all the proposers assumed the orientation shown Fig. 6.13a. But what if the tropomyosin actually existed as shown in Fig. 6.13b and how was one to know which position was correct? Seymour and O'Brien (1980) addressed this issue. They reasoned that if reconstructions were carried out on micrographs of I-segments, the assemblies of thin filaments still attached to the Z-line, the correct orientation was known because the characteristic arrowhead appearance obtained by adding S-1 to thin filaments always points away from the Z-line. When they did the experiment, they found that the tropomyosin was located as shown in Fig. 6.13b! Thus it could not possibly sterically block myosin attachment. This was a real jolt to the steric blocking model. Of course, there could still be some indirect effect of tropomyosin movement on myosin binding to actin. Finally they noted that there was no obvious way in which tropomyosin could sterically block myosin unless the shape of S-1 or its position of attachment to actin was revised.

In fact the shape of myosin S-1 and its orientation when bound to an actin filament was being revised by postdoctoral fellow Kenneth A. Taylor and Linda A. Amos (1981) at the Laboratory of Molecular Biology at Cambridge. They utilized the method of three-dimensional image reconstruction of electron micrographs to analyze the structure of thin filaments and pure F-actin filaments decorated with

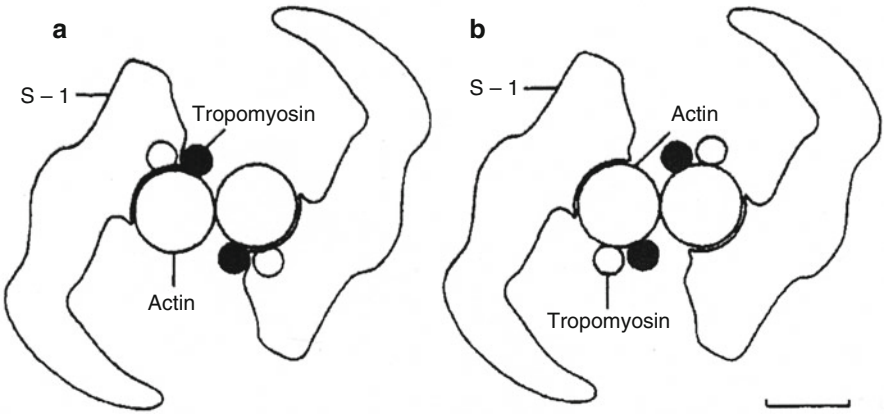


Fig. 6.13 The two ways of superimposing reconstructions of thin filaments decorated with myosin S-1 and undecorated thin filaments. (**a**) Based on Huxley (1973a). (**b**) As in (**a**) except that the thin filament has opposite orientation. The *arrowheads* are pointing out of the plane of the paper away from the *Z*-line. Tropomyosin in contracting muscles is represented by *filled circles* and in relaxed muscle by *open circles* (Seymour and O'Brien 1980. With permission Nature Publishing Group)

myosin S-1. They also made modifications in the specimen preparation that allowed the processing of straight stretches of filaments twice as long as any used by Moore et al. (1970). These modifications resulted in a corresponding improvement in the signal-to-noise ratio and the resolution. Their results led to a new assignment for the position of actin within the decorated filament structure which resulted in a radical change in the geometry of the model for myosin S-1 association with actin. Their results supported the data of Seymour and O'Brien (1980). What did these results mean for the steric blocking model? Figure 6.14 shows the new model for the interaction of myosin S-1 to actin. TMb is the position tropomyosin occupies in the inhibited filaments. In this position, tropomyosin would strongly block the actin-S-1 binding site. Thus, with respect to simple steric blocking, the reversal in relative polarity of the decorated and undecorated filament models compensates for the change in interpretation of the decorated filament structure. Tropomyosin in the activated filaments (TMa) is removed from the apparent site of direct interaction between actin and S-l. Thus the steric blocking model lived again!

It would be natural to wonder, given the potential artifacts of electron microscopy and the questionable quality of the electron microscopic preparations, if the resolution really was adequate to distinguish unambiguously actin, tropomyosin and myosin S-1. Things would get more complicated when Egelman and DeRosier (1983) determined that the actin subunits were not spherical as was assumed in the modeling of the steric blocking mechanism but rather were dumbbell shaped (Fig. 6.10b). Amos (1985) assessed the state of the art of 3D reconstruction of muscle filaments in a review. She emphasized that the quality of the reconstructed image was dependent on the quality of the electron micrographs used. Furthermore,

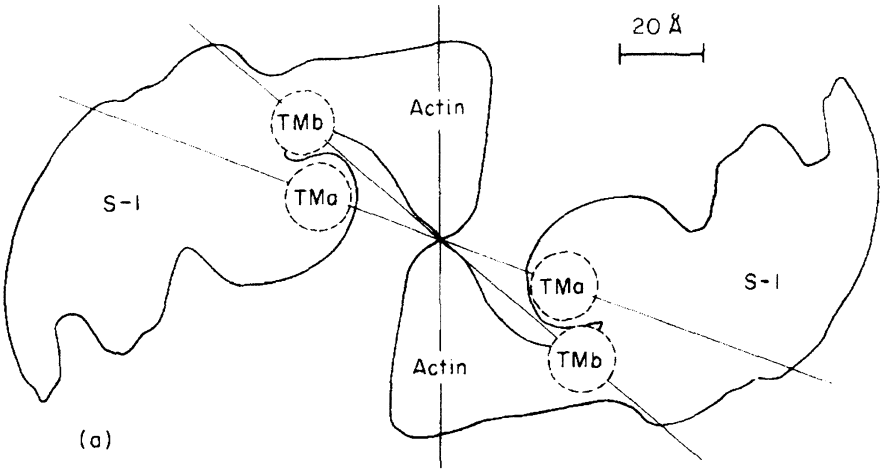


Fig. 6.14 A summary of another model of thin filaments decorated with myosin S-1 taking into account new reconstructed images and the data from previous work on the relative positions of tropomyosin. This view represents a projection down the filament axis, towards the *Z*-line, of two acto-myosin S-1 units. The probable positions of tropomyosin in the two different regulatory states of the thin filament are shown as *broken circles*. TMa is thought to be the position in activated muscle, TMb is the position in the blocked state (Taylor and Amos 1981. With permission Elsevier)

in her view, there was no obvious candidate in the reconstructions for tropomyosin. With the new model for the actin subunit, the structure thought to be tropomyosin by Seymour and O'Brien (1980) was now likely to be a subdomain of actin. Thus the structural studies to date were not a critical test of the steric blocking hypothesis. Amos emphasized what was needed. First, atomic structures of actin and myosin S-1. But also better electron microscopy was needed which could remove the potential artifacts of staining and flattening. She noted the potential of cryo-electron microscopy in this regard. Cryo-electron microscopy is a form of transmission electron microscopy where the sample is studied at cryogenic temperatures (Al-Amoudi et al 2004). It allows the observation of specimens that have not been stained or fixed in any way, showing them in their native hydrated environment. Even with negative stains, biological samples are prone to structural collapse upon dehydration of the specimen. Both of these major advances already were on the horizon.

But there was more serious trouble for the steric blocking model in the difficult 1980s. Chalovich et al. (1981) at the NIH found that in solution at low ionic strength (0.018 M) (physiological ionic strength is in the 0.17–0.20 M range), the binding of myosin S-1.ATP or S-1.ADP.Pi to regulated actin (F-actin + tropomyosin + troponin) was independent of Ca^{2+} concentration. Nonetheless the actomyosin ATPase was activated at high Ca^{2+} concentration and inhibited at low Ca^{2+} concentration as expected. These results strongly suggested that Ca^{2+} and troponin-tropomyosin regulated the actomyosin ATPase rate by affecting a kinetic step other than the attachment step. The results, of course, were totally contradictory to predictions of the

steric blocking model. The authors were careful to point out that the effects of ionic strength on their results were unknown. Chalovich and Eisenberg (1982) confirmed their previous results and extended them to a moderate ionic strength of 0.050 M. Bernhard Brenner and Mark Schoenberg in the Eisenberg laboratory followed up on these biochemical studies with an investigation of the properties of chemically skinned muscle fibers at rest and at low ionic strength (Brenner et al. 1982). A skinned muscle fiber in low Ca^{2+} concentration shows little resistance to strength. This result would seem to be incompatible with the notion that the cross-bridges could bind to thin filaments at low Ca^{2+} concentration. They reasoned that the binding was weak and thus very transient. If this was true, then the more rapidly the fibers were stretched the higher the probability of sensing attached cross-bridges. This is what they found. At an ionic strength of 0.02 M, stiffness of the resting fiber became very large with rapid stretches. Furthermore the effect was dependent on myofilament overlap, decreasing at long sarcomere length. At an ionic strength of 0.170 M, the rapid stiffness increase was greatly depressed, suggesting that cross-bridge binding was greatly inhibited in the resting state or in such a rapid equilibrium that it was almost undetectable with their methods. Thus the biochemical and physiological results were in agreement that at low ionic strength, cross-bridges attach to the thin filament in a rapid equilibrium even in the presence of low Ca^{2+} concentrations. But what of the situation at physiological ionic strength? Brenner et al. (1982) argued that it was unlikely that troponin-tropomyosin caused relaxation by two completely different mechanisms at low ionic strength and at physiological ionic strength. Therefore they concluded that the inhibition of a kinetic step between two attached states probably played a major role in relaxation at physiological ionic strength even if the equilibrium between weakly attached and unattached cross-bridges was shifted towards unattached states at higher ionic strength.

Twenty years later Hugh Huxley (Chalovich 2002) would comment with regard to cross-bridge binding at low Ca^{2+} concentrations that he was never been convinced that that was the case at normal ionic strength. Nonetheless there was a lot of uncertainty about a steric blocking mechanism in the 1980s.

6.4.3 Time Resolved X-ray Diffraction Evidence During Muscle Contraction Supports the Steric Blocking Model

There was a X-ray diffraction study in the 1986 that provided support for the concept of the steric blocking model as originally proposed (Kress et al. 1986[9]). Huxley (1996) has called this a "thrilling experience" and his "favorite experiment". What was the thrilling experiment? It started earlier with a major technological advance. Kenneth C. Holmes was able to show in 1971, with Gerd Rosenbaum and John Witz, that electron synchrotrons could be used as a powerful X-ray source for diffraction experi-

[9] Marcus Kress died tragically in a hiking accident before this paper was published.

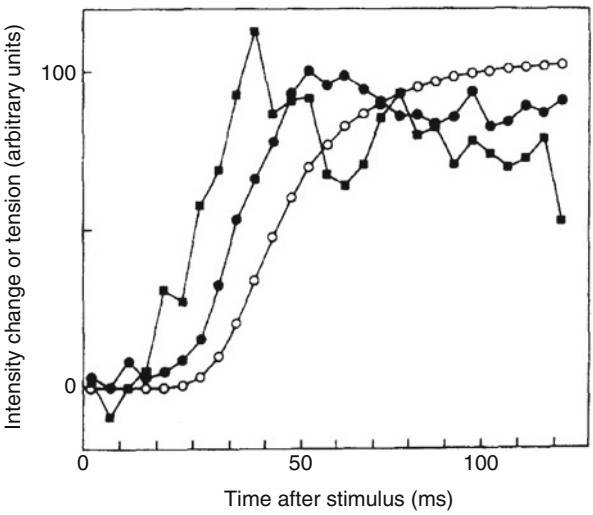


Fig. 6.15 Time course of the increase in intensity of the second actin layer-line (an indicator of tropomyosin position on the thin filament) (*filled squares*), the equatorial reflection (an indicator of cross-bridge movement toward the thin filament) (*filled circles*) and isometric force (*open squares*). Note that the tropomyosin movement leads cross-bridge movement which leads force development (Kress et al. 1986. With permission Elsevier)

ments (Huxley and Holmes 1997; Holmes 2010). This discovery meant that the X-ray diffraction studies on living muscles now could be conducted with millisecond time resolution. The idea was simple. If the steric blocking model was correct, then it was necessary that tropomyosin moved before cross-bridges attached and force was generated. The time course of the increase in intensity of the second actin layer line was taken as an indication of the time course of tropomyosin movement after stimulation. The time course of the change in the equatorial reflections was taken as an indicator of cross-bridge movement toward the thin filaments (see Chap. 3). Also the time course of isometric force was measured in twitches and tetani. An example is shown in Fig. 6.15. Instead of 500–1,000 contractions over 20 h or so as was required previously, these results were obtained from 50 twitches in 12.5 s. The increase in intensity in the second layer line clearly preceded the equatorial reflection change which preceded force. They concluded the regulatory mechanism appeared to exert a large measure of control over the initial attachment of the cross-bridges following activation in an intact muscle but this was not incompatible with it also exerting an important influence at other steps in the cycle. This then was the thrilling result that provided Hugh Huxley his favorite experiment. Whereas the results were powerful, certainly proving a change in thin filament structure, and consistent with the steric blocking model, they were not sufficient in themselves to prove the hypothesis.

6.4.4 Dynamic Equilibrium: Three State Model of Actomyosin ATPase Activation

In 1993 Michael Geeves in the department of biochemistry, University of Bristol, along with Daniel McKillop published a biochemical paper that has become influential in thinking about the activation of muscle (McKillop and Geeves 1993). They

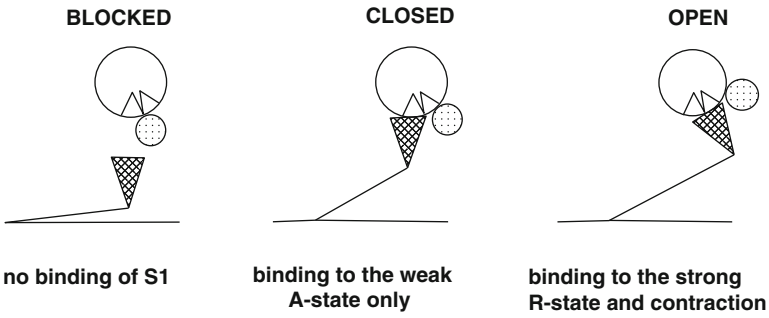


Fig. 6.16 A diagrammatic representation of the three-state model of activation of myosin ATPase by regulated thin filaments. In the blocked state, tropomyosin prevents all binding of myosin S-l to actin. In the closed state, troponin moves to uncover actin binding sites which allow myosin S-1 to form a relatively weakly bound state. In the open state the full interaction of myosin S-1 with actin occurs. (McKillop and Geeves 1993. With permission Elsevier)

examined cooperative binding of myosin S-1 to regulated actin with both steady state and transient techniques at an ionic strength of about 0.14 M. In order to explain their results, they proposed a model (Fig. 6.16) in which the thin filament was assumed to exist in a dynamic equilibrium among three states: a "blocked state" which was unable to bind myosin S-1, a "closed state" which could only bind myosin S-1 relatively weakly (electrostatic binding) and an "open state" in which the myosin S-1 could both bind and undergo an isomerization to a strongly bound (hydrophobic interactions) state that would accelerate the ATPase and would be the force generating conformation. In the absence of Ca^{2+}, about 80% of the actin binding sites would be in the blocked state, about 20% in the closed state and less than 2% in the open. In the presence of Ca^{2+}, about 5% of the sites would be in the blocked state, about 80% in the closed state and about 20% in the open state. Calcium binding alone was not sufficient to turn on the filament. Cooperative myosin S-1 binding was required to switch the filament completely into the open state. Thus this three state model had elements of steric blocking in the "blocked" state and also cooperative activation. This model is consistent with the notion that the tropomyosin molecule is flexible, subject to the thermal energy of the environment, and when linked to actin, would exist in multiple conformational states about an average position (Phillips et al. 1986). Thus regulation of contraction is seen as a dynamic and statistical mechanism.

One problem which remained unresolved was the work of Chalovich and Eisenberg (1982) which led to a serious challenge of the original steric blocking model. In the discussion of their paper, McKillop and Geeves (1993) noted that when they duplicated the low ionic conditions of Chalovich and Eisenberg (0.015 M), the rate of the myosin S-1 association reaction became independent of Ca^{2+} even though the ATPase was still Ca^{2+} dependent. They concluded that the fraction of the actin binding sites in the blocked state was ionic strength dependent, decreasing with decreasing ionic strength. The implication was that steric blocking did

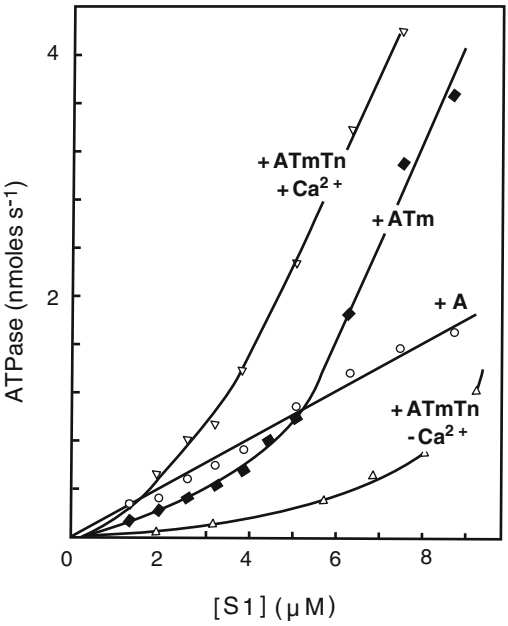


Fig. 6.17 Effect of actin, tropomyosin, troponin and Ca^{2+} on the myosin ATPase as a function of myosin S-1 concentration. Myosin S-1 ATPase with actin alone (+A), with actin-tropomyosin (+ATm), with actin, tropomyosin, and troponin ± Ca^{2+} (+ATmTn + Ca^{2+} and +ATmTn–Ca^{2+}). Note that in the case of regulated actin with Ca^{2+}, the myosin S-1 ATPase rate is slightly higher than for actin alone at low myosin S-1 concentrations but increases steeply at higher myosin S-1 concentrations. Thus the myosin S-1 ATPase rate for regulated actin + Ca^{2+} is enhanced over that for F-actin alone at any myosin S-1 concentration and is cooperatively increased at higher myosin S-1 concentrations. (Gordon et al. 2000. With permission The American Physiological Society)

occur in low Ca^{2+} concentration at high, near physiological, ionic strength but not at low ionic strength. Finally McKillop and Geeves noted that their results were compatible with the X-ray diffraction studies of Kress et al. (1986).

The studies of Lehrer and Morris (1982) at the Boston Biomedical Research Institute also emphasized the role of strong cross-bridge activation of the thin filament during cross-bridge cycling. They investigated the effects of myosin S-1 concentration on ATPase activity in the presence of actin, actin + tropomyosin, or actin + tropomyosin + troponin ± Ca^{2+} (Fig. 6.17). Their studies showed that, compared with myosin S-1 ATPase with actin alone, the ATPase activity with actin + tropomyosin + troponin + Ca^{2+} was slightly increased at low myosin S-1 concentration but dramatically increased at high myosin S-1 concentration. Thus, during cross-bridge cycling, it appeared that even in the presence of Ca^{2+}, a sufficient number of cross-bridges must be strongly bound to move the tropomyosin to a position where the actin in the regulated filament could bind strongly to myosin S-1 and activate the ATPase. These results implied that Ca^{2+} binding was sufficient to activate for low myosin S-1 but that cooperative binding of myosin S-1 could

further activate at higher myosin S-1 concentration. This behavior of myosin S-1 concentration on regulated thin filaments was expected based on the McKillop and Geeves (1993) three state model. In their model, a fraction of the actin sites would be in the open state for strong myosin S-1 binding in the presence of Ca^{2+}, but even more would be in the open state in the presence of strongly bound neighboring myosin S-1.

An important caveat of the analysis of McKillop and Geeves (1993) was that one should think of the actin states existing in a dynamic equilibrium and not as static states. From the viewpoint of possible effects of tropomyosin, this model meant that tropomyosin should be thought of as oscillating among three states, spending more or less time in one state or another depending on conditions. Thus the activation could not be thought of as a simple off or on switch. (See Chap. 8 for physiological studies on the role of strong cross-bridge binding and muscle activation.)

6.4.5 Where is Tropomyosin on the Actin Filament? Is its Position Calcium and Cross-bridge Dependent?

What was needed was an agreed upon identification of tropomyosin on the thin filament in electron micrographs and evidence of its movement in a Ca^{2+} and possibly cross-bridge dependent manner. The 3D reconstruction studies of thin filaments decorated with myosin S-1 before 1985 were contradictory and confusing. Agreement finally came on the location of tropomyosin in the thin filament from the studies of Ronald A. Milligan and colleagues at the Research Institute of Scripps Clinic in California. Milligan et al. (1990) utilized cryo-electron microscopy to examine unstained preparations in their natural hydrated state and helical image processing with extensive image averaging. Their results revealed the structure of F-actin and the binding sites of tropomyosin and myosin S-1 on actin at a resolution of 2.5–3.0 nm. They concluded that in the presence of Ca^{2+} and myosin S-1, tropomyosin resided about 3.8 nm from the central axis of the actin filament. Thus it required 20 years to reach a basic agreement of the 3D structure of the decorated thin filaments, albeit at relatively low resolution.

During the same year, Holmes (Fig. 6.18) and his colleagues at the Max Planck Institute for Medical Research, Heidelberg published the crystal structure of actin (Kabsch et al. 1990) and deduced the first atomic model of the actin filament (Fig. 6.10c) (Holmes et al. 1990). These were milestone achievements in the history of muscle contraction and motility. Thus the shape of the actin monomer in models of the actin filament had evolved from a sphere (Fig. 6.10a), to a dumbbell (Fig. 6.10b) to a disk (Fig. 6.10c). (See Chap. 9 for details about the discovery of the crystal structure of actin and building of the model of the actin filament.) Muscle structure now had entered the atomic age.

But these experiments left open the question of possible tropomyosin movement under different conditions. This difficult question was taken up by a collaboration

Fig. 6.18 Kenneth C. Holmes (1934–) obtained his Ph.D. in 1959 at Birkbeck College London working on the structure of tobacco mosaic virus with Rosalind Franklin. Franklin died during this period and the work was completed with Aaron Klug. After post-doctoral studies (1960–1961) at Children's Hospital Boston with Donald Caspar where he also started to work on muscle structure with Carolyn Cohen, he returned to the Laboratory of Molecular Biology in Cambridge. Here he developed methods for the analysis of structures by X-ray fiber diffraction. He worked with Aaron Klug on the structure of tobacco mosaic virus and with Hugh Huxley and J.D. Pringle (Oxford) on muscle. In 1968 he moved to Heidelberg to open the Department of Biophysics at the Max Planck Institute for Medical Research where he remained as director until his retirement in 2003. During this time he completed the structure of tobacco mosaic virus by fiber diffraction and solved the structures of a number of protein molecules by protein crystallography including the structure of the muscle protein actin. He solved the structure of the actin filament using X-ray fiber diffraction data in combination with crystal data. Increasingly he has worked on the molecular mechanism of muscle contraction. In 1970 he pioneered the use of synchrotron radiation as a source for X-ray diffraction. He was elected as a fellow of the Royal Society in 1981. *Left*: Ken and wife Mary Holmes, Christmas 1960, Boston, Mass (Holmes 2010. With permission Elsevier). *Right*: Holmes and wife Mary, circa 2011 (Photo: courtesy K. Holmes)

among William Lehman at Boston University, Roger Craig at the University of Massachusetts and Peter Vibert at Brandeis University (1994). Using 3D helical reconstruction techniques of *limulus* thin filaments which exhibited the same properties as vertebrate thin filaments but which where more ordered in low Ca^{2+} conditions, they were able to resolve tropomyosin strands on filaments both in the presence and absence of Ca^{2+} (Fig. 6.19). In the absence of Ca^{2+}, tropomyosin was found to cover the outer domain of actin over known myosin binding-sites (Fig. 6.19, left). Moreover, tropomyosin moved toward the inner domain of actin and away from these sites in Ca^{2+} treated filaments (Fig. 6.19, right). They concluded that the results were consistent with the tenets of the steric blocking model and for the first

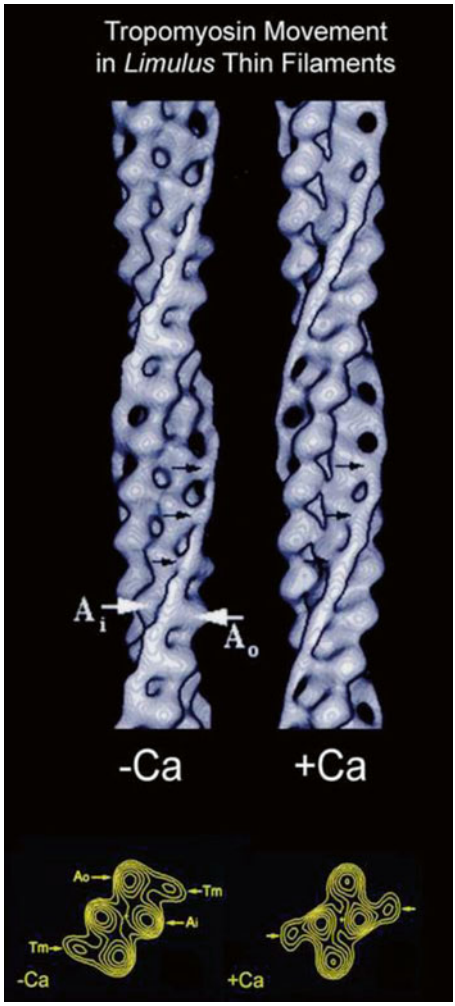


Fig. 6.19 Calcium dependent movement of tropomyosin on actin filaments. *Limulus* thin filament reconstructions (*Top*, surface views; *Bottom*, helical projections [i.e. projections down the two long-pitch actin strands]) showing tropomyosin strands (indicated by *arrows*) on the inner edge of the outer domain of actin (Ao) at low Ca^{2+} (*Left*) and on the outer edge of the inner domain of actin (Ai) at high Ca^{2+} (*Right*). The pointed end of the filament is facing up (Lehman and Craig 2004. With permission Springer)

time corroborated the proposed tropomyosin movement. Vibert et al. (1997) went on to find that the tropomyosin strands in thin filaments existed in three distinct positions in the low Ca^{2+}, high Ca^{2+} and myosin S-1 bound states. In the presence of Ca^{2+}, tropomyosin moved 25° away from its low Ca^{2+} position, exposing most, but not all, of the previously blocked myosin-binding sites. Saturation of filaments with myosin heads produced a further 10° shift in tropomyosin position, thereby exposing the entire myosin-binding site. They concluded that the results suggested that full switching-on of thin filaments by reversal of steric-blocking requires both Ca^{2+} and the binding of myosin heads, acting in sequence. Thus this structural information was consistent with the proposed three state model of muscle activation by McKillop and Geeves (1993). Furthermore it was consistent with the steric blocking model.

Their conclusions with regard to tropomyosin blocking the actin-myosin interface were derived from a near-atomic model of myosin S-1 decorated actin (Rayment et al. 1993a). This model was generated by fitting the crystal structures of actin (Kabsch et al. 1990) and myosin S-1 (Rayment et al. 1993b) into the lower resolution 3D reconstruction of thin filaments (Milligan et al. 1990) (See Chap. 9 for details of the discovery of the crystal structure of myosin S-1).

Holmes (1996) proposed a reconciliation of the sometimes confusing terms of weak binding and strong binding as they apply to myosin S-1 binding to actin. He suggested that the Ca^{2+} independent interaction of actin with myosin observed at low ionic strength which has been called weak is actually a collision complex which is a non-stereospecific, electrostatic interaction. In the view of Lehman and Craig (2004) the collision complexes formed electrostatically between charged sites on actin and myosin may reduce the diffusional freedom of myosin heads near actin but these interactions do not participate in cross-bridge cycling. Thus they are not dealt with in the three-state models. Holmes proposed that this collision complex preceded a weak, low affinity, stereospecific interaction of actin and myosin followed by a strong, high affinity, stereospecific interaction of actin and myosin. Thus he suggested using the terms weak and strong for stereospecific interaction and not collision interactions.

A question remained: was the X-ray diffraction data from muscles consistent with the new structural information and with the three-state model of steric blocking? In a collaborative study, Katrina J. V. Poole in the Holmes research group examined this problem by measuring the X-ray diffraction pattern using skinned muscle fibers in different conditions. Using powerful synchrotron radiation, they examined the X-ray diffraction pattern in the fibers at rest, stretched to a non myofilament overlap length in the presence of Ca^{2+} and at partial overlap where force was generated in the presence of Ca^{2+} (Poole et al. 2006). They compared the observed X-ray patterns to models of the thin filament by incorporating current atomic-resolution structures of actin, tropomyosin, troponin and myosin subfragment-1 and fitting these atomic coordinates to electron micrograph reconstructions. Taking the low Ca^{2+} (EGTA) state as a point of reference, their modeling of the X-ray data and the previous structural studies were consistent with an average movement of tropomyosin of 15–25° in the high Ca^{2+} state and a further movement of 10° when cross-bridges bind and generate force. These results were consistent with the three-state model of activation. They found no grounds for believing that actin itself undergoes substantial conformational changes when Ca^{2+} binds to troponin. Furthermore the modeling showed that there was substantial steric interference of myosin binding to actin by tropomyosin in the low Ca^{2+} state. In a sense this study brought investigation of the steric blocking model “full circle”. Haselgrove (1973) and Huxley (1973a) both examined the X-ray patterns of muscle under non overlap conditions and generated a model to explain their results in terms of tropomyosin movement. The same experiment was done again 30 years later with improved technology and better structural information and more definitive results.

Thus over a period of 30 years the steric blocking model has been in and out of fashion only to return to fashion once again. The activation of actomyosin

ATPase and muscle contraction is certainly more complex than originally proposed. Activation is not a simple on or off switch involving the movement of a rigid tropomyosin cable but rather likely involves three states in a dynamic mechanism where myosin itself cooperatively activates contraction. Technical improvements in methods for analyzing 3D structure in electron microscopic images and collecting fiber X-ray diffraction data, coupled with the availability of crystal structures of actin, tropomyosin, troponin and myosin S-1 have allowed a validation and elucidation of the molecular details of the steric regulatory mechanism. Lehman and Craig (2004), in a tribute to Jean Hanson, have written a lucid history of the steric blocking model from the view point of the structural biologist. Gordon et al. (2000) have written a comprehensive review of muscle activation which emphasizes the physiological aspects of the problem (see Chap. 8).

6.4.6 Sarcoplasmic Reticulum, Transverse Tubules and Excitation-Contraction Coupling

At the time of the meeting it was well understood that skeletal muscle excitation travelled down the transverse tubules and caused the release of Ca^{2+} stored in the terminal cisternae of the sarcoplasmic reticulum. This Ca^{2+} then bound to troponin to cause contraction and ultimately was returned to the sarcoplasmic reticulum by an ATP requiring Ca^{2+} pump (see Chap. 4 for details). But there were numerous uncertainties, particularly concerning the mechanism of Ca^{2+} release from the sarcoplasmic reticulum. Ultrastructural analysis by Clara Franzini-Armstrong in 1970 showed that there were "SR feet structures" that appeared to connect the sarcoplasmic reticulum to the transverse tubules (Fig. 4.8) but there was no clue as to what they might be or what they might do. There were only two presentations at the meeting that related to the sarcoplasmic reticulum and one related to the transverse tubules. There were no presentations describing how the electrical activity in the transverse tubules might cause the release of Ca^{2+} from the sarcoplasmic reticulum during muscle activation. The lack of attention to this important topic no doubt reflected the state of knowledge in the field at the time. Endo (1977) in a comprehensive review of Ca^{2+} release from the sarcoplasmic reticulum noted that questions about the kind of information transmitted from the T system to the SR and the mechanism by which Ca^{2+} was released from the SR were "almost entirely unanswered". He called this transmission the "least understood" part of the entire series of events in excitation-contraction coupling.

Nonetheless there was an important presentation at the meeting made by a young Canadian investigator who would go on to become one of the most prominent investigators in the sarcoplasmic reticulum field. David MacLennan (Fig. 6.20), from the Banting and Best Department of Medical Research at the University of Toronto, described his research goals as the resolution, characterization, and reconstitution of proteins involved in the Ca^{2+} transport process of sarcoplasmic reticulum

Fig. 6.20 David MacLennan (1937–), a Canadian biochemist and geneticist, received his Ph.D. in 1963 from Purdue University and was a postdoctoral fellow and assistant professor at the University of Wisconsin. In 1969 he went to the Banting and Best Department of Medical Research at the University of Toronto. MacLennan has made fundamental contributions to the understanding of the mechanism of ion transport by sarcoplasmic reticulum (SR) Ca^{2+} pumps [sarco(endo)plasmic reticulum calcium-ATPase (SERCA)], the storage of Ca^{2+} in the SR associated with lumenal proteins (calsequestrin, calreticulin and sarcalumenin) and the release of Ca^{2+} from the SR by Ca^{2+} release channels (ryanodine receptors). He has led teams that defined the genetic basis for the human skeletal muscle diseases, including malignant hyperthermia and central core disease (both associated with mutations in the ryanodine receptor) and Brody disease (associated with defects in SERCA) and he has demonstrated that mutations in phospholamban, a regulator of the Ca^{2+} pump, can cause cardiomyopathy. He has won numerous awards and was elected as a fellow of the Royal Society of Canada (1985) and Royal Society of London (1994) and foreign associate of the National Academy of Sciences (2001). Photo: courtesy D. MacLennan

(MacLennan et al. 1973). This research endeavor would carry on for more than 40 years leading to numerous major discoveries. At the meeting he described two recent important contributions of his laboratory. First MacLennan (1970) purified and characterized the properties of the ATPase from isolated sarcoplasmic reticulum. This Ca^{2+} pump protein had a molecular weight of about 100 kDa. The properties of the purified enzyme appeared to be the same as those of the enzyme in sarcoplasmic reticulum. In both cases the ATPase activity required Mg^{2+} and was stimulated by Ca^{2+}. The purified enzyme catalyzed an ATP-ADP exchange and was phosphorylated by ATP labeled in the terminal position with ^{32}P. MacLennan and Wong (1971) followed up this work with a major discovery. They isolated an acidic protein from sarcoplasmic reticulum vesicles of molecular weight about 44 kDa.

This protein bound Ca^{2+} with a high capacity (about 40 mmol Ca^{2+}/mol protein) and with a relatively low affinity. They concluded that the protein was likely unique to the sarcoplasmic reticulum and that it existed in the interior of the sarcoplasmic reticulum vesicles. They suggested that the protein played a role in sequestering Ca^{2+} and thus maintaining a low free Ca^{2+} concentration within the sarcoplasmic reticulum. MacLennan and Wong named the protein "calsequestrin"[10]. Calsequestrin would become one of the main protein components defining sarcoplasmic reticulum function (see Chap. 8).

6.4.7 Calcium Pump of the Sarcoplasmic Reticulum

Given the biological importance of ion pumps, it was only natural that the early work with isolated sarcoplasmic reticulum vesicles concentrated on the mechanism of sequestration of Ca^{2+} by an ATP driven process. By 1970 the calcium concept of muscle contraction and relaxation was well established. As a result of the classic studies of Ebashi, Hasselbach and Makinose and Weber the following facts were known (see Chap. 4). The relaxing factor of muscle was due to ATP dependent Ca^{2+} transport into the sarcoplasmic reticulum of muscle. This transport involved a phosphorylated intermediate of the Ca^{2+} ATPase. Two moles of Ca^{2+} were transported for each mole of ATP hydrolyzed. Furthermore the calcium pump could lower the free Ca^{2+} concentration to a level that was consistent with muscle relaxation and it operated rapidly enough to explain the kinetics of muscle relaxation. The accumulated Ca^{2+} was stored in the terminal cisternae of the sarcoplasmic reticulum (Costantin et al. 1965).

By the end of the 1970s there was a well defined kinetic scheme for the mechanism of Ca^{2+} transport by the Ca^{2+} ATPase (for reviews see de Meis and Vianna 1979; Martonosi and Beeler 1983) (Fig. 6.21). This sequence includes two distinct functional states of the enzyme, E and *E. The high affinity Ca^{2+} binding site in the E form faces the outer surface of the vesicle and has a K_m for Ca^{2+} in the range of 1 μM at pH 7.0. In the *E form the affinity for Ca^{2+} binding is low and the Ca^{2+} binding site faces the inner surface of the vesicle and has an apparent K_m for Ca^{2+} of about 1 mM at pH 7.0. The process involves the following elementary reaction steps where NTP is nucleoside triphosphate and NDP is nucleoside diphosphate. The enzyme is activated by Ca^{2+}. The physiological substrate is Mg·ATP. After Ca^{2+} and NTP bind to the enzyme (steps 1–2), a rapid phosphorylation of the enzyme occurs and NDP is released on the cytoplasmic side of the membrane (step 3). This step occurs 20–40 times faster than the steady-state rate of ATP hydrolysis or Ca^{2+} transport (Froehlich and Taylor 1975; Hasselbach 1978). The phosphate is covalently attached to the enzyme. Formation of the phosphorylated enzyme intermediate leads to decreasing Ca^{2+} affinity (step 4), followed by the eventual release of Ca^{2+}

[10] In the acknowledgement section of their paper, MacLennan and Wong (1971) state that the name calsequestrin was suggested by their colleague Philip Seeman, a neuropharmacologist at the University of Toronto.

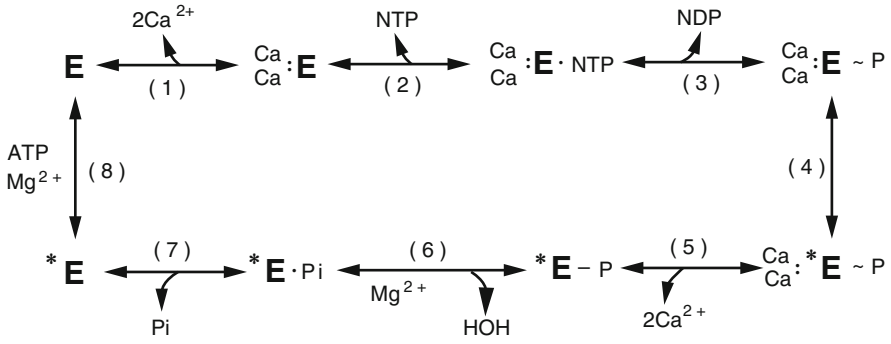


Fig. 6.21 Proposed reaction sequence involved in the process of substrate hydrolysis and Ca^{2+} transport by the sarcoplasmic reticulum Ca^{2+} pump. See text for details (de Meis and Vianna 1979. With permission Annual Reviews)

(step 5) inside the lumen of the sarcoplasmic reticulum. This change in enzyme affinity was shown directly by Ikemoto (1975, 1976) at the Boston Biomedical Research Institute who utilized a preparation of purified Ca^{2+} ATPase. He observed that concomitant with the formation of the phosphorylated enzyme (E–P) bound Ca^{2+} was released from the enzyme and concomitant with the dephosphorylation Ca^{2+} rebound to the enzyme. The hydrolysis of the phosphorylated intermediate is promoted by Mg^{2+}, requires membrane phospholipids, and yields inorganic phosphate (P_i) (steps 6–7). Reaction 8 can be the rate-limiting step depending on the NTP used. The Ca^{2+} transport is reversible and permits the synthesis of 1 mol NTP from NDP and P_i for each two Ca^{2+} released across the membrane (Hasselbach 1978).

By the end of the 1970s a major effort was directed toward the study of the conformational changes in the enzyme that accompanied transport of Ca^{2+} by analyzing the reactivity of various side-chain groups in different phases of the Ca^{2+} transport cycle. This effort would be greatly enhanced once the amino acid sequence of the calcium pump was known in the 1980s and particularly when the three-dimensional structure of the pump was solved in 2000 (see Chap. 8).

Regulation of the calcium pump: At first it was assumed that all Ca^{2+} pumps in muscle were the same but this was shown to be incorrect in the 1970s in a dramatic way. It had long been known that catecholamines accelerated relaxation in the heart. In 1972 Arnold Katz and his colleagues Michihiko Tada, Madeleine Kirchberger and Doris Repke demonstrated that cyclic AMP plus cyclic AMP-dependent protein kinase accelerated Ca^{2+} uptake by sarcoplasmic reticulum vesicles isolated from the heart (Kirchberger et al. 1972). The substrate for the cyclic AMP-dependent protein kinase was not the sarcoplasmic reticulum Ca^{2+} pump but rather a small protein of molecular weight of about 20 kDa. This protein, called phospholamban[11], inhibited

[11] The name phospholamban was suggested by Arnold Katz's wife Phyllis Katz who was educated in the classics. The term is derived from "phosphate" and a Greek work which means "to receive or to seize" (Katz 1998).

the calcium pump and its phosphorylation removed the inhibition. This system was absent in skeletal muscle. These results were viewed with a great deal of skepticism by the skeletal muscle biochemists who at the time were working hard to isolate highly pure components of the sarcoplasmic reticulum (see below). It was well known that the sarcoplasmic reticulum vesicles isolated from heart muscle were less pure than those isolated from skeletal muscle. Katz (1998) remembered that these results were "vehemently challenged" at several national and international meetings. But history has proven the correctness of the data and emphasized that not all Ca^{2+} pumps are the same. We now know that there are various isoforms of the muscle Ca^{2+} pumps.

Relaxation of fast-twitch versus slow-twitch muscle fibers and calcium sequestration: At the end of the 1960s there began an acceleration in the interest in characterizing skeletal muscle fiber types in terms of their functional, biochemical and structural properties. It had been known for over a century that some muscles contracted and relaxed more rapidly than other muscles. This property came to be attributed to differences in individual muscle fibers. It was natural to consider that the faster relaxation of fast-twitch muscle fibers than slow-twitch muscle fibers related in some way to Ca^{2+} removal by the sarcoplasmic reticulum. The obvious possibilities were that compared to slow-twitch fibers, the fast-twitch fibers had: a) more sarcoplasmic reticulum Ca^{2+} pumps and/or b) intrinsically faster Ca^{2+} pumps. Brenda R. Eisenberg (now Brenda Russell) and Aileen M. Kuda (1975) employed stereological techniques to quantitatively estimate the surface area and volume of muscle cells and cellular organelles in fast-twitch and slow-twitch fibers from the guinea pig. Relative to the volume of the myofibrils, they found that the surface area of the sarcoplasmic reticulum was 50% greater in fast-twitch muscle fibers than in slow-twitch fibers. David MacLennan and colleagues (Jorgensen et al. 1979) utilized immunofluorescent staining and phase contrast microscopy to show the distribution and intensity of the Ca^{2+} ATPase and calsequestrin in fast-twitch and slow-twitch muscle fibers of the rat. The Ca^{2+} ATPase staining was uniformly distributed throughout the sarcoplasmic reticulum whereas the calsequestrin staining was localized to the A/I junction (where the terminal cisternae are located). Furthermore the Ca^{2+} ATPase staining intensity was higher in fast-twitch fibers than in slow-twitch fibers. This result suggests a greater density of Ca^{2+} pumps in the sarcoplasmic reticulum of fast-twitch skeletal muscle. Later reconstitution experiments would show that the intrinsic pumping rate of fast-twitch Ca^{2+} pumps was about twofold faster than that of slow-twitch Ca^{2+} pumps (Sumbilla et al. 1999). Taken together these results show that fast-twitch fibers relax more rapidly than slow-twitch fibers because they have more sarcoplasmic reticulum, a greater density of Ca^{2+} pumps in the sarcoplasmic reticulum and intrinsically faster Ca^{2+} pumps. Besides these factors, the fast-twitch muscle fibers also contain more parvalbumin (Heizmann et al. 1982), a soluble protein which has been proposed to promote muscle relaxation (Gerday and Gillis 1976; Pechere et al. 1977) (see Sect. 6.3, above and Chap. 8). Thus multiple factors lead to a more rapid accumulation of Ca^{2+} during relaxation in fast-twitch muscle fibers compared to slow-twitch muscle fibers.

By the early 1980s much had been learned about the Ca^{2+} pump and muscle relaxation but there would be an explosion of knowledge with the introduction to gene cloning and mutagenesis techniques into muscle research and great advances in structural biology. Multiple isoforms the Ca^{2+} pump would be discovered along with diseases related to the Ca^{2+} pump. The muscle Ca^{2+} pumps now are known as sarco(endo)plasmic reticulum or SERCA pumps. For a recent review of the Ca^{2+} pumps in health and disease, see Brini and Carafoli (2009).

6.4.8 Ryanodine Receptor: Calcium Release Channel of Sarcoplasmic Reticulum

The early work with isolated sarcoplasmic reticulum vesicles focused on the active transport of Ca^{2+}. Only later was research also directed toward the release of Ca^{2+} from the vesicles. Nonetheless an early study that foreshadowed some of the later work was conducted by Alan S. Fairhurst (1929–1990) and Wilhelm Hasselbach (1970) at the Max-Planck-Institut fur Medizinische Forschung in Heidelberg. Earlier Jenden and Fairhurst (1959) had written a review of the pharmacology of the plant alkaloid ryanodine which was used as an insecticide. Insects exposed to ryanodine died in muscle contractures. In the case of vertebrate skeletal muscle, the evidence favored the view that ryanodine specifically interfered with muscle relaxation. It had been suggested that the interference with relaxation was due to an inhibition of the Ca^{2+} pump of the sarcoplasmic reticulum. Fairhurst and Hasselbach (1970) examined the possibility that Ca^{2+} efflux from isolated sarcoplasmic reticulum vesicles might be affected by ryanodine. Calcium efflux was measured from two different sarcoplasmic reticulum fractions isolated from rabbit skeletal muscle in the absence of ATP. These fractions were referred to as the light and heavy fraction based on centrifugation results. When loaded with Ca^{2+}, efflux was stimulated by ryanodine and caffeine from the heavy fraction but much less so from the light fraction. Both fractions contained the Ca^{2+} pumps. They concluded (Fairhurst and Hasselbach 1970. With permission John Wiley & Sons Inc):

> The effects of ryanodine and of caffeine are of particular interest, since it has been thought that these drugs inhibit net calcium uptake by inhibiting the calcium pump…but the present results suggest an alternative site of action associated with an altered membrane permeability. Ryanodine and caffeine both suppress calcium uptake but not the associated Ca-ATPase of heavy muscle fractions, so that the apparent uncoupling of calcium transport produced by these drugs could be due to the decreased ability of the vesicles to retain the transported calcium in the presence of the drugs.

They went on to suggest that the heavy fraction may be derived from the terminal cisternae of the sarcoplasmic reticulum and thus the release sensitive mechanism may be confined to this portion of the sarcoplasmic reticulum. Ryanodine would become an important tool in the isolation and characterization of the Ca^{2+} release channel of the terminal sarcoplasmic reticulum and caffeine is an often utilized agent to stimulate Ca^{2+} release in muscle fibers. This study would appear crude in

light of developments in purification of various components of the sarcoplasmic reticulum and transverse tubules but it pointed in a direction that would lead to major discoveries.

Throughout the 1970s and early 1980s the goal of the biochemists was to isolate and characterize pure subfractions of the sarcoplasmic reticulum and transverse tubule membranes. An important advance occurred when Gerhard Meissner (1975), working as a postdoctoral fellow in Sidney Fleischer's laboratory at Vanderbilt University, developed a well characterized and "clean" preparation of light and heavy sarcoplasmic reticulum vesicles. Light vesicles were composed of about equal amounts of phospholipid and Ca^{2+} pump protein which constituted about 90% of the protein. Heavy vesicles contained in addition to the Ca^{2+} pump protein (about 60% of the protein) two other major protein components, a Ca^{2+} binding protein and another protein which accounted for about 25 and 7% of the protein of these vesicles, respectively. He suggested that the light and heavy vesicles were derived from the longitudinal sections and terminal cisternae of sarcoplasmic reticulum, respectively. A further important advance came when Saito et al. (1984) reported the isolation of terminal cisternae vesicles which retained junctional feet similar to those first observed by Franzini-Armstrong (1970) in situ. The isolated terminal cisternae vesicles contained two distinct membranes: a membrane portion containing the Ca^{2+} pumps and a membrane portion containing the junctional feet. Electron microscopy showed that the feet projected about 12 nm from the surface and were distributed on the surface in a checker board like lattice of alternating square feet structures and spaces about 20 nm^2 (Fig. 6.22). Calsequestrin, inside the vesicles, appeared to be localized in strands near the junctional feet. The longitudinal cisternae contained only the Ca^{2+} pump protein membrane. In the same year, Ferguson et al. (1984) also observed junctional feet on isolated sarcoplasmic reticulum vesicles using freeze-drying, rotary-shadowing techniques. They obtained face-on three-dimensional views (stereo micrographs, Fig. 6.23) of the feet. They noted the four subunit structure of the feet. These were the first observations of junctional feet in isolated sarcoplasmic reticulum vesicles.

Fleischer et al. (1985) observed that the enhancement of Ca^{2+} uptake by isolated sarcoplasmic reticulum vesicles in the presence of ruthenium red could be blocked by ryanodine in submicromolar concentration. This effect occurred only in the terminal cisternae vesicles as neither ruthenium red nor ryanodine affected Ca^{2+} uptake in the longitudinal vesicles. Direct binding studies with [^{3}H]ryanodine localized the receptors to the terminal cisternae and not to longitudinal vesicles. Since these compounds had no effect on Ca^{2+} loading in the longitudinal vesicles, they concluded that their effects were not on the Ca^{2+} pump but rather on a Ca^{2+} release process. They speculated that ruthenium red inhibited Ca^{2+} release and that ryanodine locked the Ca^{2+} release channels in the "open state" so that Ca^{2+} was not reaccumulated and the muscle fiber could not relax.

The goal now became the isolation and characterization of the ryanodine receptor which seemed likely to be the Ca^{2+} release channel of the sarcoplasmic reticulum. From 1986 to 1988 various laboratories purified the ryanodine receptor from skeletal muscle. John E. Casida, a pesticide chemist at the University of California

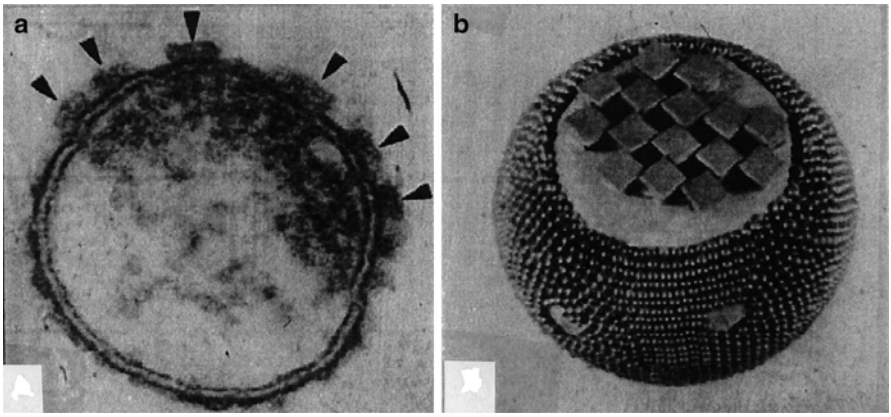


Fig. 6.22 Structure of isolated terminal cisternae. (**a**) Junctional terminal cisternae showing two types of membranes, the junctional face membrane with foot structures (labeled by *arrowheads*) and the asymmetric calcium pump membrane. (**b**) Diagrammatic representation of junctional terminal cisternae of the sarcoplasmic reticulum (Fleischer 2008. With permission Elsevier)

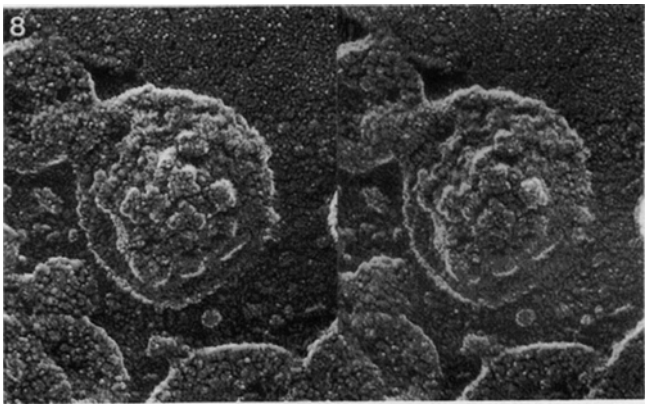


Fig. 6.23 Stereo micrograph of isolated sarcoplasmic reticulum vesicles from rat muscles. The feet are located over a raised platform. Extensive areas of membrane are covered by feet, even though the arrays are not complete (Ferguson et al. 1984. With permission Rockefeller University Press)

Berkeley, was interested in the mechanism of action of ryanodine from the viewpoint of an insecticide. He and his colleagues (Pessah et al. 1986) solubilized and partially characterized the ryanodine receptor. In Fleischer's laboratory, Makoto Inui purified the ryanodine receptor as a single high molecular weight band and Akitsugu Saito examined the purified receptor with electron microscopy and observed square-like structures about 22 nm per side (Inui et al. 1987). This shape matched the shape and size of the foot structures observed in tangential sections at

the junctional face of the terminal cisternae. Kevin Campbell's laboratory at the University of Iowa also purified the ryanodine receptor. Jeffrey S. Smith and Roberto Coronado at Baylor College of Medicine determined that the purified receptor mediated single channel activity identical to that observed in isolated vesicles of the terminal cisternae that were treated with ryanodine (Imagawa et al. 1987). The channel activity was measured after the purified receptor was reconstituted into planar lipid bilayers. Gerhard Meissner's laboratory at the University of North Carolina purified the ryanodine receptor and observed with electron microscopy that the isolated channel was a tetramer with a "four-leaf clover" structure, consisting of four 400 kDa subunits, previously described for the 'feet' that span the junction between the transverse tubules and sarcoplasmic reticulum. When the ryanodine receptor was incorporated into a lipid bilayer membrane, the channel exhibited Ca^{2+} release properties similar to those of the native sarcoplasmic reticulum (Lai et al. 1988). They concluded that the feet, the ryanodine receptor and the Ca^{2+} release channel, all seemed to be synonymous. Hansgeorg Schindler and Lin Hymel of the University of Linz, Austria, in collaboration with the Fleischer laboratory also determined the channel properties of the ryanodine receptor (Hymel et al. 1988). The competition must have been intense to determine the single channel properties of the ryanodine receptor as the three papers (Imagawa et al. 1987; Lai et al. 1988; Hymel et al. 1988) appeared within 2 months of each other. Thus there was general agreement that the ryanodine receptor was a large four subunit channel located in the junctional face membrane of the terminal cisternae and that it was the physiological Ca^{2+} release channel in muscle activation. Much more would be learned about the structure and function of the ryanodine receptor in the coming years (see Chap. 8).

6.4.9 Dihydropyridine Receptor: Voltage Sensor and Calcium Channel in Transverse Tubules

A central question in excitation-contraction coupling in skeletal muscle is how does the depolarization in the transverse tubules cause the release of Ca^{2+} from the sarcoplasmic reticulum? In the 1970s there were three general hypotheses: electrical transmission, chemical transmission and mechanical coupling. Electrical transmission seemed unlikely because the measured membrane capacitance of a skeletal muscle fiber was too small to include the very large amount of sarcoplasmic reticulum membrane. Thus it was unlikely that there was electrical continuity between the transverse tubules and sarcoplasmic reticulum. By the middle of the 1970s there was compelling evidence for chemical transmission in cardiac muscle. Alexandre Fabiato and his wife Francoise Fabiato (1975) showed that a small amount of Ca^{2+} rapidly delivered onto a skinned cardiac muscle cell triggered a much larger release of Ca^{2+} and contraction and then relaxation. This mechanism seemed less likely in skeletal muscle because of the well known observation that a skeletal muscle could

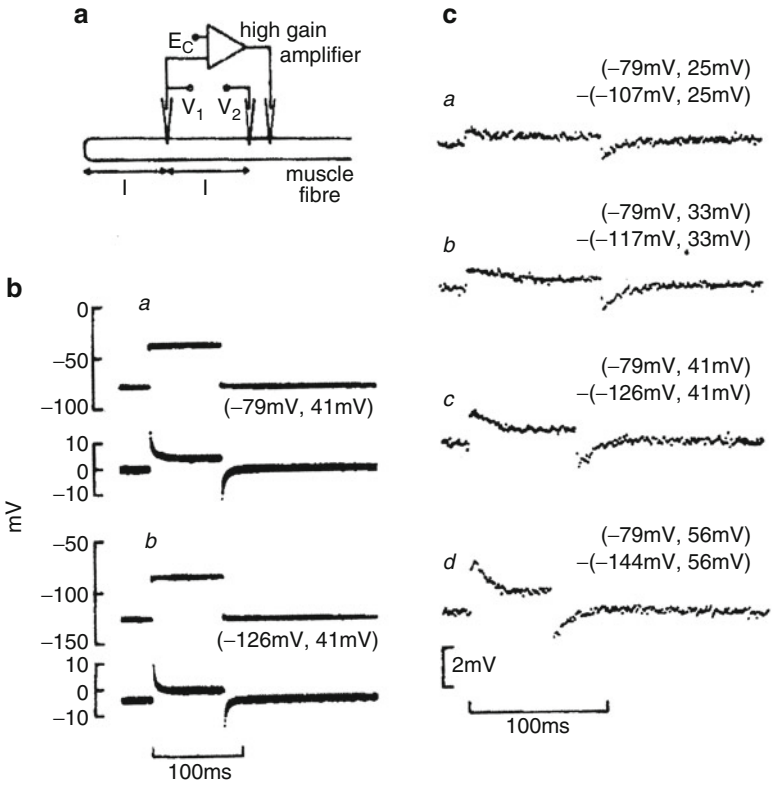


Fig. 6.24 Charge movement in skeletal muscle fibers. (**a**) diagram of the three microelectrode voltage clamp technique. (**b**) Traces of V_1 and **Δ**V from a fiber, holding potential = −79 mV: (*a*) pulse applied from holding potential; (*b*) potential stepped to −126 mV before the start of pulse. (**c**) In order to correct for the capacitative transient, the difference in **Δ**V between records such as **b**a and **b**b was determined. This difference represents the charge movement. Charge movement increased as the voltage pulse increased (Schneider and Chandler 1973. With permission Nature Publishing Group)

contract in the complete absence of extracellular Ca^{2+}. The third possibility, mechanical coupling, was put forward in 1973 by Martin Schneider, a postdoctoral fellow, and W. Knox Chandler at Yale University. Schneider and Chandler (1973) suggested that a link in excitation-contraction coupling involved the movement of a fixed amount of charge free to move between different locations across the membrane. They measured this charge movement using voltage clamp techniques in frog skeletal muscle after blocking virtually all of the time and voltage dependent changes in Na^+ and K^+ currents, preventing contraction in a hypertonic solution and subtracting off the capacitative transient. What was left upon stimulation was a small movement of charge that appeared over the same range of membrane potential that produced muscle contraction (Fig. 6.24). They noted that the time course of the

charge movement was very different from the time course of the gating current of the Na^+ channels. They speculated that (Schneider and Chandler 1973. With permission Nature Publishing Group):

> If the (charged) groups were attached to long molecules which extended to the adjacent projections of the SR terminal cysterna membrane…, they would provide a means by which the potential across the wall of the T-system could be sensed by the SR.

They were thinking in terms of the "feet" structures that were observed by Franzini-Armstrong (1970). In this regard a rough calculation suggested that it would be "numerically possible" for approximately one charged group to be associated with each "foot". Much would change in the excitation-contraction coupling field in skeletal muscle but this hypothesis would stand the test of time.

During the 1950s and 1960s voltage-gated Ca^{2+} channels were discovered and being characterized in various vertebrate and invertebrate tissues (see historical reviews by Tsien and Barrett [2000–2012]; Dolphin 2006). In the late 1960s Albrecht Fleckenstein (1917–1992) and his colleagues at the University of Freiburg discovered that the cardiac and smooth muscle contraction could be inhibited by various pharmacological compounds that acted in the same way as removal of extracellular Ca^{2+} (for a history of this work, see Fleckenstein 1983). He called this general effect "calcium antagonism". Nifedipine, a member of the dihydropyridine family of chemicals, was a particularly potent Ca^{2+} antagonist. This class of drugs inhibited Ca^{2+} movement through the voltage-gated Ca^{2+} channels. Sanchez and Stefani (1978) observed a slow inward Ca^{2+} current in frog skeletal muscle fibers. Then Wolhard Almers, R. Fink and Philip T. Palade (1981) at the University of Washington concluded that the Ca^{2+} current flows almost entirely across the membrane of the transverse tubules.

Around the same time interesting biochemical studies were occurring that would eventually lead to the isolation and characterization of the skeletal muscle Ca^{2+} channels. Noriaki Ikemoto and colleagues at the Boston Biomedical Research Institute developed a method to isolate transverse tubule membranes from rabbit skeletal muscle (Rosemblatt et al. 1981). It turned out that the transverse tubule membranes contained the richest source of receptors for the dihydropyridine Ca^{2+} antagonists and thus presumably the richest source of Ca^{2+} channels (Fosset et al. 1983). These dihydropyridine receptors (DHPR) exhibited a density 30 times greater in the transverse tubules than in the surface membrane. Curtis and Catterall (1984) at the University of Washington utilized [^{3}H]nitrendipine as a chemical probe to isolate the dihydropyridine receptor from the transverse tubules of rabbit skeletal muscle (Curtis and Catterall 1984). Analysis by gel electrophoresis demonstrated that three polypeptides termed α (130 kDa), β (50 kDa) and γ (33 kDa) quantitatively comigrated with the [^{3}H]nitrendipine-receptor complex. These three polypeptides were associated noncovalently. They concluded that the results suggested that these three polypeptides are the subunits of the Ca^{2+} antagonist receptor and are major components of the transverse tubule voltage sensitive Ca^{2+} channel. It would eventually become more complicated than this with the dihydropyridine receptor now known to consist of five subunits and to exist in multiple isoforms (see Chap. 8). Nonetheless progress was being made on the molecular characterization

of the channel. The next step came when Franz Hofmann and Wolfgang Trautwein and their colleagues at the Universitat des Saarlandes in Homburg showed that the purified dihydropyridine receptor inserted into a lipid bilayer membrane was a functional Ca^{2+} channel with properties similar to the so-called "L-type" Ca^{2+} channels (Flockerzi et al. 1986). The L-type Ca^{2+} channels[12] were so named because of the long lasting potential change in response to Ca^{2+}. Thus the Ca^{2+} current in the transverse tubules of skeletal muscle was due to Ca^{2+} channels that are blocked by dihydropyridine compounds.

But it was a major surprise when Almers and his colleagues (Schwartz et al. 1985) published in Nature a paper entitled: "dihydropyridine receptors in muscle are voltage-dependent but most are not functional calcium channels". They measured dihydropyridine binding in intact frog skeletal muscle and compared the binding capacity with voltage-clamp measurements of Ca^{2+} channel current. The number of binding sites was very large compared to the Ca^{2+} current. They concluded that there were 35–50 times more dihydropyridine binding sites than there were voltage-dependent Ca^{2+} channels that can be activated to pass current. These were confusing results: Ca^{2+} channels that do not pass Ca^{2+}? What could this mean for the understanding of excitation-contraction coupling? Rios and Brum (1987) at Rush University in Chicago made sense of these results when they observed that a dihydropyridine compound inhibited charge movement and Ca^{2+} release by the sarcoplasmic reticulum in parallel. They measured Ca^{2+} transients and charge movements in single fibers from frog skeletal muscle and concluded that the molecule that generates charge movement is the dihydropyridine receptor. They speculated that the dihydropyridine receptors were "channel-like" proteins, but not necessarily Ca^{2+} conducting proteins, that performed the voltage sensing function and were coupled to the SR release pathway by some unknown means. Thus this speculation was consistent with the original mechanical hypothesis put forward by Schneider and Chandler. The dihydropyridine receptors were voltage sensors and involved in transferring information to the Ca^{2+} release channels of the sarcoplasmic reticulum.

If the mechanical model is correct, then there must be a direct interaction of the dihydropyridine receptor and the Ca^{2+} release channel of the sarcoplasmic reticulum. That such an interaction could exist was shown as a result of an important collaboration between the Clara Franzini-Armstrong laboratory and Kevin Campbell laboratory in 1988 (Block et al. 1988). Barbara Block, a postdoctoral fellow, and Franzini-Armstrong generated freeze-fracture images of the junctional sarcoplasmic reticulum and junctional transverse tubule membranes from muscle fibers of the swimbladder of the toadfish. The freeze-fracture technique exposed the intramembrane protein components. The swimbladder of the toadfish was utilized because it is rich in sarcoplasmic reticulum and transverse tubules. They found clusters of four

[12] Various types of calcium channels have been discovered and characterized. The current classification includes: L-type, N-type, P/Q-type and R-types calcium channels. The evolution of this classification is reviewed by Richard W. Tsien and Curtis F. Barrett (2000–2012). Richard Tsien's laboratory at Stanford University made major contributions in the understanding of calcium channel function.

"bumps" or tetrads in the transverse tubule membranes. Also they observed bumps in the junctional membrane of the sarcoplasmic reticulum at the position of the feet processes. The spacing and disposition of the transverse tubule tetrads was such that they matched the position of alternate feet and sarcoplasmic membrane bumps. Toshiaki Imagawa, a postdoctoral fellow in Campbell's laboratory, purified the ryanodine receptor and dihydropyridine receptor. Block and Franzini-Armstrong made rotary-shadowed images of these purified receptors. Their dimensions were similar to those of the respective bumps in the membranes (Fig. 6.25). They also noted that the size of the isolated dihydropyridine receptor was equal to or smaller than that of the subunits of the junctional feet so that it was possible for four dihydropyridine receptors to be associated with each foot. They put these results together in the schematic representation shown in Fig. 6.26. This spatial relationship made it reasonable to suppose that the dihydropyridine receptors might initiate transduction of the voltage change across the transverse tubule membrane to cause Ca^{2+} release from the sarcoplasmic reticulum. They concluded that the results suggested the presence of a large "junctional complex" spanning the two junctional membranes and intervening gap. This junctional complex was an "ideal candidate" for a mechanical coupling hypothesis of excitation-contraction coupling at the triadic junction. With these results investigators started thinking in terms of the "junctional complex" as a macromolecular protein complex which would come to be termed the "calcium release unit". This calcium release unit consisted of the ryanodine receptor, the transverse tubule L-type Ca^{2+} channel acting as a voltage sensor to detect the action potential and the Ca^{2+} binding protein calsequestrin anchored to the ryanodine receptor to detect the environment within the sarcoplasmic reticulum.

Along with the progress during the 1970s and 1980s, the term excitation-contraction coupling also evolved narrowly within the muscle field to encapsulate only the processes that intervene between depolarization of the surface/transverse tubule membrane and Ca^{2+} release from the sarcoplasmic reticulum. Much progress was made during the 1980s toward understanding the mechanism of excitation-contraction coupling but many questions remained. In fact in many ways the "surface was just being scratched". With the advent of molecular biology approaches including gene cloning, mutagenesis and transgenic techniques along with the rapid increase in structural biology, many questions would be answered and more raised. There would be many more proteins discovered and effort would be concentrated on regulation of Ca^{2+} release, especially in cardiac muscle. This area of research is very active in the twenty-first century with the ultimate mechanisms still to be discovered (see Chap. 8 for more recent advances).

6.5 Contractile Proteins in Non-Muscle Tissue

When the discovery of the sliding filament hypothesis of muscle contraction was announced in 1954, it was generally accepted that myosin and actin were confined to muscle cells. But experiments conducted during this time in Hans H. Weber's

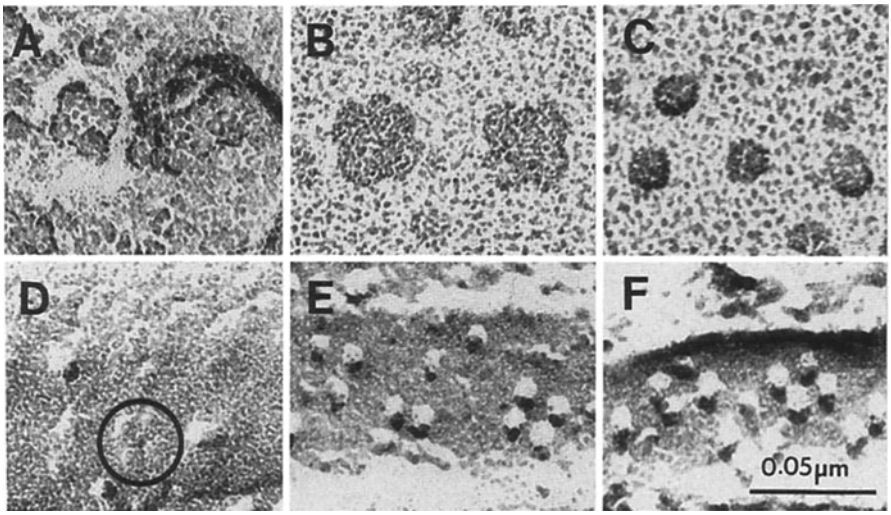


Fig. 6.25 Comparison between a foot process associated with the sarcoplasmic reticulum membrane (**a**), isolated foot proteins (**b**), isolated dihydropyridine receptors (**c**), junctional sarcoplasmic reticulum bumps (**d**), and junctional transverse tubule tetrads (**e**, **f**). Note the similarity in size and shape of a foot process of the sarcoplasmic reticulum and the isolated foot protein (ryanodine receptor) and similarity in size of the isolated dihydropyridine receptor and a junctional transverse tubule bump. Four bumps forming a tetrad are about the size of a single foot process (Block et al. 1988. With permission Rockefeller University Press)

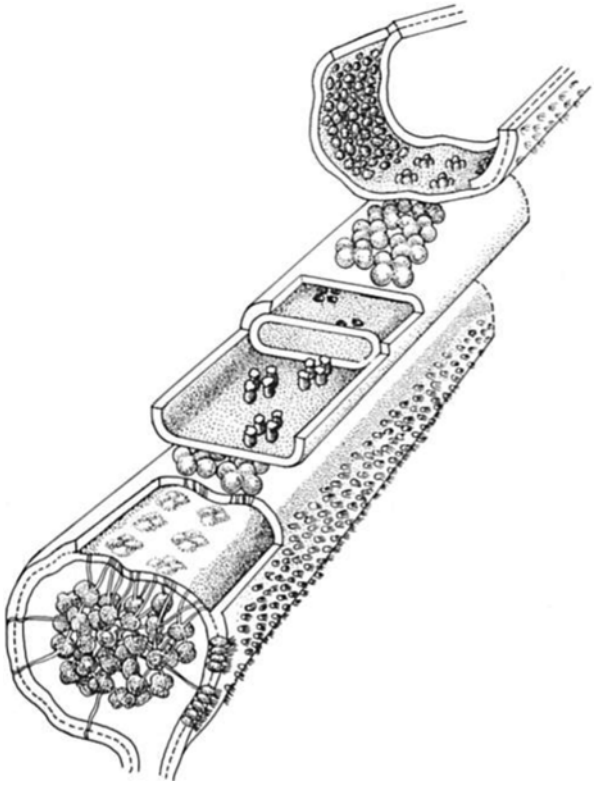


Fig. 6.26 A three-dimensional reconstruction of a triad, showing the relative positions of foot proteins, junctional transverse tubule tetrads, calsequestrin, and calcium ATPase. The feet (four balls with a central depression) (ryanodine receptors) occupy the junctional gap between the sarcoplasmic reticulum and transverse tubule, and the intramembranous portion of the foot protein penetrates into the junctional sarcoplasmic reticulum membrane, forming bumps with a fourfold structure. The *tall particles* (dihydropyridine receptors) of the junctional transverse tubule tetrads are located in the junctional transverse tubule membrane, opposite alternate feet (Block et al. 1988. With permission Rockefeller University Press)

laboratory in Heidelberg suggested otherwise. Weber developed and investigated simplified models of muscle contraction, including myosin threads and single glycerol extracted muscle fibers (see Chap. 1). He also was interested in non-muscle motility. Weber (1958) suggested that it was "an attractive idea" to assume that there was a fundamental principle common to the movements of all living creatures, to the movements of all animal cells during cell division, to the crawling and swimming movements characteristic of protozoa, and to the movements peculiar to the smooth and cross-striated musculature. Following this line of thought, Hartmut Hoffmann-Berling, in the Weber laboratory, examined the mechanical properties of glycerol extracted non-muscle cells, including amoebas, fibroblasts and other cells throughout the 1950s. For example he found that fibroblasts would shorten upon addition of ATP under conditions similar to those needed for the contraction of glycerinated muscle (Hoffmann-Berling 1954). Furthermore the extracted proteins had a similar solubility to actomyosin. He reviewed his work and that of others (Hoffmann-Berling 1960) and concluded that systematic comparisons have not yet uncovered any dissimilarity which would indicate a fundamental difference between the contraction of a muscle and the contraction of an undifferentiated cell. The differences are only quantitative. In 1959 Micheline Bettex-Galland and Ernst F. Luscher at the Theodor Koeher Institute in the University of Bern isolated from human platelets an actomyosin-like complex. Bettex-Galland and Luscher (1959) concluded that a contractile protein had been extracted from normal human thrombocytes. This protein showed characteristics of muscle actomyosin, and was most probably responsible for clot retraction. They called the actomyosin-like protein thrombosthenin, meaning "strength of the blood clot" (Bettex-Galland and Clemetson 2005). An important advance was made by Hatano and Oosawa (1966) at Nagoya University when they provided the first rigorous proof for the existence of contractile proteins in non-muscle cells. They purified actin from the slime mold *Physarum polycephalum*. They concluded that this was the first example in which the actin-like protein was obtained from organs other than muscle, or from lower organisms having no muscle, in such a pure state that its structure and function could be extensively compared with actin from muscle. Ishikawa et al. (1969) at the University of Pennsylvania utilized the concept and technique developed by Hugh Huxley in 1963 (see Chap. 3 and Fig. 3.15) wherein in the absence of ATP heavy meromyosin (HMM) bound stereospecifically to actin filaments to form "arrowhead" structures. They found arrowheads bound to filaments in a wide variety of glycerol extracted and sectioned cells, including fibroblasts, nerve cells, epithelial cells, etc. The arrowheads were formed on thin filaments but not intermediate filaments or microtubules. This technique provided a convenient way to determine if a particular non-muscle cell contained actin filaments. According to the thinking of the day, where there was actin, there must be myosin. It was more difficult to purify myosin-like protein from non-muscle cells, in part, because it combined avidly with actin. Adelman and Taylor (1969) and Hatano and Ohnuma (1970) succeeded in purifying a myosin-like enzyme from *Physarum*.

The Cold Spring Harbor meeting was certainly a muscle meeting but the strong possibility that the mechanism of contraction so highly developed in muscle may

also be present in a more rudimentary form in non-muscle cells was so appealing that a session on non-muscle contractile proteins was included in the meeting. Six papers were presented. Adelstein and Conti (1973) at the NIH described experiments in human platelets. They isolated a myosin-like molecule from platelets whose ATPase activity was stimulated by rabbit skeletal muscle actin. Also they isolated platelet actin that stimulated the ATPase activity of rabbit skeletal muscle myosin. Furthermore the activation of HMM by platelet actin was dependent on Ca^{2+} in the presence of troponin-tropomyosin. The platelet myosin had an activated ATPase activity similar to that of smooth muscle myosin. They concluded that platelet myosin was remarkably similar to muscle myosin in structure and function and similar to smooth muscle myosin. They suggested that this actomyosin system was involved in clot retraction in platelets. There could be little doubt now that actomyosin systems similar to muscle were operating in non-muscle cells.

But the paper in the session that "raised eyebrows" and generated skepticism was from Edward D. Korn's laboratory at the NIH. He and postdoctoral fellow Thomas D. Pollard searched for a myosin-like protein in the soil amoeba *Acanthamoeba castellanii* (Pollard and Korn 1973a). They utilized *Acanthamoeba* because it exhibited amoeboid movements and could be grown in virtually unlimited quantities in a defined medium, thus facilitating biochemical analysis. The Korn laboratory had previously isolated actin from *Acanthamoeba*. Reasoning that where there is actin, there must by myosin, they started to search for myosin. They weren't successful using the accepted procedures for myosin isolation. Finally they did isolate a myosin-like protein from *Acanthamoeba* but there seemed to be a problem, at least in the eyes of the skeptics. The myosin that they isolated exhibited less than half the molecular weight of muscle myosin (about 180,000 Da). It seemed to have only one heavy chain and activation of its ATPase by actin required a mysterious cofactor. Pollard and Korn (1973a) concluded that the remarkable similarities between *Acanthamoeba* actin and muscle actin suggest that the function of *Acanthamoeba* actin within the cell is to interact with myosin to generate force for movement.

Many years later Taylor (2001) recalled his surprise upon hearing the presentation. Whereas it was not understood why muscle myosin had two heads, he thought that an amoeba with a one-headed myosin must somehow be "a defective creature". Korn (2004) remembered the situation in a slightly different way. It was not so much that *Acanthamoeba* was thought to be a defective creature as that this small myosin was thought to be a degradation product of a conventional myosin. It turned out that Pollard and Korn were correct and that they had discovered the first of what became known as the unconventional myosins. The cofactor for myosin activation turned out to be a myosin heavy chain kinase. Taylor (2001) later commented that they did not realize at the time that this was the beginning of the end of the isolation of the muscle community from the rest of cell biology. What was first presented at this meeting and later published by Pollard and Korn (1973b, c) was the first of the unconventional myosins but certainly not the last. With the advent of recombinant technology, this field would expand enormously. By the beginning of the twenty-first century there would be 150 myosin heavy chain sequences grouped into 18 classes based on sequences of their head domains (Korn 2004) with, no doubt, more

to come. Korn called the *Acanthamoeba* protein myosin I. The muscle myosin and its isoforms were labeled myosin II and subsequent myosins that were discovered were numbered in the order of their discovery.

There were also two presentations on non-muscle actin at the muscle meeting. One presentation was by Dennis Bray (1973) from the Laboratory of Molecular Biology in Cambridge who described a comparative study of cytoplasmic actins. He concluded that the experimental results clearly demonstrated "the ubiquity of actin-like proteins" in non-muscle cells. Another young scientist who made a presentation was James A. Spudich (1973), then a junior faculty member at the University of California, San Francisco, who suggested that cytochalasin B might inhibit cellular movements by interacting with actin-like filaments and changing their morphology. It probably wasn't suspected at the time that Jim Spudich would go on to Stanford University and become one of the most important contributors to the understanding of the molecular basis of cell motility and muscle contraction (see Chap. 9).

In his summary of the meeting, Huxley (1973b) called contraction in non-muscle systems "an extremely active and exciting area". Soon thereafter Andrew Szent-Gyorgyi (1975) speculated that he believed that "the picking" will be more fruitful in cellular motility than in muscle. He viewed this as one of the compensations for working on systems that are experimentally more difficult. Neither one of them possibly could have guessed just how right they were. The cell motility field accelerated in the 1970s and exploded in the 1980s. When actin (Sheterline et al. 1998) and myosin (Sellers 1999) were characterized in the Protein Profile series published by Oxford University Press, a total of over 6,000 references were cited! Bray (2001) has written an excellent monograph that provides an introduction to the scope of the non-muscle motility field from cells to molecules. At first the muscle discoveries stimulated investigation with non-muscle cells. In time discoveries in non-muscle cells would feedback to stimulate further investigation into muscle myosin function at the individual molecule level (see Chap. 9).

6.6 ATPase and Contraction: Energetics and Mechanical Properties

Some of the topics presented in these two sessions at the meeting already have been considered in Chap. 5. In the ATPase and contraction session, there were a number of presentations relating to the kinetic mechanism of myosin and actomyosin ATPase. The Evan Eisenberg (Eisenberg and Kielley 1973) and Edwin Taylor (Koretz et al. 1973) laboratories described their somewhat contradictory views of the actomyosin kinetic mechanism. Noteworthy in the energetics and mechanical properties session was the presentation by Andrew Huxley describing the model that he and Robert M. Simmons developed to explain the transient mechanical behavior of muscle fibers and the origin of muscular force (Huxley and Simmons 1973). This model still is considered the modern day standard explanation of

transient muscle mechanics. Also Douglas Wilkie (Gilbert et al. 1973) and Woledge (1973) made presentations that ushered in the era of "unexplained energy" into the muscle energetics field. They described a problem that would take over a decade to solve. See Chap. 5 for further details on these topics. There also was a session on myogenesis. This important topic would blossom with the advent of molecular biology into the muscle field in the 1980s.

6.7 Muscle 1972: Progress and Problems

At the end of the Cold Spring Harbor meeting, Huxley (1973b) gave a "personal summary" of the meeting emphasizing those topics and ideas especially interesting to him. He made several important observations. One of the issues that struck him was that several of the questions that were originally restricted to muscle fibers seemed to apply to other directed biological forms of movement in nonmuscle systems. Thus he asked if the experience with muscle could be utilized to help understand the broader problems of cellular movement. He felt, correctly, that this was an extremely exciting area for future research. With regard to muscle, he emphasized the pressing need for crystalline preparations of contractile proteins for detailed X-ray studies. Another important point that he made related to a barrier that would plague muscle research for decades to come when he said (Huxley 1973b. With permission Cold Spring Harbor Laboratory Press):

> However there is a very large gap in our present knowledge, unfortunately right at the heart of the whole problem. It concerns the nature of the structural changes in the myosin head accompanying the different stages of ATP-splitting which are thought to produce the changes in the effective angle of attachment to actin during the operation of the cross-bridge.

Despite this major unknown at the heart of the problem, there was the feeling among many of the participants that the mechanism of muscle contraction had been solved in principle. Looking back on that attitude, Robert M. Simmons has commented (Simmons 1992. With permission Cambridge University Press):

> On the one hand was the view that the attachment-detachment cycle of A. F. Huxley (1957), plus the structural picture of H. E. Huxley (1969), plus the biochemical cycle of A. F. Lymn and Taylor (1971), plus the force-generating step of A. F. Huxley and Simmons (1971b) equaled the muscle problem solved. We found this astonishing, as we knew we were just scratching the surface of the problem.

Not only was the surface just being scratched in some ways but there would be trouble ahead for the sliding filament theory in the 1980s as new spectroscopic technology could not verify cross-bridge titling and even the concept of constant filament length would be challenged by naysayers. Fortunately the development of an in vitro assay of filament sliding in the late 1980s provided novel and direct evidence of the reality of actin filaments sliding along myosin heads whose tails were attached to a fixed support. These studies re-established beyond doubt the conviction that the sliding force had to be generated by the myosin heads (see Chap. 9).

Hugh Huxley also emphasized that he thought the time was right for more frequent workshop style meetings on both sides of the Atlantic Ocean. Indeed this has happened with the development of regular meetings on muscle related topics. Included in these meetings are the Gordon Research Conferences held in the north-east United States, meetings held in Alpbach, Austria (every 3 years since 1974) and the evolution of the annual European Muscle Conference first held at Liege University in 1972. Of course there have been and continue to be many other types of muscle meetings. Finally, Huxley (1973b) expressed what many at the meeting must have been thinking: "…I think we are fortunate to be working in this area at present." (Huxley 1973b. With permission Cold Spring Harbor Laboratory Press)

References

Adelman MR, Taylor EW (1969) Further purification and characterization of slime mold myosin and slime mold actin. Biochemistry 8:4976–4988

Adelstein RS, Conti MA (1973) The characterization of contractile proteins from platelets and fibroblasts. Cold Spring Harb Symp Quant Biol 37:599–605

Al-Amoudi A, Chang J-J, Leforestier A, McDowall A, Salamin LM, Norlen LPO, Richter K, Blanc NS, Studer D, Dubochet J (2004) Cryo-electron microscopy of vitreous sections. EMBO J 23:3583–3588

Almers W, Fink R, Palade PT (1981) Calcium depletion in frog muscle tubules: the decline of calcium current under maintained depolarization. J Physiol 312:177–207

Amos LA (1985) Structure of muscle filaments studied by electron microscopy. Annu Rev Biophys Biophys Chem 14:291–313

Ashton FT, Weisel J, Pepe FA (1992) The myosin filament XIV backbone structure. Biophys J 61:1513–1528

Atkinson SJ, Stewart M (1991) Expression in Escherichia coli of fragments of the coiled-coil domain of rabbit myosin: influence of different regions of the molecules on aggregation and paracrystal formation. J Cell Sci 99:823–836

Bailey K (1946) Tropomyosin: a new asymmetric protein component of muscle. Nature 157:368–369

Bailey K (1957) Invertebrate tropomyosin. Biochim Biophys Acta 24:612–619

Bennett PM (1981) The structure of spindle-shaped paracrystals of light meromyosin. J Mol Biol 146:201–221

Bennett P, Craig R, Starr R, Offer G (1986) The ultrastructural location of C-protein, X-protein and H-protein in rabbit muscle. J Muscle Res Cell Motil 7:550–567

Benzonana G, Capony J-P, Pechere J-F (1972) The binding of calcium to muscular parvalbumins. Biochim Biophys Acta 278:110–116

Bettex-Galland M, Clemetson KJ (2005) First isolation of actomyosin from a non-muscle cell: first isolated platelet protein. J Thrombosis Haemost 3:834–839

Bettex-Galland M, Luscher EF (1959) Extraction of an actomyosin-like protein from human thrombocytes. Nature 184:276–277

Block BA, Imagawa T, Campbell KP, Franzini-Armstrong C (1988) Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. J Cell Biol 107:2587–2600

Bray D (1973) Cytoplasmic actin: a comparative study. Cold Spring Harb Symp Quant Biol 37:567–571

Bray D (2001) Cell movements: from molecules to motility, 2nd edn. Garland, New York

Bremel RD, Weber A (1972) Cooperation within actin filament in vertebrate skeletal muscle. Nat New Biol 238:97–101
Bremel RD, Murray JM, Weber A (1973) Manifestations of cooperative behavior in the regulated actin filament during actin-activated ATP hydrolysis in the presence of calcium. Cold Spring Harb Symp Quant Biol 37:267–275
Brenner B, Schoenberg M, Chalovich JM, Greene LE, Eisenberg E (1982) Evidence for cross-bridge attachment in relaxed muscle at low ionic strength. Proc Natl Acad Sci U S A 79:7288–7291
Brini M, Carafoli E (2009) Calcium pumps in health and disease. Physiol Rev 89:1341–1378
Brown JH, Kim K-H, Jun G, Greenfield NJ, Dominguez R, Volkmann N, Hitchcock-DeGregori SE, Cohen C (2001) Deciphering the design of the tropomyosin molecule. Proc Natl Acad Sci U S A 98:8496–8501
Caspar DLD, Cohen C, Longley W (1969) Tropomyosin: crystal structure, polymorphism and molecular interactions. J Mol Biol 41:87–107
Chalovich JM (2002) Regulation of striated muscle contraction: a discussion. J Muscle Res Cell Motil 23:353–361
Chalovich JM, Eisenberg E (1982) Inhibition of actomyosin ATPase activity by troponin.tropomyosin without blocking the binding of myosin to actin. J Biol Chem 257:2432–2437
Chalovich JM, Chock PB, Eisenberg E (1981) Mechanism of action of tropomyosin*tropomyosin: inhibition of actomyosin ATPase activity without inhibition of myosin binding to actin. J Biol Chem 256:575–578
Chantler P (1982) Retreats from the steric blocking of muscle contraction. Nature 298:120–121
Cohen C (1975) The protein switch of muscle contraction. Sci Am 233:36–45
Cohen P (2002) The origins of protein phosphorylation. Nat Cell Biol 4:E127–E130
Cohen C, Holmes KC (1963) X-ray diffraction evidence for α-helical coiled-coils in native muscle. J Mol Biol 6:423–432
Cohen C, Longley W (1966) Tropomyosin paracrystals formed by divalent cations. Science 152:794–796
Cohen C, Szent-Gyorgyi AG (1957) Optical rotation and helical polypeptide chain configuration in α-proteins. J Am Chem Soc 79:248
Cohen C, Caspar DLD, Johnson JP, Nauss K, Margossian SS, Parry DAD (1973) Tropomyosin-troponin assembly. Cold Spring Harb Symp Quant Biol 37:287–297
Collins JH, Potter JD, Horn MJ, Wilshire G, Jackman N (1973) The amino acid sequence of rabbit skeletal muscle troponin C: gene replication and homology with calcium-binding proteins from carp and hake muscle. FEBS Lett 36:268–272
Costantin LL, Franzini-Armstrong C, Podolsky RJ (1965) Localization of calcium-accumulating structures in striated muscle fibers. Science 147:158–160
Craig R (1986) The structure of the contractile filaments. In: Engel AG, Banker BQ (eds) Myology. McGraw-Hill, New York, pp 73–123
Craig R, Offer G (1976) The location of C-protein in rabbit skeletal muscle. Philos Trans R Soc Lond B Biol Sci 192:451–461
Crick FHC (1953) The packing of α-helices: simple coiled-coils. Acta Cryst 6:689–697
Cummins P, Perry SV (1973) The subunits and biological activity of polymorphic forms of tropomoysin. Biochem J 133:765–777
Curtis BM, Catterall WA (1984) Purification of the calcium antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse tubules. Biochemistry 23:2113–2118
Davis BJ (1964) Disc electrophoresis. 2, Method and application to human serum proteins. Ann N Y Acad Sci 121:404–427
de Meis L, Vianna AL (1979) Energy interconversion by the Ca^{2+}-dependent ATPase of the sarcoplasmic reticulum. Annu Rev Biochem 48:275–292
DeRosier DJ, Klug A (1968) Reconstruction of three dimensional structures from electron micrographs. Nature 217:130–134
Dolphin AC (2006) A short history of voltage-gated calcium channels. Br J Pharmacol 147:S56–S62

Draper MH, Hodge AJ (1949) Studies on muscle with the electron microscope. 1. The ultrastructure of toad striated muscle. Austral J Exptl Biol Med Sci 27:465–504
Dubuisson M (1954) Muscular contraction. Charles C. Thomas, Springfield
Ebashi S, Kodama A (1966) Interaction of troponin with F-actin in the presence of tropomyosin. J Biochem 59:425–426
Ebashi S, Endo M, Ohtsuki I (1969) Control of muscle contraction. Q Rev Biophys 2:351–384
Egelman EH, DeRosier DJ (1983) A model for F-actin derived from image analysis of isolated filaments. J Mol Biol 166:623–629
Eisenberg E, Kielley WW (1973) Evidence for a refractory state of heavy meromyosin and subfragment-1 unable to bind to actin in the presence of ATP. Cold Spring Harb Symp Quant Biol 37:145–152
Eisenberg BR, Kuda AM (1975) Stereological analysis of mammalian skeletal muscle. II. White vastus muscle of the adult guinea pig. J Ultrastruct Res 51:176–187
Elzinga M, Collins JH (1973) The amino acid sequence of rabbit skeletal muscle actin. Cold Spring Harb Symp Quant Biol 37:1–7
Endo M (1977) Calcium release from the sarcoplasmic reticulum. Physiol Rev 57:71–108
Endo M, Nonomura Y, Masaki T, Ohtsuki I, Ebashi S (1966) Localization of native tropomyosin in relation to striation patterns. J Biochem 60:605–608
Enfield DL, Ericsson LH, Blum HE, Fischer EH, Neurath H (1975) Amino-acid sequence of parvalbumin from rabbit skeletal muscle. Proc Natl Acad Sci U S A 72:1309–1313
Fabiato A, Fabiato F (1975) Contractions induced by calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. J Physiol 249:469–495
Fairhurst AS, Hasselbach W (1970) Calcium efflux from a heavy sarcotubular fraction: effects of ryanodine, caffeine and magnesium. Eur J Biochem 13:504–509
Ferguson DG, Schwartz HW, Franzini-Armstrong C (1984) Subunit structure of junctional feet in triads of skeletal muscle: a freeze-drying, rotary-shadowing study. J Cell Biol 99:1735–1742
Fischer EH (1992) Nobel lecture: protein phosphorylation and cellular regulation. nobelprize.org
Flashman E, Redwood C, Moolman-Smook J, Watkins H (2004) Cardiac myosin binding protein C: its role in physiology and disease. Circ Res 94:1279–1289
Fleckenstein A (1983) History of calcium antagonism. Circ Res 52(Suppl 1):3–16
Fleischer S (2008) Personal recollections on the discovery of the ryanodine receptors of muscle. Biochem Biophys Res Commun 369:195–207
Fleischer S, Ogunbunmi EM, Dixon MC, Fleer EAM (1985) Localization of Ca^{2+} release channels with ryanodine in junctional terminal cisternae of sarcoplasmic reticulum of fast skeletal muscle. Proc Natl Acad Sci U S A 82:7256–7259
Flockerzi V, Oeken H-J, Hofmann F, Pelzer D, Cavalie A, Trautwein W (1986) Purified dihydropyridine-binding site from skeletal muscle t-tubules is a functional calcium channel. Nature 323:66–68
Fosset M, Jaimovich E, Delpont E, Lazdunski M (1983) [^{3}H]Nitrendipine receptors in skeletal muscle. Properties and preferential localization in transverse tubules. J Biol Chem 258:6086–6092
Frank G, Weeds AG (1974) The amino-acid sequence of the alkali light chains of rabbit skeletal-muscle myosin. Eur J Biochem 44:317–334
Franzini-Armstrong C (1970) Studies of the triad. I. Structures of the junction in frog twitch fibers. J Cell Biol 47:488–499
Frearson N, Focant BWW, Perry SV (1976) Phosphorylation of a light chain component of myosin from smooth muscle. FEBS Lett 63:27–32
Froehlich JP, Taylor EW (1975) Transient state kinetic studies of sarcoplasmic reticulum adenosine triphosphatase. J Biol Chem 250:2013–2021
Gerday C, Gillis JM (1976) The possible role of parvalbumins in the control of contractions. J Physiol 258:96–97P
Gilbert C, Kretzschmar KM, Wilkie DR (1973) Heat, work, and phosphocreatine splitting during muscular contraction. Cold Spring Harb Symp Quant Biol 37:613–618

Gordon AM, Homsher E, Regnier M (2000) Regulation of contraction in striated muscle. Physiol Rev 80:853–924
Greaser ML, Gergely J (1971) Reconstitution of troponin activity from three protein components. J Biol Chem 246:4226–4233
Greaser ML, Gergely J (1973) Purification and properties of the components from troponin. J Biol Chem 248:2125–2133
Greaser ML, Yamaguchi M, Brekke C, Potter J, Gergely J (1973) Troponin subunits and their interactions. Cold Spring Harb Symp Quant Biol 37:235–244
Hall CE, Jakus MA, Schmitt FO (1946) An investigation of cross striations and myosin filaments in muscle. Biol Bull 90:32–50
Hamoir G, Konosu S (1965) Carp myogens of white and red muscles. General composition and isolation of low-molecular-weight components of abnormal amino acid composition. Biochem J 96:85–97
Hanson J, Lowy J (1963) The structure of F-actin and actin filaments isolated from muscle. J Mol Biol 6:46–60
Hanson J, Lowy J (1964) The structure of actin filaments and the origin of the axial periodicity in the I-substance of vertebrate striated muscle. Philos Trans R Soc Lond B Biol Sci 160:449–460
Hanson J, Lednev V, O'Brien EJ, Bennett PM (1973) Structure of the actin-containing filaments in vertebrate skeletal muscle. Cold Spring Harb Symp Quant Biol 37:311–318
Harrington WF, Burk M, Barton JS (1973) Association of myosin to form contractile systems. Cold Spring Harb Symp Quant Biol 37:77–85
Hartshorne DJ, Mueller H (1968) Fractionation of troponin into two distinct proteins. Biochem Biophys Res Commun 31:647–653
Haselgrove JC (1973) X-ray evidence for a conformational change in the actin-containing filaments of vertebrate striated muscle. Cold Spring Harb Symp Quant Biol 37:341–351
Hasselbach W (1978) The reversibility of the sarcoplasmic calcium pump. Biochim Biophys Acta 515:23–53
Hatano S, Ohnuma J (1970) Purification and characterization of myosin A from the myxomycete plasmodium. Biochim Biophys Acta 205:110–120
Hatano S, Oosawa F (1966) Isolation and characterization of plasmodium actin. Biochim Biophys Acta 127:488–498
Heizmann CW, Berchtold MW, Rowlerson AM (1982) Correlation of parvalbumin concentration with relaxation speed in mammalian muscle. Proc Natl Acad Sci U S A 79:7243–7247
Henrotte JG (1955) A crystalline component of carp myogen precipitating at high ionic strength. Nature 176:1221
Hodges RS, Sodek J, Smillie LB, Jurasek L (1973) Tropomyosin: amino acid sequence and coiled-coil structure. Cold Spring Harb Symp Quant Biol 37:299–310
Hoffmann-Berling H (1954) Adenosintriphosphat als betriebsstoff von zellbewegungen. Biochim Biophys Acta 14:182–194
Hoffmann-Berling H (1960) Other mechanisms of producing movement. In: Florkin M, Mason HS (eds) Comparative biochemistry, vol 2. Academic, New York, pp 341–370
Holmes KC (1996) Muscle proteins- their actions and interactions. Curr Opin Struct Biol 6:781–789
Holmes KC (2010) 50 years of fiber diffraction. J Struct Biol 170:184–191
Holmes KC, Popp D, Gebbard W, Kabsch W (1990) Atomic model of the actin filament. Nature 347:44–49
Huxley AF, Simmons RM (1973) Mechanical transients and the origin of muscular force. Cold Spring Harb Symp Quant Biol 37:669–680
Huxley HE (1957) The double array of filaments in cross-striated muscle. J Biophys Biochem Cytol 3:631–648
Huxley HE (1963) Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. J Mol Biol 7:281–308

Huxley HE (1967) Recent X-ray diffraction and electron microscope studies of striated muscle. J Gen Physiol 50(suppl 6):71–85
Huxley HE (1969) The mechanism of muscular contraction. Science 164:1356–1366
Huxley HE (1971) Structural changes during muscle contraction. Biochem J 125:85P
Huxley HE (1973a) Structural changes in the actin- and myosin-containing filaments during contraction. Cold Spring Harb Symp Quant Biol 37:361–376
Huxley HE (1973b) Muscle 1972: progress and problems. Cold Spring Harb Symp Quant Biol 37:689–693
Huxley HE (1996) A personal view of muscle and motility mechanisms. Annu Rev Physiol 58:1–29
Huxley HE, Brown W (1967) The low-angle X-ray diagram of vertebrate striated muscle and its behaviour during contraction and rigor. J Mol Biol 30:383–434
Huxley HE, Holmes KC (1997) Development of synchrotron radiation as a high-intensity Source for X-ray diffraction. J Synchrotron Radiat 4:366–379
Hymel L, Inui M, Fleischer S, Schindler H (1988) Purified ryanodine receptor of skeletal muscle sarcoplasmic reticulum forms Ca^{2+}-activated oligomeric Ca^{2+} channels in planar bilayers. Proc Natl Acad Sci U S A 85:441–445
Ikemoto N (1975) Transport and inhibitory Ca^{2+} binding sites on the ATPase enzyme isolated from the sarcoplasmic reticulum. J Biol Chem 250:7219–7224
Ikemoto N (1976) Behavior of the Ca2+ transport sites linked with the phosphorylation reaction of ATPase purified from the sarcoplasmic reticulum. J Biol Chem 251:7275–7277
Imagawa T, Smith JS, Coronado R, Campbell KP (1987) Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+}-permeable pore of the calcium release channel. J Biol Chem 262:16636–16643
Inui M, Saito A, Fleischer S (1987) Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. J Biol Chem 262:1740–1747
Ishikawa H, Bischoff R, Holtzer H (1969) Formation of arrowhead complexes with heavy meromyosin in a variety of cell-types. J Cell Biol 43:312–328
Jeacocke SA, England PJ (1980) Phosphorylation of a myofibrillar protein of Mr 150,000 in perfused rat heart, and the tentative identification of this as C-protein. FEBS Lett 122:129–132
Jenden DJ, Fairhurst AS (1959) The pharmacology of ryanodine. Pharmacol Rev 21:1–25
Johnson JD, Charlton SC, Potter JD (1979) A fluorescence stopped flow analysis of Ca^{2+} exchange with Troponin C. J Biol Chem 254:3497–3502
Jorgensen AO, Kalnins V, MacLennan DH (1979) Localization of sarcoplasmic reticulum proteins in rate skeletal muscle by immunofluorescence. J Cell Biol 80:372–384
Kabsch W, Mannherz HG, Suck D, Pai EF, Holmes KC (1990) Atomic structure of actin: DNAase 1 complex. Nature 347:3–44
Kachur TM, Pilgrim DB (2008) Myosin assembly, maintenance and degradation in muscle: role of the chaperone UNC-45 in myosin thick filament dynamics. Int J Mol Sci 9:186–1875
Kamm KE, Stull JT (1985) The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. Annu Rev Pharmacol Toxicol 25:593–620
Katz AM (1998) Discovery of phospholamban. A personal history. Ann N Y Acad Sci 853:9–19
Kendrick-Jones J, Lehman W, Szent-Gyorgyi AG (1970) Regulation in molluscan muscles. J Mol Biol 54:313–326
Kendrick-Jones J, Szentkiralyi EM, Szent-Gyorgyi AG (1973) Myosin-linked regulatory systems: the role of the light chains. Cold Spring Harb Symp Quant Biol 37:47–53
Kensler RW, Levine RJC (1982) An electron microscopic and optical diffraction analysis of the structure of *Limulus* telson muscle thick filaments. J Cell Biol 92:443–451
Kensler RW, Stewart M (1983) Frog skeletal muscle thick filaments are three-stranded. J Cell Biol 96:1797–1802
Kirchberger MA, Tada M, Repke DI, Katz AM (1972) Cyclic adenosine 3′,5′-monophosphate-dependent protein kinase stimulation of calcium uptake by canine cardiac microsomes. J Mol Cell Cardiol 4:673–680

Konosu S, Hamoir G, Pechere J-F (1965) Carp myogens of white and red muscle. Properties and amino acid composition of the main low molecular-weight components of white muscle. Biochem J 96:98–112
Koretz JF, Hunt T, Taylor EW (1973) Studies on the mechanism of myosin and actomyosin ATPase. Cold Spring Harb Symp Quant Biol 37:179–184
Korn ED (2004) The discovery of unconventional myosins: serendipity or luck? J Biol Chem 279:8517–8525
Krebs EG (1992) Protein phosphorylation and cellular regulation, I. December 8, 1992. nobleprize.org
Krebs EG, Beavo JA (1979) Phosphorylation-dephosphorylation of enzymes. Annu Rev Biochem 48:923–959
Kress M, Huxley HE, Faruqi AR, Hendrix J (1986) Structural changes during activation of frog muscle studied by time-resolved X-ray diffraction. J Mol Biol 188:325–342
Kretsinger RH, Barry CD (1975) The predicted structure of the calcium binding component of troponin. Biochim Biophys Acta 405:40–52
Kretsinger RH, Nockolds CE (1973) Carp muscle calcium-binding protein. II. Structure determination and general description. J Biol Chem 248:3313–3326
Kretsinger RH, Dangelat D, Bryan RF (1971) Crystal data for low molecular weight albumins of carp. J Mol Biol 59:213–214
Lai FA, Erickson HP, Rousseau E, Liu Q-Y, Meissner G (1988) Purification and reconstitution of the calcium release channel of skeletal muscle. Nature 331:315–319
Lehman W, Craig R (2004) The structure of the vertebrate striated muscle thin filament: a tribute to the contributions of Jean Hanson. J Muscle Res Cell Motil 25:455–466
Lehman W, Szent-Gyorgyi AG (1975) Regulation of muscular contraction. Distribution of actin control and myosin control in the animal kingdom. J Gen Physiol 66:1–30
Lehman W, Kendrick-Jones J, Szent-Gyorgyi AG (1973) Myosin-linked regulatory systems: comparative studies. Cold Spring Harb Symp Quant Biol 37:319–330
Lehman W, Craig R, Vibert P (1994) Ca^{2+}-induced tropomyosin movement in Limulus thin filaments revealed by three-dimensional reconstruction. Nature 368:65–67
Lehrer SS, Morris EP (1982) Dual effects of tropomyosin and troponin-tropomyosin on actomyosin subfragment 1 ATPase. J Biol Chem 257:8073–8080
Locker RH, Hagyard CJ (1967) Variations in the small subunits of different myosins. Arch Biochem Biophys 122:521–522
Lowey S, Holt JC (1973) An immunochemical approach to the interaction of light and heavy chains in myosin. Cold Spring Harb Symp Quant Biol 37:19–28
Lowey S, Kucera J, Holtzer A (1963) On the structure of the paramyosin molecule. J Mol Biol 7:234–244
Lowy J, Vibert PJ (1973) Studies of the low-angle X-ray pattern of a molluscan smooth muscle during tonic contraction and rigor. Cold Spring Harb Symp Quant Biol 37:353–359
Luther PK, Winkler H, Taylor K, Zoghbi ME, Craig R, Padron R, Squire JM, Liu J (2011) Direct visualization of myosin-binding protein C bridging myosin and actin filaments in intact muscle. Proc Natl Acad Sci U S A 108:11423–11428
MacLennan DH (1970) Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. J Biol Chem 245:4508–4518
MacLennan DH, Wong PTS (1971) Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. Proc Natl Acad Sci U S A 68:1231–1235
MacLennan DH, Yip CC, Iles GH, Seeman P (1973) Isolation of sarcoplasmic reticulum proteins. Cold Spring Harb Symp Quant Biol 37:469–477
Martonosi AN, Beeler TJ (1983) Mechanisms of Ca^{2+} transport by sarcoplasmic reticulum. In: Peachey LD, Adrian RH, Geiger SR (eds) Handbook of physiology: section 10, skeletal muscle. American Physiological Society, Bethesda, pp 417–485
Maruyama K, Matsubara S, Natori R, Nonomura Y, Kimura S, Ohashi K, Murakami F, Handa S, Eguchi G (1977) Connectin, an elastic protein of muscle. Characterization and function. J Biochem 82:317–337

Masaki T, Takaiti O, Ebashi S (1968) "M-substance", a new protein constituting the M-line of myofibrils. J Biochem 64:909–910
McKillop DFA, Geeves MA (1993) Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. Biophys J 65:693–701
McLachlan AD, Karn J (1982) Periodic charge distributions in the myosin rod amino acid sequence match cross-bridge spacings in muscle. Nature 299:226–231
McLachlan AD, Stewart M (1976a) The 14-fold periodicity in alpha-tropomyosin and the interaction with actin. J Mol Biol 103:271–298
McLachlan AD, Stewart M (1976b) The troponin binding region of tropomyosin. Evidence for a site near residues 197 to 217. J Mol Biol 106:1017–1022
Meissner G (1975) Isolation and characterization of two types of sarcoplasmic reticulum vesicles. Biochim Biophys Acta 389:51–68
Milligan RA, Whittaker M, Safer D (1990) Molecular structure of F-actin and location of surface binding sites. Nature 348:217–221
Moore PB, Huxley HE, DeRosier DJ (1970) Three-dimensional reconstruction of F-actin, thin filaments and decorated thin filaments. J Mol Biol 50:279–295
Moos C (1973) Discussion: interaction of C-protein with myosin and light meromyosin. Cold Spring Harb Symp Quant Biol 37:93–95
Moos C, Mason CM, Besterman JM, Feng I-NM, Dubin JH (1978) The binding of skeletal muscle C-protein to F-actin, and its relation to the interaction of actin with myosin subfragment-1. J Mol Biol 124:571–586
O'brien EJ, Bennett PM, Hanson J (1971) Optical diffraction studies of myofibrillar structure. Philos Trans R Soc Lond B Biol Sci 261:201–208
Offer G (1973) C-protein and the periodicity in the thick filaments of vertebrate skeletal muscle. Cold Spring Harb Symp Quant Biol 37:87–93
Offer G (1974) The molecular basis of muscular contraction. In: Bull AT, Lagnado JR, Thomas JQ, Tipton KF (eds) Companion to biochemistry. Longman, London, pp 623–671
Offer G, Moos C, Starr R (1973) A new protein of the thick filaments of vertebrate skeletal myofibrils: extraction, purification and characterization. J Mol Biol 74:653–676
Ohtsuki I, Masaki T, Nonomura Y, Ebashi S (1967) Periodic distribution of troponin along the thin filament. J Biochem 61:817–819
Ornstein L (1964) Disc electrophoresis. 1, Background and theory. Ann N Y Acad Sci 121:321–349
Parry DAD (1981) Structure of rabbit skeletal myosin. Analysis of the amino acid sequences of two fragments from the rod region. J Mol Biol 153:459–464
Parry DAD, Squire JM (1973) Structural role of tropomyosin in muscle regulation: analysis of the X-ray diffraction patterns from relaxed and contracting muscles. J Mol Biol 75:33–55
Pauling L, Corey RB, Branson HR (1951) The structure of proteins: two hydrogen-bonded helical configuration of the polypeptide chain. Proc Natl Acad Sci U S A 37:205–211
Pechere J-F (1968) Muscular parvalbumins as homologous proteins. Comp Biochem Physiol 24:289–295
Pechere J-F, Capony J-P, Ryden L (1971) The primary structure of the major parvalbumin from Hake muscle. Isolation and general properties of the protein. Eur J Biochem 23:421–428
Pechere J-F, Derancourt J, Haiech J (1977) The participation of parvalbumins in the activation-relaxation cycle of vertebrate fast skeletal-muscle. FEBS Lett 75111–114
Pepe FA (1967) The myosin filament. I. Structural organization from antibody-staining in electron microscopy. J Mol Biol 27:203–225
Pepe FA (1973) The myosin filament: immunochemical and ultrastructural approaches to molecular organization. Cold Spring Harb Symp Quant Biol 37:97–108
Pepe FA, Drucker B (1975) The myosin filament. III. C-protein. J Mol Biol 88:609–617
Permyakov EA, Kretsinger RH (2011) Calcium binding proteins. Wiley, Hoboken
Perrie WT, Smillie LB, Perry SV (1973a) A phosphorylated light chain component of myosin from skeletal muscle. Cold Spring Harb Symp Quant Biol 37:17–18
Perrie WT, Smillie LB, Perry SV (1973b) A phosphorylated light-chain component of myosin from skeletal muscle. Biochem J 135:151–164

Pessah IN, Francini AO, Scales DJ, Waterhouse AL, Casida JE (1986) Calcium-ryanodine receptor complex: solubilization and partial characterization from skeletal muscle junctional sarcoplasmic reticulum vesicles. J Biol Chem 261:8643–8648

Phillips GN, Filler JP, Cohen C (1986) Tropomyosin crystal structure and muscle regulation. J Mol Biol 192:111–131

Philpott DE, Szent-Gyorgyi AG (1954) The structure of light-meromyosin: an electron microscopic study. Biochim Biophys Acta 15:165–173

Pollard TD, Korn ED (1973a) The "contractile" proteins of Acanthamoeba castellanii. Cold Spring Harb Symp Quant Biol 37:573–583

Pollard TD, Korn ED (1973b) *Acanthamoeba* myosin: I. Isolation from Acanthamoeba castellanii of an enzyme similar to myosin. J Biol Chem 248:4682–4690

Pollard TD, Korn ED (1973c) *Acanthamoeba* myosin: II. Interaction with actin and with a new cofactor protein required for activation of Mg^{2+} adenosine triphosphatase activity. J Biol Chem 248:4691–4697

Poole KJV, Lorenz M, Evans G, Rosenbaum G, Pirani A, Craig R, Tobacman LS, Lehman W, Holmes KC (2006) A comparison of muscle thin filament models obtained from electron microscopy reconstructions and low-angle X-ray fibre diagrams from non-overlap muscle. J Struct Biol 155:273–284

Potter JD, Gergely J (1974) Troponin, tropomyosin, and actin interactions in the Ca^{2+} regulation of muscle contraction. Biochemistry 13:2697–2703

Potter JD, Gergely J (1975) The calcium and magnesium binding sites on troponin and their role in the regulation of myofibrillar adenosine triphosphatase. J Biol Chem 250:4628–4633

Rayment I, Holden HM, Whittaker M, Yohn CB, Lorenz M, Holmes KC, Milligan R (1993a) Structure of the actin–myosin complex and its implications for muscle contraction. Science 261:58–65

Rayment I, Rypniewski WR, Schmidt-Base K, Smith R, Tomchick DR, Benning MM, Winkelmann DA, Wesenberg G, Holden HM (1993b) Three-dimensional structure of myosin subfragment-1: a molecular motor. Science 261:50–58

Rios E, Brum G (1987) Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. Nature 325:717–720

Rosemblatt M, Hidalgo C, Vergara C, Ikemoto N (1981) Immunological and biochemical properties of transverse tubule membranes isolated from rabbit skeletal muscle. J Biol Chem 256:8140–8148

Saito A, Seiler S, Chu A, Fleischer S (1984) Preparation and morphology of sarcoplasmic reticulum terminal cisternae from skeletal muscle. J Cell Biol 99:875–885

Sanchez JA, Stefani E (1978) Inward calcium current in twitch muscle fibres of the frog. J Physiol 283197–209

Schneider MF, Chandler WK (1973) Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. Nature 242:244–246

Schwartz LM, McCleskey EW, Almers W (1985) Dihydropyridine receptors in muscle are voltage-dependent but most are not functional calcium channels. Nature 314:747–751

Sellers JR (1999) Myosins, 2nd edn. Oxford University Press, Oxford

Seymour J, O'Brien EJ (1980) The position of tropomyosin in muscle thin filaments. Nature 83:680–682

Shapiro A, Vinuela E, Maizel JV (1967) Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem Biophys Res Commun 28:815–820

Sheterline P, Clayton J, Sparrow JC (1998) Actin, 4th edn. Oxford University Press, Oxford

Simmons RM (1992) A. F. Huxley's research on muscle. In: Simmons RM (ed) Muscular contraction. Cambridge University Press, Cambridge, pp 19–42

Simmons RM, Szent-Gyorgyi AG (1985) A mechanical study of regulation in the striated adductor muscle of the scallop. J Physiol 358:47–64

Sobieszek A (1977) Vertebrate smooth muscle myosin. In: Stephens NL (ed) The biochemistry of smooth muscle. University Park Press, Baltimore, pp 413–443

Sohn RL, Vikstrom KL, Strauss M, Cohen C, Szent-Gyorgyi AG, Leinwand LA (1997) A 29 residue region of the sarcomeric myosin rod is necessary for filament formation. J Mol Biol 266:317–330

Spudich JA (1973) Effects of cytochalasin B on actin filaments. Cold Spring Harb Symp Quant Biol 37:585–593

Spudich JA, Huxley HE, Finch JT (1972) Regulation of skeletal muscle contraction. II. Structural studies of the interaction of the tropomyosin-troponin complex with actin. J Mol Biol 72:619–632

Squire JM (1971) General model for the structure of all myosin-containing filaments. Nature 233:457–462

Squire JM (1972) General model of myosin filament structure. II. Myosin filaments and cross-bridge interactions in vertebrate striated and insect flight muscle. J Mol Biol 72:125–138

Squire JM (1981) The structural basis of muscular contraction. Plenum, New York

Squire JM (1986) Muscle: design, diversity, and disease. Benjamin/Cummings, Menlo Park

Squire JM (1994) The actomyosin interaction—shedding light on structural events: 'Plus ca change, plus c'est la meme chose'. J Muscle Res Cell Motil 15:227–231

Starr R, Offer G (1971) Polypeptide chains of intermediate molecular weight in myosin preparations. FEBS Lett 15:40–44

Starr R, Offer G (1978) The interaction of C-protein with heavy meromyosin and subfragment-2. Biochem J 171:813–816

Starr R, Offer G (1982) Preparation of C-protein, H-protein, X-protein and phosphofructokinase. In: Frederiksen DW, Cunningham LW (eds) Methods in enzymology, vol 85, Part B, Structural and contractile proteins. Academic, New York, pp 130–138

Stone D, Smillie LB (1978) The amino acid sequence of rabbit skeletal α-tropomyosin. The NH_2-terminal half and complete sequence. J Biol Chem 253:1137–1144

Stull JT, Kamm KE, Vandenboom R (2011) Myosin light chain kinase and the role of myosin light chain phosphorylation in skeletal muscle. Arch Biochem Biophys 510:120–128

Sumbilla C, Cavagna M, Zhong L, Ma H, Lewis D, Farrance I, Inesi G (1999) Comparison of SERCA1 and SERCA2a expressed in COS-1 cells and cardiac myocytes. Am J Physiol 277:H2381–H2391

Szent-Gyorgyi AG (1975) Introductory remarks. In: Inoue S, Stephens RE (eds) Molecules and cell movement, vol 30, Society of General Physiologists. Raven, New York, pp 335–338

Szent-Gyorgyi AG (2007) Regulation by myosin: how calcium regulates some myosins: past and present. Adv Exp Med Biol 592:253–264

Szent-Gyorgyi AG, Cohen C, Kendrick-Jones J (1971) Paramyosin and the filaments of molluscan "catch" muscles. II. Native filaments: isolation and characterization. J Mol Biol 56:239–258

Szent-Gyorgyi AG, Szentkiralyi EM, Kendrick-Jones J (1973) The light chains of scallop myosin as regulatory subunits. J Mol Biol 74:179–203

Taylor EW (2001) 1999 E. B. Wilson lecture: the cell as molecular machine. Mol Biol Cell 12:251–254

Taylor KA, Amos LA (1981) A new model for the geometry of the binding of myosin crossbridges to muscle thin filaments. J Mol Biol 147:297–324

Tsien RW, Barrett CF (2000–2012) A brief history of calcium channel discovery. NCBI Bookshelf ID: NBK6465. National Library of Medicine, National Institutes of Health, Madame Curie Bioscience Database [Internet]. Landes Bioscience, Austin

Vibert P, Craig R, Lehman W (1997) Steric-model for activation of muscle thin filaments. J Mol Biol 266:8–14

Wang K, McClure J, Tu A (1979) Titin: major myofibrillar components of striated muscle. Proc Natl Acad Sci 76:3698–3702

Watson JD (1973) Foreword. Cold Spring Harb Symp Quant Biol 37:xvii

Weber HH (1958) The motility of muscle and cells. Harvard University Press, Cambridge

Weber K, Osborn M (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244:4406–4412

Weeds AG, Frank G (1973) Structural studies on the light chains of myosin. Cold Spring Harb Symp Quant Biol 37:9–14
Weisberg A, Winegrad S (1996) Alteration of myosin cross bridges by phosphorylation of myosin-binding protein C in cardiac muscle. Proc Natl Acad Sci 93:8999–9003
Woledge RC (1973) In vitro calorimetric studies relating to the interpretation of muscle heat experiments. Cold Spring Harb Symp Quant Biol 37:629–634
Woodhead JL, Zhao F-Q, Craig R, Egelman EH, Alamo L, Padron R (2005) Atomic model of a myosin filament in the relaxed state. Nature 436:1195–1199

Chapter 7
Endosarcomeric and Exosarcomeric Cytoskeleton: Emergence of Cell and Molecular Biology in the Muscle Field

Titin and/or nebulin apparently provide axial continuity for the production of resting tension on stretch. (Horowits et al. 1986. With permission Nature Publishing Group)

Horowits et al. (1986)

This four-filament sarcomere model raises the possibility that nebulin and titin might act as organizing templates and length-determining factors for actin and myosin respectively. (Wang and Wright 1988. With permission Rockefeller University Press)

Wang and Wright (1988)

The realization that the M-band is elastic and serves signaling functions opens the perspective that M-band strain might also translate into modulation of metabolic activity, in addition to protein turnover and transcription regulation, and thereby regulate short-term adaptation of muscle to strain. (Gautel 2011. With permission Elsevier)

Gautel (2011)

7.1 Introduction

In the early 1970s the sliding filament, attached cross-bridge, model of muscle contraction had become dogma and attention was directed to elucidating the mechanisms of muscle activation and cross-bridge function. Of course this was a two filament model of contraction with the thin filaments anchored at the Z disc and the thick filaments held in place at the M band. But there were lingering questions. In the classic experiments of Huxley and Hanson (1954), extraction of actin and myosin did not cause the collapse of the myofibril but rather "ghost" myofibrils resulted that still exhibited elasticity. They explained this observation by suggesting that an unseen elastic structure existed that held the Z discs together. With the exciting advances in understanding of activation and cross-bridge function, this observation seemed to have been forgotten. But there was more, striated muscles are striated because of the precise lateral alignment of the myofibrils across the fiber.

J.A. Rall, *Mechanism of Muscular Contraction*, Perspectives in Physiology,
DOI 10.1007/978-1-4939-2007-5_7

The classic experiments of Huxley and Niedergerke (1954) showed that this alignment did not change when a living fiber shortened actively or was stretched while at rest. What determines this alignment? What is the molecular nature of the Z disc and M band that hold the filaments together transversely and what structure holds the sarcomeres together longitudinally even in the absence of actin and myosin (endosarcomeric structures)? What structures bind the myofibrils together transversely across a muscle fiber (exosarcomeric structures). Electron microscopic evidence provided some early clues but the results were inconsistent and there were concerns about artifacts associated with fixation.

The rapid emergence of cell biology and molecular biology techniques in the 1980s played and continues to play a major role in the advancement of our understanding of the endosarcomeric and exosarcomeric cytoskeleton. Of preeminent importance was the development of immunological approaches to molecular histology with fluorescent antibody and immunoelectron microscopy techniques. These techniques coupled with the amino acid sequence determination of proteins by complementary DNA (cDNA) techniques have led to spectacular advances in the understanding of the muscle cytoskeleton. As a result, new fields of investigation have evolved characterizing intracellular signaling pathways in regulation of muscle cell development and response to mechanical stress and disease.

7.2 Passive Force and the Third Filament of the Sarcomere: Titin First Discovered as Connectin

7.2.1 Introduction

In the early 1970s generally it was thought that the resting elasticity or passive force production observed when a resting muscle was stretched was due simply to the connective tissue that surrounded the muscle fibers and collagen fibrils in the sarcolemma (Fig. 7.1). But there were early observations that suggested that at least some of this elasticity must reside inside the fiber and within the myofibril itself. In the early 1950s, Reiji Natori at the Jikeikai Medical School in Tokyo Japan developed a single skeletal muscle fiber preparation in which the sarcolemma was mechanically removed from a frog fiber. The muscle fiber contained no extracellular collagen but yet when stretched at rest still exhibited the passive force attributed to the intact fiber (Natori 1954). Thus he concluded that much of the resting elasticity of a fiber must exist within the fiber itself. At about the same time, Hugh Huxley and Jean Hanson were describing their evidence for the sliding filament mechanism of muscle contraction. In a remarkable experiment they showed that isolated myofibrils that had their myosin and actin extracted still exhibited Z discs and a "backbone" of virtually zero optical density (see Fig. 2.8) (Huxley and Hanson 1954; Hanson and Huxley 1955). These "ghost" myofibrils were also elastic and could be stretched and released. Because of this observation Hanson and Huxley (1955) speculated that there must be a "third filament" along with the thick and thin

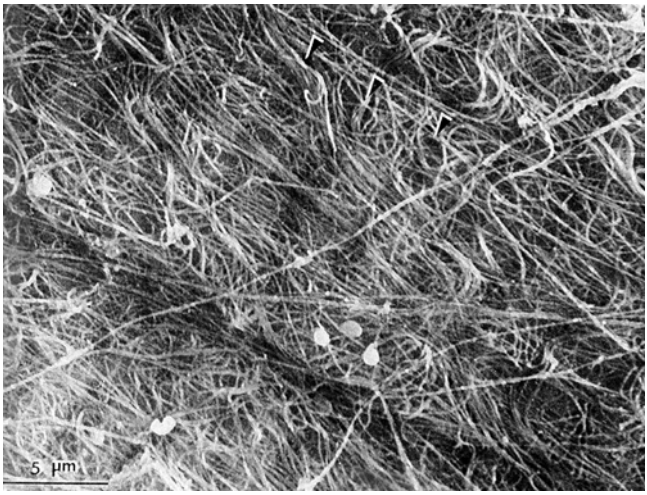


Fig. 7.1 Scanning electron micrograph showing the fibrous layer of connective tissue on the surface of a frog sartorius muscle fiber. Collagenous fibrils densely cover the muscle fiber and take a predominantly longitudinal course. Cross striations can be seen through fibrous layer (Ishikawa et al. 1983. With permission The American Physiological Society)

filaments that composed the sarcomere. They named this hypothetical third filament the "S filament", S for stretch, and envisioned that it connected the ends of thin filaments in each half of the sarcomere (see Fig. 2.9). This third filament vanished from the models of the sliding filament mechanism after 1956 most likely because in Hugh Huxley's classic electron microscopic observations of 1957 there was no visual evidence for an S filament (see Figs. 3.3, 3.4, 3.5).

7.2.2 Early Electron Microscopic Evidence for a Third Filament and Its Skeptical Reception

Despite Huxley's results, there were those scientists who believed that the two filament model of contraction was inherently unstable and that there had to be a third filament to explain the stability of the sarcomere and the elasticity of the myofibril. Electron microscopic evidence started to appear in the early 1960s that suggested a third filament in the sarcomere. For example, Fritiof S. Sjostrand (1962) at the University of California at Los Angeles (UCLA) stretched isolated frog skeletal muscle to the point where there was no overlap of thick and thin filaments. At these lengths a "gap" developed between the thick and thin filaments as seen in the electron microscope. Filaments thinner than the thin filaments could be seen bridging the gap. These filaments were of variable thickness and appeared to be continuous with thick filaments of the A band. Thus he named them "gap filaments". Graham Hoyle at the University of Oregon working with Patricia A. McNeill (McNeill and

Hoyle 1967) also saw very thin filaments in the gap when muscles were stretched to long lengths. These "superthin filaments" or T filaments as Hoyle called them also could be seen in the H zone and McNeill and Hoyle concluded that they ran the whole length of the sarcomere and connected adjacent Z discs together. In 1975 Ronald Locker and N. G. Leet at the Meat Industry Institute of New Zealand published the first of a series of papers examining the structure of highly stretched beef muscle fibers. They found that the thick filaments were staggered and that gap filaments were observed. They concluded that the filaments that "always" span the gap must be responsible for transmitting the force that dislocates and stretches the A-band. There were also other reports of third filaments, particularly in insect muscle (see Wang 1985 for a review).

These results were greeted with skepticism by Hugh Huxley and Jean Hanson (Huxley 1968; Hanson 1968). The issue was discussed extensively at a symposium on muscle in Budapest. There seemed to be multiple reasons for the skepticism. First, Hugh Huxley didn't see them in his classical 1957 study. Second, the third filament proponents could not agree on the exact location of the filaments in the sarcomere. Third, while not denying the observations, there was always the possibility of fixation artifacts. With regard to her own study of the ghost myofibrils, Hanson (1968) stated in the general discussion at the Budapest symposium that she had observed a sparse network of very thin structureless filaments connecting successive Z line but did not know if this material was present in the intact fibril or if it represented an insoluble residue of the actin and myosin filaments or was derived from interfibillar material. In short, in the view of Huxley and Hanson, the electron microscopic evidence was not convincing.

There was a lot of frustration on the part of the third filament proponents. For example, Trombitas (2000) of Washington State University who had seen a third filament in insect muscle provided in 2000 some perspective on those days (Trombitas 2000. With permission Springer):

> Although there was a general consensus about the appearance of the gap filaments in overstretched vertebrate skeletal muscle, the evidence was not convincing enough to firmly establish a third type of filament…Since the sliding filament theory explained muscle contraction so well, the muscle field became conservative and, in my experience, this hindered progress in elastic filament research. It became very difficult to publish new results from this field in high-ranking journals. Referees were not sufficiently open-minded…

Graham Hoyle was more outspoken about the lack of acceptance of the evidence of a third filament by the establishment (Hoyle 1983. With permission John Wiley & Sons Inc):

> The most glaring example of a very real attempt on the part of a majority of muscle scientists to willfully lose a vital piece of information in the last 25 years has been in regard to ultrathin (T or S) filaments…The evidence for their existence, both theoretical, based on dynamic properties, and visible, based on electron microscopy, has been amply provided… Yet for reasons I have not been able to understand, the majority of muscle scientists have ignored them completely. There have been occasional howls from the frustrated few believers, but these believers have been treated by the majority as nasty pests needing to be destroyed. Why? Without them, T filaments would certainly be getting 'lost.' With such obstinacy the majority deserve to be proved wrong!

Reading the arguments on both sides, one comes away with the impression that the majority didn't deny the possible existence of a third filament but rather that the evidence for a third filament didn't reach the level of expectation for wide acceptance. After all, the sliding filament model was greeted with much skepticism when it was proposed in 1954 but Hugh Huxley produced the structural evidence in 1957 that led to general acceptance of the model. It was up to the third filament proponents to do the same.

7.2.3 A Breakthrough: Protein Nature of a Putative Third Filament

It turned out that the required evidence of the existence of a third filament in the sarcomere did not come first from electron microscopy but rather from biochemistry. The breakthrough started with the pioneering studies on the biochemistry of muscle elasticity by Koscak Maruyama (Fig. 7.2a) in Japan. Maruyama was an expert in the isolation and characterization of muscle proteins. He had collaborated with Setsuro Ebashi on the characterization of α-actinin, a protein discovered by Ebashi 1963 (Maruyama and Ebashi 1965). Soon thereafter he discovered another sarcomeric protein, β-actinin (Maruyama 1965) and later showed that it was equivalent to the actin capping protein now known as Cap Z (Maruyama et al. 1990). Maruyama was intrigued by Natori's results on the elasticity of skinned muscle fibers and he set out to determine the protein nature of this elasticity. Along with Natori and Yoshiaki Nonomura, they first showed that skinned muscle fibers from the frog from which actin and myosin were extracted exhibited elasticity when stretched. They then isolated a protein from rabbit ghost myofibrils that was clearly different from elastin and collagen based on its amino acid composition (Maruyama et al. 1976). Furthermore a thread of this protein was elastic as it could be stretched and bear force. They entitled their Nature paper: "New elastic protein from muscle" but did not give it a name. At least in part this may have been because one of the reviewers of the paper apparently suggested that the new elastic protein could be reticulin. The month that the Nature paper was accepted, Maruyama (1976) sent a preliminary communication to the Japanese journal, Journal of Biochemistry, entitled: "Connectin, an elastic protein from myofibrils". In this communication he showed that the new elastic protein had a different amino acid composition than reticulin. Thus he felt confident that he had discovered a genuine new protein and thus gave it a name. He proposed to call the new intracellular elastic protein "connectin". These results were published in full (Maruyama et al. 1977[1]). They knew

[1] Sumiko Kimura, department of biology of Chiba University, collaborated with Koscak Maruyama on virtually all of his publications on connectin over a period of nearly three decades. Her publications span more than 40 years.

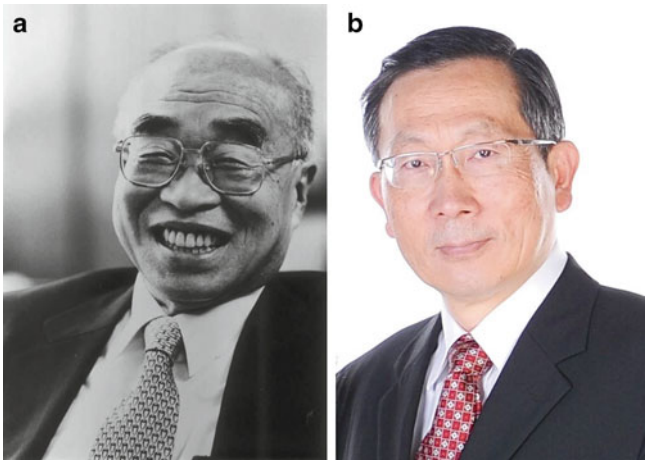


Fig. 7.2 (**a**) Koscak Maruyama (1930–2003) received a Ph.D. from Tokyo University in 1958 and was a postdoctoral fellow in John Gergely's laboratory in Boston from 1959 to 1961. He held faculty positions at the University of Tokyo, Kyoto University and was the head of the biology department at Chiba University. He discovered the protein connectin (now more generally known as titin) and was the first to recognize the elasticity of this protein. He also discovered β-actinin (now called CapZ) and contributed to the characterization of α-actinin that was discovered by Setsuro Ebashi. He had an avid interest in the history of biochemistry and wrote numerous articles on the topic. An able administrator, Maruyama served as the president of Chiba University from 1994 to 1998. For further details, see Gergely and Kimura (2005). Photo: Gergely and Kimura (2005). With permission Springer. (**b**) Kuan Wang received his Ph.D. from Yale University in 1974 and did postdoctoral training with Seymour J. Singer at the University of California at San Diego in 1975. Along with J. Frederick Ash and Singer, Wang isolated and named the protein filamin from smooth muscle. He isolated and named the proteins titin (also called connectin), nebulin and nebulette. Wang was a professor of chemistry and biochemistry at the University of Texas from 1977 to 1997 before becoming the laboratory chief of muscle biology at NIAMS, NIH until 2010. He then became the Director of the Nanomedicine Program and Distinguished Research Fellow of the Institute of Biological Chemistry and Institute of Physics, Academica Sinica, Taiwan. Photo: courtesy K. Wang

that connectin was a large protein but they thought that its size was due to covalent connections among smaller subunits. Looking back, Maruyama (Maruyama and Kimura 2000) stated that they wasted more than one year because they could not believe the presence of a single giant peptide of a few million Daltons. Therefore, it was expected that there were some crosslinks such as in collagen or elastin. It turned out that the crosslink was an artifact resulting from the alkaline treatment of muscle and in fact they were dealing with a giant protein.

Kuan Wang (Fig. 7.2b) at the University of Texas at Austin was also interested in the filamentous proteins of muscle. He had established that filamin existed in smooth muscle cells (Wang et al. 1975) and wondered whether it might also be present in skeletal muscle. It turned out that filamin was not present in skeletal muscle but in the process Wang, Janela McClure and Ann Tu (1979) made a major discovery. When purified chicken and rabbit skeletal myofibrils were analyzed with high-porosity

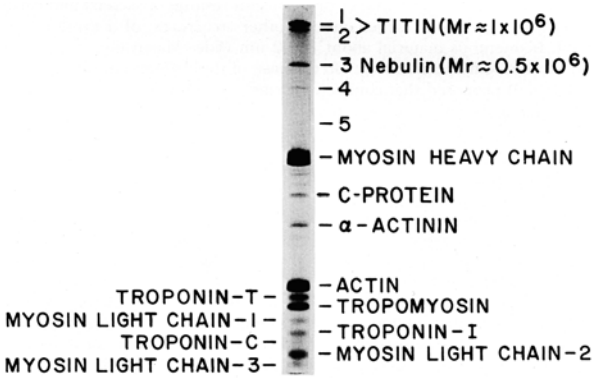


Fig. 7.3 Major myofibrillar proteins of rabbit skeletal muscle. SDS-polyacrylamide gel pattern of thiol-reduced, denatured rabbit myofibrillar proteins. A titin doublet (T1 and T2) and nebulin (originally called band 3) are major components of very low electrophoretic mobility (Wang 1985. With permission Springer)

polyacrylamide gel electrophoresis, all solubilized proteins entered the gels and there were three major bands above the myosin heavy chain band at 200 kDa: a closely spaced doublet and a single band with faster mobility (Fig. 7.3). They estimated the molecular weight of the slow moving doublet to be about 1,000 kDa[2] and the faster moving band at about 500 kDa. They tentatively named the doublet "titin" derived from Titan (Greek, a giant deity, anything of great size.). They called the 500 kDa band "band 3" but later Wang would name it "nebulin" (see below). They purified titin and determined its immunological properties. Immunofluorescent staining of glycerinated chicken breast myofibrils indicated that titin was present in M bands, Z discs, the junctions of A and I bands, and perhaps throughout the entire A bands. Thus they concluded that titin appears to be a "bona fide" structural component of myofibrils. Apparently they were unaware of Maruyama's results since they didn't cite them. Furthermore, despite Maruyama's study, they stated that previously there was no supporting biochemical evidence for a third filament. Also they did not speculate on the function of titin beyond suggesting that titin might be a candidate for the "elusive" third filament of the sarcomere. Nonetheless they discovered the largest protein known in nature. In fact it would continue to become larger as techniques for measuring molecular weights became more precise.

Maruyama naturally wondered whether connectin and titin were the same protein. He and his colleagues compared the two proteins (Maruyama et al. 1981). They found that the amino acid compositions of titin and high molecular weight connectin were almost the same. Furthermore the band 3 protein of Wang, the molecular weight of which is certainly smaller than that of connectin but much larger than that of myosin, was different in amino acid composition from connectin. They therefore concluded that titin and connectin were the same protein and that the band 3 protein (nebulin) should be regarded as an entity distinct from connectin and titin.

[2] As predicted by Wang et al. (1979), the doublet nature of titin was due to limited proteolysis of titin.

7.2.4 What's in a Name: Connectin or Titin?

To Koscak Maruyama, connectin and titin were the same protein and since his work occurred earlier it was natural that he felt that the name connectin should take precedence over the name titin. Kuan Wang did not see it that way. He felt that the original crude extract isolated by Maruyama contained titin, nebulin and other protein contaminants. He summarized his view in a comprehensive review of the subject in the following way (Wang 1985. With permission Springer):

> In 1981 Maruyama et al (1981a) purified titin from KI-extracted muscle residues and proceeded to identify connectin as titin…This redesignation has created much confusion in the literature, because titin is only one of the many components of stroma proteins. Indeed, KI residues contain titin, nebulin, myosin, actin, Z-line proteins, intermediate filament proteins, and contaminating connective tissue proteins (Wang and Ramirez-Mitchell 1983). It is unfortunate that these workers have now attributed most of the properties and structural organization of the poorly defined connectin residue to titin without undertaking a careful reevaluation of the various claims. In the following discussions, we shall adapt the original definitions and use *titin* to designate the large polypeptides near 10^6, *connectin residue* to refer to the insoluble residue, and *connectin* (*titin*) to designate titin-equivalent connectin.

This disagreement in nomenclature has not been completely resolved. Certainly in Japan the elastic protein will likely always be known as connectin in deference to Maruyama's pioneering investigations. Throughout the rest of the world, the usual designation is "titin" or sometimes "titin (also known as connectin)". Sumiko Kimura (2005), Maruyama's long-time research collaborator, avoided the controversy about names in the memorial symposium dedicated to Maruyama when she said: "This symposium commemorated the pioneering work of the late Professor Koscak Maruyama, who discovered elastic proteins in muscle." (Kimura 2005. With permission Springer) Possibly Linke and Kruger (2010) have struck the fairest compromise when in a recent review they stated: "titin, first described as connectin" and referenced Maruyama's 1977 paper and Wang's 1979 paper. Koscak Maruyama, who had an avid interest in the history of biochemistry, wrote a brief paper about a year before his death describing some "extinct names" of discovered proteins, especially the names of his first discovered proteins, β-actinin and connectin (Maruyama 2002). He stated what is, no doubt, a truism (Maruyama 2002. With permission Elsevier): "Only the creators of the extinct names really care about these losses as most scientists are not interested in the history of their fields."

7.2.5 First Comprehensive Model of Titin and Nebulin Location and Function

Based on the ultrastructural studies of Locker and Leet (1975) and his own immunocytological studies, Wang (1985) provided the first comprehensive model of titin and nebulin location and elastic function in the muscle sarcomere (Fig. 7.4). In constructing this model, he made the following predictions:

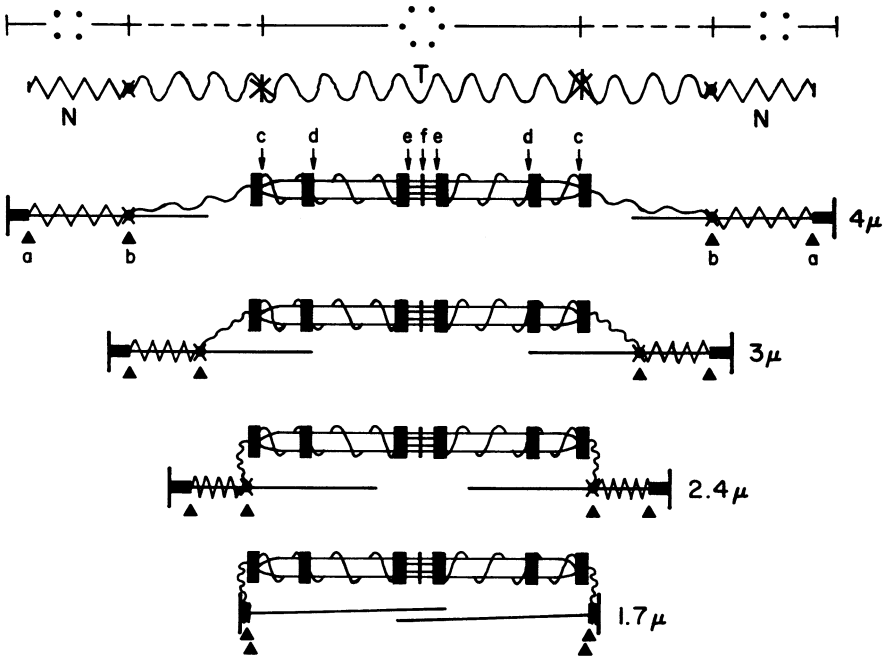


Fig. 7.4 A hypothetical three-filament sarcomere. According to this working hypothesis the third filament constitutes a three-dimensional lattice containing both longitudinal and transverse elements. The longitudinal filament is continuous from Z line to Z line and consists of two alternating filament domains, each containing either titin (T) or nebulin (N). The longitudinal filament need not surround the thick helically as depicted here. One (of many) possible mechanisms by which the third filament may respond to sarcomere length changes is shown; only the I-band domain (**a**–**c**) changes length. See text for further details (Wang 1985. With permission Springer)

1. The longitudinal filaments run parallel and external to thick and thin filaments.
2. The filaments are intrinsically elastic and/or extensible except when and where they interact with other inextensible sarcomeric structures.
3. The filaments are continuous from Z line to Z line.
4. The filaments are anchored at the Z line and M line.
5. Both titin and nebulin are components of the same filament strand.
6. Titin and nebulin are serially connected.
7. The filament is intrinsically elastic along its entire length from Z line to Z line.
8. Weaker or a different mode of interaction exists between the third filament and the thin filament since thin filaments are not displaced with stretch.
9. The filaments are linked to each other at a few spots in the I band to stabilize the sarcomeric lattices.

Wang emphasized that the important point about this model was that it was detailed and testable. Some aspects of the model did not stand the test of time but it was a very valuable guide for future investigation.

7.2.6 *Physiological Role of Titin in Muscle Fibers*

Despite the pioneering work of Maruyama and Wang and their observations that suggested that titin was the protein in the third highest concentration in myofibrils, representing about 5–8 % of the total myofibrillar protein (Maruyama et al. 1977; Wang et al. 1979), there was still great doubt about the observations. The reported gigantic molecular weight of the titin molecule was especially difficult to accept.

An important breakthrough came with the study of the function of this putative giant elastic protein in muscle fibers. Richard J. Podolsky's laboratory at the NIH had a long history of performing creative experiments on muscle. His experiments with postdoctoral fellow Robert Horowits in 1986 are some of the most striking and imaginative in the titin field. They reasoned that one way to examine titin function would be to destroy the molecule and then determine to what extent the mechanical and structural properties of a muscle fiber changed. They established a collaboration with Ellis S. Kempner, also at the NIH, who was utilizing radiation inactivation to estimate the molecular weight of various enzymes (Kempner and Haigler 1982). Since the radiation sensitivity of macromolecules was directly related to their mass, the idea was that the largest molecules would be damaged first by ionizing radiation (destruction of covalent bonds). By controlling the intensity of the irradiation it might be possible to damage titin and nebulin without appreciably destroying the other molecules in the sarcomere. This was not a trivial experiment as radiation inactivation was only effective on dried or frozen samples. They utilized chemically skinned psoas muscle fibers from rabbits (Horowits et al. 1986). They measured the maximum active force production at rest length and the passive force with stretch, then rapidly froze the fibers, irradiated them, thawed the fibers and measured the mechanical properties again. As a control some frozen fibers were not irradiated before thawing. In parallel experiments they examined the effects of irradiation of the proteins with SDS polyacrylamide gel electrophoresis. They established an irradiation dose that resulted in almost complete destruction of the titin bands with only an approximately 15 % reduction in the intensity of the myosin heavy chain, actin, and α-actinin bands. They observed an approximate 20 % decline in active force production with no change in passive force in the control fibers that were frozen and thawed. In contrast in the irradiated fibers, the active force declined but the passive force with stretched declined to a greater extent (Fig. 7.5). Thus they concluded that "Titin and/or nebulin apparently provide axial continuity for the production of resting tension on stretch…" (Horowits et al. 1986. With permission Nature Publishing Group) But what was remarkable was that with prolonged activation, the irradiated fibers exhibited misalignment of the thick filaments in the A bands as seen in the electron microscope. They stated that in some sarcomeres this led to an almost complete disappearance of the I band as individual thick filaments were actively driven toward the nearest Z disk. They envisioned that this effect was due to destruction of some of the titin filaments as shown schematically in Fig. 7.6. Thus they concluded that the findings indicated that irradiation destroyed a structure responsible for centering thick filaments within the sarcomere during both passive and active force generation. Now there was physiological evidence consistent with a role for titin in producing passive force with stretch and in centering the A band in the sarcomere at rest and during contraction.

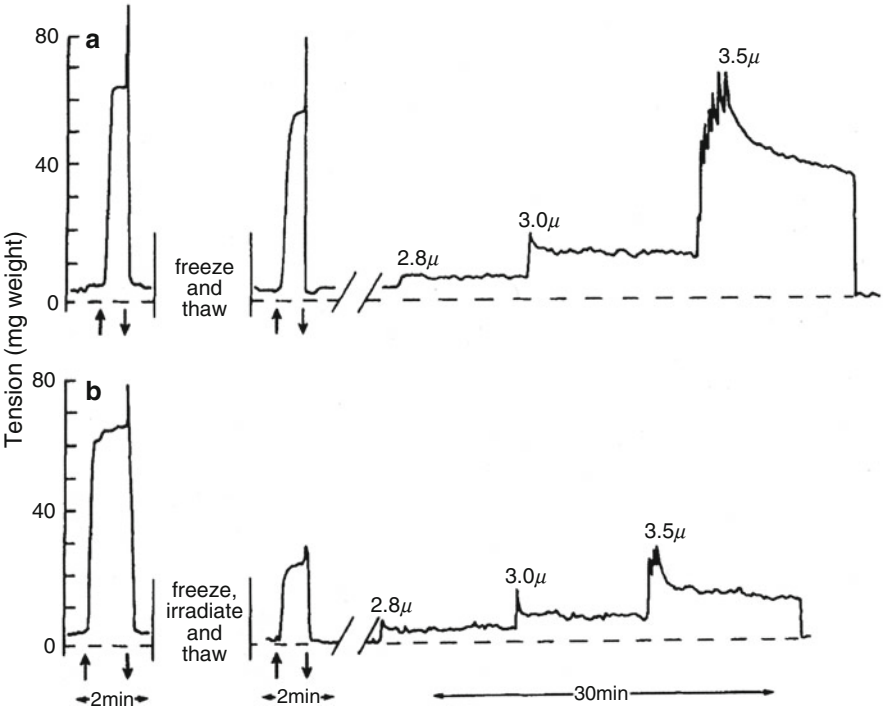


Fig. 7.5 Effects of destruction of titin on active and passive force production of single skinned muscle fibers. (**a**) Dose = 0 Mrad, (**b**) dose = 0.5 Mrad. After maximum isometric force was measured at a sarcomere length of 2.6 μm, the fiber was removed from the force measuring apparatus and frozen, irradiated and thawed. After remounting the fiber in the apparatus, maximum force was again measured at a sarcomere length of 2.6 μm. Passive force was measured by stretching the fibers to various sarcomere lengths as indicated above the force records. Freezing and thawing alone decreased active force but did not significantly affect passive force. Irradiation led to a further decrease in active force but a larger decrease in passive force with stretch (Horowits et al. 1986. With permission Nature Publishing Group)

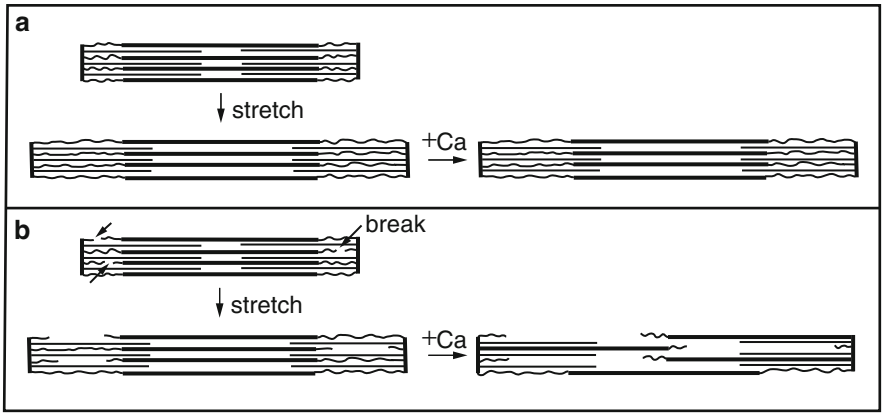


Fig. 7.6 Model of a sarcomere in which each end of the thick filament is linked to the nearest Z disc by elastic filaments made of titin and/or nebulin. (**a**) Control, (**b**) after radiation. Breakage of the elastic filaments by radiation leads to thick filament misalignment on stretch or activation (Horowits et al. 1986. With permission Nature Publishing Group)

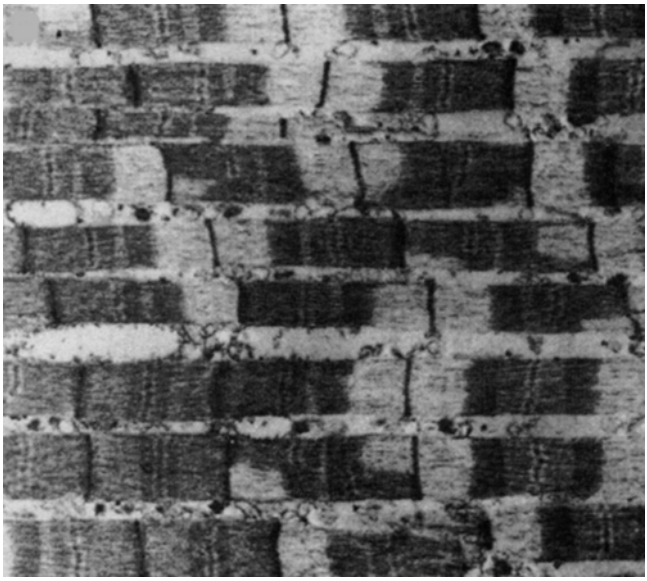


Fig. 7.7 Electron micrograph of a rabbit skinned muscle fiber that was fixed 7.5 min after the start of activation. The fiber was initially at a resting sarcomere length of 2.6 μm and then activated to produce an isometric contraction. Note the dramatic displacements of the A bands from the center of many of the sarcomeres (Horowits and Podolsky 1987. With permission Rockefeller University Press)

Horowits and Podolsky (1987) followed up this study with a more detailed electron microscopic investigation. They reasoned that if it was true that titin helped center the A band in the sarcomere that this centering function might be overcome with very long maximum activation of skinned muscle fibers at rest length. Under these conditions, skinned fibers developed a sarcomere length inhomogeneity. It was possible that a small imbalance of force production in each half of an A band might develop which would be amplified over time and would tend to pull the A band toward one end of the sarcomere. Rabbit skinned psoas fibers were activated to produce an isometric contraction for 5–7.5 min at a resting sarcomere length which would produce maximum force production. These fibers then were examined in the electron microscope. Sarcomeres were of variable length and the A bands were displaced from the center of the sarcomere even though the length of the thick and thin filaments remained constant (Fig. 7.7). This was a most dramatic result. When fibers were fixed after relaxation, the A bands had returned to the center of the sarcomeres as was observed before contraction. In contrast, if a fiber was activated for 5 min at a long sarcomere length where passive force was significant, the A bands remained in the center of the sarcomere. Horowits and Podolsky explained these results quantitatively. At longer sarcomere lengths where active force production is decreased, any imbalance in each half of the A band would not be great enough to

overcome the high resting stiffness and the A band would remain centered in the sarcomere[3]. This resting stiffness was attributed to the elasticity of the titin molecule. Thus the results of Horowits and Podolskly further supported the proposal that titin was responsible for the intracellular resting elasticity of a muscle fiber and for maintaining the A band in the center of the sarcomere upon muscle activation.

7.2.7 Sarcomeric Organization of Titin Molecules

By the mid-1980s much had been learned about the titin molecule (for a review see Ohtsuki et al. 1986). Maruyama determined that the molecular of weight of titin, initially estimated to be about 1,000 kDa by Wang, was actually closer to 3,000 kDa. As expected, the lower band in the titin doublet was a proteolytic breakdown product of the upper band. The titin molecule was isolated in its native form by the Maruyama, Wang and John Trinick laboratories in 1983 and 1984. There was general agreement that titin was the third most abundant protein in the sarcomere and constituted about 10 % of the protein content of myofibrils. In the electron microscope, individual titin molecules appeared as string-like structures up to 1 μm in length with a width of 4–5 nm. Trinick (1981) had earlier identified filaments extruding from both ends of native thick filaments and called them "end filaments" and now speculated that they were titin filaments. Trinick et al. (1984) summarized the status of the third filament models and their relationship to titin. They felt that there was not sufficient data to decide whether any of the various models of elastic filaments was correct or what the exact relationship of titin to such a system might be. What was needed was a precise location of titin in the muscle based on electron micrographs of muscle labeled with titin antibodies. They also emphasized that it would important to demonstrate elasticity in the native molecule.

Thus what clearly was needed was an agreed upon location of the titin molecule in the sarcomere. The definitive results came in a beautiful study from the laboratory of Klaus Weber at the Max Planck Institute for Biophysical Chemistry in Gottingen, Germany. Furst et al. (1988) generated a battery of ten monoclonal antibodies against the native titin molecule. The titin antibodies did not cross react with nebulin. These antibodies were distributed from the amino to the carboxyl end of the titin molecule. They then employed immunofluorescence and immunoelectron microscopy on myofibrils to determine the location of the antibodies in the sarcomere. The result was a linear epitope map for titin in the sarcomere. One antibody bound very near to the Z line, five antibodies bound to titin in the I band, one at the A/I junction and three in the A band. Each of the titin antibodies provided a pair of symmetric decoration lines per sarcomere. The position of these pairs was centrally symmetric to the M band which indicated that the titin molecules were

[3] It should be mentioned that the activation of muscle fibers continuously for 5–7.5 min would likely never occur in vivo. Furthermore it is well known that continuous activation of skinned muscle fibers will cause sarcomere length inhomogeneity.

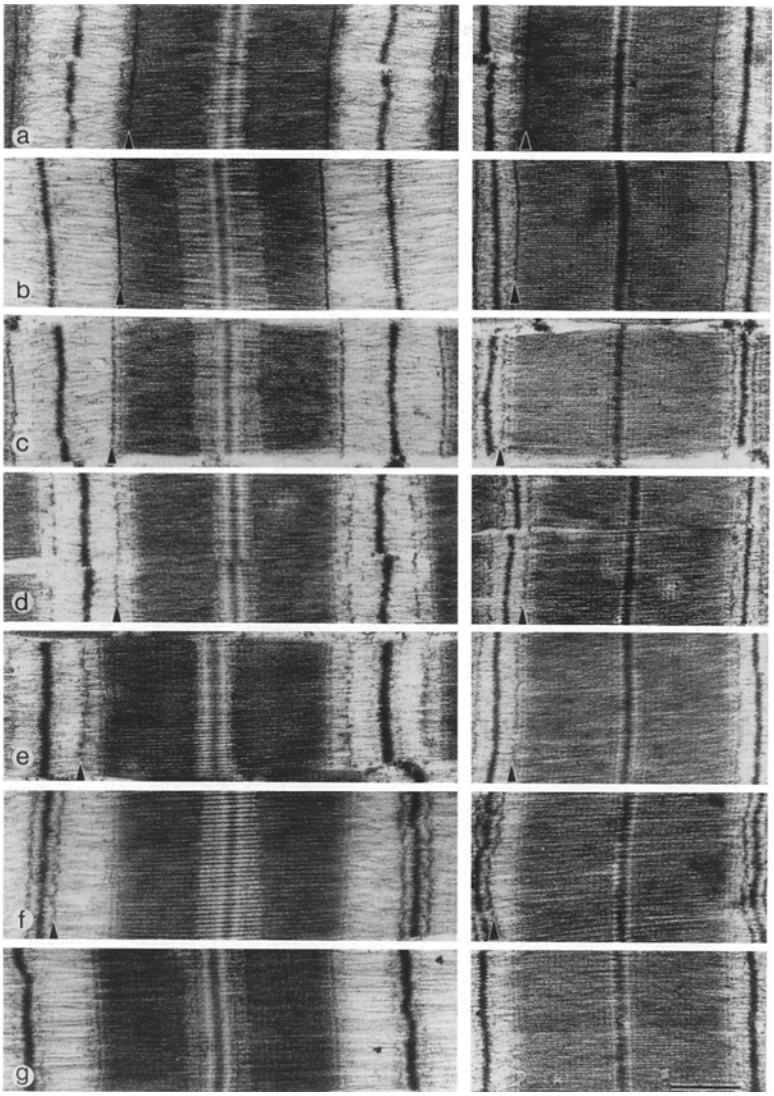


Fig. 7.8 Immunoelectron microscopical localization of titin in chicken pectoralis muscle. Results are presented in pairs. The micrographs on the left show the result on relaxed sarcomeres whereas the micrographs on the right show the result in contracted sarcomeres. Positions of antibody label are indicated by *arrowheads*. Note that each antibody gives only one specific decoration line per half-sarcomere. Six antibody locations are shown (**a**–**f**) and g shows control muscle. Only the three antibodies in the I band (**c**–**e**) closest to the A/I junction change their position upon contraction. Bar: 500 nm (Furst et al. 1988. With permission Rockefeller University Press)

polar structures reaching from the carboxyl end of the molecule at the M band to the amino end at the Z disc (Fig. 7.8). One antibody was located within 0.2 μm of the M band and at the other end of the molecule one antibody decorated the Z disc. Thus the titin molecule ran from the M band to the Z disc in the sarcomere. Since the resting sarcomere length in these experiments was about 2.7 μm, the titin molecule must be about 1.3 μm long under these conditions. They also examined how the antibody location changed when the myofibrils contracted. Only the three antibodies in the I band closest to the A/I junction changed their position upon contraction (Fig. 7.8c–e). Thus the issue of titin location was settled. Certain models of the third filament could now be rejected. A single molecule of titin did not run from Z disc to Z disc. Titin was not connected serially to nebulin but rather it connected the M band to the Z disc.

7.2.8 *Elasticity of the Titin Molecule*

The process of understanding the elasticity of titin at the myofibrillar and molecular level was greatly advanced when Labeit and Kolmerer (1995a) of the European Molecular Biology Laboratory in Heidelberg, Germany, determined the primary sequence of human cardiac titin using complementary DNA (cDNA) analysis. The results predicted a protein of nearly 27,000 residues with a molecular weight of almost 3,000 kDa. Ninety percent of the protein mass is contained in a repetitive structure composed of 100-residue repeats. These repeats encode 112 immunoglobulin (Ig)-like and 132 fibronectin-type III (FN3)-like domains. The I band region of

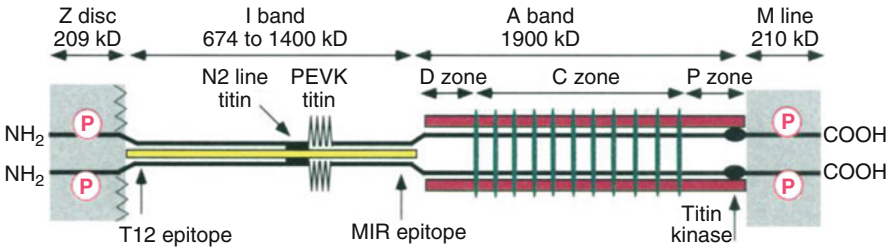


Fig. 7.9 A Model of the titin filament in the sarcomere. The titin filaments (two *thin lines*), the thin filament (actin) (extending from A band to Z disc), and the thick filament (myosin) (*thick lines*). The epitopes of the titin antibodies (T12 and MIR) have been mapped in the sarcomere by immunoelectron microscopy. Antibodies to the titin kinase domain react with the periphery of the M band. For the I band, the range of variation as predicted by the splice variants is indicated. The presumed extensible element of the I band, the PEVK element (*zig-zag* pattern) is shown. Within the thick filament, titin binds to both the C protein (*stripes*) and myosin and is likely to specify the presence of 11 copies of the 430 Å thick filament repeat in vertebrate striated muscles. Phosphorylation of the repeats in the Z disc and the M band titin (P) may control integration of the titin filament into Z discs and M bands during myogenesis (Labeit and Kolmerer 1995a. With permission American Association for the Advancement of Science)

titin is composed of three different segments: tandem Ig-like domains, nonrepetitive sequence insertions in the central region of the I band, and a complex domain architecture near the junction of the A band and the I band. In the central part of the I band, cardiac and skeletal titins branch into distinct isoforms. Alternative splicing of a single titin gene accounts for the tissue-specific variation of the I band titin structure. The four amino acids P (proline), E (glutamic acid), V (valine), and K (lysine) constitute 70 % of this element, and therefore it is referred to as the PEVK domain of titin. This domain comprises 163 or more residues in the cardiac titin sequence and 2,174 residues in the skeletal titin sequence (Fig. 7.9). They concluded that the range of isoforms (see titin isoforms below) of I band titin generated by differential splicing affects the length of the tandem Ig and the PEVK regions of titin. Furthermore the highly conserved A band region of titin could explain the location of C protein along the thick filament (see Chap. 6 for information about C protein [Myosin Binding Protein C, MyBP-C]).

They went on to speculate about the shape of the passive force-extension curve in muscle and how it might relate to the structure of the titin molecule. Labeit and Kolmerer (1995a) suggested the PEVK region might be more easily stretched than the Ig domains because the reduced complexity of the sequence together with the clusters of negative charges could prevent the formation of stable tertiary structure folds. Therefore, the PEVK domain may account for the extensibility of the titin filament at low forces. At longer lengths the stable folds of the Ig domains of I band titin would resist further extension. This would account for the rapid rise in force toward the end of the slack length of sarcomeres. The moderate levels of further extension that occur in vivo in skeletal muscles may be explained by conformational changes in the tandem Ig segments, such as bending and stretching of interdomain linkers. Thus they proposed that titin molecule acts as two springs in series. Also they suggested that the differential expression of the springs provided a molecular explanation for the diversity of sarcomere length and resting tension in vertebrate striated muscle. With this work it was now possible to begin to think about the elasticity of the titin molecule in molecular terms.

Wolfgang A. Linke, then at the University of Heidelberg, and his colleagues Marc Ivemeyer and J. Caspar Ruegg collaborated with Siegfried Labeit and Bernhard Kolmerer and their colleague Nicoletta Olivieri in an effort to locate the elastic region of the titin molecule in myofibrils (Linke et al. 1996). They characterized the elastic properties of both the tandem Ig domain and the PEVK region by a combination of immunofluorescence microscopy and single myofibril mechanics. They measured changes in passive force with stretch of single isolated myofibrils from cardiac and skeletal muscle and the stretched-induced movements of I band titin antibody epitopes that were located adjacent to the PEVK and Ig domains of titin. Since titin is expressed in different isoforms in various muscle tissues, they investigated three types of muscle expressing a long (soleus), intermediate (psoas), and short (cardiac) isoform of titin of known tandem Ig and PEVK content. They found that with myofibril stretch, I-band titin does not extend homogeneously. As an example, the results for soleus myofibrils are shown in Fig. 7.10. An antibody near the Z line (Fig. 7.10a labeled T12 and Fig. 7.10b open circles) hardly moved at

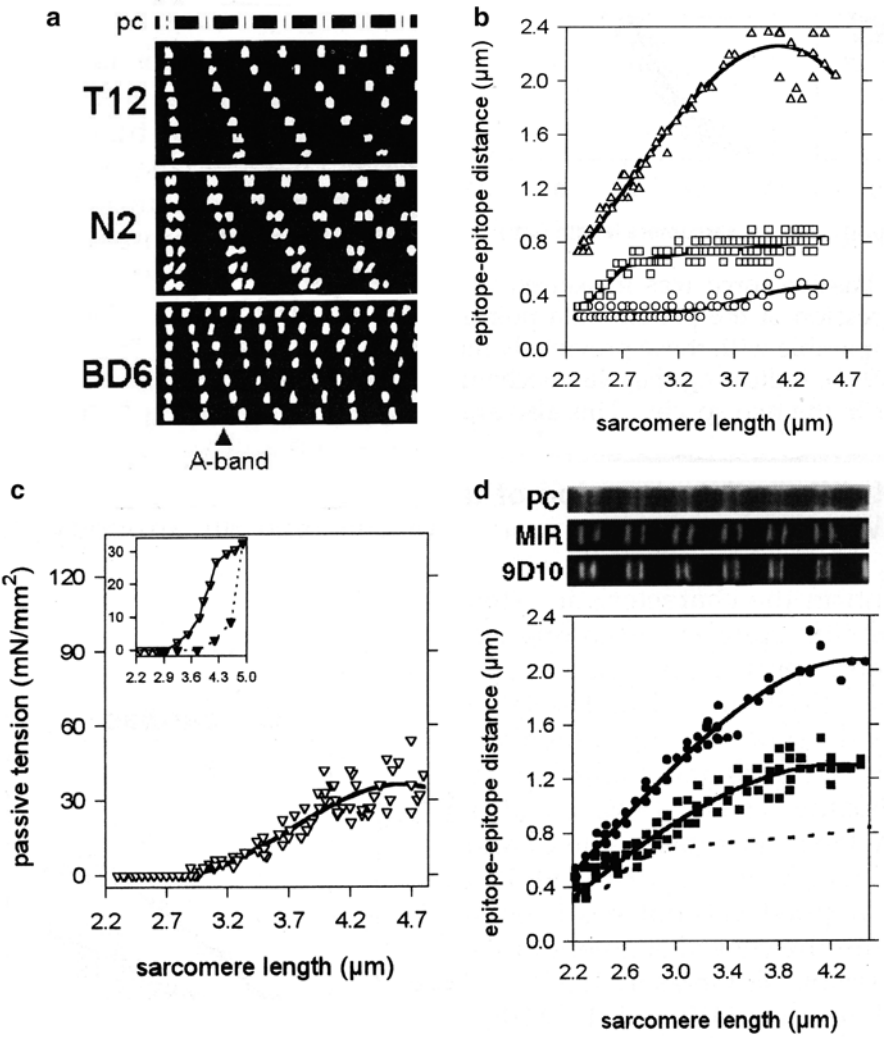


Fig. 7.10 Results of immunofluorescence measurements of titin antibody movement and passive force with stretch of soleus myofibrils. (**a**) Fluorescent antibodies labeled myofibrils near the Z disc (antibody T12), in the Ig region of titin adjacent to the PEVK region (N2 antibody), or in the A band (BD6 antibody). Myofibrils were stretched from sarcomere lengths of 2.4 to ~4.4 μm from *top* to *bottom* and the separation distance of antibody pairs measured across the Z disc. For reference a phase contrast (PC) image is shown at the *top*. (**b**) Summary of antibody movement with stretch. Note that T12 (*open squares*) hardly moves with stretch (no separation of antibodies), N2 (*open squares*) moves only at moderate sarcomere lengths and BD6 (*open triangles*) moves linearly with sarcomere length to the strain limit of titin. (**c**) Passive force versus sarcomere length. (**d**) Summary of antibody movement with stretch for 9D10 in the PEVK region (*filled squares*) and MIR near the A/I junction (*filled circles*) (*dashed line* represents N2 movement). At the *top* of (**d**), phase contrast image and fluorescence images of MIR and 9D10. See text for further explanation (Linke et al. 1996. With permission Elsevier)

all with stretch. Another antibody in the A band (Fig. 7.10a labeled BD6 and Fig. 7.10b open triangles) moved in parallel with the increasing sarcomere length. An antibody that labeled a region adjacent to the Ig domain in the I band (Fig. 7.10a labeled N2 and Fig. 7.10b open squares) initially moved linearly with stretch and then did not move at all with further stretch. This lengthening did not result in measurable passive tension (Fig. 7.10c) and was attributed to straightening, rather than unfolding, of the Ig repeats. An antibody in the PEVK domain region (Fig. 7.10d, 9D10, filled squares) moved linearly with stretch. An antibody near the A/I junction (MIR) also moved linearly with stretch but remained stationary relative to the M band (Fig. 7.10d filled circles). At moderate to extreme stretch, the main extensible region of titin was the PEVK segment whose unraveling was correlated with a steady increase in passive tension (Fig. 7.10c). Thus they concluded that the PEVK domain transition from a linearly extended to a folded state appears to be principally responsible for the elasticity of muscle fibers. Also, the length of the PEVK sequence may determine the tissue-specificity of muscle stiffness, whereas the expression of different Ig domain motif lengths may set the characteristic slack sarcomere length of a muscle type. (See more about titin isoforms below.)

A further step toward understanding the molecular nature of the elasticity of titin was taken in spectacular experiments by three groups who measured the length-force relationship of individual titin molecules or titin segments. The papers all appeared in May of 1997. Larissa Tskhovrebova and John Trinick at Bristol University collaborated with John A. Sleep and Robert M. Simmons at King's College London to measure the elasticity of and unfolding of single titin molecules using the optical trap or optical tweezer technique (see Chap. 9 for more information about optical tweezers) (Tskhovrebova et al. 1997). In this technique a titin molecule is tethered between a glass surface and a polystyrene bead (1.1 μm), using antibodies (covalently coupled to the beads) directed against epitopes located near the ends of the molecule. A bead was trapped using optical tweezers and the molecule was stretched by moving a microscope stage horizontally (Fig. 7.11). They measured the force from the displacement of the bead from the trap center. Miklos S. Z. Kellermayer, Henk L. Granzier at Washington State University collaborated with Steven B. Smith and Carlos Bustamante at the University of Oregon to also use the optical tweezer technique to measure the elastic properties of single titin molecules (Kellermayer et al. 1997). In the final study Mathias Rief, Filipp Oesterhelt, Hermann E. Gaub at the Lehrstuhl fur Angewandte Physik, Muchen, Germany collaborated with Mathias Gautel at the European Molecular Biology Laboratory, Heidelberg, Germany and with Julio M. Fernandez at the Mayo Clinic in Rochester, Minnesota (Rief et al. 1997). They utilized an atomic force microscope[4] (AFM) to

[4] The atomic force microscope (AFM) originated from the principles of scanning tunneling microscopy that were developed by Gerd Binning and Heinrich Rohrer at the IBM Zurich research laboratory. The scanning tunneling microscope (STM) was developed to reveal the structure of surfaces at the atomic level. Binning and Rohrer along with Ernst Ruska, the inventor of the electron microscope, shared the Nobel Prize in physics in 1986. Calvin F. Quate, Christoph Gerber and Binning (Binning et al. 1986) reported the invention of the atomic force microscope in 1986. The AFM can be operated in numerous modes. In the force measuring mode, a sample is attached to the cantilever tip of the microscope and the measured deflections result from the extension of the sample. The

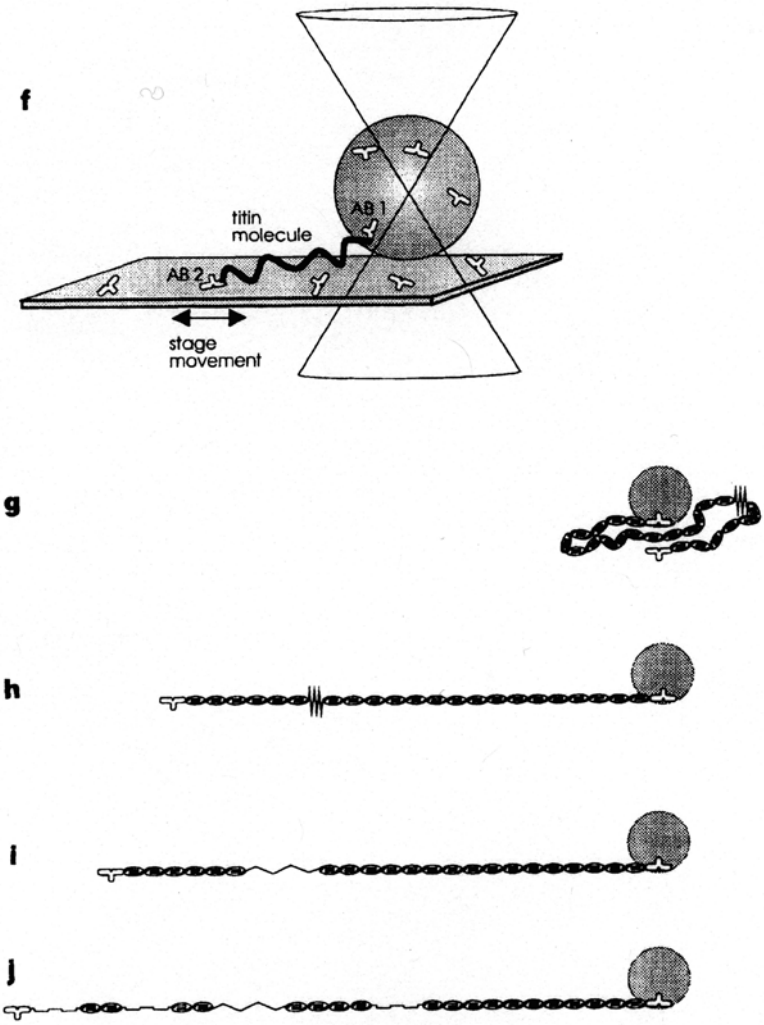


Fig. 7.11 Measurement of the force-extension relationship for a single titin molecule using an optical trap technique. Experimental procedure and mechanisms of elasticity as a single titin molecule is stretched. Extension of the molecule was obtained from the stage movement; force was obtained from the bead movement (Tskhovrebova et al. 1997. With permission Nature Publishing Group)

measure the elasticity of recombinant titin immunoglobulin segments. There was general agreement among these studies that striking elasticity of the titin molecule

AFM is capable of effecting displacements on an Angstrom scale and measuring forces of a few piconewtons. It was first utilized to measure elasticity at the molecular level with the extension of single dextran molecules in 1997. See the review by Fisher et al. (1999) for a discussion of various applications of the AFM for the measurement of the micro-mechanics of single molecules.

was due to two sources: one deriving from the straightening of the molecule and the other from extension of the PEVK region of titin (see Fig. 7.11). Kellermayer et al. (1997) furthermore concluded that scaling the molecular data up to sarcomeric dimensions reproduced many features of the passive force versus extension curve of muscle fibers. Thus by the turn of the century there was a reasonable molecular understanding of the source of titin elasticity and its role in the passive elasticity of the myofibril.

7.2.9 Titin and the Molecular Ruler Hypothesis

Whiting et al. (1989) addressed the question: "Does titin regulate the length of muscle thick filaments?" It had been a puzzle for a long time as to how the thick filaments were precisely regulated to a length of 1.6 μm containing 294 myosin molecules. They suggested that titin molecules provided a mechanism by which the length of thick filaments could be regulated accurately. They described their reasoning in the following way (Whiting et al. 1989. With permission Elsevier):

> If titin molecules are 1 μm or more long and are bound to the outside of the thick filaments, it is not difficult to envisage how, in principle, they could act to determine the length of the filament. In general terms, the simplest way accurately to regulate the length of an extended structure consisting of many similar subunits is by some form of template spanning the entire structure.

Thus among titin's other properties, they envisioned titin as a template or ruler to determine thick filament length. Tskhovrebova and Trinick (2012) have revisited the molecular ruler hypothesis for titin in a brief commentary. They emphasize the intrinsic attractiveness of the hypothesis and also state that titin is one of the first proteins expressed in myofibrillogenesis and thus is likely to form a scaffold before thick filament assembly. But they also point out the nagging problem that insect flight muscle which exhibits a precise thick filament length does not contain titin. Also some gene targeting experiments seem to argue against the molecular ruler hypothesis (see Kontrogianni-Konstantopoulos et al. 2009). Thus the hypothesis is attractive but the evidence is at best inconclusive.

7.2.10 Number of Titin Molecules per Half Thick Filament and the Sarcomere Symmetry Paradox

By the beginning of the twenty-first century, it was agreed that titin molecules constituted the third filament in the sarcomere and that titin was the third most abundant protein in the myofibril after myosin and actin. Therefore it was natural to ask: how many titin molecules were there in a titin filament? Or put another way: how many titin molecules were there per half thick filament? John Trinick and his colleagues summarized the published data on this point in 2001 (Liversage et al. 2001).

The number of titin molecules per half thick filament was very variable and ranged from 3 to 13. The variability seemed most likely to be due to variation in titin extraction and quantification from gel analysis of the amount of titin. They applied an entirely independent technique. Previously Trinick (1981) had described what he called "end filaments" protruding from each end of isolated native thick filaments. These end filaments were shown to be made of titin molecules. Liversage et al. (2001) utilized scanning transmission electron microscopy (STEM) to measure the mass per unit length of the end filaments and compared this value to the expected value for a single titin molecule. Their data was consistent with six titin molecules in an end filament and thus six titin molecules per half thick filament.

It turned out that this number led to a conceptual problem with regard to thick and thin filament symmetry, a problem that they called the "sarcomere symmetry paradox". The thick filaments exhibit three-fold symmetry (see Fig. 6.5). In order to maintain that symmetry, three titin molecules or a multiple of three would have to bind to each thick filament. Six titin molecules thus maintain the thick filament symmetry. At the Z disc titin binds to α-actinin but also at the edge of the Z disc to the thin filament (see description of the Z disc structure below). The thin filament has an approximate twofold symmetry (see Fig. 6.10) and there are twice as many thin filaments as thick filaments in the sarcomere. In order to maintain the thin filament symmetry, two titin molecules or a multiple of two would need to bind to the thin filament at the Z disc. In order to satisfy the thin filament symmetry, of the six titin molecules emanating from a half thick filament, two could bind to each of two thin filaments. This result would leave two extra titin molecules and thus a symmetry mismatch or a paradox seemed to exist. They suggested a model where the remaining two titin molecules went through the Z disc and bound to a thin filament in the adjacent sarcomere. This is an issue that remains unresolved. In conclusion, more than one titin molecule constitutes a titin filament, the likely number of titin molecules in a titin filament is six. The arrangement of these molecules in relationship to the thick and thin filaments is speculative and still a matter for further research.

7.2.11 Titin Isoforms and the Passive Mechanical Properties of Muscle

By the early 1990s it was recognized that there were multiple titin isoforms (Wang et al. 1991). The isoform nature of the titin molecule was firmly established by the cDNA analysis by Labeit and Kolmerer (1995a). Through alternative splicing of a single titin gene, isoforms of titin are generated[5]. These isoforms vary in the length

[5] The complete sequence of the human titin gene could encode a total of 38,138 residues (molecular weight of about 4.2 MDa) and differential expression gives rise to a spectrum of titin isoforms. About a third of titin's 363 exons are differentially spliced. Isoforms currently known differ in size from ~600 kDa to 3.7 MDa corresponding to molecular lengths of ~0.2 to 1.4 μm (Bang et al. 2001).

	Slack length	First Detectable Passive Force	Strain Limit
Soleus myofibrils:	**~2.25 μm**	**~2.9 μm**	**~4.2 μm**
Psoas myofibrils:	**~2.1 μm**	**~2.6 μm**	**~3.6 μm**
Cardiac myofibrils:	**~1.85 μm**	**~2.1 μm**	**~3.0 μm**

Fig. 7.12 Correlation of titin molecule length in soleus, psoas and cardiac muscle with muscle slack length, first detectable passive force and strain limit. Titin isoforms vary in molecular weight and thus length in the order of soleus > psoas > cardiac. The longer the titin molecule, the greater the muscle slack length, first detectable passive force length and strain limit length (Data from Linke et al. 1996)

and amino acid composition of their I band regions. The titin isoforms vary from the shortest (lowest molecular weight) to the longest (highest molecular weight) in the order: cardiac < psoas < soleus muscle. Three predictions arise from these facts: (1) the equilibrium resting sarcomere length of a myofibril, (2) the sarcomere length where passive force is first detectable and (3) the sarcomere length at which the strain limit is reached when a resting myofibril is stretched should all be in the order: cardiac < psoas < soleus muscle. Results describing the passive force versus extension relationships in isolated myofibrils follow this predicted pattern (Fig. 7.12) (Linke et al. 1996). These data are in general agreement with the earlier results of Kuan Wang, Roger McCarter and their colleagues (Wang et al. 1991) with skinned muscle fibers. This analysis of isolated myofibrils, of course, ignores the elasticity of the sarcolemma and extracellular connective tissue which can vary considerably among muscle types and contribute significantly to passive force with stretch. Nonetheless the isoforms of titin are an important component of the resting elasticity of a muscle.

7.2.12 Beyond the "Classical" Functions of the Titin molecule: Titin as a Mechanosensor and Integrator of Myocyte Signaling Pathways

It was generally agreed that the titin molecule connecting the thick filament to the Z disc functions as a molecular spring responsible for much of the passive elasticity of the sarcomere and also functions to maintain the A band in center of the sarcomere. Furthermore the portion of the titin molecule in the A band might act as a scaffold for controlling thick filament length. But there were indications in the 1990s that titin might exhibit more than just these "classical" properties. Titin contains a kinase domain near the carboxyl terminus, the M band end of the molecule, and both ends of the molecule also contain potential phosphorylation sites. These results suggested involvement in signaling mechanisms for both the

M band and Z disc portions of the titin molecule. The possible roles of titin as a mechanosensor and regulator of various signaling pathways has become a rapidly growing field of study. Kontrogianni-Konstantopoulos et al. (2009) have concluded that current evidence suggests that there is a complex of signaling molecules associated with titin that links its kinase activity with sarcomere assembly and maintenance, stress-sensing mechanisms, transcriptional regulation, protein turnover, and hypertrophic responses. For another contemporary review, see Linke and Kruger (2010).

What started out as a search for the source of passive sarcomeric elasticity has evolved, and continues to evolve rapidly, into the study of major regulatory mechanisms for cell growth and maintenance in health and disease. Much more will no doubt be learned about the multi-faceted roles of titin in the near future.

7.3 A Fourth Sarcomeric Filament: Nebulin, Structure and Function

In their classic study, Wang et al. (1979) not only identified the titin doublet on a gel but also observed another high molecular weight band with an approximate molecular weight of 500 kDa which they designated simply as "band 3". Band 3 was an abundant protein which constituted about 3–5 % of the total myofibrillar protein. This protein quickly was shown to be a protein different than titin (Wang and Williamson 1980). The protein seemed to be associated with the N_2 line in the I band which was sometimes seen in electron micrographs of striated muscle (Wang and Williamson 1980). Since the N_2 line seemed to be a "nebulous structure" and since band 3 was associated with it, Wang (1981, 1985) renamed band 3 as nebulin.

Originally Wang (1985) proposed (Fig. 7.4) that nebulin existed in the I band and connected the end of the titin filament to the Z disc. Furthermore he suggested that it was an elastic protein based on the observation by Sally Page (1968) that the N_2 line moved away from the Z disc when a resting muscle was stretched and their similar observations of movement of an antibody bound to nebulin. But later Wang and Wright (1988) showed that multiple antibodies bound to nebulin were located in the I band and did not change their position with respect to the Z disc when rabbit muscle fibers that were split to allow antibody access were stretched[6]. Thus nebulin was not involved in the passive elasticity of a muscle. They concluded that the nebulin molecule appeared to extend the entire length of a thin filament. Thus Wang revised his thinking and now suggested (Wang and Wright 1988) that "nebulin and titin might act as organizing templates and length-determining factors for actin and myosin filaments respectively".

[6] The movement of the N_2 line with stretch of a resting muscle as observed by Page (1968) suggests that the N_2 line is likely associated with titin and not nebulin as Wang originally suggested. Thus the name nebulin was derived from the incorrect assumption that the protein was associated with the N_2 line.

7.3.1 Nebulin as a Molecular Ruler

Siegfried Labeit and John Trinick and their colleagues (Labeit et al. 1991) were thinking along the same lines as Wang and Wright and provided evidence that was consistent with nebulin acting as a "protein-ruler". Based on the partial amino acid sequence of nebulin, derived from human cDNA clones, they found repeating motifs of about 35 residues and super repeats of 7 × 35 residues of a length of 38.5 nm. The repeat motifs were consistent with the suggestion that nebulin is associated with the actin filament since there is one molecule each of tropomyosin and troponin for every seven actin subunits, or 38.5 nm, in the filament. They also speculated that nebulin is likely to interact with tropomyosin and troponin. They went on to suggest that the amount of nebulin in a muscle is consistent with two or four nebulin molecules per thin filament with two being the most likely number. Also they pointed out that the predicted α-helical nebulin strands with a width of about 1 nm would be below the resolution of the electron microscope and thus would not be observed in three dimensional reconstruction of native thin filaments. Finally they showed data that indicated that the molecular weight of nebulin was positively correlated with the variable length of the thin filaments observed in muscles from different animals. The data was consistent with the molecular ruler hypothesis but some of the information was preliminary and discussion speculative.

During the 1990s data was accumulating that was consistent with this attractive hypothesis. Biochemical and structural characterization of the nebulin molecule was not possible because it could not be purified in the native state. Despite this limitation, short partial sequences of nebulin containing the 35 amino acid repeat motifs, derived from human cDNA clones, were examined. Jin and Wang (1991) showed that these nebulin fragments bound to actin with a high affinity under physiological conditions. Nebulin fragments were shown to be α-helical structures and a model was proposed for the structure of the thin filament nebulin complex (Pfuhl et al. 1994). In this model two nebulin molecules occupied symmetrical positions along the central cleft of the actin filament bridging the two strands of the actin helix. Kruger et al. (1991) also found a positive correlation between the molecular weight of nebulin as determined from gels (which varied from 10 to 30 %) and the thin filament lengths in different muscles from chickens, rabbits, snakes and humans (where the thin filament lengths ranged from 1.04 to 1.3 μm). Wright et al. (1993), using monoclonal antibodies, provided further evidence that nebulin extended along the entire length of the thin filament with its C terminal anchored within the Z disc and its N terminal extending out toward the center of the sarcomere. Labeit and Kolmerer (1995b) succeeded in isolating the full-length cDNA encoding human nebulin and determined its sequence. The cDNA predicted a peptide of molecular weight of 773 kDa (a 6,669 residue peptide) with 97 % of its mass consisting of 185 copies of a ~35-residue module. These results confirmed and extended earlier observations from nebulin fragments to the whole molecule. The central 154 copies (with a total length of ~ 0.8 μm) are grouped into 22 seven-module super repeats corresponding to 38.5 nm thin filament repeats. In the thin filament "ruler region", multiple isoforms are generated by alternative exon usage. They concluded that different

types of nebulin molecular rulers were expressed in different types of skeletal muscles by differential splicing. This evidence combined with later data that showed that nebulin bound to the actin filament capping proteins tropomodulin (McElhinny et al. 2001) and CapZ (Pappas et al. 2008) made the molecular ruler hypothesis even more compelling. Capping proteins effectively inhibit actin polymerization and depolymerization at the filament ends and are essential for proper thin filament assembly and length regulation. Thus, nebulin could specify the length of the thin filament by binding a specific number of actin and tropomyosin/troponin molecules and then recruiting capping proteins, which would restrict assembly to that defined length. The hypothesis has great appeal. Nonetheless the evidence, though consistent with the molecular ruler hypothesis, was not conclusive.

Pappas et al. (2010) at the University of Arizona put the molecular ruler hypothesis to a critical test in living cells, i.e., chick skeletal myocytes in culture. They designed and constructed a shorter version of human nebulin, mini-nebulin (missing 18 of the 22 super repeats and about 25 % the length of nebulin) and introduced it into the myocytes where it replaced the endogenous nebulin. The cells then were analyzed by immunofluorescence microscopy. The idea was simple. If the "strict" molecular ruler hypothesis held, then a shorter nebulin should result in shorter and possibly more variable thin filament lengths. What they found was inconsistent with the strict molecular ruler hypothesis. The thin filaments extended beyond the end of the mini-nebulin, an observation which is inconsistent with a strict ruler function. However, under conditions that promote actin filament depolymerization, filaments associated with mini-nebulin were remarkably maintained at lengths either matching or longer than mini-nebulin. This result indicates that mini-nebulin is able to stabilize portions of the filament that it does not contact. They concluded that nebulin regulated thin filament architecture by a mechanism that included stabilizing the filaments and preventing actin depolymerization. These results suggest that the action of nebulin is more complex than acting as a simple ruler. It may best be thought of as a thin filament stabilizer. The work in this area is ongoing. For a recent review see Pappas et al. (2011).

Another reason to question the molecular ruler hypothesis is that cardiac muscle does not seem to express full length nebulin. Rather a shorter version called "nebulette" by Moneman and Wang (1995) is found in cardiac muscle. Nebulette (molecular weight 107 kDa) is about one sixth the size of nebulin and it is difficult to imagine how it could function to determine the length of cardiac thin filaments that are much longer than itself.

7.3.2 Beyond the Molecular Ruler and/or Actin Stabilizer: Possible Roles of Nebulin in Muscle Contraction

One of the intriguing issues relating to nebulin function involves the expected close proximity of nebulin to tropomyosin and troponin in the thin filament and thus the possibility that nebulin may be involved in regulating muscle contraction. With the introduction of knockout mouse models deficient in nebulin, it became possible to

examine the contractile properties of muscle fibers with and without nebulin. It was found that muscle fibers deficient in nebulin developed less force due to reduced myofilament Ca^{2+} sensitivity and altered cross-bridge kinetics. Also the fibers exhibited a higher tension cost, i.e., utilized more ATP to maintain a given force for a given period of time. These novel functions suggested that nebulin might have evolved to enable vertebrate skeletal muscles to develop high levels of force efficiently. Clearly more interesting results will be forthcoming in this rapidly evolving area. See a recent review by Labeit et al. (2011) for further details.

7.3.3 Nebulin and Human Disease

Mutations in nebulin are the leading cause of the human muscle disorder nemaline myopathy. Individuals with nemaline myopathy exhibit muscle weakness, which can be severe, leading to neonatal lethality. Rod-like "nemaline" bodies, composed of Z-disc and thin filament proteins, are prevalent in the muscle fibers of nemaline myopathy patients. To date, more than 60 separate mutations have been identified in nebulin that result in nemaline myopathy. Granzier and his colleagues at the University of Arizona recently have directly linked a nebulin mutation that results in nemaline myopathy to improper assembly of the thin filament and impaired contractility. This mutation results in decreased nebulin levels, which in turn leads to decreased force production because of shorter thin filaments, less thick and thin filament overlap and thus reduced cross-bridge attachment (see Pappas et al. 2011, for a review).

7.4 Sarcomeric Organization: M Band and Z Disc Structure and Function

The regular arrangement of thin and thick filaments within the sarcomere is not simply the result of the self-assembly properties of actin and myosin alone but rather involves specific interactions with a sarcomeric cytoskeletal lattice. The most obvious structures in this context are the M bands and Z disks which are involved in packing thick and thin filaments, respectively. The component that integrates both compartments is the giant protein titin (see above).

7.4.1 M Band Structure

In the middle of the A band in the sarcomere is a structure called the M band or M line. It was observed using light microscopy at least 100 years ago. In a review in 1933, H. E. Jordan called it the "the M membrane or mesophragma", suggesting that it was a septum or partial diaphragm. Today the usual understanding is that the "M" derives from the German "Mittelscheibe". Jordan noted that the M band was

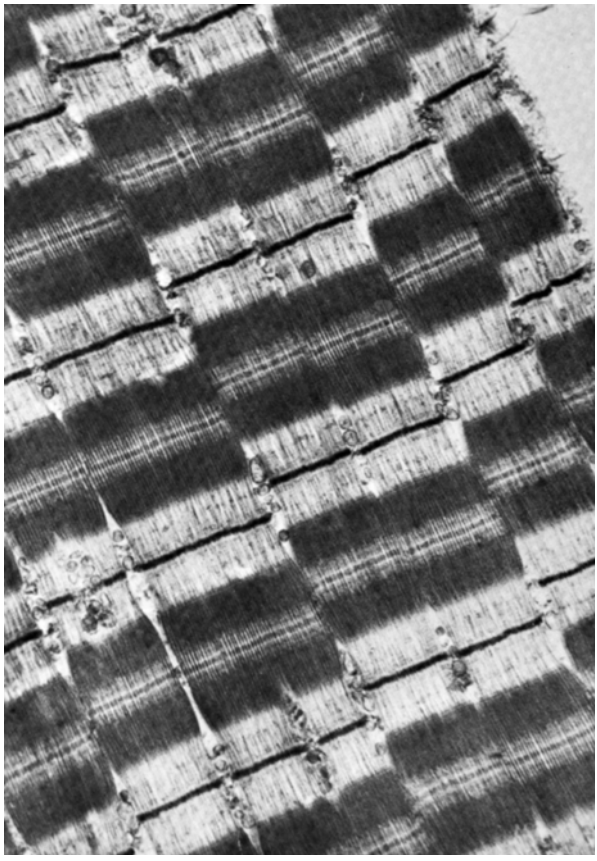


Fig. 7.13 Electron micrograph of a longitudinal section from rabbit psoas muscle. The M band is flanked on either side by a narrow zone of lower density than the rest of the H zone called the pseudo-H zone or M region. It is the area where there are no cross-bridges. Magnification: ×13,700 (Huxley 1972. With permission Elsevier)

located in the middle of a lighter staining region in the A band called the "median disc of Hensen" or H disc[7]. Today the usual designation is H zone. The H zone is that region of the A band where there are no thin filaments (Huxley and Hanson 1954). The M band was clearly seen as a dense band in the H zone in early electron microscopic studies of muscle (Hall et al. 1946; see Figs. 1.5 and 1.6). They noted that in frayed myofibrils the filaments separated laterally but adhered to one another in the region of Z and also M. Hugh Huxley (1972) showed that the M band was flanked on either side by a narrow zone of lower density than the rest of the H zone (Fig. 7.13). These lighter staining bands together with the M band have been referred

[7] With regard to the designation of the H zone after the nineteenth century microscopist V. Hensen, Andrew F. Huxley, who did a thorough investigation of nineteenth century microscopy related to muscle, has implied that Hensen in 1869 was likely looking not at the M band when he described lighter regions on either side but rather at the Z disc. Engelmann (1873) correctly described that lighter region in the middle of the A band and according to Huxley "generously referred to Hensen in this connexion". Thus apparently Hansen's name is associated with a banding region of the sarcomere that he did not discover at all (Huxley 1977, footnote page 35).

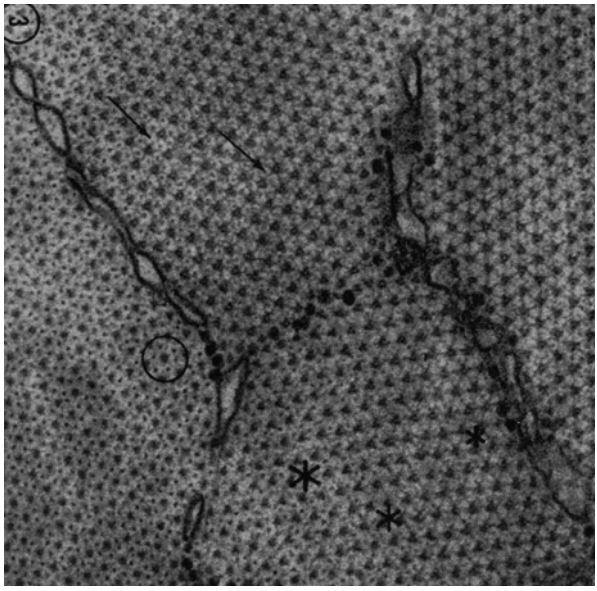


Fig. 7.14 Electron micrograph of transversely oriented bridges connecting thick filaments in the M band of fish skeletal muscle. The bridges are clearly seen in the right and upper portions of the micrograph. Magnification: ×90,000 (Franzini-Armstrong and Porter 1964. With permission Rockefeller University Press)

to as the pseudo-H zone or more recently the M region (Sjostrom and Squire 1977). The pseudo-H zone or M region is that area of the A band where there are no cross-bridges (Huxley 1965).

Details of the M band structure began to emerge after the introduction of glutaraldehyde fixation into electron microscopy which led to much better preservation of structural detail (Sabatini et al. 1963). In transverse sections through the M band, Franzini-Armstrong and Porter (1964) (Fig. 7.14), Page (1965) and Huxley (1965) all noted transversely oriented structures that connected adjacent thick filaments together. At a muscle meeting in 1964, Hugh Huxley was asked about the cross-connections between thick filaments that he observed in the electron micrographs of the muscle sarcomere. Huxley (1965) responded that they only existed in the H zone and that there were three sets of them about 200 Å apart. Knappeis and Carlsen (1968) at the University of Copenhagen performed an influential electron microscopic study of the M band in frog skeletal muscle. In longitudinal sections of the muscle, they found in the M band three, sometimes four or five, dense lines about 200 Å apart that ran transversely across the myofibril. In cross sections through the M band, they observed structures that connected each thick filament with its six neighboring thick filaments. These structures were called M bridges. The M bridges were longer than the cross-bridges observed by Hugh Huxley in 1957 (see Fig. 3.5). They concluded that the dense lines observed in the longitudinal sections of the M band were due to the M bridges. Also they observed filaments oriented parallel to the thick filaments that existed only in the M band and linked each set of M bridges together. They called these structures M filaments. Their observations were summarized in a model that formed the basis for future study

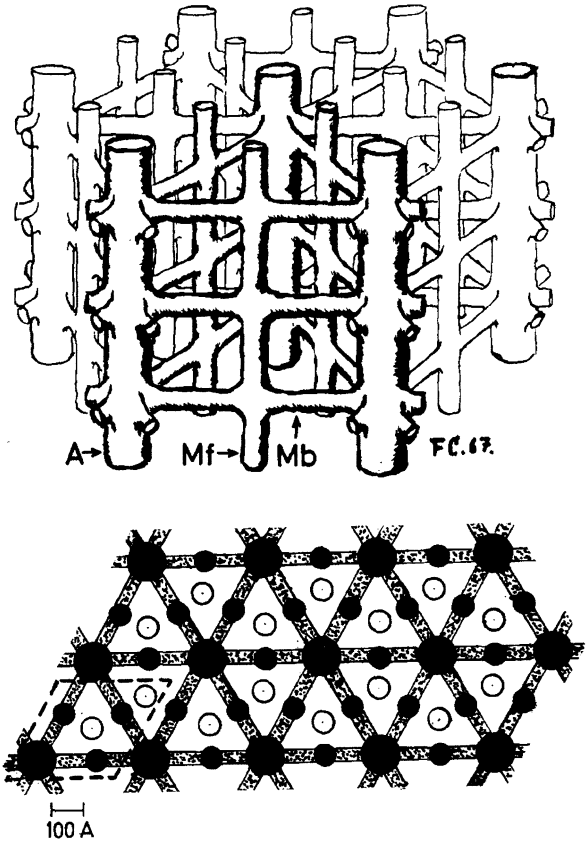


Fig. 7.15 A model of the structure of the M band in vertebrate skeletal muscle. *Top* Arrangement of M bridges (Mb) and M filaments (Mf) between the thick filaments (labeled A). *Bottom* Schematic cross-section through the M band. *Large solid circles* equal thick filaments; small solid circles equal M filaments; *open circles* equal projections of thin filaments to the level of the M region; *dotted bars* equal M bridges (Knappeis and Carlsen 1968. With permission Rockefeller University Press)

(Fig. 7.15). Knappeis and Carlsen speculated that the function of the M band was to keep thick filaments in position in the longitudinal as well as in the transverse direction. They also made the intriguing observation that an increase in the cross-sectional area of the M band must lead to a stretch of the M bridges. This suggestion was consistent with the early X-ray diffraction data that showed that the lateral spacing of the thick filaments increased with muscle shortening (Elliott et al. 1963) (see Chap. 3).

With regard to the variation from three to five in the number of transverse dense lines in the M band of frog muscle, Sjostrom and Squire (1977) observed the same variation in human muscle biopsies. The differences were related to different muscle fiber types. Densitometry scans across the M band exhibited numerous transversely oriented lines of varying intensity. They developed a nomenclature to describe these lines. Starting from the center line, labeled M1, and working outward in one direction, lines were labeled M2–M6 or outward in the other direction M2′–M6′. It was concluded that the central strongest three lines (M1, M4, M4′) represented the M bridges whereas the outer two lines (M6, M6′), in a five line fiber, were

probably non-bridging protein. In the fibers with the four line pattern (M4, M4′, M6, M6′), the central M1 bridge was missing. Since the two M bridges (M4, M4′) on either side of the central M bridge were always present, it was suggested that M4 and M4′ represented the principal cross-links that determined A band ordering. On the basis of this conclusion, Squire (1986) suggested that the central M1 bridge and M6 and M6′ non-bridging protein may not have a structural role but rather may be enzymes located to modify the physiological behavior of the fibers. In general slow twitch fibers exhibited the four line pattern, fast twitch fibers the three line pattern and intermediate fibers the five line pattern (Edman et al. 1988). (The other numbered lines in the M band were weak staining and of unknown origin.)

7.4.2 Protein Content of the M Band

Masaki and Takaiti (1974) in Setsuro Ebashi's laboratory at the University of Tokyo reported the isolation and characterization of a protein that localized to the M band of vertebrate skeletal muscle. This protein, called M-protein, had a molecular weight of 165 kDa and bound to myosin. In 1984 in Hans M. Eppenberger's laboratory at the Swiss Federal Institute of Technology in Zurich, Barbara Kay Grove and colleagues discovered a contaminant in the M-protein preparation. This contaminant was a separate protein of molecular weight 185 kDa and it too located to the M band of skeletal muscle (Grove et al. 1984). They named the protein myomesin. The titin molecule was also shown to enter the M band (see above). Earlier David C. Turner and Theo Wallimann along with Eppenberger discovered another protein that localized to the M band (Turner et al. 1973). This was not a structural protein but rather the muscle specific form of the enzyme creatine kinase (creatine + ATP ↔ creatine phosphate + ADP). This discovery meant that besides a structural role, the M band had a physiological function with a strong link to the energy supply for muscle contraction.

7.4.3 Integrating M Band Proteins with M Band Structure: A Molecular Model

Attention now turned to attempting to integrate the M band proteins into a molecular model that was consistent with M band structure. Dieter O. Furst and his colleagues at the Max-Planck-Institute for Biophysical Chemistry in Gottingen, Germany made an important contribution in this regard (Obermann et al. 1996)[8].

[8] In a tribute to Klaus Weber of the Max-Planck Institute for Biophysical Chemistry, Dieter Furst has commented that Weber's support and interest in the sarcomeric cytoskeleton gave an invaluable impact to the field. (Furst et al. 1999) Weber and his wife Mary Osborn pioneered the development of immunofluorescence microscopy for the localization of molecules in cells. Also see Footnote 11 below and Chap. 6, Footnote 1.

Since the primary sequences of M-protein, myomesin and titin were known, they approached the problem of M band structure by immunoelectron microscopy using a panel of antibodies directed against defined epitopes of each protein. There were four antibodies against the portion of titin that entered the M band, six antibodies against myomesin and six antibodies against M-protein. From experiments with fast and slow twitch fibers from the rabbit psoas, they determined that there was an overlap of titin molecules in the M band where the titin molecules interacted with each other in an antiparallel fashion. Myomesin molecules were oriented parallel to the long axis of the myofibril and neighboring molecules were oriented in an antiparallel fashion. Since the antibodies directed against the amino-terminal domains of myomesin could not be distinguished from one another, this region of the molecule was thought to bend transversely toward the surface of the thick filament and bind to myosin. Also the titin and myomesin molecules exhibited extensive interaction with each other. In contrast M-protein primarily was restricted to the M1 line in the M band. Titin and myomesin were found in both slow and fast twitch fibers but M-protein was found only in the fast twitch fibers. They integrated this information with the structural information and produced the first comprehensive molecular model of the M band shown in Fig. 7.16. In this model the M-protein constitutes the M1 line. They speculated that the M filaments running parallel to thick filaments between M4 and M4′ could be myomesin molecules forming a tight complex with two titin molecules. The M4 and M4′ positions are occupied by the transversely oriented ends of the myomesin molecule.

Irina Agarkova and Jean-Claude Perriard of the Swiss Federal Institute of Technology in Zurich proposed that myomesin was of primary importance in determining the structural and functional properties of the M band (see a review by Agarkova and Perriard 2005, and the references therein). Their proposal is based on the following facts. Myomesin is present in all fiber types at the time of formation of myofibrils during myofibrillogenesis. Myomesin forms antiparallel dimmers that bind to the thick filaments by their N-terminal domains. Myomesin also binds to M band titin and together they might form the M filaments. Furthermore myomesin binds to creatine kinase at a position near to the M4 and M′ lines of the M band. Single molecule studies indicate that the myomesin molecule exhibits an elasticity that is similar to that exhibited by titin. Thus Agarkova and Perriard viewed the M band not as a rigid construction made of transverse and longitudinal rods but rather as an elastic web of fibrillar proteins that were built in the same fashion as titin but on a much smaller scale. Recent evidence is accumulating that suggests that the M band could be a component of a mechanosensitive signaling complex (for a review, see Gautel 2011). Mathias Gautel (2011) of King's College London has speculated that:

> The realization that the M-band is elastic and serves signaling functions opens the perspective that M-band strain might also translate into modulation of metabolic activity, in addition to protein turnover and transcription regulation, and thereby regulate short-term adaptation of muscle to strain.

Even though great progress is being made in elucidating the structure and function of the M band, many questions remain unanswered in this exciting area of research.

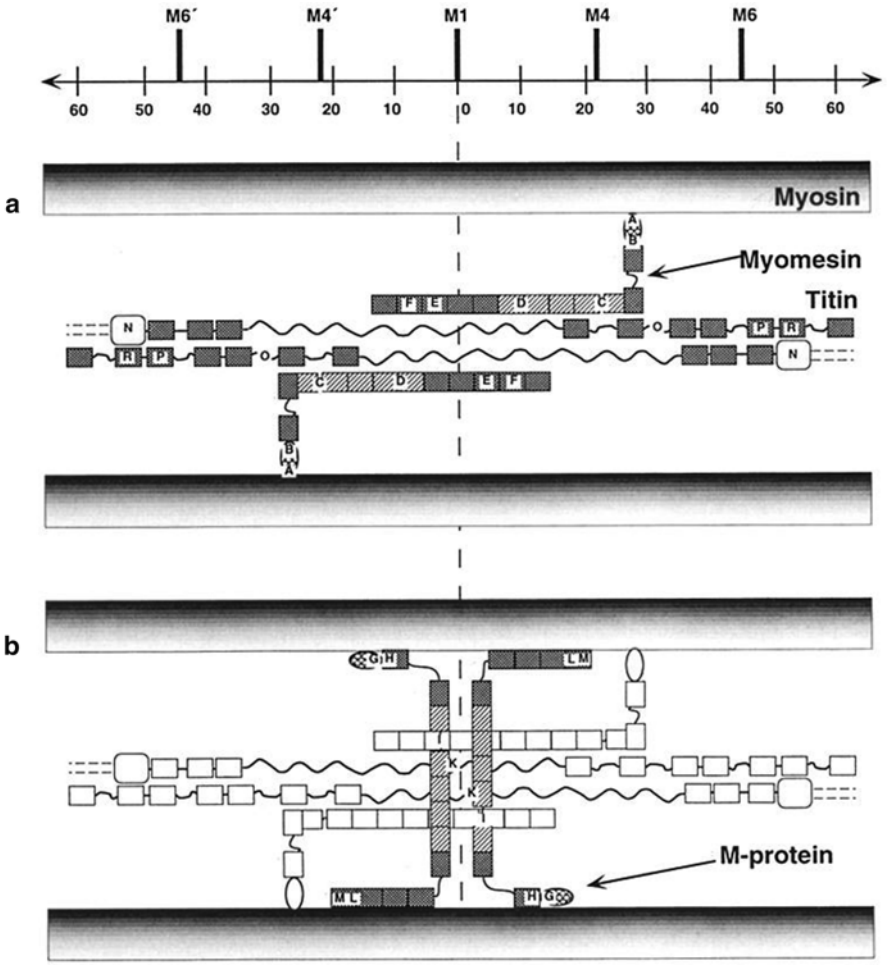


Fig. 7.16 Arrangement of titin, myomesin, and M-protein in the M band compatible with the immunoelectron microscopical results. At the *top* of the diagram, the positions of the prominent M band striations and a scale bar are indicated. Numbers give the distances in nm from the center of the A band, the M1 line. (**a**) The layout of titin and myomesin deduced from labeling data. Only two molecules of each kind are shown in order not to over burden the figure. (**b**) In addition to the structures shown in (**a**), the proposed location of M-protein around the M1 line is shown. Titin, myomesin and M-protein antibody decoration positions are indicated by *letters* (Obermann et al. 1996. With permission Rockefeller University Press)

7.4.4 Z Disc Structure

Another important component of the sarcomeric cytoskeletal lattice is the Z disc which is involved in packing of the thin filaments in the sarcomere. The Z disc or Z line (Zwischenscheibe) or Z band which bisects the I band and delineates the ends

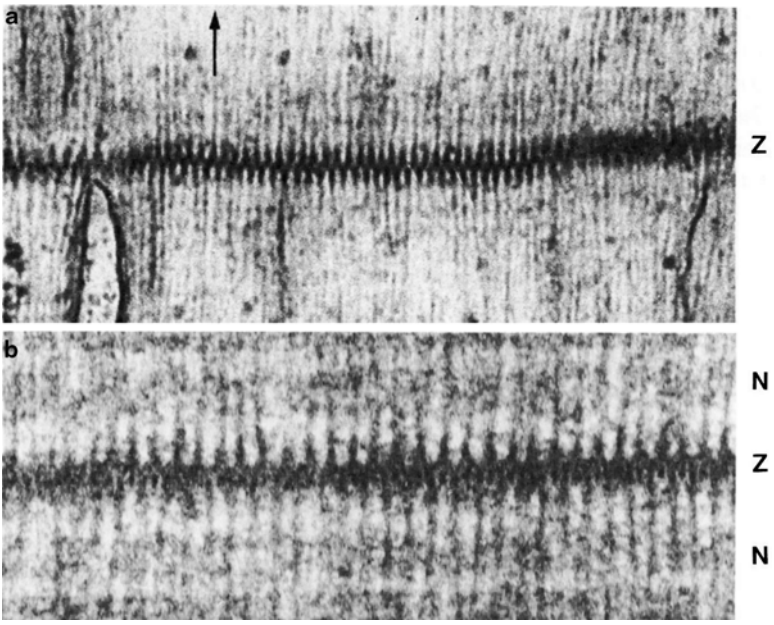


Fig. 7.17 The *zigzag* structure of the Z disc in longitudinal section. Roach white muscle fibers. Magnification: (**a**) ×133,000; (**b**) ×200,000 (Squire 1981. With permission Springer)

of a sarcomere was well known to the nineteenth century light microscopists (Huxley 1977). It also has been called Krause's membrane, Z membrane or telophragma because it was thought to be a membrane barrier between sarcomeres. It was clearly observed in the early electron microscopic studies of Hall et al. (1946) (see Fig. 1.6).

The first three dimensional model of the Z disc was developed by Knappeis and Carlsen (1962). They performed an electron microscopic study of the Z disc of frog skeletal muscle. In longitudinal sections the Z disc exhibited a zigzag line appearance (for examples from a later study, see Fig. 7.17). A thin filament from one sarcomere met the Z disc midway between two thin filaments from the adjacent sarcomere which met the same Z disc from the opposite side. The thin filaments were connected in the Z disc by structures that they called Z filaments (Fig. 7.18a, c). From cross sections through the Z disc, Knappeis and Carlsen found a tetragonal array of thin filaments from one sarcomere interdigitated with a tetragonal array of oppositely oriented thin filaments from the adjacent sarcomere (Fig. 7.18b). The arrays were displaced transversely by half a period from each other. There were extensive cross-links between the two arrays in which one thin filament was attached to four Z filaments and in turn the four Z filaments were attached to four thin filaments from the adjacent sarcomere. The pattern formed at the center of the Z disc was a smaller square lattice positioned at 45° with respect to the original thin filament lattice. Thus the thin filaments were not continuous through the Z disc from

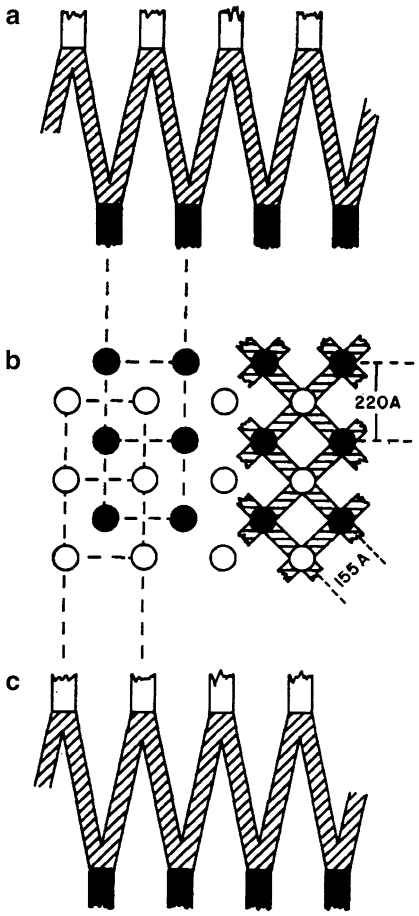


Fig. 7.18 Diagrammatic representation of Z disc structure and I filaments on either side of the Z disc. (**a**, **c**) Longitudinal sections, displaced with respect to each other so that each I filament lies between two opposite I filaments on either side of the Z disc (the all *black* and all *white bars*). The *shadowed bars* represent the Z filaments. (**b**) Projection of the square pattern formed by the cross-cut I filaments on one side of Z disc (*open circles*) onto the square pattern formed by the I filaments on the opposite side of Z disc (*black circles*) such that each I filament on one side faces the *center* of a *square* formed by four I filaments on the opposite side of Z disc (Knappeis and Carlsen 1962. With permission Rockefeller University Press)

one sarcomere to the next. This data fit well with the myosin decorated I-Z-I segments which exhibited reversed polarity of thin filaments at the Z disc as shown by the appearance of arrowheads pointing away from the Z disc (Huxley 1963). The tetragonal arrangement of thin filaments at the Z disc, of course, was in contrast to the hexagonal lattice of the thin and thick filaments in the overlap zone of the sarcomere (Fig. 7.19). Knappeis and Carlsen suggested that the thin filament transition from a hexagonal to tetragonal lattice could be accommodated by a small displacement of the thin filaments as they traverse from the overlap zone to the Z disc.

The so-called small square pattern of thin and Z filaments observed in the Z disc was not the only type of pattern that was observed. Michael Reedy (1964) described what he called a "woven" appearance, as if one were viewing a mat woven of white ribbons having black edges (Fig. 7.20). This remarkable pattern would later become known as a basketweave pattern in contrast to the small square pattern of Fig. 7.18b. Mamoru Yamaguchi and Masatoshi Izumimoto at Ohio State University and Richard

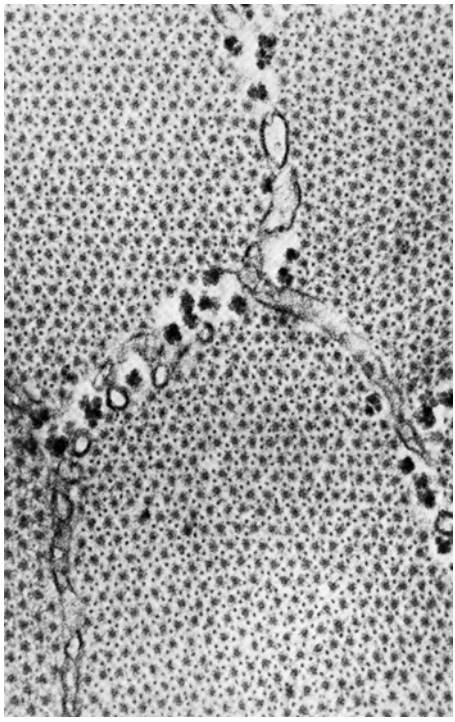


Fig. 7.19 Electron micrograph of a cross section of frog sartorius muscle showing the hexagonal lattice of thin and thick filaments. The thin filaments can be seen at the trigonal points of the hexagonal lattice of thick filaments. Magnification: ×112,000 (Huxley 1972. With permission Elsevier)

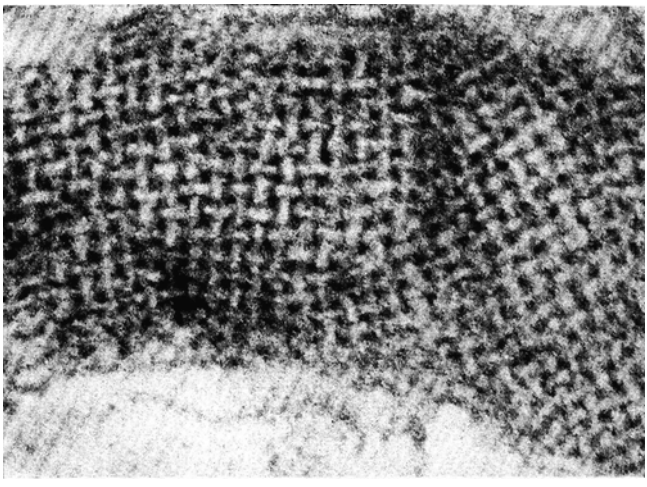


Fig. 7.20 Electron micrograph of cross-section through the Z disc of rat diaphragm muscle showing a basketweave pattern. Magnification: ×170,000 (Reedy 1964. With permission The Royal Society)

M. Robson and Marvin H. Stromer at Iowa State University developed a model of the Z disc that indicated that a small movement of the thin filaments of less than 10 nm could cause a change of appearance of the Z disc from the small square to the basketweave pattern (Yamaguchi et al. 1985). Margaret Goldstein and her colleagues at Baylor University have observed a transition from the small square pattern in resting skeletal muscle to the basketweave appearance during tetanic contraction (Goldstein et al. 1986). After relaxation, the small square pattern returned. They concluded that the Z disc lattice pattern correlated with the physiological state of the muscle. This correspondence with physiological state suggested that the small square to basketweave transition could occur dynamically during contraction. There appeared to be a connection between the molecular events of contraction and structural changes within the Z disc. Yamaguchi and Toshiharu Oba and colleagues also showed that there is a transition in the structure of the Z disc when a resting muscle fiber is stretched (Yamaguchi et al. 1999). Thus both active force production and resting force appear to be "sensed" by changes in Z disc structure.

The interest in characterizing mammalian skeletal muscle fibers into different fiber types began in earnest in the early 1960s. Physiological, metabolic and structural properties were compared in different muscles and muscle fibers in order to establish criteria for fiber type classification. With regard to structural properties, Geraldine F. Gauthier (1971) at Wellesley College, Massachusetts, noted that the width of the Z disc varied considerably with fiber type with fast twitch (white) fibers exhibiting a narrower Z disc than slow twitch (red) fibers. In fact the width of the Z disc was found to be one of the most reliable structural parameters for discriminating fiber types with a range of widths from about 60 nm (fast twitch fiber) to 140 nm for the slow twitch fibers (Eisenberg 1983). Rowe (1973) at the Meat Research Laboratory in Queensland, Australia observed that the width of the Z disc was related to a repeating number of zigzag bands or chevrons seen in longitudinal sections. In rat plantaris muscle, the white, intermediate, and red fibers have Z-bands comprised respectively two, three and four chevrons with a periodicity parallel to the fiber axis of ~40 nm. Pradeep K. Luther at Imperial College, London, has undertaken an extensive study of the three dimensional structure of Z discs of varying widths (for a review see Luther 2009). Figure 7.21 shows the three dimensional models for Z discs of varying width from different muscles. The adjacent thin filaments of opposite polarity overlap to a greater extent as the Z discs become wider. These models differ from that originally proposed by Knappeis and Carlsen in that the thin filaments enter the Z disc to varying extents depending on fiber type and the thin filament end does not attach to four Z filaments. Luther has called the structures linking the thin filaments Z links.

7.4.5 Protein Content of the Z Disc

During the time of the classic investigations that led to the discovery of troponin, Ebashi et al. (1964) described another new protein factor. This protein had an amino acid composition similar to actin but did not function like actin. The protein did not

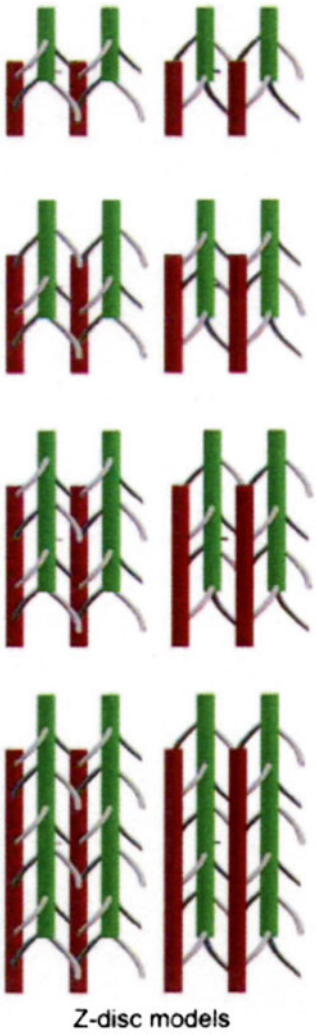


Fig. 7.21 Three dimensional models of the structure of vertebrate muscle Z discs of various widths. The figure shows (from *top* to *bottom*), 2-, 3-, 4- and 6-layer Z discs. *Top* shows the Z disc in fish body muscle which comprises single *zig-zag* links between oppositely oriented actin filaments. This Z disc is composed of two layers of α-actinin and is the minimum width of a Z disc. By adding additional layers of α-actinin, 3-, 4- and 6-layer Z discs are formed (Luther 2009. With permission Springer)

interact with myosin but did promote gelation of F-actin (Maruyama and Ebashi 1965). Ebashi and Ebashi (1965) named this protein α-actinin. α-actinin was localized to the Z disc (Masaki et al. 1967). The Z filaments were presumed to be constructed of α-actinin.

After the discovery of α-actinin and the suggestion that it presented the Z filaments in the model of Knappeis and Carlsen, the investigation of the Z disc was relatively dormant until the 1980s when the development of cell and molecular biology techniques led to an explosion of knowledge with the discovery of new proteins and the identification of their localization in the Z disc and speculation about their function. Alpha-actinin, molecular weight 97 kDa, was shown to be a rod shaped protein of length about 35 nm that formed anti-parallel homodimers

which cross linked actin filaments. The N-terminus of the titin molecule is located in the Z disc and the length of the titin molecule in the Z disc is proportional to the width of the Z disc. This portion of titin interacts with the actin filaments and also contains multiple α-actinin binding sites. Adjacent titin molecules are linked in an antiparallel fashion at their extreme N-terminal ends by the protein telethonin. The Z disc end of the actin filament is capped by a protein called CapZ (originally β-actinin) which binds to α-actinin and the C-terminus of nebulin. The Z disc also contains the actin binding protein nebulin. Numerous other proteins have been localized to the Z disc. It has been suggested that along with its structural role, the Z disc functions as a mechanosensor. Several of these other proteins have been implicated in mechanical strain signaling in skeletal and cardiac muscle (for reviews see Gautel 2011; Pyle and Solaro 2004).

Thus the Z disc and M band which were originally thought to play important but relatively mundane structural roles are now considered important players in strain sensing and signaling in health and disease. Despite the excitement in these rapidly evolving areas of research, there is still no generally agreed upon molecular models of the Z disc and the M band and their proteins.

7.5 Intermediate Filaments: Desmin and the Exosarcomeric Cytoskeleton

The structures considered above have been limited to the endosarcomeric cytoskeleton. Yet striated muscles are striated because of the precise lateral alignment of the myofibrils as can be seen clearly in light and electron micrographs (Fig. 7.22). The striated appearance is maintained when a muscle fiber shortens actively or is stretched while at rest (Huxley and Niedergerke 1954; see Figs. 2.4 and 2.5). This striking appearance implies that there exists an exosarcomeric cytoskeleton that consists of transversely oriented structures that keep the myofibrils in line laterally. The strongest early evidence that intermyofibrillar transverse structures might exist resulted from the observations of N. N. Garamvolgyi (1965) at the Institute of Biophysics, Pecs, Hungary. His electron micrographs of indirect flight muscle of the honey bee clearly showed transverse bridges connecting myofibrillar Z discs laterally. He called these structures "inter-Z bridges". Nonetheless such transverse structures were rarely seen in electron micrographs of vertebrate skeletal muscle.

The breakthrough did not come from observations of adult striated muscle but rather from investigations of structures in developing skeletal muscle and in smooth muscle. Ishikawa et al. (1968) (1935–2008)[9] at the University of Pennsylvania identified a new class of filaments with a diameter between that of the actin filaments

[9] At the time of the publication of this seminal paper, Harunori Ishikawa was a postdoctoral fellow in Lee Peachey's laboratory at the University of Pennsylvania. In a gesture that would not likely happen today, Peachey did not place his name on this paper despite his guidance of Ishikawa. Ishikawa went on to a distinguished career in cell biology (Sanger 2009).

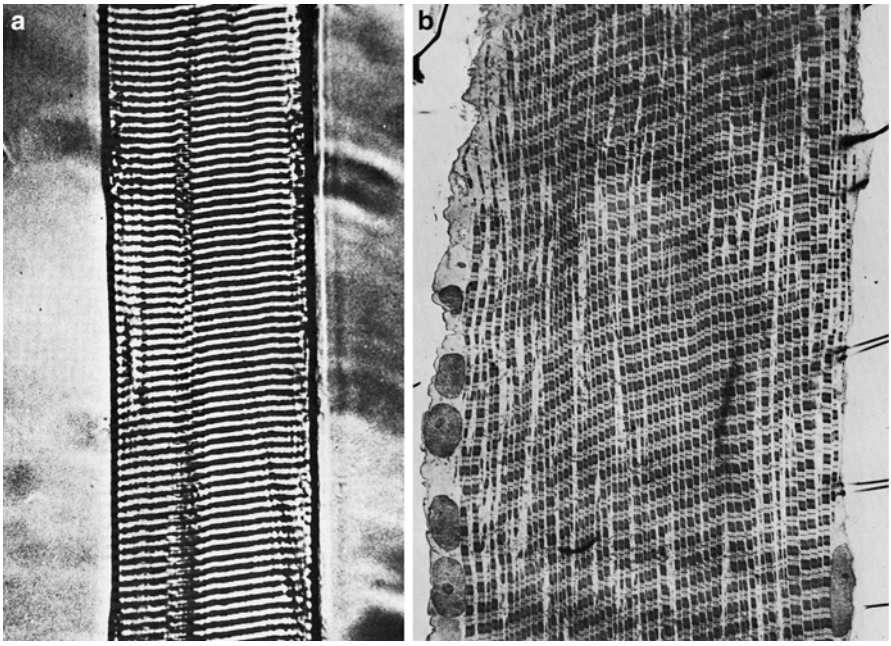


Fig. 7.22 The striated appearance of muscle as seen in the light and electron microscope. (**a**) Single muscle fiber from a rabbit psoas muscle, photographed in the phase contrast light microscopy. A bands are dark and I bands are light. The sarcomere repeat is about 2.6 μm, and the fiber diameter is about 50 μm. Magnification: ×980. (**b**) Single fiber from a human muscle sectioned longitudinally and viewed at low magnification in the electron microscope. The striations arise from the characteristic band pattern of the myofibrils which are laterally aligned. Myofibrillar diameter is about 1 μm and fiber diameter is about 55 μm. Magnification: ×1,480 (Huxley 1972. With permission Elsevier)

and myosin filaments in skeletal muscle cells cultured from chick embryos. These "intermediate filaments" had a diameter, on average, of 10 nm as compared to the 6 nm diameter of actin filaments and 15 nm diameter of myosin filaments. Unlike actin filaments, the intermediate filaments did not form arrowhead complexes in the presence of heavy meromyosin (Ishikawa et al. 1969)[10]. The intermediate filaments were most abundant in smooth muscle. Peter Cooke (1976), then at the University of Kansas, found a filamentous cytoskeleton in vertebrate smooth muscle cells which was made of intermediate filaments. The isolated intermediate filaments contained a new, unnamed, protein of apparent molecular weight of 55 kDa. This protein could form filamentous segments that closely resembled the structure of the native isolated intermediate filaments. Later that same year, Lazarides and Hubbard

[10] This paper was the first to show the utility of formation of arrowhead complexes with myosin decoration to identify actin filaments in non muscle cells.

(1976)[11], then at the University of Colorado, described the isolation of a 50 kDa protein from smooth muscle intermediate filaments and its location in chicken skeletal muscle fibers. They formed an antibody against the protein and employed indirect immunofluorescense to localize the protein in the muscle fiber. The protein was found in close association with the Z discs of the sarcomeres and extended between the Z discs of adjacent myofibrils. It also was associated with filamentous structures that ran along the length of a muscle fiber both in close association with the plasma membrane and between myofibrils. Lazarides and Hubbard (1976) concluded that this protein functioned in muscle as a three dimensional matrix which interconnected individual myofibrils to one another and to the plasma membrane at the level of the Z lines. The molecule thus provided a framework that mechanically integrated the contractile myofilaments during the contraction and relaxation of muscle. To emphasize its linking role in muscle, they named the protein desmin (from the Greek δεσμός = link, bond). Thus whereas intermediate filaments were rarely seen with electron microscopy in adult skeletal muscle fibers, the protein desmin[12] was prevalent and appeared to connect adjacent myofibrils transversely and be responsible for the striated appearance of skeletal muscle fibers.

Lazarides followed up this study with a further investigation of the location of desmin in skeletal muscle fibers. He and Bruce L. Granger isolated, from chicken skeletal muscle, planes of in-register Z discs which appeared as honeycomb-like arrays (Granger and Lazarides 1978). This unique preparation provided a "face-on-view" of Z discs in the same plane. Once again they employed indirect immunofluorescence to localize desmin and also α-actinin in the Z discs. Desmin was present at the periphery of each Z disc, forming a network of collars within the Z plane. α-Actinin was localized within each disc, resulting in a face-on fluorescence pattern that was complementary to that of desmin (Fig. 7.23). Thus the evidence was strong that desmin was a primary component of the cytoskeletal structures responsible for the striated appearance of skeletal muscle fibers. Lazarides (1980) illustrated his vision of the role of desmin in Fig. 7.24.

Wang and Ramirez-Mitchell (1983) described a network of not only transverse but also longitudinally oriented filaments in vertebrate skeletal muscle fibers. They extracted the majority of actin and myosin from rabbit skeletal muscle myofibrils with a potassium iodide solution. The "ghost" myofibrils were examined with transmission and scanning electron microscopy. They observed filaments with the

[11] In 1974 Elias Lazarides, a graduate student at Harvard University, working with Klaus Weber at the Cold Spring Harbor Laboratory, published a classic paper utilizing actin antibodies, indirect immunofluorescence and fluorescence light microscopy to visualize actin filaments in a variety of non muscle cells (Lazarides and Weber 1974). This paper had a major impact on the study of the cytoskeleton and showed the power of indirect immunofluorescence and fluorescence light microscopy for localization of a particular cellular component. The paper became a Citation Classic and is considered to be a landmark paper in cell biology. Also see Lazarides and Revel (1979). The origin of immunofluorescence microscopy can be traced back to the early 1940s and the work of Albert H. Coons at Harvard University (Coons 1961).

[12] Small and Sobieszek (1977) at Aarhus University isolated and characterized an intermediate filament protein from vertebrate smooth muscle and named it skeletin. Skeletin turned out to be equivalent to desmin and desmin is the usual designation today.

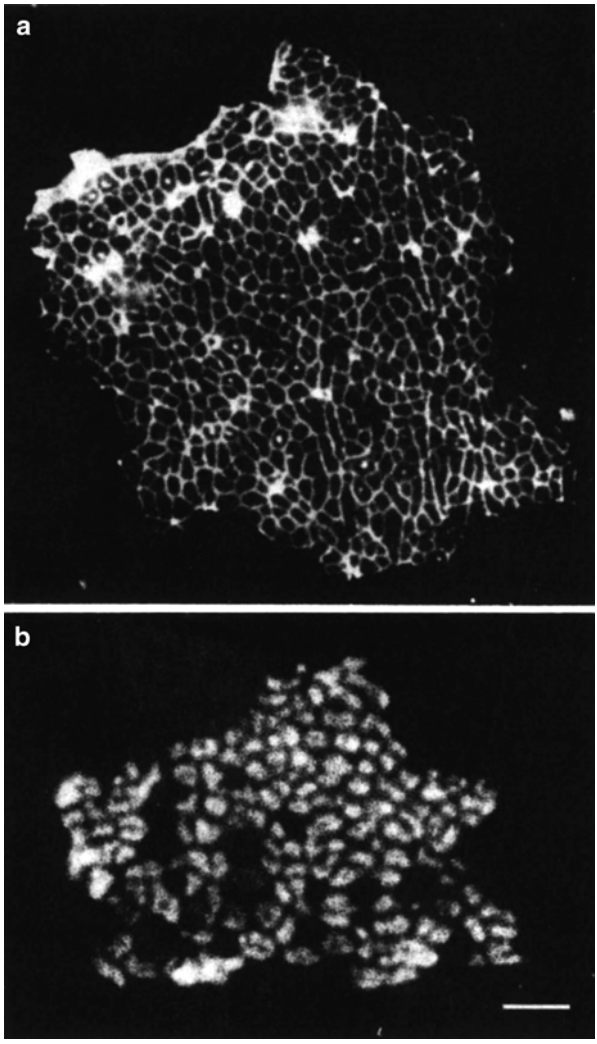


Fig. 7.23 Immunofluorescence localization of desmin and α-actinin at the Z disc of isolated Z disc sheets from chicken skeletal muscle. (**a**) Desmin is distributed at the periphery of Z discs. (**b**) α-actinin is located in the interior of the Z discs. Note the complementarity of the desmin and α-actinin fluorescence patterns. Scale bar: 2.5 μm (Lazarides 1980. With permission Nature Publishing Group)

characteristics of intermediate filaments that were oriented: (1) transversely and connected adjacent myofibrils at the level of the Z discs and M bands and (2) longitudinally and connected the peripheries of successive Z discs and seemed to ensheath the sarcomere (Fig. 7.25).

Besides connecting adjacent myofibrils at the Z discs, desmin is now known to connect myofibrils to mitochondria, the nucleus, the transverse tubular system and the cell membrane through various binding partners (Bar et al. 2004). Desmin also runs longitudinally to surround individual myofibrils. The desmin intermediate filament is only one of many different kinds of intermediate filaments. The various intermediate filaments are heterogeneous in structure and vary somewhat in diameter. The desmin containing intermediate filaments are classified as class III intermediate

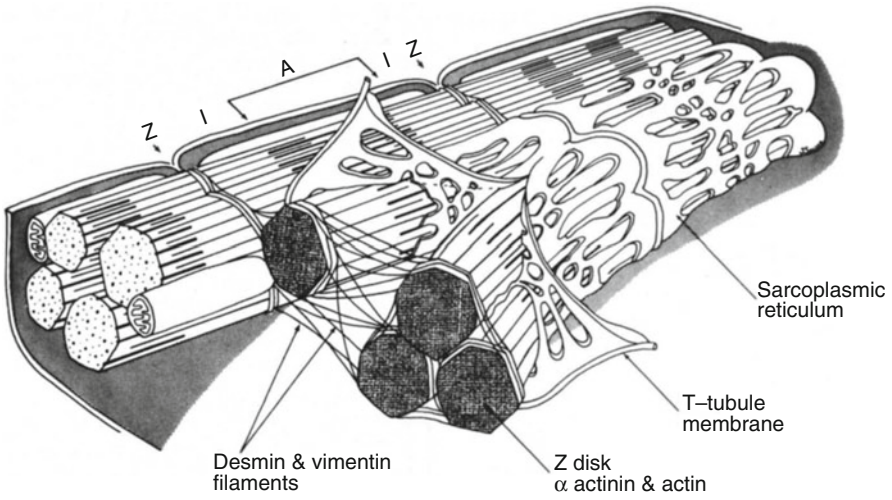


Fig. 7.24 Schematic representation of distribution of desmin, α-actinin, actin and membranous organelles in relationship to skeletal myofibril Z discs. Vimentin is seen early in muscle development and later is replaced by desmin in adult muscle (Lazarides 1980. With permission Nature Publishing Group)

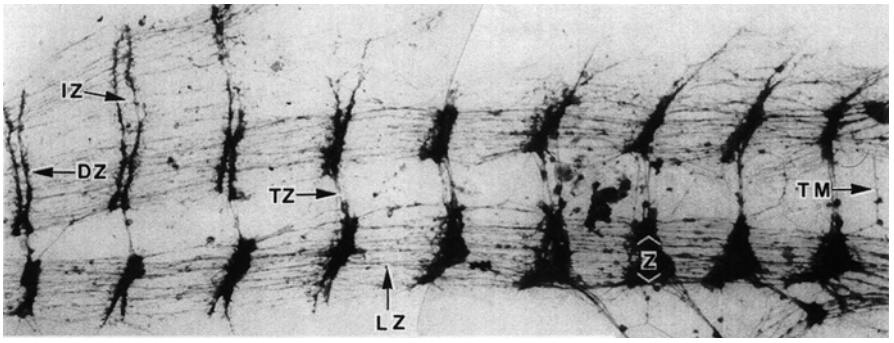


Fig. 7.25 An exosarcomeric network of transverse and longitudinal intermediate filaments. A bundle of rabbit myofibrils is shown from which actin and myosin have been extracted with a potassium iodide solution. The bundle exhibits decreasing degrees of structural integrity from *right* to *left*. Filaments connect myofibrils transversely at the Z disc (TZ) and M band (TM) and filaments connect Z discs longitudinally (LZ). Magnification: ×6,000 (Wang and Ramirez-Mitchell 1983. With permission Rockefeller University Press)

filaments. There are at least five different classes of intermediate filaments based on sequence homology. The desmin molecules form dimeric coiled coils which further associate laterally and end-to-end to form an intermediate filament about 10–12 nm in diameter (Herrmann et al. 2007). Localized at the sarcolemma is a complex lattice network that connects the outer myofibrils to the sarcolemma. The lattice network has been called a costamere (latin costa, rib; Greek, meros, part) (Pardo et al. 1983). Desmin filaments connect to the costameres which in turn connect the outer

myofibrils to the sarcolemma (for a recent review see Ervasti 2003). Investigation is ongoing to establish the molecular nature and function of the exosarcomeric cytoskeleton in greater detail. Of course all cells have a cytoskeleton and the milestones in this area of research was chronicled beautifully in Nature Milestones in 2008 (available at http://www.nature.com/milestones/cytoskeleton).

As impressive as the advancements in understanding the muscle cytoskeleton have been, it is somewhat sobering to realize that there is not yet a complete three dimensional, molecular, description of the architecture of any part of the sarcomere, be it the M band, Z disc, thin or thick filaments. Furthermore the transverse intermyofibrillar connections to other cell organelles are understood in some detail but not in detailed molecular terms. There is still much to learn about the endosarcomeric and exosarcomeric cytoskeleton of muscle.

References

Agarkova I, Perriard J-C (2005) The M-band: an elastic web that crosslinks thick filaments in the center of the sarcomere. Trends Cell Biol 15:477–485

Bang M-L, Centner T, Fornoff F, Geach AJ, Gotthardt M, McNabb M, Witt CC, Labeit D, Gregorio CC, Granzier H, Labeit S (2001) The complete gene sequence of titin, expression of an unusual ~700-kDa titin isoform, and its interaction with obscurin identify a novel Z-line to I-band linking system. Circ Res 89:1065–1072

Bar H, Strelkov SV, Sjoberg G, Aebi U, Herrmann H (2004) The biology of desmin filaments: how do mutations affect their structure, assembly, and organisation? J Struct Biol 148:137–152

Binning G, Quate CF, Gerber C (1986) Atomic force microscope. Phys Rev Lett 56:930–933

Cooke P (1976) A filamentous cytoskeleton in vertebrate smooth muscle fibers. J Cell Biol 68:539–556

Coons AH (1961) The beginnings of immunofluorescence. J Immunol 87:499–503

Ebashi S, Ebashi F (1965) α-actinin, a new structural protein from striated muscle. J Biochem 58:7–12

Ebashi S, Ebashi F, Maruyama K (1964) A new protein factor promoting contraction of actomyosin. Nature 203:645–646

Edman A-C, Squire JM, Sjostrom M (1988) Fine structure of the A-band in cryo-sections. Diversity of M-band structure in chicken breast muscle. J Ultrastruct Mol Struct Res 100:1–12

Eisenberg BR (1983) Quantitative ultrastructure of mammalian skeletal muscle. In: Peachey LD, Adrian RH, Geiger SR (eds) Handbook of physiology: section 10, skeletal muscle. American Physiological Society, Bethesda, pp 73–112

Elliott GF, Lowy J, Worthington CR (1963) An x-ray and light-diffraction study of the filament lattice of striated muscle in the living state and in rigor. J Mol Biol 6:295–305

Ervasti JM (2003) Costameres: the Achilles' heel of herculean muscle. J Biol Chem 278:13591–13594

Fisher TE, Marszalek PE, Oberhauser AF, Carrion-Vazquez M, Fernandez JM (1999) The micromechanics of single molecules studied with atomic force microscopy. J Physiol 520:5–14

Franzini-Armstrong C, Porter KR (1964) Sarcolemmal invaginations constituting the T system in fish muscle fibers. J Cell Biol 22:675–696

Furst DO, Osborn M, Nave R, Weber K (1988) The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z line extends close to the M line. J Cell Biol 106:1563–1572

Furst DO, Obermann WMJ, van der Ven PFM (1999) Structure and assembly of the sarcomeric M band. Rev Physiol Biochem Pharmacol 138:163–202
Garamvolgyi N (1965) Inter-Z bridges in the flight muscle of the bee. J Ultrastruct Res 13:435–443
Gautel M (2011) The sarcomeric cytoskeleton: who picks up the strain? Curr Opin Cell Biol 23:39–46
Gauthier GF (1971) The structural and cytochemical heterogeneity of mammalian skeletal muscle fibers. In: Podolsky RJ (ed) The contractility of muscle cells and related processes. Prentice-Hall, Englewood Cliffs, pp 131–150
Gergely J, Kimura S (2005) Forward: Memorials of Dr. Koscak Maruyama (1930–2003). J Muscle Res Cell Mot 26:277–284
Goldstein MA, Michael LH, Schroeter JP, Sass RL (1986) Z-band lattice in skeletal muscle before, during and after tetanic contraction. J. Muscle Res Cell Motil 7:527–536
Granger BL, Lazarides E (1978) The existence of an insoluble Z disc scaffold in chicken skeletal muscle. Cell 15:1253–1268
Grove BK, Kurer V, Lehner C, Doetschman TC, Perriard J-C, Eppenberger HM (1984) A new 185,000-dalton skeletal muscle protein detected by monoclonal antibodies. J Cell Biol 98:518–524
Hall CE, Jakus MA, Schmitt FO (1946) An investigation of cross striations and myosin filaments in muscle. Biol Bull 90:32–50
Hanson J (1968) Untitled. In: Ernst E, Straub FB (eds) Symposium on muscle, vol 8. Akademiai Kiado, Budapest, p 248
Hanson J, Huxley HE (1955) The structural basis of contraction in striated muscle. In: Brown R, Danielli JF (eds) Symposia of the society for experimental biology, vol 9, Fibrous proteins and their biological significance. Academic, New York, pp 228–264
Herrmann H, Bar H, Kreplak L, Strelkov SV, Aebi U (2007) Intermediate filaments: from cell architecture to nanomechanics. Nat Rev Mol Cell Biol 8:562–573
Horowits R, Podolsky RJ (1987) The positional stability of thick filaments in activated skeletal muscle depends on sarcomere length: evidence for the role of titin filaments. J Cell Biol 105:2217–2223
Horowits R, Kempner ES, Bisher ME, Podolsky RJ (1986) A physiological role for titin and nebulin in skeletal muscle. Nature 323:160–164
Hoyle G (1983) Muscles and their neural control. Wiley, New York
Huxley AF (ed) (1977) Looking back on muscle. In: The pursuit of nature: informal essays on the history of physiology. Cambridge University Press, Cambridge, pp 23–64
Huxley AF, Niedergerke R (1954) Interference microscopy of living muscle fibres. Nature 173:971–973
Huxley HE (1957) The double array of filaments in cross-striated muscle. J Biophys Biochem Cytol 3:631–648
Huxley HE (1963) Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. J Mol Biol 7:281–308
Huxley HE (1965) Structural evidence concerning the mechanism of contraction in striated muscle. In: Paul WM, Daniel EE, Kay CM, Monckton G (eds) Muscle. Pergamon Press, Oxford, pp 3–28
Huxley HE (1968) Untitled. In: Ernst E, Straub FB (eds) Symposium on muscle, vol 8. Akademiai Kiado, Budapest, p 248
Huxley HE (1972) Molecular basis of contraction in cross-striated muscle. In: Bourne GH (ed) The structure and function of muscle, vol 1, 2nd edn. Academic, New York, pp 301–387
Huxley HE, Hanson J (1954) Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. Nature 173:973–976
Ishikawa H, Bischoff R, Holtzer H (1968) Mitosis and intermediate-sized filaments in developing skeletal muscle. J Cell Biol 38:538–555
Ishikawa H, Bischoff R, Holtzer H (1969) Formation of arrowhead complexes with heavy meromyosin in a variety of cell types. J Cell Biol 43:312–328

Ishikawa H, Sawada H, Yamada E (1983) Surface and internal morphology of skeletal muscle. In: Peachey LD, Adrian RH, Geiger SR (eds) Handbook of physiology: section 10, skeletal muscle. American Physiological Society, Bethesda, pp 1–21

Jin J-P, Wang K (1991) Cloning, expression, and protein interaction of human nebulin fragments composed of varying numbers of sequence modules. J Biol Chem 266:21215–21223

Jordan HE (1933) The structural changes in striped muscle during contraction. Physiol Rev 13:301–324

Kellermayer MSZ, Smith SB, Granzier HL, Bustamante C (1997) Folding-unfolding transitions in single titin molecules characterized with laser tweezers. Science 276:1112–1116

Kempner ES, Haigler HT (1982) The influence of low temperature on the radiation sensitivity of enzymes. J Biol Chem 257:13297–13299

Kimura S (2005) Foreword- International symposium on muscle elastic proteins: Koscak Maruyama memorial meeting. J Muscle Res Cell Mot 26:275

Knappeis GG, Carlsen F (1962) The ultrastructure of the Z disc in skeletal muscle. J Cell Biol 13:323–335

Knappeis GG, Carlsen F (1968) The ultrastructure of the M line in skeletal muscle. J Cell Biol 38:202–211

Kontrogianni-Konstantopoulos A, Ackermann MA, Bowman AL, Yap SV, Bloch RJ (2009) Muscle giants: molecular scaffolds in sarcomerogenesis. Physiol Rev 89:1217–1267

Kruger M, Wright J, Wang K (1991) Nebulin as a length regulator of thin filaments of vertebrate skeletal muscles: correlation of thin filament length, nebulin size, and epitope profile. J Cell Biol 115:97–107

Labeit S, Kolmerer B (1995a) Titins: giant proteins in charge of muscle ultrastructure and elasticity. Science 270:293–296

Labeit S, Kolmerer B (1995b) The complete primary structure of human nebulin and its correlation to muscle structure. J Mol Biol 248:308–315

Labeit S, Gibson T, Lakey A, Leonard K, Zeviani M, Knight P, Warddale J, Trinick J (1991) Evidence that nebulin is a protein-ruler in muscle thin filaments. FEBS Lett 282:313–316

Labeit S, Ottenheijm CAC, Granzier H (2011) Nebulin, a major player in muscle health and disease. FASEB J 25:822–829

Lazarides E (1980) Intermediate filaments as mechanical integrators of cellular space. Nature 283:249–256

Lazarides E, Hubbard BD (1976) Immunological characterization of the subunit of the 100 Å filaments from muscle cells. Proc Natl Acad Sci U S A 73:4344–4348

Lazarides E, Revel JP (1979) The molecular basis of cell movement. Sci Am 240:100–113

Lazarides E, Weber K (1974) Actin antibody: the specific visualization of actin filaments in non-muscle cells. Proc Natl Acad Sci U S A 71:2268–2272

Linke WA, Kruger M (2010) The giant protein titin as an integrator of myocyte signaling pathways. Physiology 25:186–198

Linke WA, Ivemeyer M, Olivieri N, Kolmerer B, Ruegg JC, Labeit S (1996) Towards a molecular understanding of the elasticity of titin. J Mol Biol 261:62–71

Liversage AD, Holmes D, Knight PJ, Tskhovrebova L, Trinick J (2001) Titin and the sarcomere symmetry paradox. J Mol Biol 305:401–409

Locker RH, Leet NG (1975) Histology of highly-stretched beef muscle. I. The fine structure of grossly stretched single fibers. J Ultrastruct Res 52:64–75

Luther PK (2009) The vertebrate muscle Z-disc: sarcomere anchor for structure and signaling. J Muscle Res Cell Motil 30:171–185

Maruyama K (1965) A new protein-factor hindering network formation of F-actin in solution. Biochim Biophys Acta 94:208–225

Maruyama K (1976) Connectin, an elastic protein from myofibrils. J Biochem 80:405–407

Maruyama K (2002) β-Actinin, Cap Z, Connectin and Titin: what's in a name? Trends Biochem Sci 27:264–266

Maruyama K, Ebashi S (1965) α-Actinin, a new structural protein from striated muscle. II. Action on actin. J Biochem 58:13–19

Maruyama K, Kimura S (2000) Connectin: from regular to giant sizes of sarcomeres. In: Granzier HL, Pollack GH (eds) Elastic filaments of the cell. Kluwer Academic, New York, pp 25–33
Maruyama K, Natori R, Nonomura Y (1976) New elastic protein from muscle. Nature 262:58–60
Maruyama K, Matsubara S, Natori R, Nonomura Y, Kimura S, Ohashi K, Murakami F, Handa S, Eguchi G (1977) Connectin, an elastic protein of muscle: characterization and function. J Biochem 82:317–337
Maruyama K, Kimura S, Ohashi K, Kuwano Y (1981) Connectin, an elastic protein of muscle. Identification of "titin" with connectin. J Biochem 89:701–709
Maruyama K, Kurokawa H, Oosawa M, Shimaoka S, Yamamoto H, Ito M, Maruyama K (1990) β-actinin is equivalent to Cap Z protein. J Biol Chem 265:8712–8715
Masaki T, Takaiti O (1974) M-protein. J Biochem 75:367–380
Masaki T, Endo M, Ebashi S (1967) Localization of 6S component of α-actinin at Z-band. J Biochem 62:630–632
McElhinny AS, Kolmerer B, Fowler VM, Labeit S, Gregorio CC (2001) The N-terminal end of nebulin interacts with tropomodulin at the pointed ends of the thin filaments. J Biol Chem 276:583–592
McNeill PA, Hoyle G (1967) Evidence for superthin filaments. Am Zool 7:483–498
Moneman CL, Wang K (1995) Nebulette: a 107 kD nebulin-like protein in cardiac muscle. Cell Motil Cytoskeleton 32:205–225
Natori R (1954) The role of myofibrils, sarcoplasm and sarcolemma in muscle contraction. Jikeikai Med J 1:18–28
Obermann WMJ, Gautel M, Steiner F, van der Ven PFM, Weber K, Furst DO (1996) The structure of the sarcomeric M band: localization of defined domains of myomesin, M-protein, and the 250-kD carboxy-terminal region of titin by immunoelectron microscopy. J Cell Biol 134:1441–1453
Ohtsuki I, Maruyama K, Ebashi S (1986) Regulatory and cytoskeletal proteins of vertebrate skeletal muscle. Adv Protein Chem 38:1–67
Page SG (1965) A comparison of the fine structures of frog slow and twitch muscle fibres. J Cell Biol 26:477–497
Page SG (1968) Fine structure of tortoise skeletal muscle. J Physiol 197:709–715
Pappas CT, Bhattacharya N, Cooper JA, Gregorio CC (2008) Nebulin interacts with CapZ and regulates thin filament architecture within the Z-disc. Mol Biol Cell 19:1837–1847
Pappas CT, Krieg PA, Gregorio CC (2010) Nebulin regulates actin filament lengths by a stabilization mechanism. J Cell Biol 189:859–870
Pappas CT, Bliss KT, Zieseniss A, Gregorio CC (2011) The nebulin family: an actin support group. Trends Cell Biol 21:29–37
Pardo JV, D'Angelo Siliciano J, Craig SW (1983) A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. Proc Natl Acad Sci U S A 80:1008–1012
Pfuhl M, Winder SJ, Pastore A (1994) Nebulin, a helical actin binding protein. EMBO J 13:1782–1789
Pyle WG, Solaro RJ (2004) At the crossroads of myocardial signaling: the role of Z-discs in intracellular signaling and cardiac function. Circ Res 94:296–305
Reedy MK (1964) Discussion. Proc Roy Soc B Biol Sci 160:458–460
Rief M, Gautel M, Oesterhelt F, Fernandez JM, Gaub HE (1997) Reversible unfolding of individual titin immunoglobulin domains by AFM. Science 276:1109–1112
Rowe RWD (1973) The ultrastructure of Z disks from white, intermediate, and red fibers of mammalian striated muscles. J Cell Biol 57:261–277
Sabatini DD, Bensch K, Barnett RJ (1963) Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J Cell Biol 17:19–58
Sanger JW (2009) A remembrance of Harunori Ishikawa (1935–2008). Cell Mot Cytoskeleton 66:63–67

Sjostrand FS (1962) The connections between A- and I-band filaments in striated frog muscle. J Ultrastruct Res 7:225–246
Sjostrom M, Squire JM (1977) Fine structure of the A-band in cryo-sections. The structures of the A-band of human skeletal muscle fibres from ultra-thin cryo-sections negatively stained. J Mol Biol 109:49–68
Small JV, Sobieszek A (1977) Studies on the function and composition of the 10-nm (100-Å) filaments of vertebrate smooth muscle. J Cell Sci 23:243–268
Squire JM (1981) The structural basis of muscular contraction. Plenum, New York
Squire JM (1986) Muscle: design, diversity and disease. Benjamin/Cummings, Menlo Park
Trinick J (1981) End-filaments: a new structural element of vertebrate skeletal muscle thick filaments. J Mol Biol 151:309–314
Trinick J, Knight P, Whiting A (1984) Purification and properties of native titin. J Mol Biol 180:331–356
Trombitas K (2000) Connecting filaments: a historical prospective. In: Granzier HL, Pollack GH (eds) Elastic filaments of the cell. Kluwer, New York, pp 1–23
Tskhovrebova L, Trinick J (2012) Molecular rulers? Curr Biol 22:R317–R318
Tskhovrebova L, Trinick J, Sleep JA, Simmons RM (1997) Elasticity and unfolding of single molecules of the giant protein titin. Nature 387:308–312
Turner DC, Walliman T, Eppenberger HM (1973) A protein that binds specifically to the M-line of skeletal muscle is identified as the muscle form of creatine kinase. Proc Natl Acad Sci U S A 70:702–705
Wang K (1981) Nebulin, a giant protein component of N_2-line of striated muscle. J Cell Biol 91:355a
Wang K (1985) Sarcomere-associated cytoskeletal lattices in striated muscle: review and hypothesis. In: Shay O (ed) Cell and muscle motility, vol 6. Plenum Pub, New York, pp 315–369
Wang K, Ramirez-Mitchell R (1983) A network of transverse and longitudinal intermediate filaments is associated with sarcomeres of adult vertebrate skeletal muscle. J Cell Biol 96:562–570
Wang K, Williamson CL (1980) Identification of an N_2 line protein of striated muscle. Proc Natl Acad Sci U S A 77:3254–3258
Wang K, Wright J (1988) Architecture of the sarcomere matrix of skeletal muscle: immunoelectron microscopic evidence that suggests a set of parallel inextensible nebulin filaments anchored at the Z line. J Cell Biol 107:2199–2212
Wang K, Ash JF, Singer SJ (1975) Filamin, a new high-molecular-weight protein found in smooth muscle and non-muscle cells. Proc Natl Acad Sci U S A 72:4483–4486
Wang K, McClure J, Tu A (1979) Titin: major myofibrillar components of striated muscle. Proc Natl Acad Sci U S A 76:3698–3702
Wang K, McCarter R, Wright J, Beverly J, Ramirez-Mitchell R (1991) Regulation of skeletal muscle stiffness and elasticity by titin isoforms: a test of the segmental extension model of resting tension. Proc Natl Acad Sci U S A 88:7101–7105
Whiting A, Wardale J, Trinick J (1989) Does titin regulate the length of muscle thick filaments? J Mol Biol 205:263–268
Wright J, Huang Q-Q, Wang K (1993) Nebulin is a full-length template of actin filaments in the skeletal muscle sarcomere: an immunoelectron microscopic study of its orientation and span with site-specific monoclonal antibodies. J Muscle Res Cell Motil 14:476–483
Yamaguchi M, Izumimoto M, Robson RM, Stromer MH (1985) Fine structure of wide and narrow vertebrate muscle Z-lines. A proposed model and computer simulation of Z-line architecture. J Mol Biol 184:621–644
Yamaguchi M, Fuller GA, Klomkleaw W, Yamano S, Oba T (1999) Z-line structural diversity in frog single muscle fiber in the passive state. J Muscle Res Cell Mot 20:371–381

Chapter 8
Excitation-Contraction Coupling and Regulation of Contraction in Skeletal Muscle: The Modern Synthesis

…despite the identification of the key players in skeletal EC coupling, the same mechanistic questions facing investigators 15 years ago persist today. (Beam and Bannister 2010. With permission Rockefeller University Press)

K. G. Beam and R. Bannister (2010)

There is little doubt that the amphiphilic helix H3(I) works as a molecular switch that transmits the initial signal of Ca^{2+} binding to the N lobe of TnC to the other components in the thin filament, and thus has a central role in initial steps of troponin-tropomyosin based regulation. (Takeda et al. 2003. With permission Nature Publishing Group)

Takeda et al. (2003)

The data can be interpreted in terms of a model in which strong cross-bridges activate the thin filament, this activation being modulated by Ca^{2+} binding to troponin. (Millar and Homsher 1990. With permission American Society for Biochemistry and Molecular Biology)

N. C. Millar and E. Homsher (1990)

We can now say that the mechanism of active transport by SERCA1a is roughly understood, as crystal structures of most of the intermediates in the reaction cycle have been determined… (Toyoshima and Cornelius 2013. With permission Elsevier)

C. Toyoshima and F. Cornelius (2013)

8.1 Introduction

Excitation–contraction coupling, as the name implies, was originally defined to include the events from the sarcolemma action potential to the contraction of the muscle. But with the rapidly increasing knowledge concerning the mechanisms of muscle activation, sub-disciplines developed. Now excitation-contraction coupling usually is defined in a more limited way as the events that link surface membrane depolarization

J.A. Rall, *Mechanism of Muscular Contraction*, Perspectives in Physiology,
DOI 10.1007/978-1-4939-2007-5_8

to the release of Ca^{2+} from the sarcoplasmic reticulum in muscle cells (Paolini et al. 2004). The contraction part of excitation-contraction coupling is more likely now to be called regulation of contraction in striated muscle (Gordon et al. 2000).

8.2 Molecular Basis of Excitation-Contraction Coupling

By the late 1980s there was a strong basis for exploring the mechanism of excitation-contraction coupling in skeletal muscle. It was established that ryanodine receptor (RyR) located in the junctional face of the terminal cisternae of the sarcoplasmic reticulum (SR) was the channel through which Ca^{2+} was released during muscle activation. Furthermore the RyR contained four subunits which together constituted the so called "foot" structure that traversed the 12 nm gap between the SR and the transverse tubular membrane. Also L-type Ca^{2+} channels, also called dihydropyridine receptors (DHPR), were located in the transverse tubular membrane at the triadic junction. There was a very precise relationship between the two channels in skeletal muscle. Four DHPRs, organized as tetrads, were superimposed on alternate RyRs (see Fig. 6.26). This structural information was consistent with an earlier proposal that excitation-contraction coupling in skeletal muscle was mediated via a movement of intramembrane charge in the transverse tubular system. The speculation in the 1980s was that the DHPR acted as a voltage sensor transferring information to the RyRs of the SR by direct protein-protein interaction causing the release of Ca^{2+} from the SR and thus muscle activation. (See Chap. 6 for a description of the development of these ideas.) The challenge then became the discovery of the mechanism of this protein-protein interaction. Progress toward unraveling this mechanism in the late 1980s and early 1990s would at first be spectacular but then things would get complicated and 20 years later the molecular mechanism would still remain elusive.

8.2.1 Transverse Tubule-Sarcoplasmic Reticulum Communication

Starting in the late 1960s Jeanne A. Powell in the department of biological sciences at Smith College in Massachusetts was studying skeletal muscle development and disease. In 1983 she and her collaborators were exploring muscular dysgenesis in mouse myotubes in culture (Klaus et al. 1983). The dysgenesis was a spontaneous, recessive lethal mutation expressed in utero as progressive degeneration of skeletal muscle. The severe abnormalities of intercostal and diaphragm muscles led to immediate postnatal death. Dysgenic embryos and newborns never exhibited spontaneous movements nor did they respond to mechanical or electrical stimulation. She and Douglas M. Fambrough (1973) had earlier observed that the dysgenic myotubes in primary culture exhibited normal passive and active membrane properties but did not contract upon stimulation. In the 1983 study, they observed that the dysgenic myotubes contracted

in response to extracellular Ca^{2+} in the presence of a Ca^{2+} ionophore but not in response to K^+ depolarization. They concluded (Klaus et al. 1983. With permission Elsevier):

> The present results offer new evidence for a possible defect in the regulation of Ca^{2+} levels in response to depolarization. The exact site of the defect may be in the physical and/or electrical coupling between plasma membrane and SR or possibly a specific molecular defect at the level of transduction of the electrical message into a permeability change in the sarcoplasmic reticulum.

Thus there seemed to be a defect in excitation-contraction coupling.

Powell's paper caught the attention of Kurt G. Beam[1] at the University of Iowa. He reasoned that the dysgenic myotubes were missing the Ca^{2+} current associated with the DHPR. Beam and Powell established a collaboration to explore this possibility (Beam et al. 1986). Calcium currents were recorded as a function of voltage using the whole-cell patch-clamp technique in myotubes form normal and dysgenic mice. They found that the slow Ca^{2+} current characteristic of L-type Ca^{2+} channels was absent in the dysgenic myotubes. They suggested possible explanations for the results. The mutation might: directly alter the structural gene for the slow calcium channel, interfere with transcription of the gene, disrupt the post-translational processing and/or insertion of the channel protein, or affect a soluble regulatory protein necessary for channel function. They understood that the slow Ca^{2+} current was not likely to be directly involved in activating contraction in skeletal muscle since it evolved so slowly, over a 100 ms or so. Nonetheless the channel protein itself might still be intimately involved since the slow Ca^{2+} channel had been proposed to serve as the voltage-sensor for the process governing SR Ca^{2+} release.

During the 1980s and early 1990s Shosaku Numa[2] and his collaborators at Kyoto University were making spectacular progress in elucidating the primary structures of membrane proteins (receptors, ion channels and ion pumps) by cloning the DNA complementary to mRNA (cDNA) coding for the proteins (Imura 1995). First they determined the primary structure of the α subunit of the nicotinic acetylcholine receptor (nAchR) in 1982 and then all of the other subunits of the receptor. They then cloned the Na^+ channel in 1984. The group also elucidated the primary

[1] Kurt G. Beam received a Ph.D. from the University of Washington in 1976 with Charles F. Stevens and did postdoctoral training at Yale University with Paul Greengard who would later win the Nobel Prize in 2000. His first faculty position was at the University of Iowa in the late 1970s from which he moved to Colorado State University and eventually to the University of Colorado. His research has concentrated on elucidation of the mechanism of excitation-contraction in skeletal muscle. Beam was elected to the National Academy of Sciences in 2012.

[2] Shosaku Numa (1929–1992) received his M.D. degree from Kyoto University in 1952. He did postdoctoral training at Harvard University and at the Max-Planck Institute in Munich. Numa returned to Kyoto University in 1961 and was appointed professor in the department of medical chemistry in 1968 where he remained throughout his research career. He was a pioneer of molecular neurobiology and he and his group were responsible for cloning the genes and studying the function of a large number of membrane proteins (receptors, ion channels and ion pumps, see text). Because of his outstanding achievements, he received many prizes and honors, including the Japan Academy Prize and nomination as a Person of Cultural Merit of Japan. He was a foreign member of the Royal Society and a foreign associate of the National Academy of Sciences. He died prematurely of colon cancer at the age of 63 at the height of his spectacular research career (Imura 1995).

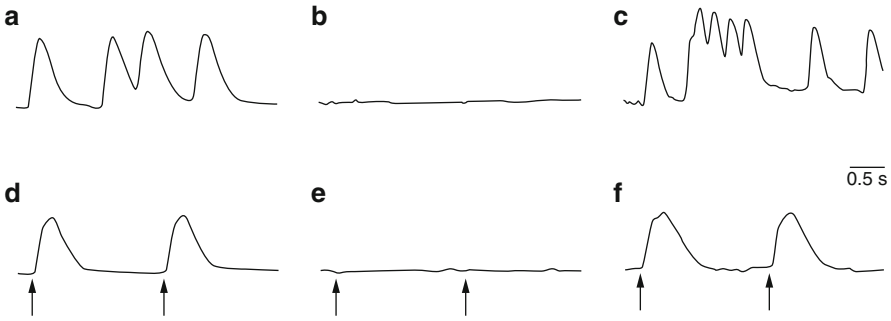


Fig. 8.1 Restoration of contractile activity in dysgenic mouse myotubes with injection of a plasmid carrying DHPR cDNA. Comparison of contractile activity in primary cultures of normal myotubes (**a**, **d**), dysgenic myotubes (**b**, **e**) and dysgenic myotubes that were injected with DHPR cDNA (**c**, **f**). The contractile activity in (**c**, **f**) was measured 3 days after injection with DNA. Spontaneous contractile activity is illustrated in (**a**–**c**); electrically evoked contractile activity is shown in (**d**–**f**). *Arrows* indicate the time of application of electrical stimulus (Tanabe et al. 1988. With permission Nature Publishing Group)

sequences of the muscarinic acetylcholine receptor, ryanodine and dihydropyridine receptors, photoreceptor cyclic GMP-gated channel and the (Na^+, K^+) ATPase. Along with determining the primary sequences of receptors and channels, Numa was interested in performing functional studies utilizing molecular biology techniques. The primary structure of the DHPR from rabbit skeletal muscle was elucidated in Numa's laboratory by Tsutomu Tanabe and their collaborators in 1987 (Tanabe et al. 1987)[3]. Structural and sequence similarities to the voltage-dependent Na^+ channel suggested that in the transverse tubule membrane of skeletal muscle the DHPR acted both as a voltage sensor in excitation-contraction coupling and as a Ca^{2+} channel.

Numa contacted Beam and suggested a collaboration to investigate the function of the cloned DHPR in dysgenic mice. The first question was: could DHPR complementary DNA restore excitation-contraction coupling in dysgenic mouse myotubes? They found that microinjection into dysgenic myotubes of an expression plasmid that carried complementary DNA encoding the DHPR of skeletal muscle restored both electrically induced and spontaneous contraction (Fig. 8.1) and also slow Ca^{2+} current (Fig. 8.2) (Tanabe et al. 1988). But they did not measure charge movement. Measurement of charge movement is difficult because one must immo-

[3] In order to clone the gene coding for the skeletal muscle voltage-gated Ca^{2+} channel, the following procedure was employed: (1) Rabbit skeletal muscle DHPR was purified, (2) Photoaffinity labeling of DHPR with dihydropyridine ^{3}H-azidopine showed that the ligand was specifically incorporated into the 170 kDa polypeptide, (3) 170 kDa polypeptide was purified, (4) 170 kDa polypeptide was digested with trypsin and the resulting peptides were fractionated, (5) Amino acid sequences of peptides were determined, (6) Synthetic oligodeoxyribonucleotides for one of the peptides were prepared, (7) Then used as a specific primer for reverse transcription and as a probe for selecting the resulting cDNA clones, (8) The initial DNA clone thus isolated was used to probe for cloning longer cDNA sequences (Tanabe et al. 1987).

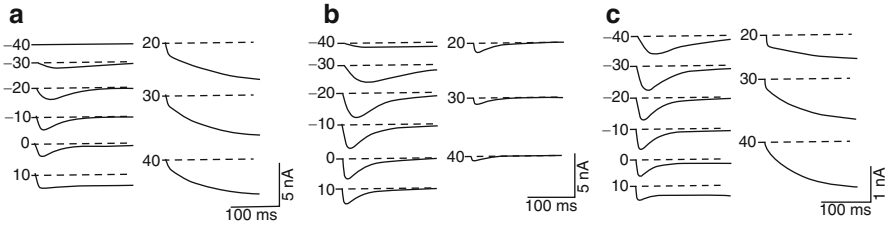


Fig. 8.2 Restoration of calcium currents in dysgenic mouse myotubes with injection of a plasmid carrying DHPR cDNA. (**a**) Comparison of Ca^{2+} currents in a normal myotube, (**b**) dysgenic myotube and (**c**) dysgenic myotube that was injected with the expression plasmid. Ca^{2+} currents were measured at various test potentials using the whole-cell patch-clamp technique. Note the lack of slow evolving Ca^{2+} currents at high voltages in the dysgenic myotube before DNA injection (**b**, *right panel*) and the large slow currents after injection (**c**, *right panel*) (Tanabe et al. 1988. With permission Nature Publishing Group)

bilize charge movements from all other sources, i.e., Na^+ and K^+ channels, and also block Ca^{2+} currents. What is measured as immobilization-resistant charge movement is assumed to be charge movement in the DHPR channel. Nonetheless in a follow-up study Brett A. Adams in Beam's laboratory along with Tanabe, Mikami and Numa showed that intramembrane charge movement was restored in dysgenic myotubes by injection of DHPR cDNA (Adams et al. 1990). Taken together these results strongly supported the hypothesis that the DHPR in the transverse tubule membrane of skeletal muscle functioned both as the voltage sensor for excitation-contraction coupling and as the slow Ca^{2+} channel.

Tanabe et al. (1990) wanted to discover the regions of the DHPR that were necessary for excitation-contraction coupling. The cloned DHPR exhibits four putative membrane spanning repeats or regions. Each repeat has five hydrophobic segments (S1, S2, S3, S5 and S6) and one positively charged segment (S4) that may function as the voltage sensor. The four regions are connected by four cytoplasmic loops (Fig. 8.3). It was natural to think that the cytoplasmic loops pointing to the feet of the RyRs might be crucial in promoting excitation-contraction coupling. They developed a powerful strategy. The took advantage of the fact that injection into dysgenic myotubes of an expression plasmid carrying the cardiac DHPR cDNA produced cardiac-type excitation-contraction coupling which required entry of extracellular Ca^{2+} whereas excitation-contracting coupling in the presence of skeletal DHPR was independent of extracellular Ca^{2+}. To identify the regions responsible for this functional difference, they expressed various chimeric DHPR cDNAs in dysgenic myotubes (Fig. 8.3). They utilized the cardiac DHPR as the "backbone" and inserted skeletal muscle cytoplasmic regions into the molecule. They found that the putative cytoplasmic region between repeats II and III of the skeletal muscle DHPR was a major determinant of skeletal-type excitation-contraction coupling. These were exciting results that showed the power of the molecular genetic approach in exploring mechanism but there would be complications ahead.

Hiroshi Takeshima and collaborators utilized a targeted mutation in the skeletal muscle ryanodine receptor (RyR1) gene to abolish excitation-contraction coupling in

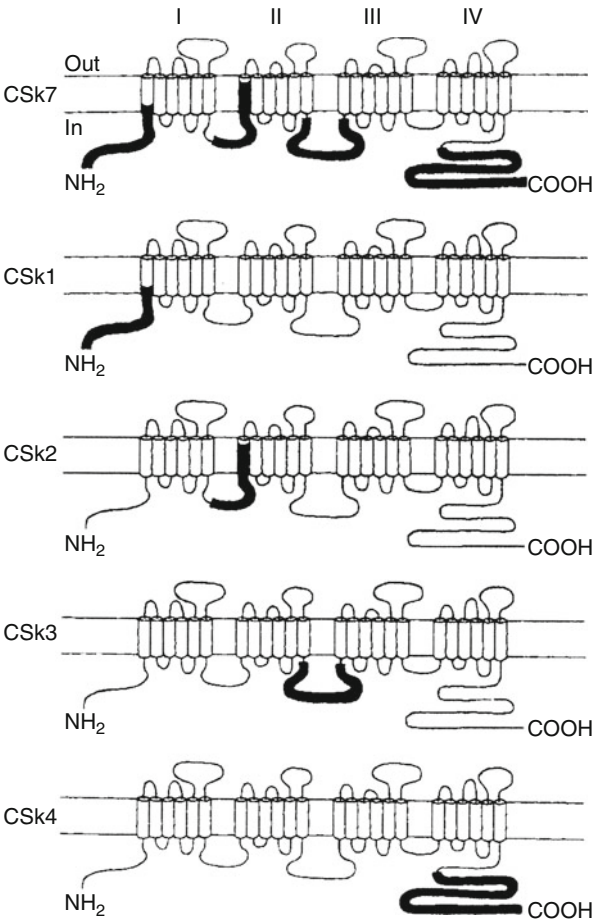


Fig. 8.3 Schematic representation of the structures of chimeric DHPRs composed of cardiac backbone and skeletal muscle loop sequences. For each chimeric DHPR, the four units of homology (repeats I–IV) are displayed linearly and the six putative transmembrane segments (S1–S6 from *left* to *right*) in each repeat are shown by cylinders. The *darkly shaded areas* indicate the regions of the cardiac DHPR that have been replaced by the corresponding portions of the skeletal muscle DHPR. The results indicated that the region between repeats II and III (CSk3) was a major determinant of skeletal muscle-type excitation-contraction coupling (Tanabe et al. 1990. With permission Nature Publishing Group)

mice (Takeshima et al. 1994). In the myotubes of this so-called dyspedic mouse, DHPR channel activity was reduced as observed by a 30-fold reduction in L-type Ca^{2+} current. Injection with skeletal muscle RyR1 complementary DNA restored excitation-contraction coupling and caused the density of L-type Ca^{2+} current to increase toward normal (Nakai et al. 1996). These results supported the possibility of a retrograde signal by which RyR1s enhance the function of DHPRs as Ca^{2+} channels. Thus the communication between the DHPR and RyR1 appeared to be bidirectional: both orthograde and retrograde. The speculation was that orthograde signaling was responsible for excitation-contraction coupling and retrograde signaling was responsible for regulating the ability of DHPRs to function as Ca^{2+} channels.

The DHPR molecule consists of multiple subunits under the control of different genes. The subunit destination in skeletal muscle is: α_{1S} (the Ca^{2+} channel), β_1, α_2-δ and γ (Takahashi et al. 1987). The α_2-δ subunit is actually two polypeptides linked by disulfide bonds that are generated by post-translational cleavage of a single poly-

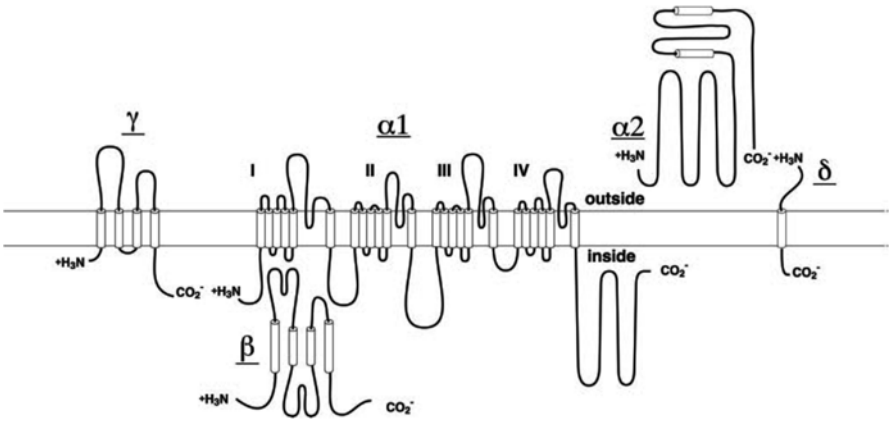


Fig. 8.4 Subunit structure of skeletal muscle voltage sensitive calcium channel (Ca_v1). Predicted **α** helices are depicted as cylinders. The lengths of lines correspond approximately to the lengths of the polypeptide segments represented (Catterall et al. 2005. With permission American Society for Pharmacology and Experimental Therapeutics)

peptide. Of the accessory subunits only the β subunit is wholly cytoplasmic in its location (Fig. 8.4). Thus it is said that the DHPR is a heteropentamer in contrast to the RyR which is a homotetrametric channel with four subunits under the control of the single gene.

Until the mid 1990s, only the role of the α_1 subunit of the DHPR was being explored in excitation-contraction coupling. This approach was natural since the α_1 subunit of both the skeletal and cardiac DHPR was the Ca^{2+} channel which contained the putative charge movement. But it was also natural to wonder what role(s) other DHPR subunits might play. Of particular interest was the β subunit since it existed in the cytoplasmic space and might conceivably influence the interaction of the DHPR and the RyR. By the mid-1990s gene targeting techniques were becoming prevalent in the study of muscle function. At the University of Wisconsin, Ronald G. Gregg and Patricia A. Powers generated a knockout of the mouse β_1 gene and the effects of the knockout were characterized by Roberto Coronado and his collaborators (Gregg et al. 1996). The phenotype of this knockout animal was essentially the same as that of the dysgenic mouse. The β_1-null mice died at birth from asphyxia. Electrical stimulation of β_1-null skeletal muscle failed to induce twitches, however, contractures were induced by caffeine. In isolated β_1-null myotubes, action potentials were normal, but they failed to elicit a Ca^{2+} transient. The L-type Ca^{2+} current was decreased 10- to 20-fold in the β_1-null cells compared with litter-mate controls. Immunohistochemistry of cultured myotubes showed that not only was the β_1 subunit absent, but the amount of the a_{1S} subunit in the membrane also was undetectable. In contrast, the β_1 subunit was localized appropriately in dysgenic (a_{1S}-null) cells. They concluded that the β_1 subunit may not only play an important role in the transport/insertion of the a_{1S} subunit into the membrane but may be vital for the targeting of the muscle dihydropyridine receptor complex to the

transverse tubule/SR junction. The picture would become more complex as Coronado and his collaborators published a series of papers (reviewed by Coronado et al. 2004) that led them to conclude that the domains of the α_{1S} II-III loop are necessary but not sufficient to recapitulate skeletal-type EC coupling. Rather the structural unit that controls the excitation-contraction coupling signal appears to be the α_{1S}/β_{1a} pair. They suggested that domains of the DHPR β_1 subunit, similar to elements present in the α_{1S} subunit, might be directly involved in activation of RyR1 channels. Thus the β_1 might play a dual role. First a trafficking/targeting function to locate the α_{1S} subunit in the appropriate position in the junctional membrane and a second function to modulate excitation-contraction coupling directly.

Manfred Grabner and his colleagues at the Innstruck Medical University in Austria collaborated with Clara Franzini-Armstrong to explore the role of the β_{1a} in excitation-contraction coupling (Schredelseker et al. 2005). In a comprehensive investigation, they examined the myotubes of zebrafish that lacked the DHPR β_{1a} subunit (called zebrafish *relaxed*) utilizing biophysical, molecular biology, protein biochemistry, immunocytochemistry and ultrastructural techniques. These myotubes exhibited the same phenotype as seen previously in mouse myotubes null for the β_{1a} subunit. There was a lack of depolarization-induced but not caffeine-induced Ca^{2+} transients and a large reduction of intramembrane charge movement. An interesting observation was that L-type Ca^{2+} currents were absent in both normal and *relaxed* myotubes. Thus the zebrafish DHPR is a non-Ca^{2+}-conducting voltage sensor and as such this organism provides in vivo evidence that skeletal muscle excitation-contraction coupling is fully functional without Ca^{2+} influx through the DHPR[4]. Freeze-fracture analysis of the DHPR clusters in *relaxed* myotubes revealed an approximate 2-fold reduction in cluster size with a normal density of DHPR particles within the clusters. Most important, DHPR particles in the junctional membranes of the zebrafish *relaxed* lacked entirely the normal arrangement in arrays of tetrads. The data indicated that the lack of the β_{1a} subunit did not prevent triad targeting of the DHPR α_{1S} subunit but precluded the skeletal muscle-specific arrangement of DHPR particles opposite the RyRs. They concluded that the inability to form DHPR tetrads probably explains the complete deficiency of skeletal muscle excitation-contraction coupling in β_1-null model organisms. Thus not only was it necessary for normal excitation–contraction coupling to have functional α_{1S} (with the loop connecting repeats II and III) and β_{1a} subunits of the DHPR at the junctional membrane but it also was necessary for the DHPRs to be arranged in tetrads to obtain the proper stereo specific alignment with the RyRs.

Questions remained. Some of which included the following. What roles might the α_2-δ-1 and γ subunits play? The α_{1S} and β_{1a} subunits and tetrad formation were necessary but were they sufficient to explain normal excitation-contraction coupling? Could the β subunit also modulate excitation-contraction coupling as suggested by the Coronado group? This modulation was a definite possibility since the

[4] What then is the function of the DHPR Ca^{2+} calcium current in skeletal muscle excitation-contraction coupling? In short, no one knows. It has been speculated that the Ca^{2+} current through the DHPR may act to ensure long-term Ca^{2+} replenishment of the SR (Tanabe et al. 1987).

β subunit binds to the cytoplasmic linker between repeats I and II of the α subunit of the skeletal muscle DHPR (Pragnell et al. 1994).

Apparently targeted deletions of the α_2-δ-1 and γ subunits do not critically interfere with excitation-contraction coupling (Obermair et al. 2008). Normal excitation-contraction coupling has never been observed without tetrad formation but tetrad formation can occur with impaired excitation-contraction coupling (Takekura et al. 2004). This result suggests that the molecular determinants of the functional coupling between DHPR and RyR are not necessarily the same as those required for a specific anchoring of the two proteins. Thus DHPR tetrad formation is necessary but not sufficient for normal excitation-contraction coupling. Recently Grabner and Franzini-Armstrong and their colleagues examined the role of β_{1a} in inducing the voltage-sensing function of α_{1S} (Dayal et al. 2013). Despite the ability to fully target α_{1S} into triadic junctions and tetradic arrays, the neuronal isoform β_3 was unable to restore considerable charge movement (a measure of α_{1S} voltage sensing) upon expression in β_1-null zebrafish *relaxed* myotubes. They utilized β_3 for chimera formation with β_{1a} to investigate whether any of the five distinct molecular regions of β_{1a} was dominantly involved in inducing the voltage-sensing function of α_{1S}. This strategy was similar to the one employed by Tanabe et al. (1990) to examine the role of the α_{1S} loops of the DHPR. Systematic domain swapping between β_{1a} and β_3 revealed a cooperative role for two of the β_{1a} domains in restoring charge movement and intracellular Ca^{2+} release. Furthermore, substitution of a proline with alanine in the polyproline motif in the proximal C terminus of β_{1a} fully obstructed charge movement. They proposed a model according to which β subunits adapt a discrete conformation required to modify the α_{1S} subunit conformation appropriate for voltage sensing in skeletal muscle. Thus the β subunit of the DHPR is thought to be important in not only DHPR tetrad formation at the junctional membrane but also in DHPR voltage sensing.

During the past 20 years much has been learned about skeletal muscle excitation-contracting coupling. All of the described studies are consistent with the hypothesis that there is a direct protein (DHPR)-protein (RyR) interaction in skeletal muscle excitation-contracting coupling but they have not proven the hypothesis. Probably the strongest evidence that the two receptors are linked either directly or indirectly came from a remarkable study by Paolini et al. (2004). They treated a skeletal muscle cell line with ryanodine at concentrations that blocked RyRs and determined whether this treatment affected the distance between DHPRs in the tetrad. The position of DHPRs was determined in rotary-shadowed freeze–fracture replicas. With this approach the highest points of the fractured DHPRs (or particles) appear in the electron micrographs as pale circles surrounded by dark rings of metal. The average intratetrad distance in untreated cells was 19.5 nm. In the treated cells, the intratetrad distance decreased significantly by ~2 nm. They concluded that the fact that the effect of ryanodine was transmitted from the RyR (on which it acts) to the tetrads (composed of DHPRs) indicated a conformational coupling between the two Ca^{2+} channels that required a very specific intermolecular interaction. This coupling could be achieved only if the two channels were in molecular contact, even though this may be through an intermediary protein. This evidence unequivocally demonstrated

that the skeletal DHPRs are linked either directly or indirectly with the RyRs but it did not delineate mechanism.

Beam and Bannister (2010) reviewed the state of knowledge regarding the mechanism of excitation-contraction coupling in skeletal muscle in an unusually candid article entitled: "Looking for answers to EC coupling's persistent questions." They start out by stating that "despite the identification of the key players in skeletal EC coupling, the same mechanistic questions facing investigators 15 years ago persist today". These questions include: What parts of the DHPR α_{1S} subunit trigger EC coupling? How does the essential DHPR β_{1a} subunit participate in EC coupling? How is the EC coupling signal transmitted from the voltage-sensing regions of the α_{1S} to RYR1? They point out that while the functional analysis of chimeric DHPR subunits has been effective in identification of regions of the molecule that are important for excitation-contracting coupling, it is not possible to conclude whether a region of demonstrated importance is an actual site of interaction with other junctional proteins or allosterically affects such interactions. Furthermore the in vitro biochemical studies can demonstrate direct interactions but the identified interactions may not have a physiological correlate in vivo. They felt that the traditional experimental approaches almost had been exhausted. How then does one get to the mechanism of skeletal excitation-contraction coupling? They suggested that the mechanism would only be resolved when high resolution structures of the DHPR and RyR1 were elucidated. Determination of DHPR and RyR1 structures is a major on-going challenge.

8.2.2 Calcium Release Channel of Sarcoplasmic Reticulum: Structure, Function and Regulation

Calcium release channel structure: By the late-1980s, some general features of the structure of the Ca^{2+} release channel or ryanodine receptor (RyR1) of skeletal muscle were established. First of all, it was big. The channel consisted of four identical subunits of high molecular weight. Thus it is a homotetramer. It exhibited an ~20 nm square-like structure which constituted the "foot" seen in the electron microscope (see Figs. 4.8, 6.22, 6.23, 6.25 and 6.26). Numa's laboratory had determined the primary sequence of the RyR1 through gene cloning (Takeshima et al. 1989). Each subunit contained ~5,000 amino acids with a calculated molecular weight of 565 kDa. Thus the total channel molecular weight was ~2,200 kDa. The RyR1 would become the largest known ion channel, for instance ten times larger than a Na^+ channel.

The large size of the channel precluded any consideration of generating a crystal structure of the channel at atomic resolution. Nonetheless considerable process has been made in the determination of the RyR1 structure with electron microscopy and three dimensional reconstruction techniques. Sidney Fleischer at Vanderbilt University established a collaboration with Joachim Frank at the Wadsworth Center,

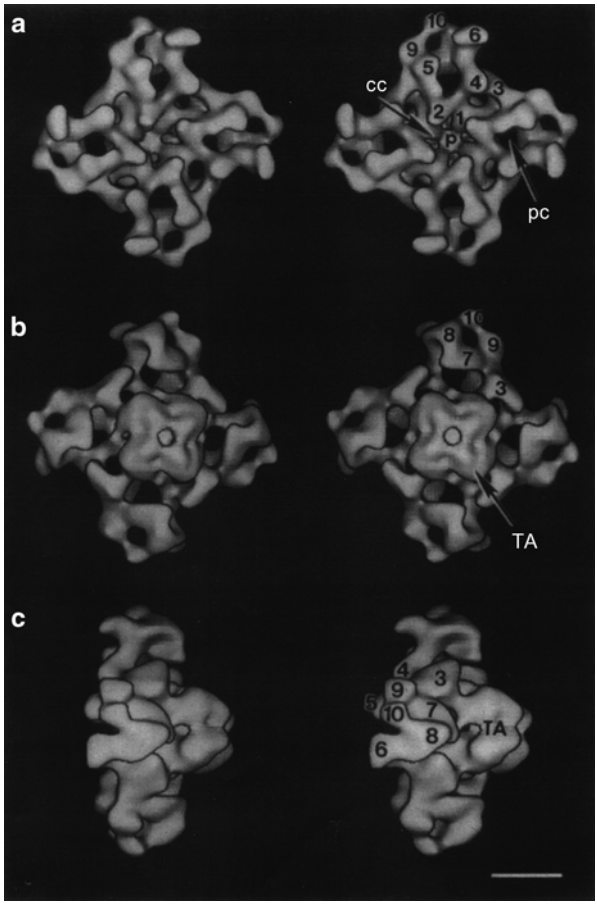


Fig. 8.5 Surface representations of the reconstruction of the Ca^{2+} release channel (RyR1) shown as stereo pairs. (**a**) View showing the surface that would face the cytoplasm and the apposing transverse tubule in a triad junction. (**b**) View onto the face that would interact with the sarcoplasmic reticulum. (**c**) Side view. Putative structural domains are labeled with numerals. The calibration bar = 100 Å (Radermacher et al. 1994. With permission Rockefeller University Press)

New York State Department of Health, Albany, New York. The Wadsworth Center has a long history of innovation and excellence in electron microscopy and Frank was interested in developing the technology for three dimensional reconstruction of large protein structures, particularly ribosomes. In 1989 together with Terence Wagenknecht, their groups produced the first three dimensional structure of the purified RyR1 using negative staining and image analysis of single particles (Wagenknecht et al. 1989). Because there was a concern that the negative staining procedure could lead to a collapse or flattening of the structure with dehydration, they shifted to cryo-electron microscopy. In this technique the specimen is rapidly frozen in vitreous ice in a hydrated state without stains or fixatives. In October of 1994 they published three dimensional 30 Å resolution images of the Ca^{2+} release channel (Radermacher et al. 1994) (Fig. 8.5). There was a clear demarcation between the cytoplasmic assembly (about 80 % of the total protein) and the channel assembly (about 20 % of the protein) (clearly seen in the side view, Fig. 8.5c). The

large cytoplasmic assembly measuring 290×290×120 Å represented the foot process connecting the sarcoplasmic reticulum and transverse tubular system. The smaller transmembrane assembly protruded 70 Å from one of its faces. A cylindrical low-density region, 20–30 Å in apparent diameter, extended down the center of the transmembrane assembly and possibly corresponded to the transmembrane Ca^{2+} conducting pathway. At its cytoplasmic end this channel-like feature appeared to be plugged by a globular mass of density. The cytoplasmic assembly appeared to be constructed from ten or more domains that were loosely packed together such that greater than 50 % of the volume of the assembly was occupied by solvent (Fig. 8.5a, b). Regarding the unusual structure of the channel protein, they speculated that one of the roles of the cytoplasmic assembly was a mechanical one to hold the transverse tubule and SR membrane systems in close proximity at the triad junction and to maintain the junction during the potentially disruptive forces generated during cycles of muscle contraction.

Just 3 months later, Irina I. Serysheva, Susan L. Hamilton and Wah Chiu and their collaborators at the Baylor College of Medicine and Max Planck Institute, Berlin, also published a three dimensional structure of the RyR1 at 30 Å resolution. They utilized a different reconstruction procedure but came to virtually identical conclusions (Serysheva et al. 1995).

By 2005, resolution of the RyR1 had improved to ~10 Å (Samso et al. 2005; Ludtke et al. 2005). At this resolution it was possible to begin to deduce some of the secondary structure in parts of the molecule using a powerful computational program developed by Baker et al. (2007). This quantitative tool could detect α helices and β sheets in structures resolved to 5–10 Å. This tool was utilized to identify α helices and β sheets in the RyR1 (Serysheva et al. 2008) (see the movie associated with the paper). It was now possible to begin to compare the RyR1 pore structure with that of other resolved ion channels. Further improvements in resolution are likely.

Even at 30 Å resolution it was possible, employing difference mapping, to begin to locate sites in the three dimensional structure where various modulators of channel activity might bind to the channel protein. The activities of all three RyR isoforms are modulated by an amazing number of agents, including: ATP, Ca^{2+}, Mg^{2+}, DHPR, calmodulin, FKBP12, junctin, triadin, calsequestrin, S100A, sorcin, etc. Also channel activity is affected by post-translational modifications such as phosphorylation, oxidation, and *S*-nitrosylation and disease mutations (see below and also see the review by Fill and Copello 2002). A prerequisite for the delineation of the mechanisms of channel regulation by individual modulators and modifications is the identity of the sites of modulator interaction or modification in the primary sequence and the location of these sites in the three-dimensional structure. Because most channel modulators bind in the cytoplasmic assembly, there must be mechanisms whereby changes in the conformation of specific domains in the cytoplasmic assembly are transduced into changes in the transmembrane helices of the pore and thus regulating the opening of the Ca^{2+} conduction pathway. Progress in elucidation of the RyR1 structure has been reviewed recently by Hamilton and Serysheva 2009).

Calcium release channel function: Over the years the release of Ca^{2+} from the SR has been studied with a variety of techniques in various experimental preparations. ^{45}Ca fluxes have been recorded from intact muscle, skinned muscle fibers and isolated SR. With the advent of single channel recording developed by Neher and Sakmann (1976), it became a goal to measure the Ca^{2+} currents through single Ca^{2+} release channels. This goal has greatly facilitated by the pioneering investigations of Christopher Miller, then at Cornell University, now at Brandeis University, that showed that SR vesicles could be fused with planar lipid bilayers and functional properties measured (Miller and Racker 1976). There were two general strategies for investigating the properties of the Ca^{2+} release channel in bilayers. One way was to purify the heavy fraction of the SR which contains the Ca^{2+} release channel and then fused these "native" SR vesicles into the planar lipid bilayers. This approach had the advantage that any accessory components necessary for channel conductance would likely still be in the SR. But it also had the disadvantage that other channels, K^+ and Cl^- channels, would also exist in the bilayer and these channels would have to be blocked pharmacologically in order to measure pure Ca^{2+} current. The other approach was to purify the Ca^{2+} release channel (RyR1 from skeletal muscle) and then insert it into the lipid bilayer. This approach had the advantage of simplicity in that no other channels would be involved but also the disadvantage that important accessory components affecting the Ca^{2+} currents might be missing. Roberto Coronado and postdoctoral fellow Jeffrey S. Smith at the Baylor College of Medicine made the first single channel recordings of the Ca^{2+} release channel by fusing the heavy SR vesicles supplied by Gerhard Meissner at the University of North Carolina into planar lipid bilayers (Smith et al. 1985). When applied to the cytoplasmic side of the channel, channel currents were stimulated by Ca^{2+} (Fig. 8.6) and ATP and inhibited by Mg^{2+}. The channels exhibited a surprisingly large conductance (~100 pS) to divalent cations while still discriminating against monovalent cations. Smith and Coronado, now in collaboration with Kevin Campbell's laboratory at the University of Iowa, went on to perform a detailed comparison of the single channel properties of the Ca^{2+} release channels in the heavy SR and the purified RyR1s (Smith et al. 1988). The channels exhibited very similar properties. Both exhibited a high conductance of ~100 pS to divalent cations (compared to the L-type Ca^{2+} channels of ~20 pS) with a relatively poor permeability ratio of divalent to monovalent cations of P_{Ca}/P_K ~6 (compared to the L-type Ca^{2+} channels of P_{Ca}/P_{Na} >35). They concluded that the ryanodine receptor channels were identical to the Ca^{2+} release channels described in native sarcoplasmic reticulum using the same techniques. Thus these high conductance channels are relatively poor discriminators between divalent and monovalent cations. Smith et al. (1988) speculated that this property of Ca^{2+} release channels could be of physiological importance in that along with allowing large efflux of Ca^{2+} for muscle activation, they also could provide a pathway for charge compensation with inward cation, e.g., K^+, movement during Ca^{2+} release.

Much was learned about Ca^{2+} release channels and their various modulators from the single channel studies. Nonetheless it was not possible to duplicate the intracellular environment in these studies and thus not possible to know how the intracellular

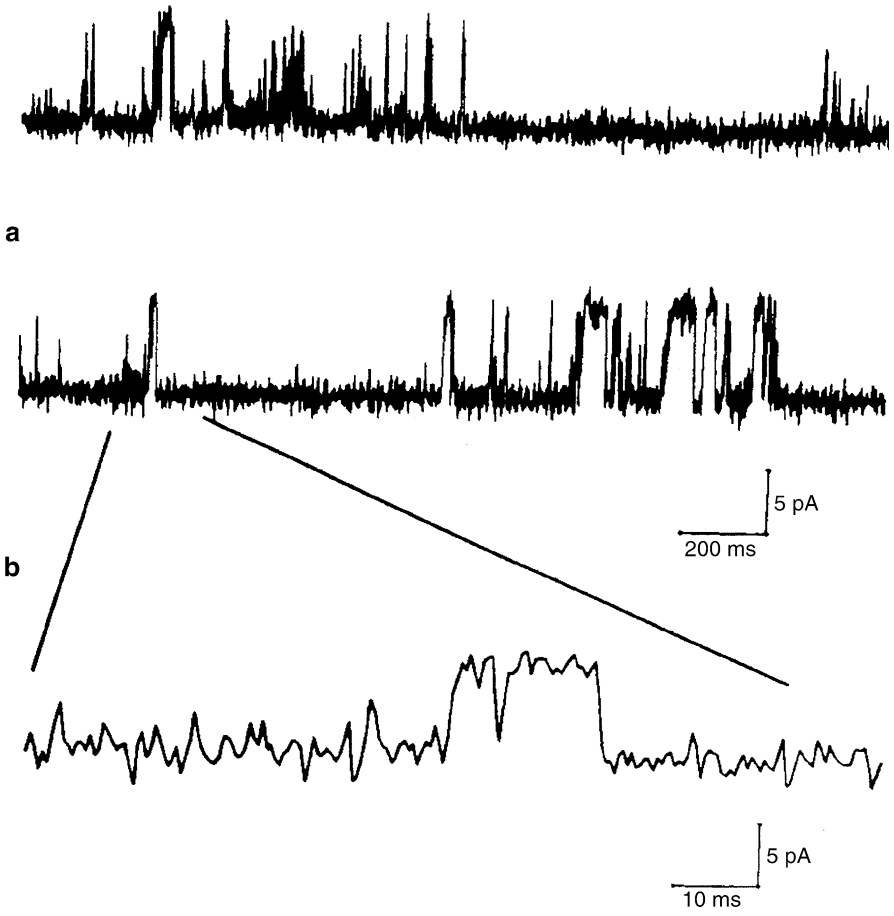


Fig. 8.6 Single channel current fluctuations triggered by Ca^{2+} when heavy sarcoplasmic reticulum vesicles are fused into planar lipid bilayers. Current versus time trace on a slow (**a**) and fast (**b**) time scale. The cytoplasmic side of the channel was perfused with a solution containing 2.5 **μ**M free [Ca^{2+}] whereas the luminal side was perfused with a solution containing 50 mM [Ca^{2+}]. The current trace is oriented so that open-channel events are directed upwards and represent divalent ion flow from the luminal to cytoplasmic side of the channel (Smith et al. 1985. With permission Nature Publishing Group)

environment would affect channel function. Clearly there was a pressing need to establish a way to study Ca^{2+} channel function at a fundamental level in muscle cells. The breakthrough came in the laboratory of W. Jonathan Lederer at the University of Maryland. Graduate student Heping Cheng and postdoctoral fellow Mark Cannell along with Lederer were utilizing isolated rat cardiac myocytes to monitor intracellular free Ca^{2+} concentration with the fluorescent Ca^{2+} indicator fluo-3 (Cheng et al. 1993). Employing a laser scanning confocal microscope, they examined a thin (<1 μm) section of the myocyte. Under resting conditions, they observed

spontaneous local, transient, increases in the concentration of intracellular Ca^{2+}. They called these responses "calcium sparks". Their calculations suggested that the calcium sparks were of a magnitude consistent with the Ca^{2+} currents measured from single RyR in planar lipid bilayers. Thus they concluded that the Ca^{2+} spark may be explained by the opening and closing of a single SR Ca^{2+} release channel or a small number of channels acting in concert. They believed that the experiments provided in situ measurements of the gating of an intracellular channel by an optical method that was analogous to the electrophysiological recording of single channels. But were these really measurements of single channel activity? The answer to that question is probably no. But nonetheless the technique became very popular and useful in investigating local Ca^{2+} responses in various muscle preparations.

The measurement of localized Ca^{2+} gradients was extended to skeletal muscle fibers by Alexander Tsugorka, Eduardo Rios and Lother A. Blatter (1995). The resting frog muscle fibers did not produce spontaneous Ca^{2+} responses. Thus the authors utilized so-called "cut" muscle fibers and voltage clamp techniques to control membrane potential. They blocked the action potential and stretched the fibers to minimize force production. In confocal images of Ca^{2+} from voltage stimulated fibers containing a fluorescent Ca^{2+} indicator, they observed elementary events that apparently derived from the opening of single SR channels. They refrained from calling these elementary events calcium sparks because they were five to tenfold smaller than the calcium sparks observed in cardiac muscle. Thus they concluded that the cardiac calcium sparks derived not from a single RyR channel but likely from a cluster of channels. Were the elementary events in skeletal muscle derived from a single Ca^{2+} release channel? Probably not. Despite the differences in magnitude, these elementary events generally became known as calcium sparks in cardiac and skeletal muscle.

In order to examine the calcium sparks in a truly natural state, Stephen Hollingworth, J. Peet, W. Knox Chandler and Stephen M. Baylor (2001) utilized intact frog skeletal muscle fibers into which a fluorescent Ca^{2+} indicator was microinjected. These fibers were depolarized with K^+. They observed calcium sparks but of a smaller amplitude than those observed in cut frog fibers. Were these sparks now from a single Ca^{2+} release channel? One could not be sure. The answer to this question was important for the following reason. If the calcium sparks in these preparations were not from single Ca^{2+} release channels but from a cluster of channels, how was the response coordinated? This then became an important, still unresolved, question in understanding excitation-contraction coupling mechanisms. Fill and Copello (2002) have written a useful general review of the ryanodine receptor Ca^{2+} release channels and Baylor (2005) has reviewed the data on calcium sparks in skeletal muscle fibers.

Calcium release channel regulation: The mechanical hypothesis of excitation-contraction coupling of Schneider and Chandler (1973) has been strongly supported by subsequent evidence and is now generally accepted as the trigger for Ca^{2+} release from the SR in skeletal muscle (see Chap. 6). But is it the only trigger? This question arises because of the interesting relationship between the tetrads of DHPRs and the RyRs. The classic experiments of Block et al. (1988) indicated that only about 50 % of the RyRs are in close apposition to tetrads (see Fig. 6.26). Thus only 50 %

of the RyRs would be activated directly by charge movement. What happens to the other 50 %? Are they quiescent or are they activated in some other manner? Rios and Pizarro (1988) proposed that those other channels are activated by the release of Ca^{2+} from the channels activated by charge movement. In other words, they proposed that Ca^{2+} release was supplemented by a Ca^{2+} induced calcium release (CICR) mechanism. This hypothesis seemed plausible in light of the structural arrangement of DHPRs and RyRs and since it was well known that the Ca^{2+} release channels of the SR were Ca^{2+} sensitive, i.e., Ca^{2+} could stimulate or inhibit Ca^{2+} release depending on concentration. The idea that CICR release might be involved in Ca^{2+} release with muscle activation was not a new proposal. In fact CICR was first discovered in skinned muscle fibers of the frog by Endo et al. (1970) and independently by Ford and Podolsky (1970). Furthermore Fabiato and Fabiato (1972) in their hallmark experiments provided convincing evidence that cardiac muscle was activated by a CICR mechanism. In contrast the accumulating evidence suggested that CICR did not operate under physiological conditions in skeletal muscle and thus the hypothesis was discarded. The original experiments in skeletal muscle were performed in the presence of very low concentrations of free Mg^{2+}. Mg^{2+} inhibited CICR at normal physiological concentrations, i.e., at about 1 mM free Mg^{2+}. Furthermore it was reasoned that if a CICR mechanism was operating in an intact muscle fiber, then a high concentration of a fast Ca^{2+} buffer injected into the fiber should inhibit calcium release by inhibiting CICR. The opposite was found. Baylor and Hollingworth (1988) microinjected a high concentration of the Ca^{2+} indicator fura-2 in frog muscle fibers in order to rapidly buffer the Ca^{2+} release upon stimulation. Their results and model calculations showed that the amount and rate of Ca^{2+} released with an action potential was increased, not decreased, in the presence of high intracellular Ca^{2+} buffering. They concluded (Baylor and Hollingworth 1988. With permission John Wiley & Sons Inc):

> The observed effects of Fura-2 on SR Ca^{2+} release are consistent with the hypothesis that a rising level of free [Ca^{2+}] feeds back on a rapid time scale to inhibit continued SR Ca^{2+} release, and that Fura-2, by its buffering action on $\Delta[Ca^{2+}]$, suppresses this negative feedback. Moreover, since buffering of myoplasmic [Ca^{2+}] by Fura-2 did not inhibit SR Ca^{2+} release, the experiments argue against the idea that messenger [Ca^{2+}] transients (i.e. 'Ca^{2+}-induced Ca^{2+} release') play a physiological role in the normal excitation-contraction coupling sequence.

Thus they believed that "calcium inactivation of calcium release" prevented excessive release of Ca^{2+} from the SR under normal physiological conditions in skeletal muscle.

How then might the other 50 % of the RyRs be activated? In 1992 Andrew R. Marks and collaborators discovered that a protein, FKBP12[5], co-migrated with the RyR during purification (Jayaraman et al. 1992). Subsequently the Marks laboratory observed simultaneous openings and closings of two or three RyRs activated

[5] FKBP12 is a member of the immunophilin family. It was originally discovered based on its ability to bind and mediate the immunosuppressive effects of the drug FK506. It is named according to its ability to bind to FK506 and its molecular mass of 12 kDa (Marks 1996).

by caffeine in planar lipid bilayers. They termed this effect "coupled gating" and found that it depended on the presence of FKBP12 but did not require the presence of Ca^{2+} (Marx et al. 1998). They suggested that coupled gating provided a mechanism by which RyR1 channels that were not associated with voltage-dependent Ca^{2+} channels could be regulated.

But that hasn't been the end of the story as the Rios laboratory has produced results consistent with CICR in frog skinned muscle fibers by the local release of small amounts of Ca^{2+} using flash photolysis of a Ca^{2+} chelator (Figueroa et al. 2012). They found that local Ca^{2+} release generated propagated responses that had the characteristics of CICR. The threshold $[Ca^{2+}]$ for triggering a response was ~0.5 μM. Since this value is likely much lower than concentrations prevailing near channels during normal activity, they concluded that the result supported participation of CICR in the physiological control of contraction in amphibian muscle. Surprisingly, the effect was not observed in mammalian muscle fibers and thus they were led to conclude that CICR is unlikely to contribute to excitation-contraction coupling in mammalian muscle. There are some potential complications with these experiments. The skeletal muscle fibers of the frog, the preferred in situ preparation, contain two isoforms of RyR in about equal proportions: RyR1 and RyR3 (Ogawa 1994). In contrast mammalian skeletal muscle fibers contain essentially only RyR1. Besides lacking CICR, for unknown reasons, the mammalian skeletal muscle fibers do not appear to produce calcium sparks. Thus the issue of the physiological importance of CICR in skeletal muscle excitation-contraction coupling has not been settled conclusively yet.

Nonetheless Endo (2009) has provided a detailed review of investigations of CICR in skeletal muscle and has concluded that CICR, with its inherently regenerative nature, does not seem to be used in skeletal muscle but instead is utilized in cardiac muscle that must regulate Ca^{2+} release in a finely graded manner to meet its physiological requirements. Furthermore he suggested that the low probability of opening of RyR channels in CICR in skeletal muscle under physiological conditions, together with the Ca^{2+} inactivation of Ca^{2+} release, might be nature's way of avoiding unnecessary and excessive Ca^{2+} release in skeletal muscle.

Overall persistent questions remain in the excitation-coupling field. What is the molecular mechanism of the mechanical link between DHPR and RyR? What is the mechanism of the presumed coordination between adjacent RyRs that leads to stereotypical calcium sparks? To what extent do the RyR isoforms in skeletal muscle modulate excitation-contraction coupling? What roles do the numerous proteins bound to the RyR play in muscle function? This last question arises from the observations that a large number of proteins, over 20, bind to the RyR (Song et al. 2011). Amongst these proteins is the Ca^{2+} binding protein calmodulin. The physiological roles played by most of these substances are largely unknown.

The numerous proteins at the triadic junction and the structural complexity is reminiscent of the many proteins and structural complexity of the M band and Z band in skeletal muscle (see Chap. 7). These complexities could not have been imagined in the 1950s and 1960s when these structures were first elucidated. Much more still is to be learned.

8.2.3 The Calcium Transient

By the late 1960s it was becoming apparent that changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) played critical roles in many biological processes. In muscle, it was important to monitor changes in $[Ca^{2+}]_i$ during contraction and relaxation. The first comprehensive experiments in this regard by Ashley and Ridway (1968), using the photoprotein aequorin, were spectacular (see Fig. 4.21). Nonetheless there were major problems with the use of aequorin as an indicator of $[Ca^{2+}]_i$. Some of the disadvantages of using aequorin as an indicator of $[Ca^{2+}]_i$ in muscle included the fact that the protein had to be pressure injected in a fiber without damaging the fiber, the kinetics of the luminescent reaction were not fast enough to track Ca^{2+} changes without a delay, its response to $[Ca^{2+}]_i$ was nonlinear and of course availability was also an issue. As it would turn out, aequorin also had a major advantage. The light responses to changes in $[Ca^{2+}]_i$ were not influenced by muscle force production, i.e., there was no movement artifact which would be a difficulty for more modern Ca^{2+} indicators. Nevertheless the disadvantages out weighed the advantages and clearly other types of Ca^{2+} indicators were needed.

Calcium indicators utilized in the muscle field evolved throughout the later part of the twentieth century. In the mid 1970s, the metallochromic indicator dyes arsenazo III and antipyrylazo III were introduced to monitor free Ca^{2+} in squid giant axons (Brown et al. 1975) and soon thereafter in muscle fibers (Miledi et al. 1977). Basically the method involved injecting a cell with arsenazo III and monitoring changes in light absorption at a wave-length where the absorption spectrum was maximally changed in the presence of Ca^{2+}. This technique permitted quantitative estimates of changes in $[Ca^{2+}]_i$ with a fast time resolution. But the signals were small and sensitive to movement artifacts.

The real break through in the evolution of techniques to monitor $[Ca^{2+}]_i$ came from the efforts of an American graduate student in the famous Physiological Laboratory at the University of Cambridge. Roger Y. Tsien[6] wasn't interested in muscle contraction. In fact he considered muscle research "a backwater" field (Tsien 2008). This opinion must have been somewhat disconcerting to his Ph.D. mentor Richard H. Adrian, the eminent cardiac muscle electrophysiologist. Nonetheless Adrian allowed Tsien to pursue his interest in developing voltage sensitive dyes in order to monitor electrical activity in groups of neurons. Tsien found the project difficult and decided, without informing Adrian, to switch to the development of indicators and buffers for measuring intracellular Ca^{2+}. He knew that action

[6] Roger Yongjian Tsien (1952–) received a Ph.D. in physiology from the University of Cambridge in 1978. After a faculty position in physiology at the University of California, Berkeley, he moved to the University of California, San Diego in 1989 where he has remained throughout his research career. Worrying that he might become "trapped" in a career imaging inorganic ions, Tsien (2008) began a career exploring macromolecular interactions with fluorescent proteins. His laboratory is best known for designing and building molecules that either report or perturb signal transduction inside living cells. This work led to the discovery and development of the green fluorescent protein for which he shared the Nobel Prize in 2008.

potentials generated increases in intracellular Ca^{2+} to exert biological effects such as the release of neurotransmitters to excite or inhibit the next neuron in a pathway. He felt that designing a dye to measure Ca^{2+} would be an easier problem than designing a dye to track fast changes in neuronal membrane potentials (Tsien 2008). Thus he initiated the field of rational design of intracellular Ca^{2+} buffers and indicators. The major problem was that inside cells, the free Mg^{2+} concentration was about four orders of magnitude higher than that of Ca^{2+} concentration, so that an intracellular Ca^{2+} indicator needed yet higher selectivity for Ca^{2+} over Mg^{2+}. He started with the well known Ca^{2+} chelator EGTA and modified it to make it a fluorescent molecule. His first efforts resulted in the synthesis of a number of fluorescent Ca^{2+} tetracarboxylate chelators, including BAPTA[7] and quin2 (Tsien 1980). These high Ca^{2+} affinity molecules were good for buffering Ca^{2+} but not for tracking changes in intracellular free Ca^{2+} without temporal distortion. But they were a start. An accompanying question was how to get these membrane impermeant molecules into cells? One could microinject them but this would not easily allow experiments with large groups of cells. Tsien (1981) described a "chemical trick" to get the molecules into cells in a brief two page letter to Nature that would revolutionize the measurement of $[Ca^{2+}]_i$. He knew that intestinal absorption of some antibiotics could be enhanced by ester formation. The Ca^{2+} chelators were made membrane permeable by masking their four carboyxlates with special esterifying groups resulting in an acetoxymethyl tetraester, e.g. designated quin2/AM. But the real beauty of the "trick" was since many cells contained acetyesterases the chelators would be hydrolyzed inside the cells, regenerating and trapping the original chelators. He concluded that intracellularly hydrolysable esters offered the first method for trapping membrane-impermeable chelators and indicators inside large populations of small intact cells without disrupting their membranes. This chemical trick has allowed the convenient use of Ca^{2+} indicators in thousands of published papers.

Grzegorz Grynkiewicz in Tsien's laboratory synthesized six new Ca^{2+} indicators, among which fura-2 and indo-1 were most successful (Grynkiewicz et al. 1985). These new dyes, compared to quin2, exhibited much stronger fluorescence, higher wavelength shift on Ca^{2+} binding, weaker affinity for Ca^{2+} and better selectivity against other ions. Then in 1989, Tsien and colleagues further developed the visible wavelength Ca^{2+} indicators rhod-2, fluo-2 and fluo-3 which are often utilized as non-ratiometric Ca^{2+} indicators (Minta et al. 1989). There would be further advancements in the rational design of intracellular Ca^{2+} indicators in the field that Roger Tsien opened up as a graduate student in 1980. Tsien has summarized his laboratory's efforts in designing Ca^{2+} indicators and Ca^{2+} buffers (Tsien 1992).

With regard to the utility of these Ca^{2+} indicators and buffers in skeletal muscle research, it depended on the nature of the planned experiment. Stephen M. Baylor and Stephen Hollingworth at the University of Pennsylvania extensively examined the properties of a wide range of Ca^{2+} indicators and buffers in intact frog muscle

[7] Names are derived from the first four letters of the chemical name of the chromophore or most prominent substituent. The following numeral indicates the chronological order of introduction. For example, 1,2-bis(o-aminophenoxy)ethane-N,N,-N′,N′-tetraacetic acid was called BAPTA.

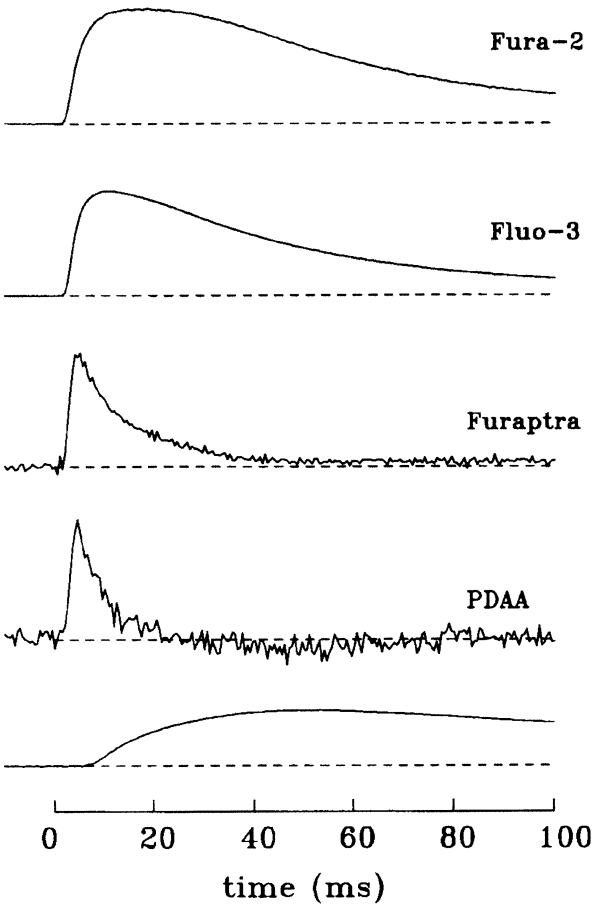


Fig. 8.7 Calcium transients in response to a single action potential in intact muscle fibers from the frog. The lowermost record is a representative twitch response 16 °C. Note the dramatic slowing of the kinetics of the calcium transient when recorded with a high affinity calcium indicator (fura-2 and fluo-3) compared to the calcium transient recorded with a low affinity calcium indicator (furaptra or PDAA) (Baylor and Hollingworth 2000. With permission The American Physiological Society)

fibers. They did not utilize the AM form of an indicator because of the possibility that the AM indicator might also penetrate intracellular organelles. Instead they injected the dye. Baylor and Hollingworth were interested in tracking the changes in $[Ca^{2+}]_i$ with as little kinetic delay as possible.[8] For this purpose the high affinity Ca^{2+} indicators were inadequate. What was needed was a low affinity Ca^{2+} indicator. A potential problem with this type of indicator is that it would not discriminate as accurately between Ca^{2+} and Mg^{2+}. Ironically the low affinity Ca^{2+} indicator that they utilized, furaptra (or mag-fura-2), originally was designed as a Mg^{2+} indicator

[8] There are complications in using Ca^{2+} indicators to accurately track $[Ca^{2+}]_i$. All of the Ca^{2+} indicators bind to intracellular proteins to a greater or lesser extent and this binding changes their kinetic properties. Furthermore in order to minimize any artifacts due to muscle movement during contraction, Baylor and Hollingworth (2011) perform experiments on fibers stretched to sarcomere lengths of 3.5–4 μm. This condition eliminates the possibility of examining the relationship of the calcium transient to various mechanical responses.

(Raju et al. 1989). The difference in the kinetics of the Ca^{2+} transient measured with high and low Ca^{2+} indicators can be very dramatic as shown in Fig. 8.7. The high affinity Ca^{2+} indicators exhibit a large delay in the decline of the Ca^{2+} transient due to the slow off rate of Ca^{2+} from the indicator. With accurate tracking of the Ca^{2+} transient in frog intact muscle fibers at 16 °C, the following characteristics of the Ca^{2+} transient were observed: time to peak, ~6 ms; half width, ~10 ms; peak amplitude, ~10–15 μM (Baylor and Hollingworth 2011). The resting $[Ca^{2+}]_i$ is difficult to measure but is about ~100 nm. Thus the $[Ca^{2+}]_i$ increases about 100–150 fold in a matter of a few milliseconds in frog muscle twitch.

They also developed a quantitative model of Ca^{2+} movements in frog muscle fibers based on the earlier model of Cannell and Allen (1984). This three dimensional "reaction-diffusion model" took into account the structure of the sarcomere, the source of Ca^{2+}, its diffusion within the sarcomere and the molecules to which Ca^{2+} bound: troponin, ATP, and parvalbumin (Baylor and Hollingworth 1998). From the measured Ca^{2+} transient and the model calculations, they could estimate the total amount of Ca^{2+} released and the rate of Ca^{2+} release, i.e., the Ca^{2+} flux, from the SR with a single stimulus. Their calculations showed that the peak $[Ca^{2+}]_i$ represented less than 10 % of the total Ca^{2+} released. The rest of the released Ca^{2+} rapidly bound to intracellular molecules. As described in the section above, buffering intracellular Ca^{2+} led to a large increase in the amount of Ca^{2+} released in a twitch (Baylor and Hollingworth 1988).

When they compared the Ca^{2+} transient in response to a single action potential in mammalian slow- and fast-twitch muscle fibers, they found that the change in $[Ca^{2+}]_i$ was smaller and slower in the slow-twitch fibers (Baylor and Hollingworth 2003). Also the estimated amount of total Ca^{2+} released in the slow-twitch fibers was only one third of the amount in the fast-twitch fibers. This value agreed reasonably well with structural studies that indicated that slow-twitch fibers contained less SR, fewer RyR1s and fewer DHPRs than the fast-twitch fibers (see references in Baylor and Hollingworth 2003). Despite the differences in total Ca^{2+} released, the half width of the Ca^{2+} flux was the same in both fiber types. This observation indicated that the rate of Ca^{2+} release was the same in both fiber types. Furthermore the decline of the Ca^{2+} transient was slower in the slow-twitch fibers because of a lower concentration of Ca^{2+} chelators in the myoplasm, i.e., no parvalbumin and only one regulatory Ca^{2+} binding site on troponin (as opposed to two regulatory sites on troponin in fast-twitch fibers). The model calculations also showed that troponin was rapidly and nearly completely saturated with Ca^{2+} in response to a single stimulus in both fiber types. Thus they concluded that mechanism of Ca^{2+} release was fundamentally the same in both fiber types and the differences in the Ca^{2+} transient were due to fewer Ca^{2+} release channels in the slow-twitch fibers and a lower concentration of Ca^{2+} chelators in the fibers. Further observations were made with a train of five closely spaced action potentials. The total Ca^{2+} released by a second and subsequent stimulus was equally diminished in both fiber types to above 20 % of the first stimulus. This decrease was attributed to Ca^{2+} inactivation of Ca^{2+} release described in the section above. The time course of decline of $[Ca^{2+}]_i$ was slowed considerably after the fifth and final stimulus. According to the

simulations, this decline was due to a reduction in available Ca^{2+} binding sites on troponin and parvalbumin. Baylor and Hollingworth (2003) concluded that the differences in the contractile speed of the two fiber types appeared to be related to differences in fiber properties and events subsequent to SR Ca^{2+} release and the association of Ca^{2+} with troponin. Thus the Ca^{2+} release mechanisms, though quantitatively different, are fundamentally the same in slow- and fast-twitch muscle fibers.

Even though these studies have reached a high level of accuracy in monitoring and modeling the calcium transient, it does not mean that other more qualitative and more convenient approaches to monitoring $[Ca^{2+}]_i$ are not valuable. As Baylor and Hollingworth (2011) have pointed out, many studies seek a qualitative answer to a biological question of interest, for example, is $\Delta[Ca^{2+}]$ important in the process under study or does $\Delta[Ca^{2+}]$ change as a result of some experimental manipulation. The simplest way to monitor $\Delta[Ca^{2+}]$ qualitatively is usually with a high-affinity indicator that has been AM-loaded into a preparation even if the information obtained is subject to quantitative interpretation difficulties.

Somewhat surprisingly more that 40 years after the initial introduction of Ca^{2+} indicators into muscle fibers, there is no perfect indicator yet for measuring $[Ca^{2+}]_i$.

8.3 Regulation of Contraction in Striated Muscle

8.3.1 The Troponin Complex and Protein-Protein Interaction in Regulation of Contraction

By the early 1980s it was well established that striated muscle contraction was triggered by Ca^{2+} binding to troponin (Tn). The Tn molecules are distributed along the thin filaments at 38.5 nm intervals with a stoichiometry of one troponin molecule to one tropomyosin (Tm) molecule to seven actin molecules (see Fig. 4.17). The Tn complex consists of three subunits that were named for their first observed function. Troponin C (TnC) binds Ca^{2+}, troponin I (TnI) inhibits actomyosin ATPase in the presence or absence of Ca^{2+} and troponin T (TnT) binds to tropomyosin (Tm). Actomyosin ATPase was known to be inhibited by a peptide fragment from fast skeletal muscle TnI containing residues 96–116. This so-called TnI inhibitory peptide also binds to TnC (Syska et al. 1976). Talbot and Hodges (1981) at the University of Alberta narrowed the effective inhibitory region of skeletal TnI to residues 105–114. In 1982 it was shown with electron microscopy that the Tn complex was ~26.5 nm long and consisted of a globular and rod-like domain (Flicker et al. 1982). The 16 nm long tail of Tn was attributed to TnT, an asymmetric molecule ~18 nm long. TnT could be divided into two subfragments with chymotryptic digestion, TnT1 (residues 1–159) and TnT2 (residues 159–258). Tn binds approximately a third of the way along a 40 nm Tm molecule and the TnT1 tail extends to the head-to-tail overlap region of two Tm molecules. These results led to a more realistic schematic representation of Tn on the thin filament in the 1980s as shown in Fig. 8.8a. TnC contains four Ca^{2+} binding sites. Two of these sites are Ca^{2+}-specific sites and, based on kinetic studies, are thought to exchange Ca^{2+} rapidly enough to

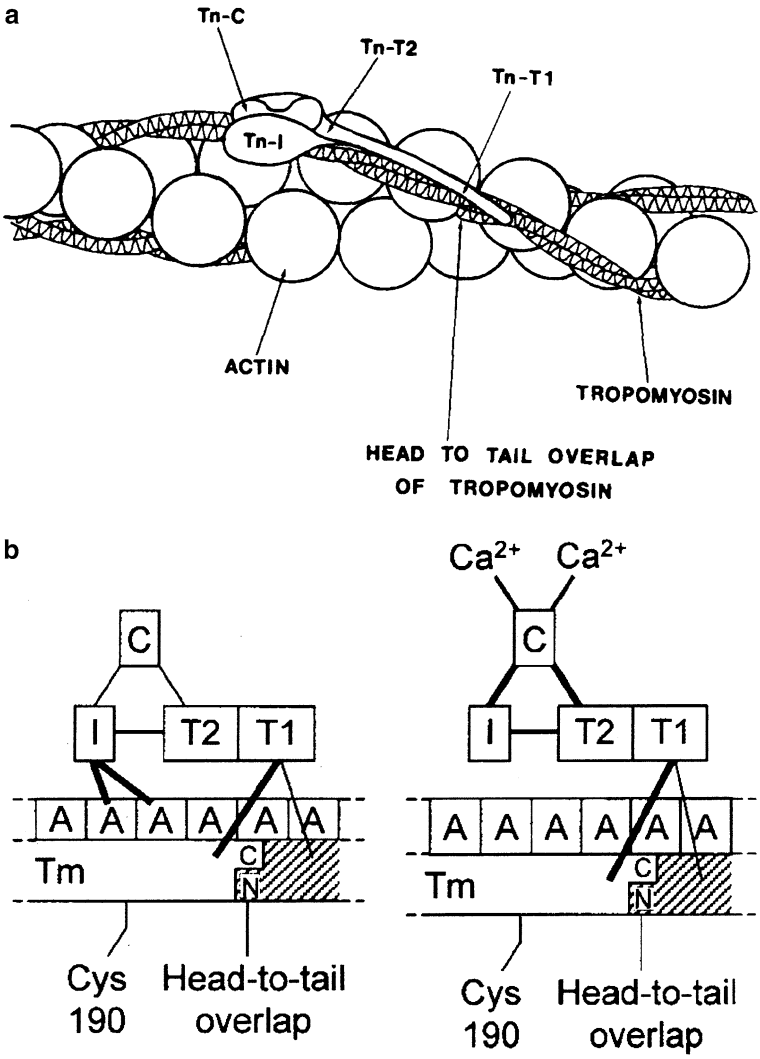


Fig. 8.8 (**a**) Model of the molecular arrangement of troponin, tropomyosin, and actin in the skeletal muscle thin filament. The various troponin subunits are indicated [TnC, TnT (TnT1 and TnT2) and TnI] as they lie along the two-stranded tropomyosin that in turn lies along an actin strand, spanning seven actin monomers (Heeley et al. 1987. With permission The American Society for Biochemistry and Molecular Biology). (**b**) Diagram indicating the effect of Ca^{2+} binding to TnC on the interaction between the various thin filament proteins. A is actin, I is TnI, C is TnC, T1 is the N-terminal portion of TnT, T2 is the C-terminal portion of TnT, Tm is tropomyosin with the N- and C-terminals indicated in the head-to-tail overlap region. *Thicker lines* imply stronger binding, and *thinner lines* imply weaker binding. *Right*: Ca^{2+} bound to TnC. *Left*: Without Ca^{2+} bound to TnC. Note that Ca^{2+} binding to TnC enhances the TnC–TnI and TnC–TnT2 interactions and weakens the TnI-A interactions. This effect allows the Tm to move on the surface of the actins opening up myosin binding residues (see Fig. 8.11) (Gordon et al. 2000. With permission The American Physiological Society)

explain muscle contraction and relaxation. The other two sites are Ca^{2+}–Mg^{2+} sites and they are thought to be structural sites (see Sect. 6.3 for further details). It was proposed that the Ca^{2+} induced conformational change in TnC was transferred to TnI and TnT through a series of protein-protein interactions. The information then went from TnT to Tm where subsequent Tm movement allowed myosin to bind to actin and contraction to ensure (see Sect. 6.4.5 and Fig. 6.8). It was, and still is, a major challenge to elucidate the molecular details of the protein-protein interactions that occur during muscle activation.

Crystal structure of TnC: In order to understand the complicated protein-protein interactions when Ca^{2+} binds to Tn during muscle activation, detailed structural information was needed. In 1973 the atomic structure of the Ca^{2+} binding protein parvalbumin was solved and soon thereafter it was predicted, based on amino acid sequence, that TnC would exhibit four EF hand Ca^{2+} binding sites (see Fig. 6.8 and associated text). Nonetheless it was not until 1985 that the atomic structure of TnC would be solved independently by two research groups. In fact all indications are that it was a race in the end to get the structure published since the papers appeared in the journals Nature and Science within one day of each other in February of 1985. Both groups solved the crystal structure of avian (turkey and chicken) skeletal muscle TnC. The crystals grew best under low pH conditions (pH 5) but under these conditions only the two Ca^{2+}–Mg^{2+} sites contained divalent cations whereas the Ca^{2+} specific sites were Ca^{2+} free. Postdoctoral researcher Osnat Herzberg and Michael M. N. James (1985) in the department of biochemistry at the University of Alberta solved the turkey TnC structure at a resolution of 2.8 Å (published in Nature, February 21, 1985) and a group led by Muttaiya Sundaralingam[9] at the University of Wisconsin solved the crystal structure of chicken TnC at 3 Å resolution (published in Science, February 22, 1985) (Sundaralingam et al. 1985). The structures are essentially identical with the turkey structure exhibiting somewhat more detail since it was obtained at a higher resolution than the chicken structure. The TnC molecule is 7.5 nm long with a somewhat unusual "dumbbell" shape with the most distinctive feature being the long helical stem (about nine turns) that connects the two globular N-terminal and C-terminal domains (Fig. 8.9). The N-terminal domain contains the first 85 amino acid residues and the C-terminal domain contains residues 97–162. The molecule contains eight helices corresponding to the homologous helical regions in parvalbumin (labeled A-H) and an additional helix in the N-terminal region (labeled N helix). For example the N-terminal domain contains: helix A–Ca^{2+} binding loop (I)–helix B–linker–helix C–Ca^{2+} binding loop (II)–helix D. The connection between the two domains is made by the central helical linker (D/E linker) from helix D of the N-domain to helix E of the C-domain. The segments of polypeptide chains forming the Ca^{2+}-binding loops are labeled I–IV sequentially. In these crystal structures the Ca^{2+} ions were found only in sites III and IV. Both groups noted the difference in conformation between the Ca^{2+} binding

[9] Muttaiya Sundaralingam (1931–2004) and his wife died tragically in the tsunami that hit the coast of Sri Lanka on December 26, 2004 following the submarine earthquake which had occurred a couple of hours earlier off the coast of Sumatra (Westhof 2005).

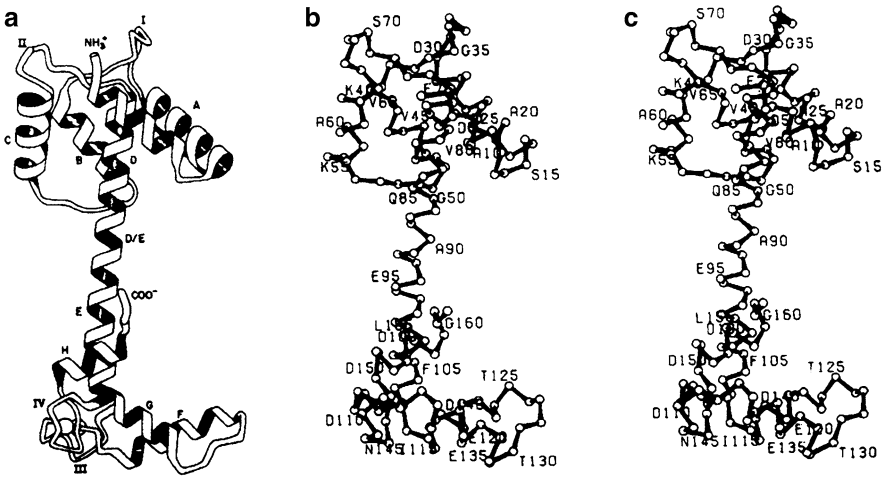


Fig. 8.9 Crystal structure of TnC. (**a**) Ribbon representation of the polypeptide chain of turkey skeletal muscle TnC. The upper helical domain is the Ca^{2+} regulatory domain; the lower domain is the high-affinity Ca^{2+}/Mg^{2+} binding domain. Helices are labeled A-H sequentially (plus N helix). The segments of polypeptide chain forming the Ca^{2+}-binding loops are labeled I to IV sequentially. Only sites III and IV contain Ca^{2+}. (**b**) Stereo representations of TnC showing the α-carbon positions in the molecule. The Ca^{2+} have been omitted for clarity (Herzberg and James 1985. With permission Nature Publishing Group)

loops with (sites III and IV, the Ca^{2+}–Mg^{2+} sites) and without (sites I and II, Ca^{2+}-specific sites) Ca^{2+} bound and went on to suggest the functional importance of these differences. Sundaralingam et al. (1985) suggested that when Ca^{2+} was not bound, the geometry of the helix-loop-helix was somewhat different from the standard EF-hand. Such differences in the conformation accompanying the binding of Ca^{2+} may provide a molecular basis for the understanding of the Ca^{2+}-induced conformational changes in TnC and of the propagation of these changes to TnI, TnT, tropomyosin, and actin leading to muscle contraction. Herzberg and James (1985) suggested that the N-domain loop conformations may be more like the state in relaxed muscle. In contrast the C-domain is always saturated with Ca^{2+} or Mg^{2+} in physiological conditions. Therefore, they believed that its structure in the troponin complex would not differ significantly from that determined crystallographically. They also noted that comparison of the conformation of loops I and II with those of loops III and IV in which Ca^{2+} ions were bound showed the kinds of conformational changes in loops I and II that must accompany Ca^{2+} binding. Finally both groups pointed out that the hydrophobic residues in the core of the N-terminal domain might become exposed upon Ca^{2+} binding. The elucidation of the crystal structure of TnC was an important first step in understanding the structure and function of whole Tn molecule.

Herzberg and James went on to collaborate with computational biologist John Moult to develop an influential model for the Ca^{2+}-induced conformational change in the N-terminal domain of TnC (Herzberg et al. 1986). This model became known

as the "HMJ" model. The basis for the model was the assumption that upon binding of Ca^{2+} to the regulatory domain of TnC the molecule underwent a conformational transition to become closely similar in structure to the C-terminal domain which always binds Ca^{2+} or Mg^{2+} under physiological conditions. In the crystal structure of TnC, there are four hydrophobic residues in helix B that are either partially or completely buried from solvent. In the model of Herzberg et al. (1986) all four amino acids became more exposed to solvent, thus creating a "hydrophobic patch" on the surface of the protein. Its presence indicated a possible contact surface with other members of the muscle system.

James and Lawrence B. Smillie and collaborators at the University of Alberta next determined the crystal structure of the N-terminal domain fragment of TnC bound with Ca^{2+} at a resolution of 1.75 Å (Strynadka et al. 1997). Compared to the crystal structure of the N-terminal domain without Ca^{2+}, upon Ca^{2+} binding helices B and C moved as a unit away from helices A and D to expose an inner hydrophobic core of residues that were likely part of the TnI binding site on TnC. The exposure of a hydrophobic patch in the N-terminal domain of TnC upon Ca^{2+} binding was experimentally verified at lower resolution in the nuclear magnetic resonance (NMR) structures of intact TnC by Carolyn M. Slupsky and Brian D. Sykes at the University of Alberta[10] in 1995. This was an important study because it confirmed the crystal structure of TnC in solution. Whereas in general the NMR study confirmed the crystal structure data on TnC, a notable difference observed in the NMR structure was that the central D/E linker was a flexible linker between the two domains. This flexible linker allowed the two domains of TnC to adopt any orientation with respect to one another such that they could interact with a variety of targets. Finally, skeletal muscle TnC was crystallized with four Ca^{2+} bound and the structure determined at a resolution of 2 Å (Houdusse et al. 1997). The results showed that the conformation of the N-terminal domain of TnC was similar to that predicted by the HMJ model.

Throughout the 1980s and 1990s many studies were directed at determining the interactions between and among the subunits of Tn and Tm and actin. Some of the techniques employed included: (a) bacterial expression and characterization of mutants of TnC, TnI, and TnT, (b) cross-linking and fluorescence studies and (c) determination of the crystal and NMR structures of Tn fragments and complexes with TnC. Results from these studies have been reviewed comprehensively by Chuck S. Farah and Fernando C. Reinach in 1995 and Larry S. Tobacman in 1996. By the end of the twentieth century, these studies resulted in a representation of the Ca^{2+} dependent interactions of various proteins of the thin filament as shown in Fig. 8.8b. Because TnI binds to both actin-Tm and to TnC, Ca^{2+}-induced changes in the relative affinities of TnI for actin-Tm and TnC have long been a central theme in

[10] During the latter part of the twentieth century investigators in the protein structure and function group in the department of biochemistry at the University of Alberta made major contributions to the understanding of protein-protein interactions in troponin and the structure of troponin C. The main investigators included: Lawrence B. Smillie and Robert S. Hodges (biochemistry of troponin and its subunits), Michael N. G. James (crystal structures of troponin C) and Brian D. Sykes (NMR solution structures of troponin C).

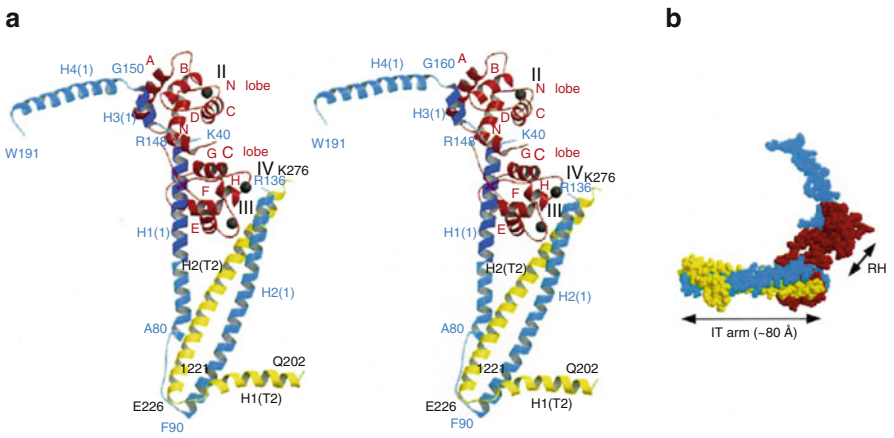


Fig. 8.10 Crystal structure of the core domain of human cardiac troponin. (**a**) Stereo view of core domain. TnC (*dumbbell shaped*), TnI (*dark gray*) and TnT (*light gray*) are shown. The three Ca^{2+} bound to the Ca^{2+}-binding sites (II–IV) are represented by *black spheres*. Each helix within TnI and TnT is indicated by the helix number whereas each helix of TnC is indicated by a capital letter (N and A–H). (**b**) Space-filling model of the core domain of Tn. RH, regulatory head (Takeda et al. 2003. With permission Nature Publishing Group)

understanding regulation by Tn. Syska et al. (1976) observed that the TnI proteolytic fragment (residues 96–116) that bound actin and inhibited the actomyosin ATPase also bound to TnC. They therefore suggested that at the heart of the regulatory mechanism was a Ca^{2+}-dependent movement of the TnI inhibitory region from actin to TnC (see Fig. 8.8b). But it must be more complicated than this as it was shown that Ca^{2+} dependent interactions occurred between both TnC domains and the inhibitory and also C-terminal regions of TnI (Farah et al. 1994).

Structure of the core domain of Tn: Diagrams like the one in Fig. 8.8b were useful in summarizing a great deal of research on the interactions among the thin filament proteins but provided no structural information regarding these interactions. Clearly what was needed was the structure of the whole Tn molecule but no one was able to crystallize whole Tn. An enormous breakthrough occurred in Yuichiro Maeda's laboratory at the RIKEN Harima Institute in Kawaguchi, Japan in 2003. Takeda et al. (2003) succeeded in crystallizing what they called the core domain of human cardiac Tn in the Ca^{2+} bound state and solving the structures at 2.6 and 3.3 Å resolution. They reasoned that whole Tn did not crystallize because of the extended structure of TnT1 (see Fig. 8.8a). Also better crystals were grown when the first 30 amino acids were removed from TnI (which made the cardiac TnI very similar to the skeletal TnI). The core domain of Tn thus contained TnC (residues 1–161), TnI (31–163 or 31–210) and TnT2 (183–288). Four crystal structures were solved. Figure 8.10a shows a stereo view of the Tn core domain. The core domain is dominated by α-helices and can be divided into two structurally distinct subdomains (Fig. 8.10b), denoted as the regulatory head (consisting of TnC residues 3–84 and TnI residues

150–159) and the IT arm (consisting of TnC residues 93–161, TnI residues 42–136 and TnT residues 203–271). TnC is shown with Ca^{2+} bound at the three Ca^{2+} binding sites (labeled II, III and IV) of cardiac TnC (site I is inactive and does not bind Ca^{2+}). The central D/E linker of TnC is no longer an α-helix but is unraveled. Tracing TnI (dark gray or blue) from the N-terminal end, starting with amino acid residue 40, the H1(I) helix interacts with the C-terminal domain of TnC (the first TnC binding site in TnI) then interacts with TnT2. The TnI helix H2(I) forms a parallel α-helical coiled-coil with the H2(T2) helix. Downstream from the coiled-coil is the inhibitory region of TnI. This flexible region (residues 137–148 [equivalent to residues 107–118 in skeletal TnI) was not observed in the crystal structure most likely because of its flexible nature. Further downstream is the α-helix H3(I) which interacts with the hydrophobic patch of the N-terminal domain of TnC (the second TnI binding site on TnC). Finally there is the protruding α-helix H4(I) which has no direct interaction with the rest of the molecule. As for TnT (light gray or yellow), starting from the C-terminal region (denoted as C-TnT or T2), the polypeptide chain moves upstream through H2(T2) to α-helix H1(T2) which is kinked by about 60° relative to H2(T2). Further upstream would be residues that were not defined in the crystal structure and the remainder of TnT, TnT1, which was not part of the crystal.

As exciting as this structure was, it is important to note that most of the electron densities associated with the potential actin-Tm interfaces were ambiguous. These densities included the inhibitory region and C-terminus of TnI and the C-terminal region of TnT. These ambiguities were likely associated with flexibility in these regions. In all, 12–20 % of the amino acid residues in the structures were not included in the models. In this regard Takeda et al. (2003) commented that that the unusual flexibility was a remarkable characteristic of the troponin molecule. Nonetheless from their results and other studies in the literature, Takeda et al. (2003) drew a number of important conclusions. Based on the differences observed in the four solved crystal structures, they suggested that the D/E linker in TnC which connects the two subdomains of Tn "works as a universal joint". This action would allow the Tn molecule to adopt multiple conformations with variable subdomain orientations on the thin filament. Since the amphiphilic helix H3(I) binds specifically to the hydrophobic patch of the Ca^{2+}-saturated N-terminal domain of TnC, they concluded:

> There is little doubt that the amphiphilic helix H3(I) works as a molecular switch that transmits the initial signal of Ca^{2+} binding to the N lobe of TnC to the other components in the thin filament, and thus has a central role in the initial steps of troponin–tropomyosin based regulation.

Furthermore the H3(I) segment of TnI is located in the sequence between the two putative actin-binding sites, the inhibitory region (residues 137–148) and the C terminus of TnI (residues 169–210). Both of these regions were shown previously to be essential for the inhibitory binding of TnI to actin in the absence of Ca^{2+} and to change the distances to the nearby actin molecules depending on the Ca^{2+} concentration. Thus they further concluded that the binding of H3(I) to the hydrophobic patch of the N lobe of TnC must induce the detachment of the extended C-terminal portion of TnI, which they denoted as the regulatory segment of TnI

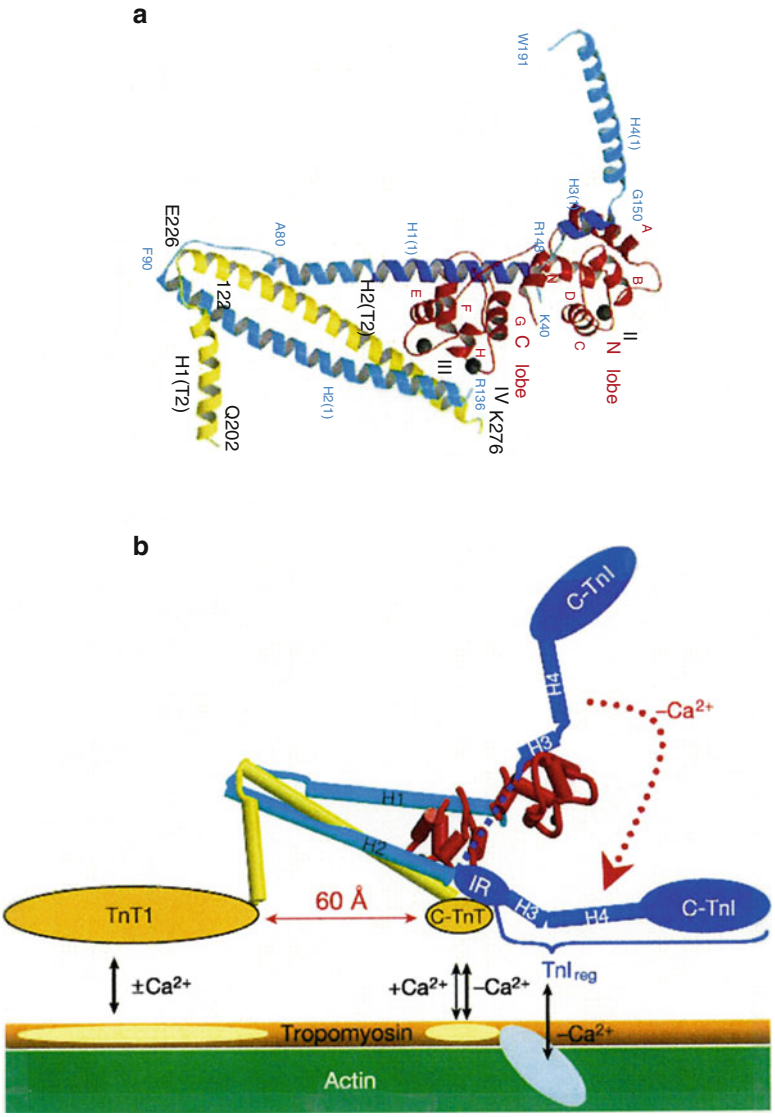


Fig. 8.11 Proposed model of the interactions between troponin and other thin filament components. (**a**) Crystal structure of the core domain of Tn (Fig. 8.10a) rotated clockwise by 90° in order to parallel the model shown in (**b**). (**b**) The potential actin–tropomyosin-binding portions, which are not included in the current crystal model, are schematically drawn: TnT1 and C-TnT are light colored ellipsoids and the inhibitory region (IR) and the C-terminal region of TnI (C-TnI) are *dark ellipsoids*. The actin and the tropomyosin strands are shown. The *arrows* indicate the interactions between troponin and tropomyosin–actin. The *dashed line* indicates the proposed movement of TnI when Ca^{2+} is removed (Takeda et al. 2003. With permission Nature Publishing Group)

(TnI_{reg}, residues 137–210), from the actin filament. Previous studies had shown that Tn is anchored to the thin filament mainly through Tm binding of two distinct portions of TnT; that is, TnT1 and C-TnT (TnT2). The former interaction was believed to be the major Ca^{2+} insensitive anchoring site of Tn onto Tm whereas the interaction through the latter site was stronger at low, rather than high, Ca^{2+} concentrations. Also no part of the IT arm was known to have direct interactions with actin or with Tm. On the basis of the current crystal structures and the previous studies, Takeda et al. (2003) proposed the model shown in Fig. 8.11b for the action of Ca^{2+} on the interactions of the Tn subunits with actin and Tm. According to the model, during Ca^{2+} regulation, TnI_{reg} undergoes major changes both in position and conformation. At higher Ca^{2+} concentrations, TnI_{reg} (dark gray or blue) detaches from actin-Tm and becomes associated with the N-terminal domain of TnC in an extended form as shown in the crystal structures. This detachment would remove restraints on the position of Tm on actin. The released Tm would move aside making the myosin binding sites more available and facilitating cooperative binding of myosin to the thin filament (see Fig. 6.19 and accompanying text). At lower Ca^{2+} concentrations, TnI_{reg} forms an extra attachment to actin-Tm so that the Tn–Tm strand is tied down onto the actin filament.

In a commentary on the Takeda et al. (2003) paper, Sykes (2003) pointed out that the structure of the core domains of Tn validated earlier work on isolated fragments, domains and binary complexes, and has led to greater understanding of the mechanism of action. As he saw it, the challenge in the field now was to bring all of this structural data together to form a consistent picture of the structural changes within and movement of troponin during the contraction cycle. But how does one take the next step in elucidating the mechanism of action? First of all, it should be noted that the main features of the core domain of human cardiac Tn also were observed in the crystal structure of the core domain of chicken skeletal Tn (Vinogradova et al. 2005). In order to take the next step, some of the following approaches are being pursued. Utilization of electron microscopy and three-dimensional image reconstruction to visualize the location and movement of Tn in muscle fibers in the active and relaxed states (Galinska-Rakoczy et al. 2008). Application of fluorescence resonance-energy transfer (FRET) to measure distances from donor-labeled Tn-Tm, to acceptor-labeled actin in the active and relaxed states of muscle fibers (Kimura-Sakiyama et al. 2008). Determination of the structure and position of the C-terminal domain of TnC using polarized fluorescence spectroscopic measurements of bifunctional rhodamine-labeled TnC in relaxed and contracting muscle fibers (Knowles et al. 2012). (See Sect. 9.7.1 in Chap. 9 for information about the use of this approach in the study of cross-bridge movement during contraction.) It has been 40 years since Greaser and Gergely (1973) summed up their classic studies on the subunits of Tn by stating that if it is assumed that TnC is the site of Ca^{2+} interaction during contraction, then there appears to be "a rather complicated series of protein-protein interactions." Much has been learned in the interim about these protein-protein interactions and whereas the problem may be solved in principle many of the molecular details still remain elusive.

8.3.2 *Regulation of Muscle Force and Kinetics of Contraction by Calcium and Strongly Bound Cross-Bridges*

Results from structural and biochemical studies: By the late 1960s it was well established that the regulation of skeletal muscle contraction by Ca^{2+} was exerted through effects on the thin filament which controlled the binding of force generating cross-bridges to actin. The basic regulatory unit was one Tn, one Tm and seven actions (see Chap. 4). By the early 1990s the following picture of skeletal muscle activation emerged based on biochemical and structural evidence (see Chap. 6). The position of Tm on the thin filament determined the interaction of myosin with the binding sites on actin. According to the biochemical model of McKillop and Geeves (1993) (see Fig. 6.16 and related text), these actin binding sites existed in a dynamic equilibrium and could be characterized as blocked (unable to bind to cross-bridges), closed (able to weakly bind cross-bridges) or open (able to bind cross-bridges so that they subsequently isomerize to become strongly bound and release ATP hydrolysis products). In this model, the Tm position on the actin filament is regulated by the occupancy of the N-terminal Ca^{2+} binding sites on TnC and changes in the interactions among Tn, Tm, and actin and as well as by strong cross-bridge binding to actin. Ca^{2+} increases the probability of formation of the closed state and strongly bound cross-bridges increase the probability of formation of the open state in a cooperative manner. Strongly bound cross-bridges promote additional Tm movement. This movement stabilizes Tm in the open position and allows cooperative activation of additional actins in that and possibly neighboring regulatory units through Tm-Tm overlap. This three state model had elements of steric blocking in the blocked state and also cooperative activation. Thus regulation of contraction was seen as dynamic and statistical rather than static. The important new conclusion from this model was that Ca^{2+} binding alone was not sufficient to completely turn on the thin filament. Cooperative cross-bridge binding was required to switch the thin filament completely into the open state and result in maximum activation. Thus muscle activation could not be thought of as a simple on or off switch.

Physiological studies: The question became how to test the hypothesis that strongly bound cross-bridges activate contraction in a cooperative manner in muscles. In order to have the flexibility of controlling the intracellular environment, the muscle preparation of choice was the skinned muscle fiber. This preparation was first the mechanically skinned frog muscle fiber preparation developed by Natori (1954) but later the glycerinated mammalian muscle fiber preparation originally conceived by Szent-Gyorgyi (1949). The skinned muscle fiber preparation allowed great flexibility in physiological studies of contraction. Measurements could be made of the steady state force production and kinetics of contraction as a function Ca^{2+} concentration. With skinned muscle fibers, it was possible to extract proteins and, with the advent of molecular genetics, replace the extracted proteins with mutant proteins. Richard L. Moss in the physiology department of the University of Wisconsin pioneered the methods for extraction of a number of proteins from skinned muscle

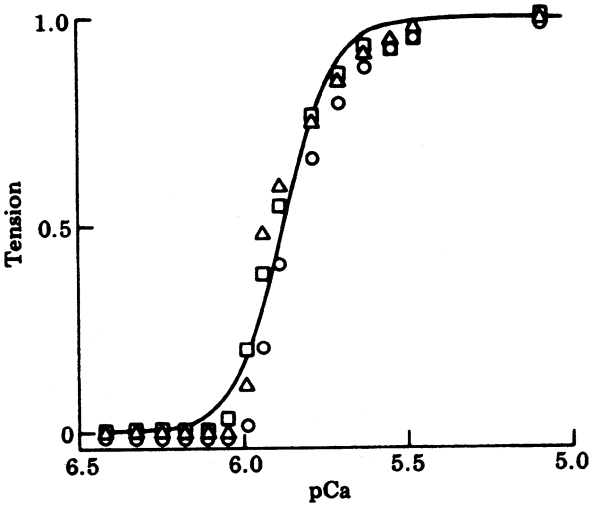


Fig. 8.12 Normalized isometric force production in skinned psoas muscle fibers from rabbits plotted against free Ca^{2+} concentration expressed as pCa. The Hill coefficient, $n_H = 5.8$, indicates a very high level of cooperativity of force production and $pCa_{50} = 5.87$ (Brandt et al. 1980. With permission of M. Kawai)

fibers, including TnC, whole Tn, C-protein (MyBP-C) and myosin light chain 2 (LC2, the regulatory light chain) (Moss 1992).

Steady force production versus Ca^{2+} concentration (*pCa*): The first force-pCa relationship was established in frog skinned muscle fibers by Hellam and Podolsky (1969). They noted that the muscle was activated over a similar range of Ca^{2+} concentrations as seen in the biochemical studies of the Ca^{2+} dependence of ATPase in rabbit myofibrils. Fred J. Julian (1971) appeared to be the first to explicitly comment on the steepness of the force-pCa relationship and its implications for cooperative activation of muscle contraction. How steep is the force-pCa relationship and what are the implications for cooperative activation of muscle contraction? Graduate student Susan Donaldson and Glenn Kerrick (1975) noted the similarity of the force-pCa relationship to the "S" shaped oxygen-hemoglobin dissociation curve and thus analyzed their results in an analogous way using the Hill (1913) equation[11]. This analysis gave a Ca^{2+} affinity or sensitivity expressed as pCa_{50} and the steepness of the relationship expressed as the Hill coefficient, n_H. Brandt et al. (1980) determined that the Hill coefficient in mammalian skinned psoas fibers was between 5 and 6 (Fig. 8.12). This relationship is extremely steep[12]. By comparison, Ca^{2+} binding to the regulatory sites of isolated TnC or to whole Tn or to regulated

[11] The Hill equation is expressed as: $F = F_o\,[Ca^{2+}]^n/(K^n + [Ca^{2+}]^n)$ or in the more usual form: $F = F_o/(1 + 10^{n(pCa50-pCa)})$ where n (or n_H, the Hill coefficient) is a measure of the steepness of the force versus pCa relationship and an indicator of the extent of cooperativity and pCa_{50} is a measure of the calcium concentration giving 50 % of maximum force and an indicator of calcium sensitivity of force of contraction (Gordon et al. 2000).

[12] The Hill coefficient, n_H, is the most variable parameter in the force versus pCa relationship. The values of n_H can vary from ~2 to 6 depending on experimental conditions and technical aspects of the measurement.

thin filaments exhibits a value of n_H of 1–2. The ATPase of regulated thin filaments plus myosin exhibits a value of n_H of ~4 (Grabarek et al. 1983).

What mechanisms could explain the extensive cooperativity seen in the force-pCa relationship in skeletal muscle? Gordon et al. (2000) discussed the possible mechanisms in a comprehensive review. They listed four possibilities: (1) coupling between Ca^{2+} binding at the two N-terminal sites on skeletal TnC; (2) coupling between Ca^{2+} binding sites along the thin filament; (3) cross-bridge-induced increase in Ca^{2+} binding in that regulatory unit or neighboring regulatory units; and/or (4) cross-bridge-induced movement of Tm to activate the thin filament in a direct or allosteric manner in that regulatory unit and neighboring regulatory units. They concluded that while all of these factors may contribute to the cooperativity seen in the force-pCa relationship, the most important factor was likely cross-bridge inducement of movement of Tm.

What is the evidence that cross-bridge binding to the thin filament activates contraction? An early suggestion that this was possible came from the biochemical experiments measuring actomyosin ATPase by Bremel and Weber (1972). They showed that the formation of some rigor complexes of myosin S-1 with actin "turned on" the regulated actin filaments to allow further binding of myosin with actin. They observed that at low ATP concentrations the rate of ATP hydrolysis in the absence of Ca^{2+} was as high as in the presence of Ca^{2+}. At higher ATP levels the rate of ATP hydrolysis in the absence of Ca^{2+} declined with increasing ATP concentration. Bremel and Weber (1972) concluded that if some actin molecules have formed rigor complexes in the absence of Ca^{2+}, the remaining actin monomers not combined with myosin may be "turned on" (i.e., interact with myosin in the presence of ATP causing contraction) although no Ca^{2+} is bound to Tn. Earlier analogous results were found in glycerinated insect flight muscle (White 1970) and in skinned crayfish muscle fibers (Reuben et al. 1971). In the crayfish fibers in the absence of Ca^{2+}, force increased at low ATP concentration and then declined at higher ATP concentrations as observed in the regulated thin filaments. Thus the evidence supported the contention that rigor cross-bridges activated regulated thin filaments and also skinned muscle fibers. But rigor cross-bridges are not cycling cross-bridges.

What is the evidence that cycling cross-bridges activate the thin filaments? The experimental dilemma was how to separate activation by strongly bound cross-bridges from force generation by cross-bridges when the "readout" for muscle activation was force generation. Postdoctoral fellow Darl R. Swartz and Moss (1992) developed a novel approach to this dilemma. They knew from biochemical studies that N-ethylmaleimide-(NEM) modified myosin subfragment-1 (Sl) bound strongly to thin filaments and dissociated only very slowly from thin filaments. They reasoned that soaking a skinned psoas fiber in NEM-S1 would allow investigation of the effects of varied concentrations of strong-binding myosin heads to modulate Ca^{2+} activation independent of the effect of intrinsic myosin heads to develop force. They further reasoned that if NEM-S1 bound tightly to actin and did not activate adjacent actins to allow interaction with endogenous heads, then it should act solely as an inhibitor of tension independent of the Ca^{2+} concentration. In contrast if binding of NEM-S1 activated adjacent actins, then it should increase tension especially

at low Ca^{2+} concentrations. They found that maximum isometric force development was little affected at low concentrations of NEM-S1. Maximum force declined at progressively higher NEM-S1 concentrations presumably because NEM-S1 competed with intrinsic cross-bridges for actin binding site. In dramatic contrast, NEM-S1 markedly increased force production when the fiber was activated to produce submaximal force (~10 % of maximum isometric force). Even though NEM-S1 was not a cycling cross-bridge, nonetheless these results were the clear evidence that strong binding cross-bridges activated the thin filaments.

An important feature of the McKillop and Geeves (1993) model was that Ca^{2+} alone does not result in full activation of the thin filament but rather that full activation requires strong S1 binding. Swartz et al. (1996) devised a test of this model. They incubated rigor myofibrils with a fluorescently labeled S1 and observed the location of S1 binding using fluorescence microscopy. This approach incorporated the native structure of the thin filament and the three-dimensional organization of the thin and thick filaments present in the intact myofibril. They predicted that the S1 would bind preferentially to the highest affinity actin sites (i.e., "on" or open states) in the myofibril and thus would demonstrate regions where the thin filament is activated. They found that at low Ca^{2+} and low S1 concentration, the fluorescence was restricted to the actin-myosin overlap region. At high Ca^{2+} and very low S1, the fluorescence was still predominantly in the overlap region. These results suggested that Ca^{2+} alone did not fully activate the thin filament for rigor S1 binding and that, even at high Ca^{2+}, the thin filament was not activated along its entire length. In contrast increases in the S1 concentration resulted in a shift in maximum fluorescence from the overlap to the nonoverlap region at both low and high Ca^{2+}. This result was consistent with the interpretation that rigor cross-bridges activated the thin filament. Thus the results in skinned muscle fibers confirmed the biochemical predictions and strongly suggested that an important component of the cooperativity in the force-pCa relationship was due to the activating action of strongly bound cross-bridges. Gordon et al. (2000) have discussed further evidence supporting this conclusion in their comprehensive review.

Regulation of the kinetics of contraction in skinned muscle fibers: The studies described above concerned the effects of strongly bound cross-bridges on the steady isometric force development. It also was of considerable interest to determine the effects of strongly bound cross-bridges on the kinetics of muscle contraction. The standard activation of skinned muscle fibers by rapidly transferring a fiber from a low to high Ca^{2+} concentration solution did not allow a determination of the true kinetics of contraction because of the slow inward movement of Ca^{2+}. Bernhard Brenner, then at the Institute of Physiology II at the University of Tubingen, devised a novel technique to determine the true kinetics of contraction in skinned muscle fibers (Brenner and Eisenberg 1986; Brenner 1988). This technique would become very important and popular. A skinned fiber was activated at a fixed Ca^{2+} concentration to produce a steady force and then rapidly shortened isotonically to detach the cross-bridges and drop the force to zero and then rapidly re-stretched to its original length (Fig. 8.13a). The kinetics of the force redevelopment, popularly called k_{tr}

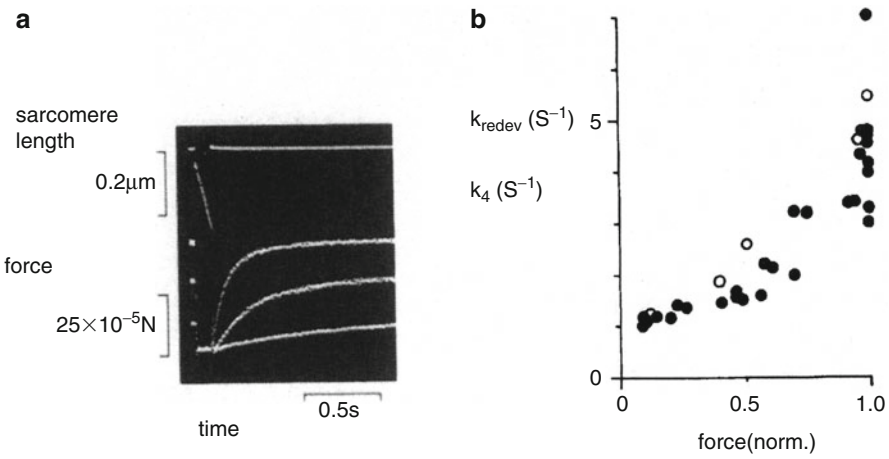


Fig. 8.13 Kinetics of force redevelopment at various levels of Ca^{2+} activation in skinned muscle fibers. (**a**) Protocol for measurement of force redevelopment after release and restretch during an isometric contraction at three different levels of Ca^{2+} activation. The *top trace*: sarcomere length. For clarity, only the trace for maximum activation is shown. The *bottom traces* represent force. (**b**) Effect of Ca^{2+} on the rate constant of force redevelopment (k_{redev}) (k_{tr}). k_{redev} is plotted versus isometric steady-state force recorded immediately before the period of isotonic shortening (Brenner 1988. With permission of B. Brenner)

(kinetics of tension recovery), was then measured. This rate of force recovery reflected the true kinetics of the contraction. One now had the convenience of the skinned fiber preparation and a means of generating meaningful kinetics data. Brenner (1988) determined that k_{tr} increased dramatically as a function of steady force development at increasing Ca^{2+} concentrations (Fig. 8.13b). He speculated that the increase in k_{tr} was due to a direct effect of Ca^{2+} on cross-bridge turnover kinetics ("regulation through turnover kinetics") and not on an increase in the number of turning-over cross-bridges ("regulation through recruitment"). This effect could occur through an acceleration of the transition from weakly bound to strongly bound cross-bridges which would be expected to lead to an acceleration of phosphate release by Ca^{2+}.

Millar and Homsher (1990) utilized laser flash photolysis of caged P_i to study the effects of P_i on force generation in isometrically contracting skinned muscle fibers (see Chap. 9 for a discussion of the use of caged compounds in muscle research). They confirmed the well known observation that increases in P_i concentration decreased steady isometric force production. The important observation was that the force transient generated by a rapid increase in P_i concentration during an isometric contraction was Ca^{2+} independent. They concluded that Ca^{2+} concentration does not directly affect the P_i release or force-generating steps of the cross-bridge cycle. Furthermore they proposed that "the data can be interpreted in terms of a model in which strong cross-bridges activate the thin filament with this activation being modulated by Ca^{2+} binding to Tn". Thus they interpreted the Ca^{2+}

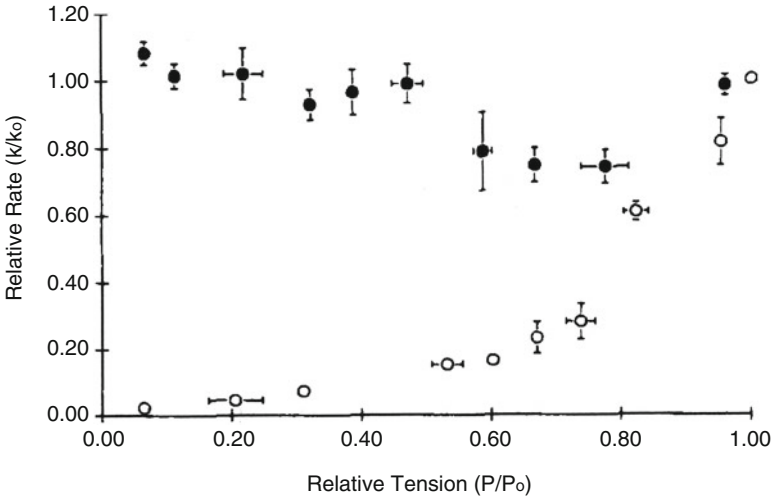


Fig. 8.14 Variation in the rate of isometric force redevelopment (k_{tr}) with relative force in untreated and NEM-S1-treated skinned muscle fibers. Force redevelopment following re-stretch was measured before (*open symbols*) and after incubation in NEM-S1 (*closed symbols*). Note the dramatic potentiation of submaximal k_{tr} by NEM-S1 suggesting that strongly bound cross-bridges activate contraction (Schwartz and Moss 1992. With permission The American Society for Biochemistry and Molecular Biology)

dependence of k_{tr} in the following way. If Ca^{2+} binding to Tn alone cannot displace Tm, Tm would return to its inhibitory position in a k_{tr} measurement while the cross-bridges are detached and will effectively switch off the thin filament whatever the [Ca^{2+}]. After restretch, the cross-bridges are allowed to attach and must again displace Tm so that the observed rate of tension development is slow and Ca^{2+} sensitive, just as activation from relaxation. According to this hypothesis, Ca^{2+} was not altering the intrinsic rate of cross-bridge cycling but rather recruiting strongly bound cross-bridges that would facilitate thin filament activation.

Another observation by Swartz and Moss (1992) is also consistent with a role of strongly bound cross-bridges in the activation of the thin filaments. They could duplicate Brenner's basic result of the dependence of k_{tr} on force development (Fig. 8.14). If the skinned fiber was incubated in a NEM-S1 solution and the k_{tr} measurements repeated, they found that k_{tr} reached its maximum value at submaximal levels of force production (Fig. 8.14). This result was consistent with the hypothesis that the strongly bound NEM-S1 activated the thin filament and thus increased k_{tr} by cross-bridge recruitment.

Thus by the beginning of the twenty first century it was reasonably well established that strongly bound cross-bridges contributed to the activation of muscle contraction. Nonetheless questions remained. Was the activation by cross-bridges confined to a single regulatory unit or did it spread to neighboring units and if so how far did it spread? Brandt et al. (1984) and Moss et al. (1985) showed that partial

extraction of TnC from skinned fibers decreased the observed cooperativity of the force-pCa relationship. The results of Brandt et al. (1984) were particularly dramatic. They suggested, according to their calculations, that extraction of TnC equivalent to only about one regulatory unit per thin filament drastically decreased cooperativity. They concluded that the thin filament was activated as a single unit. Not everyone agrees with this interpretation (see Gordon et al. 2000 for discussion) and this issue is still unresolved. Overall Moss and Daniel P. Fitzsimons in a perspective review in 2010 were forced to conclude that the molecular basis for cooperation was not well understood. Finally the most direct evidence that strongly attached cross-bridges can play a role in thin filament activation in muscle comes from results indicating that rigor-like cross-bridges and NEM-S1 activate contraction. These cross-bridges are strongly attached for a long time compared to the cycling, force generating, cross-bridges under physiological conditions. Whereas it seems highly likely that cycling cross-bridges activate contraction, it still must be pointed out that there is not direct evidence that cycling cross-bridges actually activate contraction.

Velocity of unloaded shortening and its relation to sarcomere length and calcium concentration: After Barany's (1967) classic study linking the myosin ATPase in solution to the maximum velocity of muscle shortening (V_{max}), it became important to be able to measure accurately V_{max} under a variety of experimental conditions. K. A. Paul Edman (1979) at the University of Lund in Sweden introduced a simple technique to measure the velocity of unloaded shortening in muscle fibers. After isometric force reached a steady state in a tetanically stimulated muscle fiber from the frog, the fiber was rapidly shortened a fixed distance sufficient to reduce force to zero (allowing the fiber to become "slack"). During this release, force falls to zero and the fiber shortens under zero load taking up the slack. Once the slack is taken up, isometric force then begins to rise at the new shorter sarcomere length (Fig. 8.15a). The time to the onset of force redevelopment after the release is called the "slack time" (Δt). The greater the distance released, the greater the slack time. Plots of the distance released (ΔL) against the slack time (Δt) in fully activated fibers are linear, and the slope of this line estimates the unloaded shortening velocity (V_o) (Fig. 8.15b). Edman found that V_o was not significantly different from V_{max} as estimated traditionally by extrapolation of the force versus velocity relationship.

Using this technique, Edman (1979) made a number of important observations. He found that V_o was constant between sarcomere lengths of 1.7 μm and approximately 2.7 μm. Over this sarcomere length range the isometric force varies considerably (see Fig. 3.20). This observation confirmed and extended earlier observations of Gordon et al. (1966). Furthermore it confirmed the prediction in Andrew F. Huxley's 1957 model of muscle contraction that V_{max} does not depend on the number of cycling cross-bridges but rather on the balance of positive and negative force generating cross-bridges. Huxley suggested that the drag from the attached cross-bridges opposes thin filament sliding and thus limits V_{max}. As the velocity of thick and thin filament sliding increases, some attached cross-bridges are dragged passed their equilibrium position into a negative force-bearing region (see Fig. 3.6). Accumulation of attached

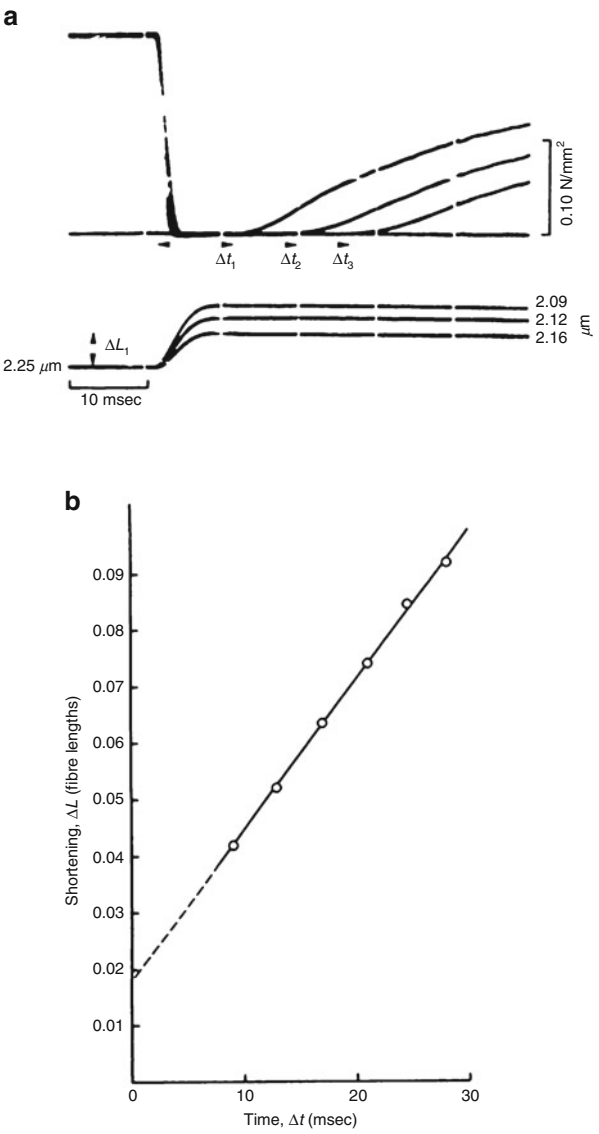


Fig. 8.15 Determination of the unloaded velocity of shortening in a muscle fiber using the slack step technique. (**a**) *Superimposed traces* showing three rapid releases of different amplitudes during the plateau of a tetanus. *Upper traces*: force. *Lower traces*: release steps. (**b**) Relationship between amplitude of shortening (**Δ**L) and time from onset of release to beginning of force redevelopment (**Δ**t). The slope of line equals V_o. The intersection of the line with the ordinate is a measure of the total series compliance (Edman 1979. With permission John Wiley & Sons Inc)

cross-bridges in the negative force-bearing region produces a force opposing continued sliding. When the force exerted by "negatively" strained cross-bridges is equal and opposite to the force exerted by cross-bridges generating positive force, the sliding velocity reaches its maximum steady-state velocity, V_{max}. Thus the detachment rate of the cross-bridges in the negatively strained region limits unloaded shortening velocity. This view is supported by the Edman's observation that V_o is independent of

the number of force generating cross-bridges in the mid range of the force versus length relationship. (See Chap. 3 and Figs. 3.6 and 3.7 for a description of Huxley's kinetic model of muscle contraction.)

Edman (1979) also explored the possible effects of Ca^{2+} on V_o by soaking a muscle fiber in dantrolene, an agent known to inhibit Ca^{2+} release from the sarcoplasmic reticulum. Under these conditions, the isometric twitch amplitude declined to 10 % of the control value but there was no significant reduction of V_o. Edman concluded that the results implied that the shortening velocity at zero load was not dependent on the degree of activation of the contractile system. Thus Ca^{2+} did not influence V_o in intact fibers. Edman's results are compelling. Nonetheless the influence of Ca^{2+} on V_{max} or V_o has been a controversial topic for more than 30 years (for a review see Gordon et al. 2000).

8.3.3 Myosin Light Chains and Muscle Function

Regulation of contraction in skeletal muscle by phosphorylation of the myosin regulatory light chain: W. T. Perrie, Lawrence B. Smillie and Samuel V. Perry at the University of Birmingham were the first to show that a myosin light chain (called LC2, DTNB or now regulatory light chain) could be phosphorylated (Perrie et al. 1973). They speculated that this light chain existed in a phosphorylated form in resting muscle. Since the regulatory light chain could be removed without altering maximum actomyosin ATPase activity in solution, the role of this phosphorylation in muscle was puzzling. Skeletal muscle contained a specific Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK) and light chain dephosphorylation occurred by a specific phosphatase in vitro. In a sense, it was a mechanism looking for a function. Graduate student David R. Manning and James T. Stull in the department of physiology at the University of Texas Southwestern Medical Center at Dallas were the first to provide evidence in vertebrate skeletal muscles that a process other than Ca^{2+} binding to TnC contributed to the regulation of contraction (Manning and Stull 1979). They showed that the transient increase in twitch amplitude observed after an isometric tetanus (post-tetanic twitch potentiation) in an isolated fast-twitch skeletal muscle was correlated in time with the extent of phosphorylation of the myosin regulatory light chain. Light chain phosphorylation was low at rest and during the brief tetanus itself, there was little or no light chain phosphorylation. Thus unlike Ca^{2+} binding to TnC, phosphorylation of the light chain was not required for the activation of contraction[13]. They concluded that light chain phosphorylation was not obligatory for contraction but might play a role in post-tetanic potentiation. This was the start of an idea that has subsequently directed research in this area. Moore and Stull (1984) confirmed the correlation of myosin light chain phosphorylation with post-tetanic twitch potentiation in fast-twitch skel-

[13] Unlike skeletal muscle, phosphorylation of the myosin regulatory light chain is obligatory for contraction in smooth muscle. See Kamm and Stull (1985) for a review.

etal muscle and also further demonstrated that there was little phosphorylation or post-tetanic twitch potentiation in slow-twitch skeletal muscle. Continuous low frequency stimulation of a muscle also led to twitch potentiation that was correlated temporally with myosin light chain phosphorylation (Klug et al. 1982). Thus, while not obligatory for contraction, the slow, but long lasting, myosin light chain phosphorylation correlated with potentiation of subsequent contractions. Stull has called this proposed modulation a "biochemical memory" (Kamm and Stull 2011).

The question then became: how does myosin light chain phosphorylation modulate contraction? The first important clue came from a study of skinned muscle contraction by Persechini et al. (1985). They showed that myosin light chain phosphorylation resulted in a reversible potentiation of submaximal force in skinned muscle fibers without an effect on maximal force production. Subsequent work by Metzger et al. (1989) showed that phosphorylation of the regulatory light chain in rabbit skinned muscle fibers accelerated the rate of force re-development as measured by k_{tr} at intermediate force levels, but not at maximum force. They proposed that phosphorylation mediated an increased rate of cross-bridge attachment to binding sites along the thin filament. This possibility was envisioned as a modulation by phosphorylation of the movement of cross-bridges away from the thick filament backbone and toward the thin filament. They speculated that the molecular basis for this possibility involved phosphorylation-induced alterations in the net charge balance of myosin. They further speculated that this movement of the cross-bridges toward the thin filaments with phosphorylation could account for post-tetanic increases in twitch force. This proposal has been supported by recent X-ray diffraction studies that have shown, in cardiac muscle, that phosphorylation of the regulatory light chain causes the myosin head to move closer to the thin filament, presumably due to charge repulsion with the surface charge of the thick filament, thereby increasing the probability of cross-bridge binding to actin (Colson et al. 2010).

The Kristine E. Kamm-Stull laboratory tested these ideas rigorously by ablating the gene expressing the Ca^{2+}-calmodulin-dependent myosin light chain kinase in skeletal muscle (Zhi et al. 2005). There was no significant increase in myosin light chain phosphorylation in response to repetitive stimulation in isolated fast-twitch skeletal muscles from the knockout mice. Furthermore, isometric twitch potentiation after a brief tetanus or low-frequency twitch potentiation (staircase) was attenuated relative to responses in muscles from wild-type mice. These results identified myosin light chain phosphorylation by the skeletal muscle Ca^{2+}-calmodulin-dependent MLCK as a primary biochemical mechanism for tension potentiation due to repetitive stimulation in fast-twitch skeletal muscle. But it has not the only mechanism modulating force as the staircase phenomenon was reduced by 50 % but not completely abolished. This result suggests that other mechanisms, likely related to Ca^{2+} handling with repeated stimulation, are also involved. Thus 40 years after the discovery of the phosphorylation of the myosin regulatory light chain, its role in modulation of contraction in fast-twitch skeletal muscle appears to be firmly established. For an excellent review of the earlier evidence, see Sweeney et al. (1993) and for a recent review, see Stull et al. (2011).

Role of the essential light chain in muscle contraction: The myosin molecule contains two globular heads and associated with each head are two light chains, the regulatory and essential light chains. A possible role in the modulation of contraction by the regulatory light chain was discussed above. The essential (or alkali) light was named based on the observation that its removable destroyed the myosin ATPase activity. (See Chap. 3 for a description of the discovery of the light chains of myosin.) It was realized early on that the essential light chain existed in two isoforms, LC1 with a molecular weight of about 25 kDa and LC3, about 16 kDa. These proteins were identical with the exception of an extra 40 or so amino acids at the N-terminal end of LC1. What role might the essential light chains play in muscle contraction? In a classic study postdoctoral fellow Peter Reiser working with Moss and Greaser determined that the maximum velocity of unloaded shortening in single muscle fibers isolated from rabbit soleus muscle was highly correlated with the myosin heavy composition (Reiser et al. 1985). H. Lee Sweeney and colleagues confirmed these results and also discovered that in fibers with the same myosin heavy chain composition, the maximum velocity of shortening correlated with the essential light chain ratio, i.e., the higher the ratio of LC1 to LC3, the slower the velocity of shortening (Sweeney et al. 1988). Thus they concluded that the primary determinant of the maximum velocity of fiber shortening was the myosin heavy chain composition but that the light chain heterogeneity provided "fine tuning" of myosin function. They suggested that the purpose of the multiplicity of myosin isozymes, and in particular alkali light chain heterogeneity, may be the "fine tuning" of the contractile characteristics of a fiber, resulting in a continuum of fiber types in terms of shortening velocity. This interpretation of the role of the essential light chains was strengthened considerably by the study of Lowey et al. (1993). Using the in vitro motility assay (see Chap. 9), they measured the velocity of actin filament movement caused by myosin containing either LC1 or LC3. Myosin containing LC1 moved actin filaments more slowly than myosin containing LC3. They concluded that the principal factor in determining velocity was the heavy chain with the light chain playing a modulatory role. Thus there was general agreement that the maximum velocity of muscle shortening was determined primarily by the myosin heavy chain composition but that the light chains provided a modulatory role to fine tune myosin activity. The molecular diversity of myofibrillar proteins has been reviewed comprehensively by Schiaffino and Reggiani (1996).

By what mechanism do the essential light chain isoforms affect muscle shortening velocity? Why do fibers containing LC1 exhibit a slower maximum velocity of shortening? Two general hypotheses have emerged (Timson 2003). Either LC1 could provide an additional direct contact with actin or the LC1 isoform may induce changes in the myosin heavy chain that strengthen its affinity for actin. There is considerable biochemical evidence that the LC1 isoform but not LC3 can bind to the C-terminus of actin (reviewed by Timson 2003 and Hernandez et al. 2007). This actin binding is associated with the amino acids at the extreme N-terminal end of the 40 amino acid extension of LC1. The binding of LC1 to actin during cross-bridge cycling could result in a decrease in velocity of muscle shortening. Nonetheless the effects of LC1 on the affinity of myosin for actin, ATPase, force,

and the kinetics of force generating myosin cross-bridges are as yet inconclusive. Since the light chains are known to wrap around the long α-helix of myosin S-1, called myosin lever arm (see Chap. 9), they have been hypothesized to provide stability to the lever arm (Rayment et al. 1993). Thus it is possible that LC1 could induce its effects on contraction by providing extra stability to the myosin motor domain through its N-terminal extension. Unfortunately the N-terminal amino acids of the essential light chain are not resolved in the crystal structure of myosin. Or possibly LC1 could provide a non-contractile function (Timson 2003; Sweeney 1995). Sweeney (1995) has suggested a possible "tethering" function for LC1. The tethering of LC1 to actin might help orient the filaments during sarcomere formation. The general impression that one gets is that the role of the essential light chains in muscle function is still to be elucidated.

8.4 Molecular Mechanism of Skeletal Muscle Relaxation

8.4.1 Introduction

The research that led to Ebashi's (1961) proposal of the "Ca concept" of muscle contraction actually resulted from his investigation of the mechanism of relaxation. In the late 1950s Ebashi and Lipmann (1962) showed that the fragmented vesicles of the sarcoplasmic reticulum induced relaxation by binding Ca^{2+} in the presence of ATP. Hasselbach and Makinose (1961, 1962) found similar results and concluded that the microsomes functioned as a "calcium pump" (Die Calciumpumpe) and the energy of the pump was derived from ATP. They further concluded that this pump became a carrier by phosphorylation and that the Ca^{2+} complex of the phosphorylated carrier diffuses to the inner surface of the membrane of the vesicles and there the phosphate group is split off from the carrier whereby the Ca^{2+} affinity of the carrier is strongly diminished. Two moles of Ca^{2+} were proposed to be transported for each mole of ATP hydrolyzed with H^+ counter transported in exchange for Ca^{2+}. However, fewer than four H^+ were released into the cytoplasm per two Ca^{2+} pumped, indicating that the transport reaction was electrogenic. Also the calcium pump could lower the free Ca^{2+} concentration to a level that was consistent with muscle relaxation and it operated rapidly enough to explain the kinetics of muscle relaxation. Furthermore the accumulated Ca^{2+} was shown to be stored in the terminal cisternae of the sarcoplasmic reticulum (Costantin et al. 1965). (See Chap. 4 for further details of these classic studies.)

By the 1970s there was a well defined kinetic scheme for the proposed mechanism of Ca^{2+} transport by the Ca^{2+}-ATPase of the sarcoplasmic reticulum (see Fig. 6.21). According to this scheme the enzyme exists in two distinct functional states, E (or E1) and *E (or E2). The high affinity Ca^{2+} binding site in the E form faces the outer surface of the vesicle and exhibits an affinity for Ca^{2+} of about 1 μM. In the *E form the affinity for Ca^{2+} binding is low, about 1 mM, and the Ca^{2+} binding site faces

the inner surface of the vesicle. The process involves the following elementary reaction steps. The enzyme is activated by Ca^{2+}. The physiological substrate is Mg·ATP. After Ca^{2+} and ATP bind to the enzyme, a rapid phosphorylation of the enzyme occurs and ADP is released on the cytoplasmic side of the membrane. This step occurs much faster than the steady-state rate of ATP hydrolysis or Ca^{2+} transport. The phosphate is covalently attached to the enzyme. Formation of the phosphorylated enzyme intermediate leads to decreasing Ca^{2+} affinity, followed by the eventual release of Ca^{2+} inside the lumen of the sarcoplasmic reticulum. By the 1990s much had been learned about the structure and mechanism of the calcium pump because of the introduction of gene cloning and mutagenesis techniques into muscle research and great advances in structural biology (progress was discussed in a brief review by MacLennan et al. 1997). By the end of the twentieth century what clearly was needed was the tertiary structure of the calcium pump, preferably at different stages of the pumping process.

8.4.2 Tertiary Structures of the Sarcoplasmic Reticulum Calcium Pump and Mechanism of Calcium Transport

The Ca^{2+}-ATPase of fast-twitch skeletal muscle [sarco(endo)plasmic reticulum Ca^{2+}-ATPase 1a or SERCA1a] is an integral membrane protein composed of a single polypeptide chain of ~994 residues and molecular weight 110 kDa (MacLennan et al. 1985). It is a member of the large superfamily of P-type (or E1/E2-type) ion translocating ATPases (for example, Na^+, K^+-ATPase, gastric H^+, K^+-ATPase, H^+-ATPase). The name derives from the fact that these enzymes are autophosphorylated at a key aspartate residue during the reaction cycle forming a high energy intermediate. The energy of this phosphoenzyme is postulated to fuel a conformational change that closes the ion gate from the cytoplasm, reduces the affinity of these transport sites for Ca^{2+}, and opens the ion gate toward the luminal side of the membrane. David H. MacLennan (see Fig. 6.20), who was the first to isolate the Ca^{2+}-ATPase in 1970, and colleagues Christopher J. Brandl, Bozena Korczak and N. Michael Green (1985) cloned and sequenced complementary DNA encoding the Ca^{2+}-ATPase from rabbit skeletal muscle sarcoplasmic reticulum. From the primary sequence, they predicted a secondary structure of the protein with four major domains. Their model contained three cytoplasmic domains joined to a set of ten transmembrane helices by a narrow stalk. An updated version of the predicted secondary structure is shown in Fig. 8.16. According to this model, the majority of the protein is found in the cytoplasm, approximately 70 %, with only 5 % in the lumen.

In the early 1990s Chikashi Toyoshima (Fig. 8.17), now at the University of Tokyo, started his pioneering 20 year quest to determine the tertiary structures of the Ca^{2+}-ATPase during various stages of the Ca^{2+} translocating process. Toyoshima et al. (1993) described the three-dimensional structure of the Ca^{2+}-ATPase in the native sarcoplasmic reticulum membrane at 14 Å resolution using cryo-electron

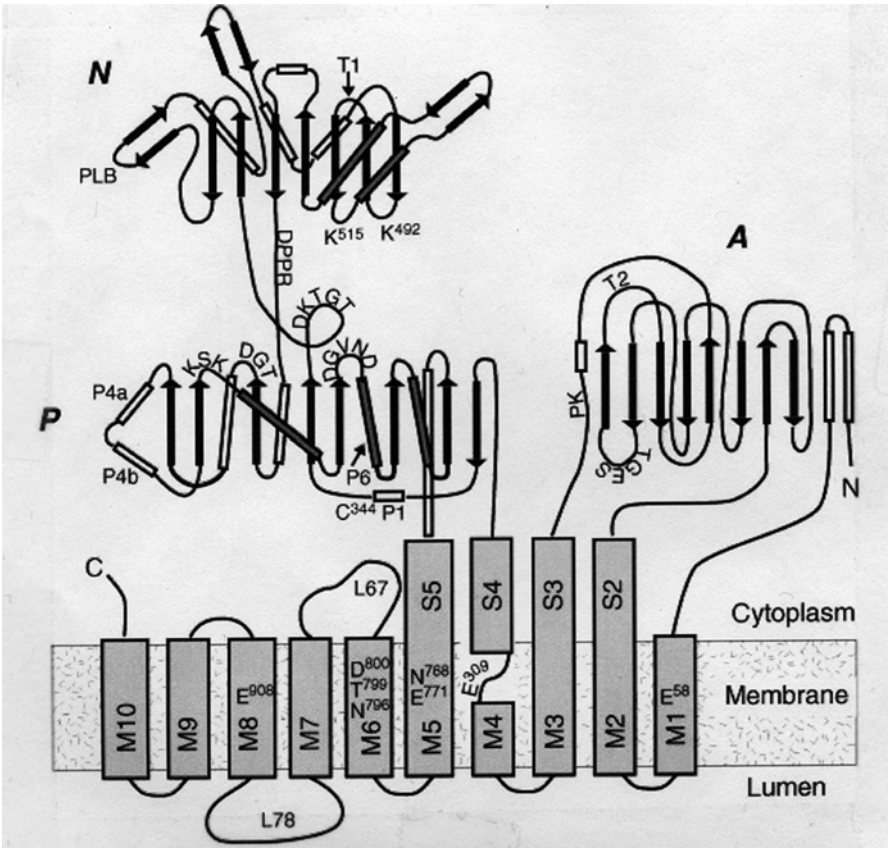


Fig. 8.16 Secondary structure of the Ca^{2+}-ATPase of the sarcoplasmic reticulum. Sequence motifs are indicated by the single-letter code with numbers corresponding to the sequence of rabbit fast-twitch muscle Ca^{2+}-ATPase (SERCA1). Cytoplasmic domains are denoted N, P, and A. *Shaded helices* in the P and N domains are in front of the central sheet whereas *unshaded helices* are behind (Stokes and Green 2003. With permission Annual Reviews)

microscopy and image reconstruction. In a colorful description of the structure, they noted that the cytoplasmic part has a complex structure consisting of several domains, and resembles the "head and neck of a bird."

The real breakthrough came when Toyoshima et al. (2000) solved the crystal structure of the Ca^{2+}-ATPase isolated from skeletal muscle sarcoplasmic reticulum at 2.6 Å resolution with two Ca^{2+} bound in the transmembrane domain. Electron microscopy showed that the crystals grown in the presence of exogenous lipid were embedded in lipid bilayers. Since ATP binding caused a conformational change that destroyed the crystals, the crystals were grown in the presence of an ATP analogue in order to obtain this "snapshot" of the Ca^{2+}-ATPase in what was presumed to represent the $E1{\cdot}2Ca^{2+}$ state (Fig. 8.18). The molecule could be fit into a box of 100 Å by 80 Å by 140 Å. The cytoplasmic headpiece has a split appearance consisting of

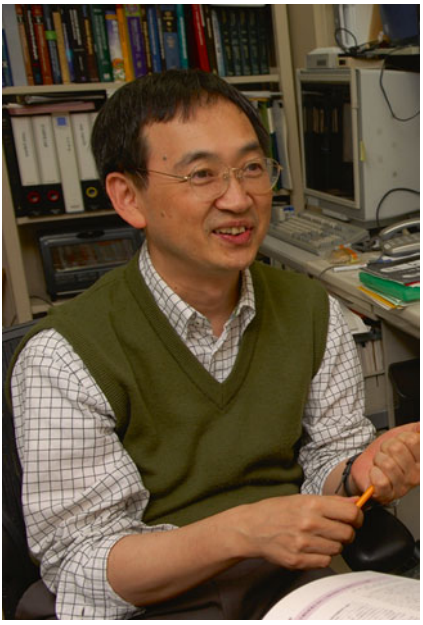


Fig. 8.17 Chikashi Toyoshima received a Ph.D. working with Taki Wakabayashi in Setsuro Ebashi's research group studying the structure of muscle thin filaments and myosin heads using electron microscopy. Toyoshima learned the cryomicroscopy technique as a postdoctoral researcher with biophysicist Nigel Unwin at Stanford University and at the MRC Laboratory of Molecular Biology in Cambridge. In Cambridge he also worked with biophysicist David L. Stokes who was studying the Ca^{2+}-ATPase. Toyoshima moved back to Japan in 1989 and became a full professor at the University of Tokyo in 1994. Toyoshima then became a professor at the Institute of Molecular and Cellular Biosciences at the University of Tokyo. He was elected to the National Academy of Sciences (USA) as a Foreign Associate in 2005. Photo: courtesy C. Toyoshima

three well separated domains. They are designated as N for nucleotide binding domain, P for the phosphorylation domain and A for the actuator of transmembrane gates domain (formerly called the transducer or beta domain). The transmembrane region (M) comprises ten α helices (M1–M10). The lengths of the helices and the inclination to the membrane vary substantially. Two Ca^{2+} binding sites (I and II) could be deduced from the crystal structure. They are located side by side in the transmembrane region near the cytoplasmic surface of the lipid bilayer and are surrounded by four transmembrane helices, M4–M6 and M8. The two sites have different coordination geometry. A striking feature of the molecule is that the phosphorylation site (Asp 351) is more than 25 Å away from the nucleotide binding site. This distance means that there must be a hinge region in the N domain to allow for a large domain motion to occur during ATP hydrolysis. Furthermore the phosphorylation site is ~50 Å from the Ca^{2+} binding sites. A key question was how does the phosphorylation site communicate with the Ca^{2+} binding sites?

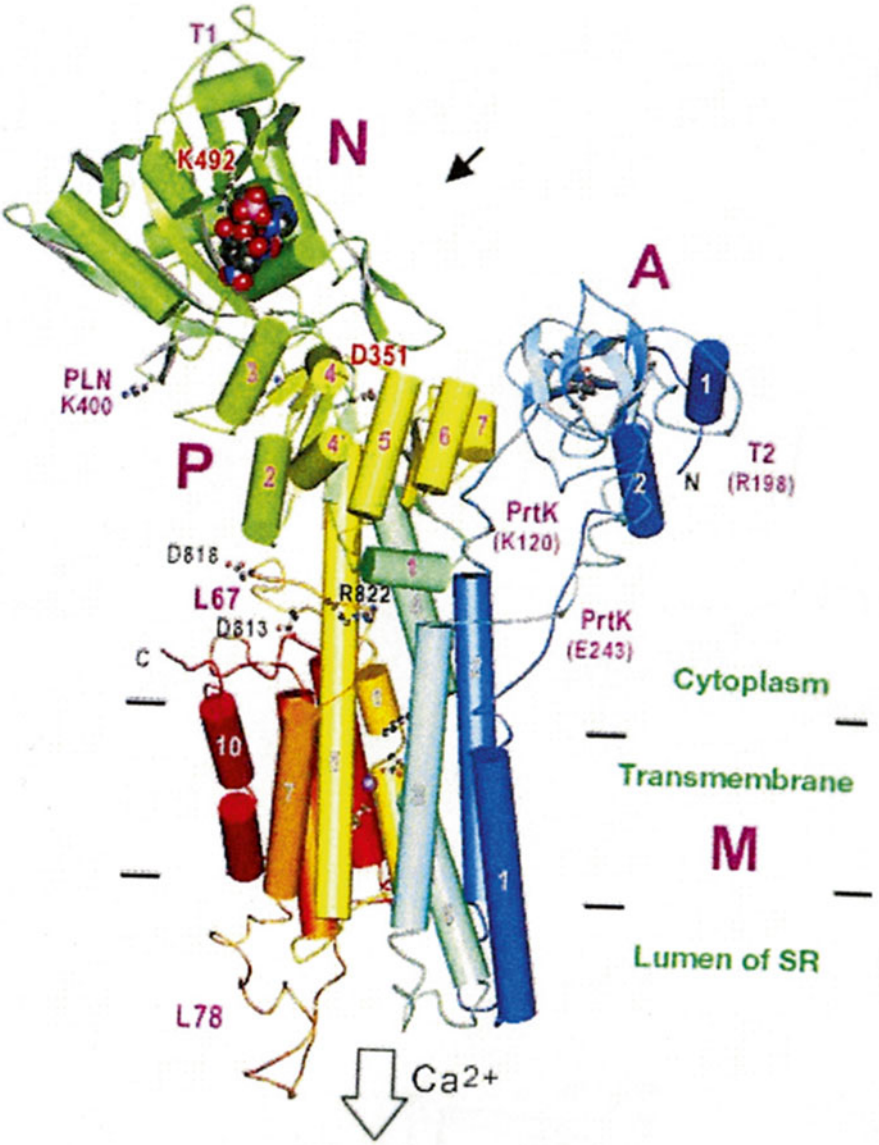


Fig. 8.18 Architecture of the sarcoplasmic reticulum Ca^{2+}-ATPase. α-Helices are represented by cylinders and β-strands by *arrows*. Three cytoplasmic domains are labeled (A, N and P). Transmembrane helices are numbered M1–M10. The M5 helix is 60 Å long and serves as a scale. D351 is the residue of phosphorylation. Note that most of the single polypeptide chain of the Ca^{2+}-ATPase protrudes into the cytoplasm and the Ca^{2+} binding sites are in the transmembrane region (Toyoshima et al. 2000. With permission Nature Publishing Group)

As spectacular as this first crystal structure was, it was clear that crystal structures in other parts of the reaction sequence would be needed in order to construct a clear picture of the mechanism of calcium pumping. Toyoshima and Nomura (2002) took the next important step when they described the crystal structure of the Ca^{2+}-ATPase in the Ca^{2+} free state at 3.1 Å resolution. This structure of the enzyme was stabilized by the potent inhibitor of the calcium pump, thapsigargin (TG). It was necessary to include thapsigargin in the specimen to keep the enzyme from denaturing in the absence of Ca^{2+}. It was assumed that thapsigargin locks the structure in a form analogous to E2. During Ca^{2+} binding and translocation, a number of large conformational changes switch the "compact" structure of the cytoplasmic portion of the pump to a more "open" structure. The E2(TG) structure showed large conformational differences compared to the E1·Ca^{2+} structure. For example the N domain moved by more than 50 Å. The three cytoplasmic domains gathered to form a single headpiece and six of the ten transmembrane helices exhibited large-scale rearrangements (see the animation in the supplementary information accompanying the original paper). These rearrangements disrupted the coordination of the Ca^{2+} and ensured the release of Ca^{2+} into the lumen of sarcoplasmic reticulum and, on the cytoplasmic side, created a pathway for entry of new Ca^{2+}. These results both confirmed widely held concepts about the mechanism of the calcium pump and provided fresh insights into its action.

Subsequent effort was directed toward identifying the crystal structures of other states of the Ca^{2+}-ATPase reaction cycle using substrate analogues and various ligands (inhibitors). By 2013 more than 50 crystal structures have been described by four different laboratories (Toyoshima and Cornelius 2013). Thus it was now possible to provide a reasonable picture of the complete pumping mechanism of the Ca^{2+}-ATPase. The full reaction cycle based on crystal structures of nine different states can be described by consideration of the four principal structures shown in the cartoon of Fig. 8.21 (Toyoshima 2008)

$E2 \rightarrow E1 \rightarrow E1{\cdot}2Ca^{2+}$: binding of Ca^{2+}

The E2 state which is the state subsequent to the release of Ca^{2+} into the lumen and hydrolysis of aspartylphosphate is the ground state of the enzyme. In the E2 state the headpiece is compact with the N- and A-domains tightly associated (Fig. 8.19, E2). M5 is bowed towards M1 to bring the P-domain underneath the A-domain. The transmembrane Ca^{2+}-binding cavity is filled with water molecules. Although the three cytoplasmic domains are associated most closely in this state, thermal agitation opens the headpiece allowing Ca^{2+} binding in exchange for H^+ release into the cytoplasm. Two Ca^{2+} enter the high affinity sites through a gating residue (Glu309) on M4 in single file. The Ca^{2+} binding straightens the M5 helix and breaks the closed configuration of the headpiece by bringing the P-domain apart from the A-domain (Fig. 8.19, E1·2Ca^{2+}).

$E1{\cdot}2Ca^{2+} \rightarrow E1P$ transition: formation of the occluded state

ATP binds near the hinge between the P- and N-domains and crosslinks them, so that the γ-phosphate of ATP and a Mg^{2+} (or Ca^{2+}) bind to the P-domain to bend it in two directions (Fig. 8.19, E1·ATP~E1P). The bending of the P-domain tilts the

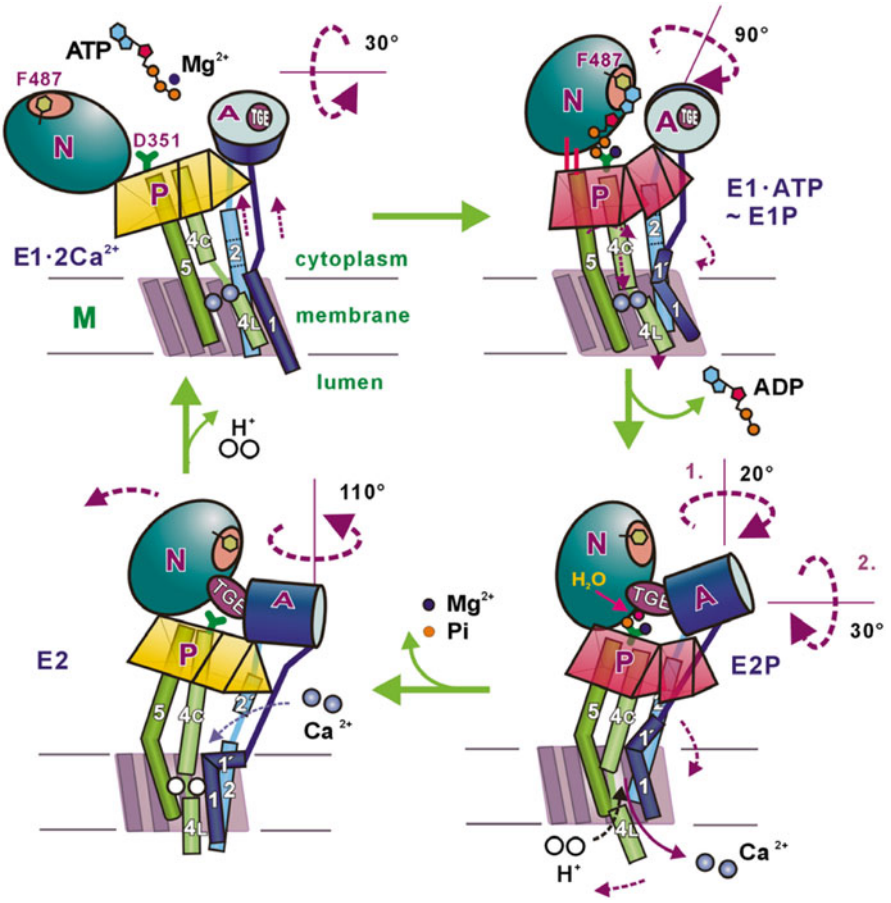


Fig. 8.19 A cartoon illustrating the structural changes of the Ca^{2+}-ATPase during the reaction cycle based on the crystal structures in the nine different states. See text for explanation (Toyoshima 2008. With permission Elsevier)

A-domain. This movement leads to closure of the cytoplasmic gating residue of the Ca^{2+} binding sites. Thus, the two Ca^{2+} are occluded in the transmembrane binding sites. The closure of the gating residue is transmitted to the phosphorylation site some 50 Å away. Phosphoryl transfer from the γ-phosphate to Asp351 fixes the N-domain in a highly inclined position so that a mechanical couple is formed between the N- and A-domains.

E1P → E2P transition: release of Ca^{2+} into the lumen of SR

Phosphoryl transfer to Asp351 allows the dissociation of ADP, which triggers the opening of the N- and P-domain interface. The A-domain rotates and brings a loop (TGES) of the A-domain deep into the gap between the N- and P-domains above the aspartylphosphate (Fig. 8.19, E2P). This A-domain rotation causes a

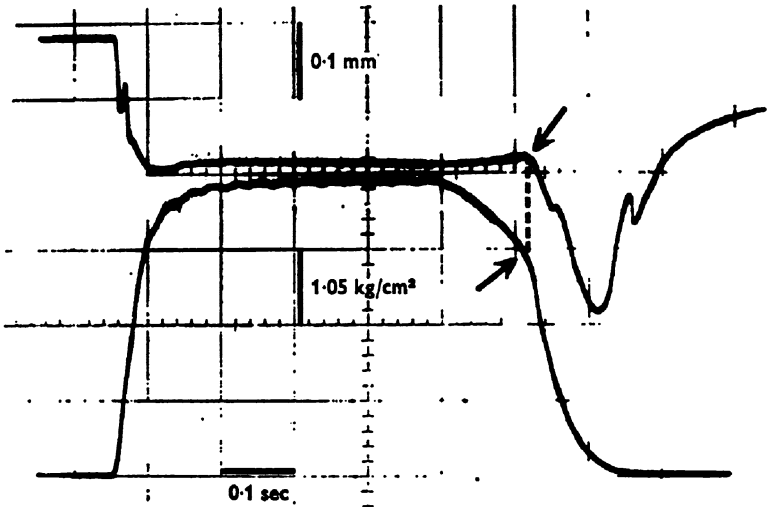


Fig. 8.20 Development of sarcomere length inhomogeneity during relaxation from an isometric tetanus in a single muscle fiber from the frog. *Upper trace*: length between two markers stuck on the fiber, shortening is downwards. *Lower trace*: tension. *Arrows* and a *dotted line* indicate the "shoulder" of tension and the simultaneous change in length. Relaxation rate is greatly accelerated when the sarcomeres become non-uniform (Huxley and Simmons 1970. With permission John Wiley & Sons Inc)

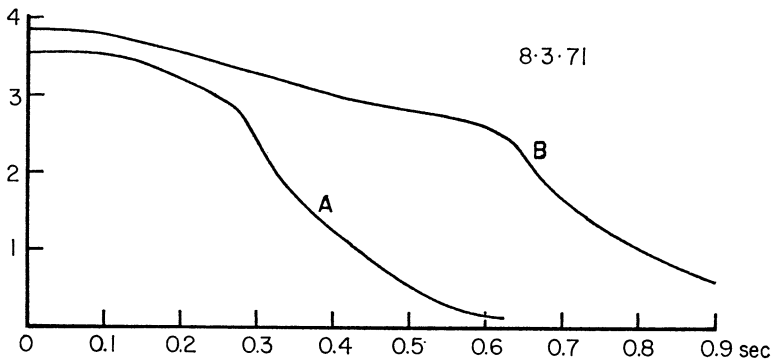


Fig. 8.21 Prolongation of the time to the shoulder of relaxation from an isometric tetanus in a single muscle fiber when the central portion of the fiber is held at a constant length during relaxation. (**a**) Time course of relaxation with tendons held stationary. (**b**) Servo system and spot-follower in operation so that the middle 50 % of the fiber is kept at a constant length in spite of elongation on one end of the fiber which begins at about the time of the "shoulder" in the corresponding (**a**) record. Some part of the fiber within the controlled segment begins to elongate when the "shoulder" occurs in the (**b**) record (Huxley and Simmons 1973. With permission Cold Spring Harbor Laboratory Press)

drastic rearrangement of the transmembrane helices M1–M6 which destroys the Ca^{2+}-binding sites and releasing the bound Ca^{2+} into the lumen. This will allow protons and water molecules to enter and stabilize the empty Ca^{2+}-binding sites.

E2P → E2 transition: hydrolysis of aspartylphosphate and closing of the luminal gate

The TGES loop of the A-domain now fixes a water molecule in the phosphorylation site to hydrolyze the aspartylphosphate. The release of the phosphate and Mg^{2+} relaxes the P-domain. This in turn releases the M1 and M2 helices so that M4 closes the luminal gate completely.

In essence, the P- and N-domains change interfaces and thereby control the position of the A-domain, which in turn transmits information to the transmembrane Ca^{2+} gates. ATP, phosphate, Mg^{2+} and Ca^{2+} are the modifiers of these interfaces. For further details, see Toyoshima (2008).

Even though much has been learned, the story is not complete. The crystal structures are by necessity equilibrium approximations of transient intermediate states. Consequently, speculation remains regarding the precise structural details of the true intermediate states. Furthermore a surprise came when it was realized that all the Ca^{2+}-ATPase preparations isolated from skeletal muscle contained significant amounts of sarcolipin (SLN), a small (31 amino acid residues) protein that regulates the activity of SERCA in fast twitch skeletal muscle[14]. Possible effects of SLN on the structural analysis were not taken into account previously. Fortunately it is possible to express the Ca^{2+}-ATPase protein in an adenovirus/COS cell system. Despite some issues still to be resolved, Toyoshima and Cornelius (2013) have concluded: "We can now say that the mechanism of active transport by SERCA1a is roughly understood, as crystal structures of most of the intermediates in the reaction cycle have been determined…"

The advances in the understanding of the pumping mechanism of the Ca^{2+}-ATPase have been truly spectacular. That so much of the understanding of this structural mechanism can be attributed to the pioneering efforts of Chikashi Toyoshima, who started as a student in Setsuro Ebashi's research group, must have been very gratifying to Ebashi who initiated his own quest to understand the mechanism of sequestration of Ca^{2+} by the sarcoplasmic reticulum 50 years earlier.

[14] Sarcolipin (SLN), originally characterized and named by Wawrzynow et al. (1992) has been shown to uncouple ATP hydrolysis from Ca^{2+} transport by the Ca^{2+}-ATPase of the sarcoplasmic reticulum of skeletal muscle (SERCA) (Smith et al. 2002). Interestingly it was also shown to be necessary for muscle-based non-shivering thermogenesis. When challenged to acute cold, SLN knockout mice were not able to maintain their core body temperature and developed hypothermia. Over-expression of SLN in the SLN-null background fully restored muscle-based thermogenesis, suggesting that SLN is the basis for SERCA-mediated heat production. Ryanodine receptor 1 (Ryr1)-mediated Ca^{2+} leak was shown to be an important mechanism for SERCA-activated heat generation. It was suggested that SLN can continue to interact with SERCA in the presence of Ca^{2+} and can promote uncoupling of the SERCA pump and cause futile cycling and heat production (Bal et al. 2012).

8.4.3 *Other Factors Influencing the Time Course of Skeletal Muscle Relaxation*

Sarcomere length inhomogeneity during muscle relaxation: Thus muscle relaxation was attributed to Ca^{2+} sequestration by the calcium pump. But in the 1970s other factors also appeared to be involved in determining the time course of muscle relaxation. Cleworth and Edman (1969) were the first to note that during relaxation from an isometric contraction in single muscle fibers, the sarcomeres of the fibers became non-uniform. Later they noted (Cleworth and Edman 1972) that the sarcomere movements during relaxation differed along the length of the fiber. As the tension declined smoothly, sarcomeres in some parts of the fiber underwent shortening while other parts of the fibers were extended. These data suggested that the duration of the mechanical activity differed in different regions along the length of the fiber.

This curious phenomenon also was described by Huxley and Simmons (1970) and related specifically to the observed change in rate of relaxation (Fig. 8.20). When a muscle fiber was stimulated to produce an isometric tetanus, internal shortening occurred due to the compliance of the tendons and connections to the force measuring device. Once the force was maximum and stable, no further change occurred in sarcomere length. Also the sarcomere length remained constant during the first 25 % of decline in force during relaxation. But at the "shoulder" of relaxation, there was a rapid change ("give") in sarcomere length in a region of the fiber. Thereafter relaxation was accelerated about ten fold. In the region of the fiber where the sarcomere length was monitored in Fig. 8.20 (upper trace), there occurred a rapid internal shortening. Thus elsewhere in the fiber sarcomeres must have been stretched since the external length of the fiber was held constant. Huxley and Simmons (1973) tested whether or not the acceleration of relaxation was due to the sudden appearance of sarcomere length inhomogeneity. Using the fiber segment length clamp apparatus that Huxley and colleagues devised previously (Gordon et al. 1966), they held the middle 50 % of a muscle fiber at a constant length during relaxation (Fig. 8.21). Compared to the normal time course of relaxation (Fig. 8.21a), the same fiber clamped exhibited a greatly prolonged time to the shoulder and longer relaxation time (Fig. 8.21b). Thus the sudden occurrence of sarcomere length inhomogeneity accelerated relaxation. The shoulder in mechanical relaxation had been observed for many years in mechanical studies of whole muscle (e.g., Hartree and Hill 1921) and thus was not an artifact of the single muscle fiber preparation. Edman and Flitney (1982) made an extensive study of the characteristics of the sarcomere length inhomogeneity during relaxation. They concluded that the complex patterns of sarcomere length changes seen during relaxation appeared to be due to variations in the duration of mechanical activity in different fiber segments. The idea was that small differences in the rate of Ca^{2+} uptake in different regions of the fiber would lead in some regions to stronger sarcomeres (where Ca^{2+} uptake was slower) stretching and forcibly detaching cross-bridges in weaker regions (where Ca^{2+} uptake was faster) with the overall effect of accelerating relaxation.

The decline in free Ca^{2+} concentration during the time period before the shoulder of relaxation is much more rapid than the decline in force (Cannell 1986). Therefore the rate of decline in force before the shoulder likely is determined by the kinetics of cross-bridge detachment and the kinetics of cross-bridge detachment is accelerated during the development of sarcomere length inhomogeneity.

Role of parvalbumin in promoting skeletal muscle relaxation: Another possible component of muscle relaxation was introduced when a soluble intracellular Ca^{2+} binding protein called parvalbumin, found in high concentration in fast contracting (and relaxing) muscle fibers (Heizmann et al. 1982), was proposed to promote muscle relaxation (Gerday and Gillis 1976; Pechere et al. 1977) (see Chap. 6). Although parvalbumins are Ca^{2+} binding proteins, they are not Ca^{2+} regulated proteins like, for example, calmodulin. Rather, they seem to act only as Ca^{2+} buffers. Parvalbumin binds two moles of Ca^{2+} per mole of protein with high affinity (association constant ~10^8 M^{-1}). In equilibrium experiments with isolated myofibrils, parvalbumin inhibited the ATPase activity by removing Ca^{2+} from the myofibrils and in turn the fragmented sarcoplasmic reticulum removed Ca^{2+} from parvalbumin (Gerday and Gillis 1976). Thus the relative Ca^{2+} affinities were appropriate for parvalbumin to promote relaxation. But there was a problem. The total parvalbumin concentration in frog muscle fibers, for example, is about 8 fold greater than the troponin concentration (Hou et al. 1991). This condition led to a puzzling question. How could frog muscle contract at all? Why is not the Ca^{2+} so effectively buffered as to prevent contraction in these muscles? Obviously, some important piece of the puzzle was missing. The solution to this dilemma stems from the observations that (1) Mg^{2+} competes for the Ca^{2+} binding sites on parvalbumin but with an affinity ~10^4 less than that observed for Ca^{2+}, (2) even so the free Mg^{2+} concentration in resting muscle (~1 mM) is ~10^4 times greater than the free Ca^{2+} concentration, (3) Mg^{2+} dissociates only slowly from parvalbumin, and (4) the Ca^{2+} regulatory sites on troponin C do not bind Mg^{2+} under in vivo conditions but rather bind Ca^{2+} at a rate limited only by the rate of diffusion of Ca^{2+} to troponin C. These observations led to the realization that Mg^{2+} is bound to parvalbumin in resting muscle. Thus, during muscle activation, Ca^{2+} will bind first to troponin C and activate the contractile machinery and only later will Ca^{2+} bind to parvalbumin at a rate limited by the rate of Mg^{2+} dissociation from parvalbumin.

The proposal was that parvalbumin, acting in parallel with the sarcoplasmic reticulum calcium pump, would promote relaxation by binding Ca^{2+} in exchange for Mg^{2+} during relaxation. The calcium pump would then remove Ca^{2+} more slowly from parvalbumin after relaxation. Graduate student Tien-Tzu Hou working with J. David Johnson and Jack A. Rall at Ohio State University put this hypothesis to a test (Hou et al. 1991). They reasoned that if parvalbumin promoted relaxation, the rate of muscle relaxation from an isometric tetanus should slow progressively with increasing duration of contraction, since parvalbumin would progressively become saturated with Ca^{2+} as the contraction proceeds and thus would be unable to promote relaxation. Furthermore, the relaxation rate should slow exponentially with increasing contraction duration with a time course that is limited by the rate of dissociation of Mg^{2+} from parvalbumin. They measured the time course of relaxation during the true isometric period before the shoulder of relaxation as a function of tetanus duration in single frog muscle fibers and the rate of Mg^{2+} dissociation from isolated parvalbumin

under similar conditions. They found that relaxation slowed exponentially at a rate of ~1 s^{-1} which was the same as the rate of Mg^{2+} dissociation from isolated parvalbumin. Also Cannell (1986) observed that the rate of fall of the free Ca^{2+} concentration during relaxation in frog skeletal muscle slowed progressively with increasing contraction duration with a time course that was equal to the rate of dissociation of Mg^{2+} from parvalbumin. A further prediction was that the rate of relaxation of a brief tetanus after a prolonged tetanus should depend on the time interval between contractions because the effectiveness of parvalbumin as a Ca^{2+} buffer would depend on the time necessary for Mg^{2+} to rebind to parvalbumin that was previously loaded with Ca^{2+}. This rebinding process should be limited by the rate of Ca^{2+} dissociation from parvalbumin. Hou et al. (1991) found that the time course of recovery of isometric relaxation rate in a brief tetanus with rest after a prolonged tetanus exhibited a rate constant of 0.12 s^{-1}. Thus the muscle recovered the ability to relax rapidly with rest ~10 times more slowly than it lost that ability with increasing contraction duration. Calcium dissociated from isolated parvalbumin with a similar rate constant of 0.2 s^{-1}, consistent with proposal that Mg^{2+} bound parvalbumin promoted relaxation rate. These and other results supporting a role for parvalbumin in promotion of skeletal muscle relaxation have been reviewed by Rall (1996).

Whereas these results supported the proposal that parvalbumin promoted relaxation, they were not direct proof. Subsequent experiments have shown that direct gene transfer of parvalbumin into rat soleus muscle that normally does not express parvalbumin leads to expression of parvalbumin and to a significantly shortened twitch half relaxation time (Muntener et al. 1995). Transfer of a gene that encodes a mutant parvalbumin that does not bind Ca^{2+} was without effect on relaxation time. This study directly proves that parvalbumin can promote relaxation in mammalian skeletal muscle. Furthermore parvalbumin gene inactivation (Raymackers et al. 2000) or gene knockout (Schwaller et al. 1999) led to a decrease in relaxation rate in fast-twitch muscle of the mouse. It is now generally accepted that parvalbumin can promote relaxation in fast contracting and relaxing skeletal muscles.

Thus relaxation in skeletal muscle is a complex process. The overall picture of relaxation is that it is initiated at the cessation of stimulation when the rate of Ca^{2+} sequestration by the calcium pump becomes greater than the rate of Ca^{2+} release from the sarcoplasmic reticulum. Along with the continued pumping of Ca^{2+}, the subsequent time course of relaxation is accelerated by the presence of parvalbumin bound to Mg^{2+} in fast-twitch muscles and by development of sarcomere length inhomogeneity.

8.5 Conclusion

In the 50 years since Ebashi (1961) put toward his “Ca concept” of muscle contraction and relaxation, there has been an absolute explosion of knowledge concerning the role of Ca^{2+}. The knowledge was first driven by advances in biochemistry and physiology and then greatly accelerated by advances in molecular and structural biology and molecular genetics. It has been a relentless quest for a molecular understanding of the role of the transverse tubules in the release of Ca^{2+} from the

sarcoplasmic reticulum, the function and regulation of the Ca^{2+} release channels, the role of the Ca^{2+} transient in the control of Ca^{2+} release, the role of Ca^{2+} binding to troponin and cross-bridges in activation of muscle contraction and the mechanism of muscle relaxation. The primary proteins have been identified and functions characterized but in no case can one state assuredly that the molecular mechanism is completely understood. Nonetheless Ebashi would have been pleased with the progress.

References

Adams BA, Tanabe T, Mikami A, Numa S, Beam KG (1990) Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs. Nature 346:569–572

Ashley CC, Ridway EB (1968) Simultaneous recording of membrane potential, calcium transient and tension in single muscle fibres. Nature 219:1168–1169

Baker ML, Ju T, Chiu W (2007) Identification of secondary structure elements in intermediate-resolution density maps. Structure 15:7–19

Bal NC, Maurya SK, Sopariwala DH, Sahoo SK, Gupta SC, Shaikh SA, Pant M, Rowland LA, Bombrdier E, Goonasekera SA, Tupling AR, Molkentin JD, Periasamy M (2012) Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. Nat Med 18:1575–1580

Barany M (1967) ATPase activity of myosin correlated with speed of muscle shortening. J Gen Physiol 50(Pt. 2):197–218

Baylor SM (2005) Calcium sparks in skeletal muscle fibers. Cell Calcium 37:513–530

Baylor SM, Hollingworth S (1988) Fura-2 calcium transients in frog skeletal muscle fibres. J Physiol 403:151–192

Baylor SM, Hollingworth S (1998) Model of sarcomeric Ca^{2+} movements, including ATP Ca^{2+} binding and diffusion, during activation of frog skeletal muscle. J Gen Physiol 112:297–316

Baylor SM, Hollingworth S (2000) Measurement and interpretation of cytoplasmic [Ca^{2+}] signals from calcium-indicator dyes. News Physiol Sci 15:19–26

Baylor SM, Hollingworth S (2003) Sarcoplasmic reticulum calcium release compared in slow-twitch and fast-twitch fibres of mouse muscle. J Physiol 551:125–138

Baylor SM, Hollingworth S (2011) Calcium indicators and calcium signaling in skeletal muscle fibres during excitation-contraction coupling. Prog Biophys Mol Biol 105:162–179

Beam KG, Bannister RA (2010) Looking for answers to EC coupling's persistent questions. J Gen Physiol 136:7–12

Beam KG, Knudson CM, Powell JA (1986) A lethal mutation in mice eliminates the slow calcium current in skeletal muscle cells. Nature 320:168–170

Block BA, Imagawa T, Campbell KP, Franzini-Armstrong C (1988) Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. J Cell Biol 107:2587–2600

Brandt PW, Cox RN, Kawai M (1980) Can the binding of Ca^{2+} to two regulatory sites on troponin C determine the steep pCa/tension relationship of skeletal muscle? Proc Natl Acad Sci U S A 77:4717–4720

Brandt PW, Diamond MS, Schachat FH (1984) The thin filament of vertebrate skeletal muscle co-operatively activates as a unit. J Mol Biol 180:379–384

Bremel RD, Weber A (1972) Cooperation within actin filament in vertebrate skeletal muscle. Nat New Biol 238:97–101

Brenner B (1988) Effect of Ca^{2+} on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: implications for regulation of muscle contraction. Proc Natl Acad Sci U S A 85:3265–3269

Brenner B, Eisenberg E (1986) Rate of force generation in muscle: correlation with actomyosin ATPase activity in solution. Proc Natl Acad Sci U S A 83:3542–3546
Brown JE, Cohen LB, De Weer P, Pinto LH, Ross WN, Salzberg BM (1975) Rapid changes of intracellular free calcium concentration: detection by metallochromic indicator dyes in squid giant axon. Biophys J 15:1155–1160
Cannell MB (1986) Effect of tetanus duration on the free calcium during the relaxation of frog skeletal muscle fibres. J Physiol 376:203–218
Cannell MB, Allen DG (1984) Model of calcium movements during activation in the sarcomere of frog skeletal muscle. Biophys J 45:913–925
Catterall WA, Perez-Reyes E, Snutch TP, Striessnig J (2005) International union of pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. Pharmacol Rev 57:411–425
Cheng H, Lederer WJ, Cannell MB (1993) Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. Science 262:740–744
Cleworth D, Edman KAP (1969) Laser diffraction studies on single skeletal muscle fibers. Science 163:296–298
Cleworth DR, Edman KAP (1972) Changes in sarcomere length during isometric tension development in frog skeletal muscle. J Physiol 227:1–17
Colson BA, Locher MR, Bekyarova T, Patel JR, Fitzsimons DP, Irving TC, Moss RL (2010) Differential roles of regulatory light chain and myosin binding protein-C phosphorylation in the modulation of cardiac force development. J Physiol 588:981–993
Coronado R, Ahern CA, Sheridan DC, Cheng W, Carbonneau L, Bhattacharya D (2004) Functional equivalence of dihydropyridine receptor α1S and β1a subunits in triggering excitation-contraction coupling in skeletal muscle. Biol Res 37:565–575
Costantin LL, Franzini-Armstrong C, Podolsky RJ (1965) Localization of calcium-accumulating structures in striated muscle fibers. Science 147:158–160
Dayal A, Bhat V, Franzini-Armstrong C, Grabner M (2013) Domain cooperativity in the β_{1a} subunit is essential for dihydropyridine receptor voltage sensing in skeletal muscle. Proc Natl Acad Sci U S A 110:7488–7493
Donaldson SKB, Kerrick WGL (1975) Characterization of the effects of Mg^{2+} on Ca^{2+}- and Sr^{2+}-activated tension generation of skinned skeletal muscle fibers. J Gen Physiol 66:427–444
Ebashi S (1961) Calcium binding activity of vesicular relaxing factor. J Biochem 50:236–244
Ebashi S, Lipmann F (1962) Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. J Cell Biol 14:389–400
Edman KAP (1979) The velocity of unloaded shortening and its relation to sarcomere length and isometric force in vertebrate muscle fibres. J Physiol 291:143–159
Edman KAP, Flitney FW (1982) Laser diffraction studies of sarcomere dynamics during 'isometric' relaxation in isolated muscle fibres of the frog. J Physiol 329:1–20
Endo M (2009) Calcium-induced release in skeletal muscle. Physiol Rev 89:1153–1176
Endo M, Tanaka M, Ogawa Y (1970) Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. Nature 228:34–36
Fabiato A, Fabiato F (1972) Excitation-contraction coupling of isolated cardiac fibers with disrupted or closed sarcolemmas: calcium-dependent cyclic and tonic contractions. Circ Res 31:293–307
Farah CS, Miyamoto CA, Ramos CHI, da Silva ACR, Quaggio RB, Fujimori K, Smillie LB, Reinach FC (1994) Structural and regulatory functions of the NH_2- and COOH-terminal regions of skeletal muscle troponin I. J Biol Chem 269:5230–5240
Farah CS, Reinach FC (1995) The troponin complex and regulation of muscle contraction. FASEB J 9:755–767
Figueroa L, Shkryl VM, Zhou J, Manno C, Momotake A, Brum G, Blatter LA, Ellis-Davies GCR, Rios E (2012) Synthetic localized calcium transients directly probe signaling mechanism in skeletal muscle. J Physiol 590:1389–1411
Flicker PF, Phillips GN, Cohen C (1982) Troponin and its interactions with tropomyosin. J Mol Biol 162:495–501

Fill M, Copello JA (2002) Ryanodine receptor calcium release channels. Physiol Rev 82:893–922
Ford LE, Podolsky RJ (1970) Regenerative calcium release within muscle cells. Science 167:58–59
Galinska-Rakoczy A, Engel P, Xu C, Jung H, Craig R, Tobacman LS, Lehman W (2008) Structural basis for the regulation of muscle contraction by troponin and tropomyosin. J Mol Biol 379:929–935
Gerday C, Gillis JM (1976) The possible role of parvalbumins in the control of contraction. J Physiol 258:96–97P
Gordon AM, Homsher E, Regnier M (2000) Regulation of contraction in striated muscle. Physiol Rev 80:853–924
Gordon AM, Huxley AF, Julian FJ (1966) The variation in isometric tension with sarcomere length in vertebrate muscle fibres. J Physiol 184:170–192
Grabarek Z, Grabarek J, Leavis PC, Gergely J (1983) Cooperative binding to the Ca^{2+}-specific sites of troponin C in regulated actin and actomyosin. J Biol Chem 258:14098–14102
Greaser M, Gergely J (1973) Purification and properties of the components from troponin. J Biol Chem 248:2125–2133
Gregg RG, Messing A, Strube C, Beurg M, Moss R, Behan M, Sukhareva M, Haynes S, Powell JA, Coronado R, Powers PA (1996) Absence of the β subunit (*cchb1*) of the skeletal muscle dihydropyridine receptor alters expression of the α_1 subunit and eliminates excitation-contraction coupling. Proc Natl Acad Sci 93:13961–13966
Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
Hamilton SL, Serysheva II (2009) Ryanodine receptor structure: progress and challenges. J Biol Chem 284:4047–4051
Hartree W, Hill AV (1921) The nature of the isometric twitch. J Physiol 55:389–411
Hasselbach W, Makinose M (1961) Die calciumpumpe der "erschlaffungsgrana" des muskels und ihre abhangigkeit von der ATP-spaltung. Biochem Z 333:518–528
Hasselbach W, Makinose M (1962) ATP and active transport. Biochem Biophys Res Commun 7:132–136
Heeley DH, Golosinska K, Smillie LB (1987) The effects of troponin T fragments T1 and T2 on the binding of nonpolymerizable tropomyosin to F-actin in the presence and absence of troponin I and troponin C. J Biol Chem 262:9971–9978
Heizmann CW, Berchtold MW, Rowlerson AM (1982) Correlation of parvalbumin concentration with relaxation speed in mammalian muscle. Proc Natl Acad Sci U S A 79:7243–7247
Hellam DC, Podolsky RJ (1969) Force measurements in skinned muscle fibres. J Physiol 200:807–819
Hernandez OM, Jones M, Guzman G, Szczesna-Cordary D (2007) Myosin essential light chain in health and disease. Am J Physiol 292:H1643–H1654
Herzberg O, James MNG (1985) Structure of the calcium regulatory muscle protein troponin-C at 2.8 Å resolution. Nature 313:653–659
Herzberg O, Moult J, James MNG (1986) A model for the Ca^{2+}-induced conformational transition of troponin C. A trigger for muscle contraction. J Biol Chem 261:2638–2644
Hill AV (1913) XLVII. The combinations of haemoglobin with oxygen and with carbon monoxide. I. Biochem J 7:471–480
Hollingworth S, Peet J, Chandler WK, Baylor SM (2001) Calcium sparks in intact skeletal muscle fibers of the frog. J Gen Physiol 118:653–678
Hou T-T, Johnson JD, Rall JA (1991) Parvalbumin content and Ca^{2+} and Mg^{2+} dissociation rates correlated with changes in relaxation rate of frog muscle fibres. J Physiol 441:285–304
Houdusse A, Love ML, Dominguez R, Grabarek Z, Cohen C (1997) Structures of four Ca^{2+}-bound troponin C at 2.0 Å resolution: further insights into the Ca^{2+}-switch in the calmodulin superfamily. Structure 5(12):1695–1711
Huxley AF (1957) Muscle structure and theories of contraction. In: Butler JAV, Katz B (eds) Progress in biophysics and biophysical chemistry, vol 7. Pergamon Press, London, pp 255–318

Huxley AF, Simmons RM (1970) Rapid 'give' and the tension 'shoulder' in the relaxation of frog muscle fibres. J Physiol 210:32–33P

Huxley AF, Simmons RM (1973) Mechanical transients and the origin of muscular force. Cold Spring Harb Symp Quant Biol 37:669–680

Imura H (1995) Shosaku Numa. 7 February 1929–15 February 1992. Biogr Mem Fellows R Soc 41:310–314

Jayaraman T, Brillantes A-M, Timerman AP, FleischerS E-BH, Tempst P, Marks AR (1992) FK506 binding protein associated with the calcium release channel (ryanodine receptor). J Biol Chem 267:9474–9477

Julian FJ (1971) The effect of calcium on the force-velocity relation of briefly glycerinated frog muscle fibres. J Physiol 218:117–145

Kamm KE, Stull JT (1985) The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. Ann. Rev. Pharmacol Toxicol 25:593–620

Kamm KE, Stull JT (2011) Signaling to myosin regulatory light chain in sarcomeres. J Biol Chem 286:9941–9947

Kimura-Sakiyama C, Ueno Y, Wakabayashi K, Miki M (2008) Fluorescence resonance energy transfer between residues on troponin and tropomyosin in the reconstituted thin filament: modeling the troponin–tropomyosin complex. J Mol Biol 376:80–91

Klaus MM, Scordilis SP, Rapalus JM, Briggs RT, Powell JA (1983) Evidence for dysfunction in the regulation of cytosolic Ca^{2+} in excitation-contraction uncoupled dysgenic muscle. Dev Biol 99:152–165

Klug GA, Botterman BR, Stull JT (1982) The effect of low frequency stimulation on myosin light chain phosphorylation in skeletal muscle. J Biol Chem 257:4688–4690

Knowles AC, Irving M, Sun Y-B (2012) Conformation of the troponin core complex in the thin filaments of skeletal muscle during relaxation and active contraction. J Mol Biol 421:125–137

Lowey S, Waller GS, Trybus KM (1993) Function of skeletal muscle myosin heavy and light chain isoforms by an *in vitro* motility assay. J Biol Chem 268:20414–20418

Ludtke SJ, Serysheva II, Hamilton SL, Chiu W (2005) The pore structure of the closed RyR1 channel. Structure 13:1203–1211

MacLennan DH, Brandl CJ, Korczak B, Green NM (1985) Amino-acid sequence of a Ca^{2+} + Mg^{2+}-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. Nature 316:696–700

MacLennan DH, Rice WJ, Green NM (1997) The mechanism of Ca^{2+} transport by sarco(endo) plasmic reticulum Ca^{2+}-ATPases. J Biol Chem 272:28815–28818

Manning DR, Stull JT (1979) Myosin light chain phosphorylation and phosphorylase A activity in rat extensor digitorum longus muscle. Biochem Biophys Res Commun 90:164–170

Marks AR (1996) Cellular functions of immunophilins. Physiol Rev 76:631–649

Marx SO, Ondrias K, Marks AR (1998) Coupled gating between individual skeletal muscle Ca^{2+} release channels (ryanodine receptors). Science 281:818–821

McKillop DFA, Geeves MA (1993) Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. Biophys J 65:693–701

Metzger JM, Greaser ML, Moss RL (1989) Variations in cross-bridge attachment rate and tension with phosphorylation of myosin in mammalian skinned skeletal muscle fibers. J Gen Physiol 93:855–883

Millar NC, Homsher E (1990) The effect of phosphate and calcium on force generation in glycerinated rabbit skeletal muscle fibers. A steady-state and transient kinetic study. J Biol Chem 265:20234–20240

Miledi R, Parker I, Schalow G (1977) Measurement of calcium transients in frog muscle by the use of arsenazo III. Proc R Soc Lond B Biol Sci 198:201–210

Miller C, Racker E (1976) Ca^{++}-induced fusion of fragmental sarcoplasmic reticulum with artificial planar bilayers. J Membr Biol 30:283–300

Minta A, Kao JP, Tsien RY (1989) Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. J Biol Chem 264:8171–8178

Moore RL, Stull JT (1984) Myosin light chain phosphorylation in fast and slow skeletal muscle in situ. Am J Physiol 247:C462–C471

Moss RL (1992) Ca^{2+} regulation of mechanical properties of striated muscle. Mechanistic studies using extraction and replacement of regulatory proteins. Circ Res 70:865–884
Moss RL, Fitzsimons DP (2010) Regulation of contraction in mammalian striated muscle—the plot thick-ens. J Gen Physiol 136:21–27
Moss RL, Giulian GG, Greaser ML (1985) The effects of partial extraction of TnC upon the tension-pCa relationship in rabbit skinned skeletal muscle fibers. J Gen Physiol 86:585–600
Muntener M, Kaser L, Weber J, Berchtold MW (1995) Increase of skeletal muscle relaxation speed by direct injection of parvalbumin cDNA. Proc Natl Acad Sci U S A 92:6504–6508
Nakai J, Dirksen RT, Nguyen HT, Pessah IN, Beam KG, Allen PD (1996) Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. Nature 380:72–75
Natori R (1954) The property and contraction process of isolated myofibrils. Jikeikai Med J 1:119–126
Neher E, Sakmann B (1976) Single-channel currents recorded from membrane of denervated frog muscle fibres. Nature 260:799–802
Obermair GJ, Tuluc P, Flucher BE (2008) Auxiliary Ca^{2+} channel subunits: lessons learned from muscle. Curr Opin Pharmacol 8:311–318
Ogawa Y (1994) Role of ryanodine receptors. Crit Rev Biochem Mol Biol 29:229–274
Paolini C, Fessenden JD, Pessach IN, Franzini-Armstrong C (2004) Evidence for conformation coupling between two calcium channels. Proc Natl Acad Sci 101:12748–12752
Pechere J-F, Derancourt J, Haiech J (1977) The participation of parvalbumins in the activation-relaxation cycle of vertebrate fast skeletal-muscle. FEBS Lett 75:111–114
Perrie WT, Smillie LB, Perry SV (1973) A phosphorylated light-chain component of myosin from skeletal muscle. Biochem J 135:151–164
Persechini A, Stull JT, Cooke R (1985) The effect of myosin phosphorylation on the contractile properties of skinned rabbit skeletal muscle fibers. J Biol Chem 260:7951–7954
Powell JA, Fambrough DM (1973) Electrical properties of normal and dysgenic mouse skeletal muscle in culture. J Cell Physiol 82:21–38
Pragnell M, De Waard M, Mori Y, Tanabe T, Snutch TP, Campbell KP (1994) Calcium channel β-subunit binds to a conserved motif in the I-II cytoplasmic linker of the α_1-subunit. Nature 368:67–70
Radermacher M, Rao V, Grassucci R, Frank J, Timerman AP, Fleischer S, Wagenknecht T (1994) Cryo-electron microscopy and three-dimensional reconstruction of the calcium release channel/ryanodine receptor from skeletal muscle. J Cell Biol 127:411–423
Raju B, Murphy E, Levy LA, Hall RD, London RE (1989) A fluorescent indicator for measuring cytosolic free magnesium. Am J Physiol 256:C540–C548
Rall JA (1996) Role of parvalbumin in skeletal muscle relaxation. NIPS 11:249–255
Raymackers JM, Gailly P, Colson-Van Schoor M, Pette D, Schwaller B, Hunziker W, Celio MR, Gillis JM (2000) Tetanus relaxation of fast skeletal muscles of the mouse made parvalbumin deficient by gene inactivation. J Physiol 527:355–364
Rayment I, Rypniewski WR, Schmidt-Base K, Smith R, Tomchick DR, Benning MM, Winkelmann DA, Wesenberg G, Holden HM (1993) Three-dimensional structure of myosin subfragment-1: A molecular motor. Science 261:50–58
Reiser PJ, Moss RL, Giulian GG, Greaser ML (1985) Shortening velocity in single fibers from adult rabbit soleus muscles is correlated with myosin heavy chain composition. J Biol Chem 260:9077–9080
Reuben JP, Brandt PW, Berman M, Grundfest H (1971) Regulation of tension in the skinned crayfish muscle fiber. I. Contraction and relaxation in the absence of Ca (pCa>9). J Gen Physiol 57:385–407
Rios E, Pizarro G (1988) The voltage sensors and calcium channels of excitation-contraction coupling. News Physiol Sci 3:223–228
Samso M, Wagenknecht T, Allen PD (2005) Internal structure and visualization of transmembrane domains of the RyR1 calcium release channel by cryo-EM. Nat Struct Biol 12:539–544
Schiaffino S, Reggiani C (1996) Molecular diversity of myofibrillar proteins: gene regulation and functional significance. Physiol Rev 76:371–423

Schneider MF, Chandler WK (1973) Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. Nature 242:244–246

Schredelseker J, Di Biase V, Obermair GJ, Felder ET, Flucher BE, Franzini-Armstrong C, Grabner M (2005) The β_{1a} subunit is essential for the assembly of dihydropyridine-receptor arrays in skeletal muscle. Proc Natl Acad Sci 102:17219–17224

Schwaller B, Dick J, Dhoot G, Carroll S, Vrbova G, Nicotera P, Pette D, Wyss A, Bluethmann H, Hunziker W, Celio MR (1999) Prolonged contraction-relaxation cycle of fast-twitch muscles in parvalbumin knockout mice. Am J Physiol 276:C395–C403

Serysheva II, Ludtke SJ, Baker ML, Cong Y, Topf M, Eramian D, Sali A, Hamilton SL, Chiu W (2008) Subnanometer-resolution electron cryomicroscopy-based domain models for the cytoplasmic region of skeletal muscle RyR channel. Proc Natl Acad Sci U S A 105:9610–9615

Serysheva II, Orlova EV, Chiu W, Sherman MB, Hamilton SL, van Heel M (1995) Electron cryomicroscopy and angular reconstitution used to visualize the skeletal muscle calcium release channel. Nat Struct Biol 2:18–24

Slupsky CM, Sykes BD (1995) NMR solution structure of calcium-saturated skeletal muscle troponin C. Biochemistry 34:15953–25964

Smith JS, Coronado R, Meissner G (1985) Sarcoplasmic reticulum contains adenine nucleotide-activated calcium cannels. Nature 316:446–449

Smith JS, Imagawa T, Ma J, Fill M, Campbell KP, Coronado R (1988) Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. J Gen Physiol 92:1–26

Smith WS, Broadbridge R, East JM, Lee AG (2002) Sarcolipin uncouples hydrolysis of ATP from accumulation of Ca^{2+} by the Ca^{2+}-ATPase of skeletal-muscle sarcoplasmic reticulum. Biochem J 361:277–286

Song DW, Lee J-G, Youn H-S, Eom SH, Kim DH (2011) Ryanodine receptor assembly: a novel systems biology approach to 3D mapping. Prog Biophys Mol Biol 105:145–161

Stokes DL, Green NM (2003) Structure and function of the calcium pump. Annu Rev Biophys Biomol Struct 32:445–468

Strynadka NCJ, Cherney M, Sielecki AR, Li MX, Smillie LB, James MNG (1997) Structural details of a calcium-induced molecular switch: x-ray crystallographic analysis of the calcium-saturated N-terminal domain of troponin C at 1.75 Å resolution. J Mol Biol 273:238–255

Stull JT, Kamm KE, Vandenboom R (2011) Myosin light chain kinase and the role of myosin light chain phosphorylation in skeletal muscle. Arch Biochem Biophys 510:120–128

Sundaralingam M, Bergstrom R, Strasburg G, Rao ST, Roychowdhury P, Greaser M, Wang BC (1985) Molecular structure of troponin C from chicken skeletal muscle at 3-angstrom resolution. Science 227:945–948

Swartz DR, Moss RL (1992) Influence of a strong-binding myosin analogue on calcium-sensitive mechanical properties of skinned skeletal muscle fibers. J Biol Chem 267:20497–20506

Swartz DR, Moss RL, Greaser ML (1996) Calcium alone does not fully activate the thin filament for S1 binding to rigor myofibrils. Biophys J 71:1891–1904

Sweeney HL (1995) Function of the N terminus of the myosin essential light chain of vertebrate striated muscle. Biophys J 68:112s–119s

Sweeney HL, Bowman BF, Stull JT (1993) Myosin light chain phosphorylation in vertebrate striated muscle: regulation and function. Am J Physiol 264:C1085–C1095

Sweeney HL, Kushmerick MJ, Mabuchi K, Sreter FA, Gergely J (1988) Myosin alkali light chain and heavy chain variations correlate with altered shortening velocity of isolated muscle fibers. J Biol Chem 263:9034–9039

Sykes BD (2003) Pulling the calcium trigger. Nat Struct Biol 10:588–589

Syska H, Wilkinson JM, Grand RJA, Perry SV (1976) The relationship between biological activity and primary structure of troponin I from white skeletal muscle of the rabbit. Biochem J 153:375–387

Szent-Gyorgyi A (1949) Free-energy relations and contraction of actomyosin. Biol Bull 96:140–161

Takahashi M, Seagar MJ, Jones JF, Reber BFX, Catterall WA (1987) Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle. Proc Natl Acad Sci 84:5478–5482

Takeda S, Yamashita A, Maeda K, Maeda Y (2003) Structure of the core domain of human cardiac troponin in the Ca^{2+}-saturated form. Nature 424:35–41

Takekura H, Paolini C, Franzini-Armstrong C, Kugler G, Grabner M, Flucher BE (2004) Differential contribution of skeletal and cardiac II-III loop sequences to the assembly of dihydropyridine receptor arrays in skeletal muscle. Mol Biol Cell 15:5408–5419

Takeshima H, Iino M, Takekura H, Nishi M, Kuno J, Minowa O, Takano H, Noda T (1994) Excitation-contraction uncoupling and muscular degeneration in mice lacking functional skeletal muscle ryanodine-receptor gene. Nature 369:556–559

Takeshima H, Nishimura S, Matsumoto T, Ishida H, Kangawa K, Minamino N, Matsuo H, Ueda M, Hanaoka M, Hirose T, Numa S (1989) Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. Nature 339:439–445

Talbot JA, Hodges RS (1981) Synthetic studies on the inhibitory region of rabbit skeletal troponin. I. Relationship of amino acid sequence to biological activity. J Biol Chem 256:2798–2802

Tanabe T, Beam KG, Adams BA, Niidome T, Numa S (1990) Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. Nature 346:567–569

Tanabe T, Beam KG, Powell JA, Numa S (1988) Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. Nature 336:134–139

Tanabe T, Takeshima H, Mikami A, Flockerzi V, Takahashi H, Kangawa K, Kojima M, Matsuo H, Hirose T, Numa S (1987) Primary structure of the receptor for calcium channel blockers from skeletal muscle. Nature 328:313–318

Timson DJ (2003) Fine turning the myosin motor: the role of the essential light chain in striated muscle myosin. Biochimie 85:639–645

Tobacman LS (1996) Thin filament-mediated regulation of cardiac contraction. Annu Rev Physiol 58:447–481

Toyoshima C (2008) Structural aspects of ion pumping by Ca^{2+}-ATPase of sarcoplasmic reticulum. Arch Biochem Biophys 476:3–11

Toyoshima C, Cornelius F (2013) New crystal structures of PII-type ATPases; excitement continues. Curr Opin Struct Biol 23:507–514

Toyoshima C, Nakasako M, Nomura H, Ogawa H (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. Nature 405:647–655

Toyoshima C, Nomura H (2002) Structural changes in the calcium pump accompanying the dissociation of calcium. Nature 418:605–611

Toyoshima C, Sasabe H, Stokes DL (1993) Three-dimensional cryo-electron microscopy of the calcium ion pump in the sarcoplasmic reticulum membrane. Nature 362:469–471

Tsien RY (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. Biochemistry 19:2396–2404

Tsien RY (1981) A non-disruptive technique for loading calcium buffers and indicators into cells. Nature 209:527–528

Tsien RY (1992) Intracellular signal transduction in four dimensions: from molecular design to physiology. Am J Physiol 263:C723–C728

Tsien RY (2008) Roger Y. Tsien-biographical. nobelprize.org.

Tsugorka A, Rios E, Blatter LA (1995) Imaging elementary events of calcium release in skeletal muscle cells. Science 269:1723–1726

Vinogradova MV, Stone DB, Malanina GG, Karatzaferi C, Cooke R, Mendelson RA, Fletterick RJ (2005) Ca^{2+}-regulated structural changes in troponin. Proc Natl Acad Sci U S A 102:5038–5043

Wagenknecht T, Grassucci R, Frank J, Saito A, Inui M, Fleischer S (1989) Three-dimensional architecture of the calcium channel/foot structure of sarcoplasmic reticulum. Nature 338:167–170

Wawrzynow A, Theibert JL, Murphy C, Jona I, Martonosi A, Collins JH (1992) Sarcolipin, the "proteolipid" of skeletal muscle sarcoplasmic reticulum, is a unique, amphipathic, 31-residue peptide. Arch Biochem Biophys 298:620–623

Westhof E (2005) Muttaiya Sundaralingam (1931–2004): a pioneering stereochemist and crystallographer of nucleic acids. Structure 13:343–345

White DCS (1970) Rigor contraction and the effect of various phosphate compounds on glycerinated insect flight and vertebrate muscle. J Physiol 208:583–605

Zhi G, Ryder JW, Huang J, Ding P, Chen Y, Zhao Y, Kamm KE, Stull JT (2005) Myosin light chain kinase and myosin phosphorylation effect frequency-dependent potentiation of skeletal muscle contraction. Proc Natl Acad Sci U S A 102:17519–17524

Chapter 9
Molecular Mechanism of Force Production: From the Difficult 1980s to the Supercharged 1990s and Beyond

…by the mid-l980s, confidence in a straightforward sliding filament mechanism for muscle contraction had been significantly eroded… (Huxley 1996. With permission Annual Reviews)

H. E. Huxley (1996)

The structure of the myosin head, along with the fit to the actomyosin complex, represented an immense breakthrough in the field, which now can be subdivided into pre- and poststructural periods…A second major breakthrough in the field of motility was the development of in vitro measurements of the force and displacement produced by single myosin molecules. (Cooke 2004. With permission Rockefeller University Press)

R. Cooke (2004)

The main complication is that the action of cross-bridges is never truly synchronized, so what is observed comes from the overlapping cycles of many cross-bridges working in parallel. (Simmons 1992. With permission Elsevier)

R. M. Simmons (1992)

…I really do believe that, altogether, there is now incontrovertible evidence for the correctness of the tilting lever-arm model, although of course many important details still remain to be worked out. (Huxley 2004. With permission John Wiley & Sons Inc.)

H. E. Huxley (2004)

9.1 Introduction

At the time of the Cold Spring Harbor muscle meeting in 1972 there was much euphoria and great enthusiasm. Essentially all of the investigators there accepted the sliding filament, attached cross-bridge, model of muscle contraction as dogma and many even felt that the problem was solved. Throughout the 1970s and beyond experiments in the muscle field were designed with the underlying assumption of the correctness of the proposed model. After all there was Huxley's (1969) swinging-tilting cross-bridge model of muscle contraction based on electron microscopic and

J.A. Rall, *Mechanism of Muscular Contraction*, Perspectives in Physiology,
DOI 10.1007/978-1-4939-2007-5_9

X-ray diffraction evidence, the transient kinetic mechanical studies of Huxley and Simmons (1971) and the natural way that ATP was proposed to fit into the cross-bridge cycle by Lymn and Taylor (1971). The sliding filament model even appeared in introductory textbooks of physiology. Nonetheless there were difficult but essential issues that had to be addressed. Could the kinetic data derived from the biochemical studies of the actomyosin ATPase reaction mechanism be applied to contracting muscle fibers? Furthermore Huxley (1973) issued a cautionary note at the Cold Spring Harbor meeting when he said that "there is a large gap in our present knowledge, unfortunately right at the heart of the whole problem." The gap concerned the lack of structural evidence for the proposed changes in the angle of cross-bridge attachment to actin during muscle contraction. This was a enormous problem that would hold up progress in much of the muscle field throughout the 1970s and early 1980s. In fact looking back on this period Huxley (1996) has commented that "…by the mid-1980s, confidence in a straightforward sliding filament mechanism for muscle contraction had been significantly eroded…". What happened? Why was there skepticism about the sliding filament mechanism of contraction? Between the stagnant 1980s and early twenty-first century there was a spectacular revolution in muscle research that could not have been predicted by even the most ardent dreamer. The investigation of the mechanism of contraction moved from the study of the behavior of billions of cross-bridges in muscle fibers to the investigation of the mechanical properties of individual molecular motors. This work combined with the elucidation of the atomic structures of actin and myosin and the advent of mutagenesis approaches supercharged what would now be called the motility field. But it was still a major challenge to elucidate the mechanism of action of cross-bridges in muscle fibers. In 2004, Hugh Huxley finally proclaimed (Huxley 2004): "…I really do believe that, altogether, there is now incontrovertible evidence for the correctness of the tilting lever-arm model, although of course many important details still remain to be worked out." (Huxley 2004. With permission John Wiley & Sons Inc) What was the incontrovertible evidence? Was the mechanism of muscle contraction finally solved? These and other issues will be considered in this chapter.

9.2 Kinetics of Actomyosin ATPase in Muscle Fibers: Relationship to the Cross-Bridge Cycle

Based on transient kinetic studies, by the early 1980s there was a well developed scheme of the reaction sequence of actomyosin ATPase in solution (see Chap. 5). A condensed version of this scheme is:

$$\begin{array}{l} \mathrm{AM + ATP \leftrightarrow AM.ATP \leftrightarrow AM.ADP.P_i \leftrightarrow AM.ADP \leftrightarrow AM} \\ \mathrm{M + ATP \leftrightarrow\ M.\overset{\updownarrow}{A}TP \leftrightarrow\ M.AD\overset{\updownarrow}{P}.P_i \leftrightarrow M + ADP + P_i} \end{array}$$

[At physiological ionic strengths, the rate of dissociation of the AM.ATP complex to A and M.ATP is so rapid and complete that virtually all cleavage occurs on the dissociated myosin. Thus ATP (the actual substrate is Mg.ATP, abbreviated here as ATP for convenience) cleavage by AM is often omitted from the above scheme (Gordon et al. 2000) (see Chap. 5).] It was generally agreed that in solution and in resting muscle fibers ATP hydrolysis by myosin alone was rapid and that the rate limiting step in the reaction sequence was the release of P_i and then ADP. The challenge became the determination of the extent to which the solution scheme could be applied to muscles when myosin, i.e., the cross-bridge, interacted with actin and force was generated and/or work performed. There were two fundamental questions to consider. First, is the solution actomyosin ATPase mechanism the same as that occurring in a contracting muscle fiber? The mechanism might be different since fibers contain a specific geometric arrangement of the contractile proteins in a fiber lattice along with other regulatory and structural proteins. Second, do the mechanical constraints (force and displacement) modify specific reaction rate constants? This almost certainly must be the case since muscle force and the rates of enthalpy production (heat + work) and ATP hydrolysis of the muscle vary with shortening velocity (the Fenn Effect, see Chap. 5). Plus based on thermodynamic arguments, it has been concluded that the rate constants governing the transitions of force-generating cross-bridges must be affected by cross-bridge strain (Hill 1974 and for a more readable account see Goldman and Brenner 1987). A cross-bridge that is generating force is strained, and its free energy content will vary with the amount of strain. This means that the change in free energy in the transition from the nonforce-producing state to the force producing state will depend on the amount of strain and with it the equilibrium constant for the transition. Since $Keq = k_+/k_-$, the cross-bridge strain will alter the rate constants of the transition. For example, if the strain is increased by imposing a load on a fiber, then the force-generating reaction will tend to be driven backwards, and Keq will decrease. If the strain is decreased by allowing the fiber to shorten, then Keq will increase. A change in the equilibrium constant must be reflected in changes in either or both the forward and reverse rate constants (since $Keq = k_+/k_-$) (Homsher and Millar 1990). Thus it was essential to determine actomyosin ATPase kinetics in muscle preparations capable of generating force and doing work.

9.2.1 Generation of Caged ATP

A core feature of the kinetic studies in solution is the ability to rapidly mix reactants, in a couple of milliseconds, and then monitor the time course of the subsequent reaction. The logical choice of muscle preparations would be the Szent-Gyorgyi glycerol extracted muscle fiber or the Natori skinned muscle fiber preparation. These single muscle fibers generate force comparable to intact fibers and allow control of the intracellular environment. The main limitation is that due to the typical fiber diameters of 50–100 μm, solution changes result in equilibration limited by

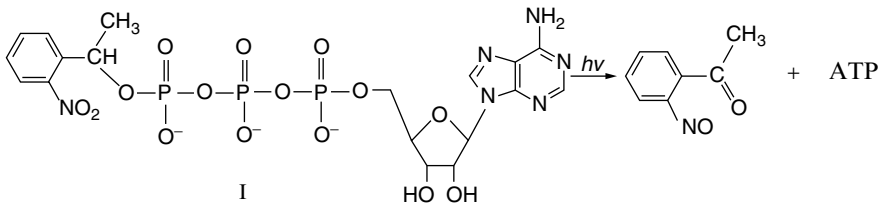


Fig. 9.1 The inert precursor of ATP, P^3-1-(2-nitro)phenylethyladenosine-5′-triphosphate, I, termed caged ATP in response to a pulse of laser light at 347 nm produces ATP and 2-nitrosoacetophenone at a rate of about 100 s^{-1} at 20 °C (Goldman et al. 1982. With permission Nature Publishing Group)

diffusion of hundreds of milliseconds. Thus diffusion precluded the investigation of actomyosin kinetics in fibers.

An ingenious technique was developed that circumvented this problem. The breakthrough came not from the muscle field but rather from the transport field. In 1978 Jack H. Kaplan working with Bliss Forbush III and Joseph F. Hoffman in the department of physiology at Yale University reported the generation of a compound that they called "caged ATP" (Kaplan et al. 1978). They based their approach on established chemical procedures where functional groups were protected during chemical synthesis and subsequently unprotected by photolytic irradiation. They protected ATP, i.e., made it biologically inert, by attaching a 2-nitrobenzyl derivative as a photolabile protecting group to the terminal phosphate of ATP. The resulting compound was P^3-1-(2-nitro)phenylethyl-ATP (caged ATP or NPE-caged ATP). With illumination in aqueous solution with 340 nm light, 50 % of the ATP was set free in about 6–9 s. Also generated were 2-nitrosoacetophenone and a proton, depending on the ionization state of ATP. It was found that the addition of glutathione was necessary to protect against the deleterious effects of the 2-nitrosoacetophenone which apparently interacted with protein sulfhydryl groups. They examined the biological function of this caged ATP as a stimulant of the Na,K-ATPase by introducing it into permeabilized red blood cells, then resealing the cells and irradiating them. The liberated ATP activated the pump. Figure 9.1 shows caged ATP and the reaction products upon photolysis.

Enzyme kineticist David R. Trentham, then at the University of Pennsylvania, quickly realized that caged ATP and related reactions had great potential for structural and kinetic studies of both intact and soluble biological systems. Thus it was important to define the kinetic characteristics of the photolytic reaction. He and Leo Herbette collaborated with physicists James A. McCray and Toru Kihara at Drexel University in Philadelphia to determine the kinetic characteristics of the photolytic reaction of caged ATP (McCray et al. 1980). The first thing that they did was to examine ways to trigger the reaction as rapidly as possible. They settled on the utilization of a laser that generated a 347 nm pulse in 30 ns. The rate of ATP release is controlled by the so-called "dark reactions", those reactions occurring after the flash photolysis, and for the solutions utilized for the muscle fiber experiments, this rate was about 100 s^{-1} (Goldman et al. 1982). Thus 50 % of the caged

ATP was released upon flash photolysis in about 5–10 ms. This rate is not as fast as the rapid mixing in kinetic experiments in solution but fast enough for most experiments in muscle fibers.

9.2.2 Transient Kinetic Studies of Actomyosin ATPase in Muscle Fibers

ATP binding and cross-bridge detachment Trentham then established a collaboration with biophysicist Yale E. Goldman (Fig. 9.2), also at the University of Pennsylvania. Goldman was an expert in investigating muscle mechanics utilizing single skinned muscle fibers. Together with Mark G. Hibberd and James A. McCray, they reported the first experiments utilizing caged ATP in muscle fibers in 1982 (Goldman et al. 1982). The experimental question was simple but the experiments were technically difficult and the results complex. The question: how does the rate of ATP binding and cross-bridge detachment in muscle fibers compare to the rate of actomyosin dissociation by ATP in solution? The rate in solution was very rapid

Fig. 9.2 Yale E. Goldman received his M.D. and Ph.D. degrees from the University of Pennsylvania in 1975. After postdoctoral training with Robert M. Simmons at University College London, Goldman returned to a faculty position in the department of physiology at the University of Pennsylvania in 1978 and has remained there throughout his career. He has a great interest in designing novel instruments and experiments to address important questions in muscle and motility. He is the past director of the internationally renowned Pennsylvania Muscle Institute and past president of the Biophysical Society (2003–2004). Photo: courtesy Y. Goldman

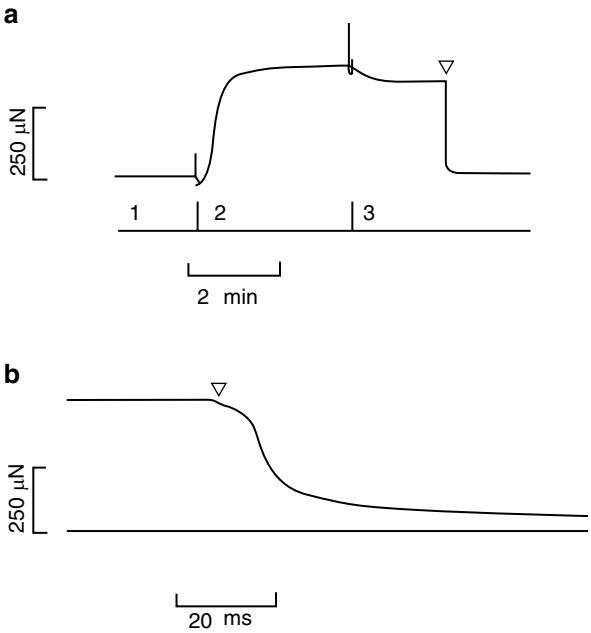


Fig. 9.3 (**a**) Force transient upon photolysis of caged ATP recorded from a skinned psoas muscle fiber in rigor in the absence of Ca^{2+} at 20 °C. The slow time base force record illustrates the experimental protocol. (1) Relaxing solution containing ATP. (2) Rigor solution—same as (1) without ATP. (3) Photolysis solution—same as (1) but without ATP and including caged ATP. (**b**) Fast time base recording of the force transient illustrated in (**a**). The *arrow* indicates the time of the laser pulse. After an initial slow decay, force falls rapidly and then slowly returns to the baseline (Goldman et al. 1982. With permission Nature Publishing Group)

with a second order rate constant of ~10^6 M^{-1} s^{-1} (White and Taylor 1976). A single glycerinated muscle fiber initially was placed in a relaxing solution containing ATP but no added Ca^{2+} (Fig. 9.3). The fiber then was transferred to a solution without ATP which resulted in slow force development as the cross-bridges accumulated in the rigor state. Then caged ATP was added to the solution and allowed to diffuse throughout the fiber. Finally mechanical relaxation was induced by flash photolysis of caged ATP (Fig. 9.3a, slow time base and Fig. 9.3b, fast time base). Unlike the experiments in solution where a single exponential was observed when ATP dissociated actomyosin, the relaxation pattern in the muscle fibers was more complex and more interesting. The relaxation time course exhibited multiple components. During the first phase there was a brief delay only partly due to the photochemical reaction rate of ATP formation, then during the second phase force decreased with a half-time of ~5 ms at 20 °C. Thus ATP detaches cross-bridges rapidly in muscle fibers. To make a kinetic comparison of cross-bridge detachment rate with the rate of actomyosin dissociation in solution, the amount of ATP liberated was varied by altering the intensity of the light pulse. The shape and duration of the first phase depended strongly on ATP concentration whereas the rate of the second phase was

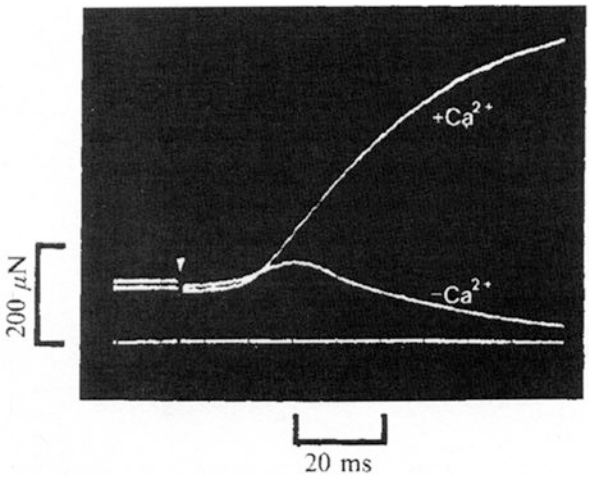


Fig. 9.4 Force transients in a skinned muscle fiber initially in rigor after liberation of ATP in the presence of ($+Ca^{2+}$) or absence ($-Ca^{2+}$) of Ca^{2+}. ATP release in the absence of Ca^{2+} leads to an initial increase in force and then a decay as observed in Fig. 9.3. In the presence of Ca^{2+}, after a brief delay, force increases rapidly to a maximum value. This result suggests that cross-bridge detachment and subsequent force development are rapid steps in the cross-bridge cycle (Goldman et al. 1982. With permission Nature Publishing Group)

independent of ATP concentration. The force during the first phase might remain nearly constant (as in Fig. 9.3b) or if less ATP was released by the pulse, the force in this phase might transiently increase even in the absence of Ca^{2+} (see Fig. 9.4). Goldman et al. (1982) explained this unexpected force generation in the following way. They developed a model in which there is cooperative cross-bridge binding to the thin filament as first suggested by Bremel and Weber (1972) (see Chap. 6). After ATP generation, cross-bridges detach but can then immediately reattach and proceed to generate force even in the absence of Ca^{2+} because the thin filament is still switched on by the remaining attached cross-bridges. The slow final phase of relaxation was likely due to some detached cross-bridges re-attaching again and delaying the final force decay. Their results could be explained if the second order rate constant for the detachment process of rigor bridges induced by ATP was $\sim 2 \times 10^5\ M^{-1}\ s^{-1}$. Thus the rate of cross-bridge detachment from rigor by ATP in muscle fibers was very rapid as predicted by the solution studies. Detachment rate was predicted to be about $600\ s^{-1}$ at a typical ATP concentration in a muscle fiber of 3 mM. Quantitatively the second order rate constant was within an order of magnitude of that observed with solubilized proteins ($\sim 10^6\ M^{-1}\ s^{-1}$). They found that cross-bridge stress had a small but significant effect on the detachment rate, i.e., greater stress led to a faster cross-bridge detachment rate.

They also did experiments in the presence of Ca^{2+}. The fiber in rigor was transferred to a caged ATP solution that contained Ca^{2+}. A typical result is shown in Fig. 9.4. In this example force during the first phase after photolysis remained about the same as observed in the absence of Ca^{2+} but in the second phase force increased rapidly until full isometric force was developed. The rate of force generation in the

second phase was 80–100 s^{-1} at 20 °C. These rapid rates of cross-bridge detachment and reattachment were much faster than the 1–3 s^{-1} steady state turnover rate of ATP hydrolysis measured in fibers. The results were published in full in 1984 (Goldman et al. 1984a; b). The main conclusions that can be drawn from these first studies utilizing caged ATP to the characterize the kinetics of actomyosin in fibers are: (a) ATP binds rapidly to the cross-bridge and produces cross-bridge detachment, (b) cross-bridge detachment in the absence of Ca^{2+} and subsequent cross-bridge reattachment in the presence of Ca^{2+} are much faster than the steady state rate of ATP hydrolysis and thus are not rate limiting, (c) thus the rate limiting step in the cross-bridge cycle occurs somewhere between force generation by the cross-bridges and ATP rebinding and (d) a cooperative mechanism allows transient force generation even in the absence of Ca^{2+}. Finally the results were in general qualitative agreement with the observations of actomyosin kinetics in solution.

Transient kinetics of ATP hydrolysis in muscle fibers Michael A. Ferenczi and David Trentham at the University of Pennsylvania working with Earl Homsher (see Fig. 5.16) at UCLA collaborated to perform "heroic" experiments to measure the kinetics of ATP cleavage in skinned single muscle fibers or small bundles (Ferenczi et al. 1984; Ferenczi 1986). This study was analogous to the solution kinetic studies of Lymn and Taylor in 1971 (see Fig. 5.20). Ferenczi and colleagues incubated muscle fibers that were in rigor in a tritiated caged ATP solution in the presence of Ca^{2+}. The tritiated ATP was released by flash photolysis and, at various times following photolysis, the fibers were frozen rapidly in a miniaturized freeze clamp device. The amounts of ATP, ADP, AMP, and caged ATP were measured in the frozen fiber extract. These so-called "flash and smash" studies showed the following features. ATP splitting occurred in a biphasic fashion: a rapid ATP hydrolysis (a phosphate burst) followed by a slower steady-state rate. The phosphate burst rate was similar in the presence or absence of Ca^{2+} and this rate was more than 20-fold greater than the steady state hydrolysis rate in the presence of Ca^{2+} and more than 350-fold greater than the steady state rate in the absence of Ca^{2+}. The rate of the phosphate burst was greater than the rate of force development in the presence of Ca^{2+} or the rate of force decline in the absence of Ca^{2+}. Thus the ATP hydrolysis step must precede cross-bridge attachment. Further, since the steady state hydrolysis rate is less than the rate of cross-bridge attachment, the rate-limiting step must occur after cross-bridge attachment. These results and conclusions were consistent with the observation of transient kinetics of actomyosin ATPase in solution (Lymn and Taylor 1971).

The general conclusion based on the above results is that the ATP binding, cross-bridge detachment and ATP hydrolysis steps in muscle fibers are similar to those in solution.

Phosphate release and force generation in muscle fibers In the classic studies of Lymn and Taylor (1971), they suggested that it was natural to propose a relationship between the kinetics of actomyosin ATPase activity in solution and the cross-bridge cycle in muscle as conceived by Hugh Huxley (see Fig. 5.21). In this depiction, the power stroke was associated with the release of products (first P_i and then ADP) from actomyosin. White and Taylor (1976) determined in solution studies that more

than half of the chemical free energy of ATP was dissipated during the release of P_i. This step was strongly favored and thus irreversible. They suggested that it was during the release of P_i that the power stroke occurred. Furthermore Cooke and Pate (1985) showed that isometric force production in skinned fibers was depressed by increases in P_i in the physiological range. They went on to suggest that muscular fatigue may be related to increases in P_i and H^+ with prolonged muscle activity.

Therefore there was considerable interest in determining the kinetics of P_i release and its possible relationship to force generation in fibers. Mark G. Hibberd working with Jody Dantzig, Yale Goldman and David Trentham examined the effects of P_i on the kinetics of contraction and relaxation in muscle fibers (Hibberd et al. 1985). They employed a protocol similar to that used for the earlier cross-bridge detachment studies with caged ATP. A fiber in rigor was incubated in a caged ATP solution with or without Ca^{2+} and in the presence of varying concentrations of P_i in the physiological range. Upon photolysis of ATP in the absence of Ca^{2+}, the initial rate of cross-bridge detachment was not influenced by P_i but the slower final phase of force decline was accelerated by P_i. In the presence of Ca^{2+}, P_i accelerated the rate of force development upon photolysis of caged ATP but reduced the final force to a level predicted by the concentration of P_i. To explain these results, they proposed that force production accompanied P_i release and that the force generating step in muscle was reversible. If attachment and force generation were reversible, then the observed rate constant for force redevelopment in the presence of Ca^{2+} would be given by the sum of the forward and reverse reaction rates. An increase in P_i concentration would then increase the rate constant for force redevelopment by increasing the rate of reversal of P_i release and would decrease steady state force by shifting the distribution of cross-bridge states toward AM.ADP.P_i. In the absence of Ca^{2+}, P_i accelerates the final (slow) rate of force decline by accelerating the reversal of the force generating step. Thus they concluded that during energy transduction, the formation of the force-generating cross-bridge state was coupled to the release of P_i in a reaction that was readily reversible. This was a novel conclusion and contrary to the results in solution.

Clearly P_i was intimately associated with force development and more information was needed about its role. Neal Millar, Joan Lacktis and Earl Homsher at UCLA and Jody Dantzig and Yale Goldman at the University of Pennsylvania started to the examine the kinetics of P_i release in skinned fibers utilizing the novel compound caged P_i. They joined forces and produced an influential paper (Dantzig et al. 1992). This approach was a kinetic perturbation study in fibers as done in solution. Unlike the caged ATP studies where the reaction was started from rigor, they activated skinned muscle fibers to produce a steady isometric force in the presence of caged P_i. They then examined the time course of the decrease in force (the P_i transient) upon photolytic release of P_i. The kinetics of the force decline should relate to the kinetics of the forward and reverse reactions for P_i release. The traces were complex. After a brief delay, there was an exponential fall of force and a later small rebound of force. It was the exponential fall of force that they examined in detail and related to the amount of P_i released upon photolysis. The rate of the P_i transient increased as P_i concentration increased. The increase was not linear but rather approached an asymptote. This hyperbolic result ruled out a single step process for P_i release. They proposed that phosphate

release was a two-step process, an initial protein isomerization step, producing force, that was followed by the release of P_i. They proposed the following scheme:

$$AM.ADP.P_i \leftrightarrow AM^*ADP.P_i \leftrightarrow AM^*.ADP$$

(An isomerization does not result in a change in the atoms in the molecule but some internal rearrangement, a conformation change.) At the same time Kawai and Halvorson (1991) analyzing sinusoidal length perturbations in muscle fibers came to the same conclusion that P_i release must be a two step process.

Homsher et al. (1997) went on to determine the rate of the P_i transient during muscle shortening. In these experiments a muscle fiber was fully activated and then allowed to shorten at a fixed velocity. Since it was known from the work of Ford et al. (1985) that fiber stiffness decreased less than force during shortening, the average strain on the cross-bridges also must decrease during shortening. They observed that the P_i transient rate increased dramatically during shortening. This result suggested that the sum of the forward and backward rates of the force generating isomerization increased as the strain on the cross-bridge was reduced. Thus the "P_i release step" (force generating isomerization + P_i release) was highly strain-dependent. The thermodynamic argument above indicated that as the strain on the cross-bridge falls (as in shortening), Keq ($=k_+/k_-$) must rise, either by an increase in the rate of the force-generating step or a fall in the rate of reversal of the force step. Since the rate of the P_i transient increased during shortening, Homsher et al. (1997) concluded that the rate of the force-generating step probably increased during shortening. Thus these results provided a qualitative explanation of the increased rate of ATP hydrolysis seen during muscle shortening (the Fenn Effect). Also the data strongly supported the idea that the production of force by the muscle is intimately related to the P_i release step.

ADP release It is natural to ask: what is the rate-limiting step in the cross-bridge cycle during an isometric contraction and during muscle shortening? Following force generation there must be a step (or steps) that controls the lifetime of the attached cross-bridge, and the rate of this step must depend on whether or not the muscle is shortening. In a fiber contracting isometrically, the lifetime of the attached state should be long to minimize the ATP consumption. If the muscle is shortening, however, the lifetime must be reduced, otherwise the attached cross bridges would inhibit shortening and power output. In a muscle shortening near its maximum velocity, it has been estimated that that cross-bridges remain attached for only a few milliseconds. In an influential study, Raymond F. Siemankowski, Meganne O. Wiseman and Howard White (1985) suggested that ADP release was the rate limiting step during the maximum velocity of muscle shortening. They found that the rate of ADP dissociation from actomyosin in solution (AM.ADP) was positively correlated with the maximum shortening velocity in muscle fibers over a wide range of velocities. They argued that a comparison of the solution kinetics and maximum velocity of shortening was reasonable because cross-bridges would by under minimum strain during rapid shortening and thus somewhat similar to the solution conditions. It should be emphasized that AM.ADP is not the same as AM*.ADP in the

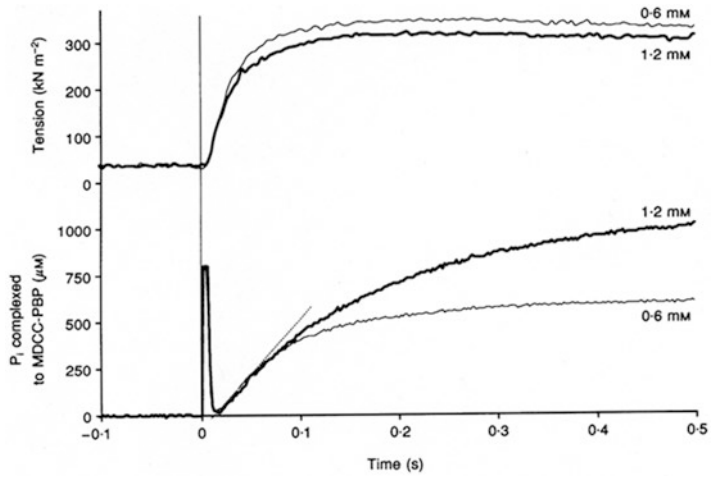


Fig. 9.5 Time course of force and P_i release in a skinned muscle fiber initially in rigor after photolysis of caged ATP in the presence of Ca^{2+} at 20 °C. Records were superimposed at the time of the laser flash. After the laser artifact, the fluorescence signal from the phosphate binding protein (in two different concentrations) indicates that Pi release increases at an initial rate slower than the rate of rise of force. This result suggests that cross-bridge force development occurs before P_i release (He et al. 1997. With permission John Wiley & Sons Inc.)

scheme above as was shown by Sleep and Hutton (1980). Thus this "ADP release step" may include first an isomerization step (AM*.ADP to AM.ADP) and then ADP release. Dantzig et al. (1991) provided evidence that suggested that the isomerization step preceding ADP release was strain dependent, i.e., ADP release was accelerated with decreased cross-bridge strain. It often has been stated that the ADP release step is slow but in reality it might be the preceding conformational change that is slow. Is the ADP release step also rate limiting during an isometric contraction or possibly could it be the strain dependent rate of release of P_i or some other step? There seems to be no general agreement.

Things were getting complicated. Steps were being added in the cross-bridge cycle and the elusive rate limiting steps could not be measured directly. Models were constructed that required assumptions that sometimes were difficult to test. Nonetheless it was clear that the P_i release step was strain dependent and closely associated with muscle force generation.

Phosphate binding protein and measurement of ATPase kinetics in muscle fibers
What was needed was a direct measure of P_i release kinetics. Such a measure would not only be useful in assessing various cross-bridge states during contraction but would also provide a time-resolved measurement of ATP hydrolysis during contraction. Martin R. Webb and his colleagues at the National Institute for Medical Research, Mill Hill, London developed a fluorescent phosphate binding protein (FPBP), which bound P_i at rates of ~1,000 s^{-1} with a Michaelis constant (Km) of <1 μM (Brune et al. 1994). Michael Ferenczi and his collaborators at Mill Hill were the first to utilize the phosphate binding protein to achieve a time-resolved

measurement of phosphate release in skinned muscle fibers (Fisher et al. 1995; He et al. 1997). Fibers could be incubated in FPBP in the absence of Ca^{2+} and activated by photolysis of caged calcium or put into rigor and contraction induced in the presence of high Ca^{2+} by photolysis of caged ATP. They found that the generation of force following the photolysis of caged ATP in high Ca^{2+} preceded P_i release (Fig. 9.5). This finding suggested that cross-bridge state AM.ADP.P_i preceding the release of P_i actually generated force which supported the idea that force development involved a transition prior to the release of P_i as proposed by Dantzig et al. (1992). This phosphate binding protein also has permitted measurement of the time course of P_i release from the strongly bound AM.ADP.P_i state in actomyosin solutions and myofibrils. Takagi et al. (2004) have discussed the results of some of these experiments as they relate to the coupling of P_i release to force development.

By the beginning of the twenty-first century much had been learned about the kinetics of actomyosin ATPase in solution and in muscle fibers. The introduction of the use of caged compounds[1] and a phosphate binding protein allowed the solution studies to be transposed into muscle fibers. The results from solution studies were generally confirmed in muscle fibers with the important addition of the strain dependence of P_i release. Nonetheless there are certain unresolved issues of importance still to be addressed. There does not seem to be a general agreement as to the rate-limiting step(s) in the cross-bridge cycle during isometric and working contractions. Also there are no probes that monitor directly the proposed isomerization steps in the cross-bridge cycle. Gordon et al. (2000) have provided an overview of the myosin and actomyosin ATPase cycles in solution and in muscle fibers.

9.3 Testing the Swinging-Tilting Cross-Bridge Model of Muscle Contraction: The Difficult Early 1980s

9.3.1 Probes of Cross-Bridge Movement: Structural Techniques Designed to Sense Cross-Bridge Movement During Muscle Contraction

The challenge after 1972 was to prove that cross-bridges attached to actin and went through a swinging-tilting power stroke during contraction. It wasn't feasible to extend electron microscopy to contracting muscle and interpretation of X-ray diffraction experiments required a detailed model of the structure of the cross-bridge. New ideas were needed. If it was possible to monitor a probe, either intrinsic or extrinsic, that sensed the orientation of the cross-bridge in space, it might then be

[1] Some other caged compounds besides caged ATP and caged P_i utilized in muscle research include: caged ADP, caged AMP, caged ATPγS, caged IP_3, caged calcium (DM-nitrophen and nitre-5), caged calcium chelators (caged BAPTA and diazo-2) (Homsher and Millar 1990). A variety of biophysical signals, including mechanical, biochemical and structural changes, have been recorded with photolysis of caged compounds (Dantzig et al. 1998).

possible to determine the change in orientation of the cross-bridge during contraction. The probes that were most extensively investigated possessed either fluorescent or electron paramagnetic resonance (EPR) properties.

Starting in the late 1960s, Manuel F. Morales and his colleagues[2] at the University of California at San Francisco pioneered the use of intrinsic and extrinsic fluorescent probes to monitor cross-bridge orientation. John F. Aronson and Morales (1969) suggested that polarized tryptophan fluorescence of muscle fibers might provide a means of detecting differences in cross-bridge attitudes in different physiological states. This possibility was explored in depth by Cristobal G. dos Remedios, Ronald G. C. Millikan and Morales (1972a). Muscle fibers, glycerinated or intact, were illuminated with polarized ultraviolet light perpendicular to the long axis of the fiber. The emitted polarization of tryptophan fluorescence was recorded. The magnitude of the perpendicular polarization of tryptophan fluorescence varied with the state of the fiber, that is, the magnitude during relaxation>contraction>rigor. Since they deduced that majority of tryptophans in the muscle were located in the S-1 portion of myosin and none in the S-2 portion of myosin, they concluded that the changes observed resulted from changes in orientation of the S-1 segments of myosin. These results provided the first definitive demonstration of differences in the rotational motions of myosin heads in muscle fibers during relaxation, contraction and rigor. Although the tryptophan signal was strong and its use did not require chemical perturbation of the proteins, it was hard to interpret the results in detailed terms, basically because myosin S-1 contains several tryptophans (about 40) in unknown arrangement. Labeling of fibers with an extrinsic fluorescent probe that was specifically and uniquely attached to the reactive thiol (SH1) of S-1 in fibers offered a promising alternative, and was soon used in a full-scale fiber study by Julian Borejdo and Susan Putnam (1977).

The first polarized fluorescence investigation that suggested that cross-bridges may not rotate during contraction was reported by Toshio Yanagida (1981) (Fig. 9.6) at Osaka University. He had been working with Fumio Oosawa utilizing fluorescent nucleotides to study the flexibility of F-actin filaments. Yanagida applied fluorescent nucleotides to investigate cross-bridge orientation in rigor and during contraction of glycerinated muscle fibers. The nucleotides included 1:N^6-etheno-ATP and -ADP (ε-ATP and ε-ADP). In resting fibers in the presence of ε-ATP, the distribution of angles of the fluorescent nucleotides was almost random. In contrast when fibers were put in rigor with a low concentration of ε-ADP, the polarized fluorescence indicated the cross-bridges were at a fixed angle with respect to the long axis of the fibers. During development of isometric force in the presence of ε-ATP and Ca^{2+}, the nucleotide angle was unchanged. Yanagida concluded that if the rotational movement occurred, the life-time of the bound state of the cross-bridge with a fixed angle to F-actin is long or the angle of rotation was very limited. There was more trouble ahead for the straightforward rotating cross-bridge model.

[2] Manuel Francisco Morales (1919–2009) was a strong supporter of Japanese muscle biochemists. He was one of a small number of scientists, including John Gergely, from the United States who attended the first international conference on the chemistry of muscular contraction held in Japan in 1957. For his many contributions to Japanese science, he received the Order of the Rising Sun in Japan in 1989. He was elected to membership in the National Academy of Sciences in 1975. His publications span 66 years with his last publication at 88 years old. (Cooke and Highsmith 2011)

Fig. 9.6 Toshio Yanagida (1946–) received his Ph.D. in engineering science in 1976 working with Fumio Oosawa at Osaka University. Yanagida has pioneered the development of numerous techniques to monitor and quantify motion and force at the single molecule level. His ideas are sometimes controversial but his technical expertise is unquestioned. Many of his publications have appeared in the most visible and important journals, including Nature, Science and Cell. He has remained at Osaka University throughout his research career and is a specially appointed professor and head of the laboratory of nanobiology. Yanagida has received many awards for his research including the US Genomic Award for Outstanding Investigator in the Field of Single Molecule Fluorescence Microscopy. Photo: courtesy T. Yanagida

In the 1960s Harden McConnell at Stanford University introduced the idea of utilizing stable free radicals bound to biomolecules to monitor molecular motion of macromolecules and supramolecular assemblies, such a membranes (McConnell and McFarland 1970). These nitrosyl-type free radicals, called spin labels or spin probes, sense the orientation of a molecule with respect to an applied magnetic field because the stable free radicals absorb microwave radiation when they are placed in a magnetic field. In the late 1960s, Roger Cooke came to the Morales laboratory as a postdoctoral fellow and they explored the possibility of using spin labels and EPR to determine cross-bridge orientation. Cooke and Morales (1969) published the first experiments utilizing spin-labels attached to the SH1 of myosin S-1 in muscle fibers. David D. Thomas received a Ph.D. from Stanford University under the guidance of McConnell in 1975. He then went to the Boston Biomedical Research Institute to work with John Gergely and John C. Seidel who had been employing EPR to investigate the motion of isolated myosin. Thomas moved on to a faculty position at the University of Minnesota and Cooke to a faculty position at the University of California at San Francisco.

Cooke and Thomas came together in a collaboration in the early 1980s that resulted in two papers that have been very influential in the history of cross-bridge investigation. They utilized EPR spectra to study the orientation of spin labels selec-

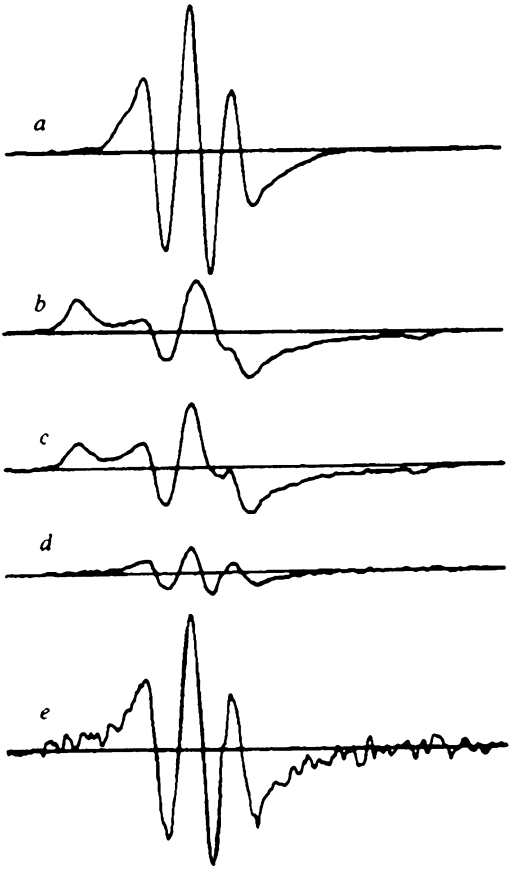


Fig. 9.7 Spectra of spin labels attached to the reactive sulfhydryl (SH1) of myosin S-1 in glycerinated psoas muscle fibers in (**a**) rigor, (**b**) at rest and (**c**) during contraction. Data is plotted as the first derivative of energy absorption by the spin labels with respect to magnetic field strength versus the strength of the magnetic field with the muscle fibers oriented parallel to the field. (**d**) A difference spectrum obtained by subtracting 81 % of spectrum (**b**) from (**c**). (**e**) Spectrum d magnified by 5.3-fold so that it represents the same number of probes as spectra (**a**)–(**c**). Fibers in rigor exhibit a sharp spectrum (**a**) indicating that the spin probes were well ordered with a narrow distribution of angles. Fibers at rest displayed a spectrum (**b**) consistent with nearly complete orientational disorder of the spin probes. The spectrum of contracting fibers (**c**) could be explained if 20 % of the spin labels were well ordered as in rigor (spectrum a) and 80 % disordered as at rest (spectrum **b**) (Cooke et al. 1982. With permission Nature Publishing Group)

tively and rigidly attached to myosin heads in glycerinated psoas muscle fibers isolated from rabbits. Spectra from labeled fibers oriented parallel to the magnetic field yielded directly the distribution of spin label orientations relative to the fiber axis. In rigor fibers at complete filament overlap, the spin labels displayed a narrow angular distribution (Fig. 9.7a). Thus it appeared that virtually all myosin heads in rigor fibers had the same orientation relative to the fiber axis, and this orientation was determined by the actomyosin bond. In light of the mismatch of actin and myosin

helices, this uniform probe orientation could only be possible if the portion of the cross-bridge between the probe (at SH1) and the thick filament core was flexible. Addition of ATP to the rigor fibers under relaxing conditions produced orientational disorder, resulting in a spectrum almost indistinguishable from that of an isotropic distribution of probes (Fig. 9.7b) (Thomas and Cooke 1980). But it was the second paper that promoted further erosion in confidence in the swinging-tilting cross-bridge model of contraction as proposed by Huxley (1969). Cooke et al. (1982) now investigated the orientation of cross-bridges during muscle contraction with EPR (Fig. 9.7c–e). What they found was striking. In their words (Cooke et al. 1982. With permission Nature Publishing Group):

> We show here that during the generation of isometric tension, ~80 % of the probes display a random angular distribution as in relaxed muscle while the remaining 20 % are highly oriented at the same angle as found in rigor muscle. These findings indicate that a domain of the myosin head does not change orientation during the power stroke of the contractile interaction.

These results and conclusion "flew directly in the face of dogma". They were careful to state that "a domain of the myosin head does not change orientation". They went on to speculate:

> There are many different models that fit the conclusion of this study. One possibility is that the entire myosin head maintains a constant orientation during the power stroke, while some other part of myosin shortens; one example of such a model has been proposed… Alternatively, the probed region of myosin may remain rigidly oriented on actin, while another domain of the myosin head does rotate. There is, as yet, insufficient evidence to exclude either possibility.

The proposed model that they referred to was the one developed by Harrington (1971) at Johns Hopkins University. He proposed a mechanism of contraction where the force-generating site was located not within the myosin head but rather within the core of the thick (myosin) filament, specifically within the trypsin-sensitive hinge region of the myosin rod. The force-developing mechanism was suggested to be the transfer of energy from ATP splitting in the globular head of one molecule directly to the hinge region of an adjoining molecule, resulting in a phase transition from crystalline to amorphous within the hinge segment of the second molecule. This "melting" of the myosin structure during contraction would generate a relative sliding motion of the filaments.

Thus the results of two different kinds of probes, spin probes attached to SH1 and fluorescent nucleotides, agreed in suggesting that the regions of the molecule that they monitored did not appear to move during muscle contraction.

9.3.2 A Fundamental Problem: Asynchronous Cross-Bridge Movement

A basic problem with all the studies that examined cross-bridge motion was the realization that the cross-bridges acted asynchronously. Thus the results would give an average over the whole range of cross-bridge states. This effect would not explain

the results of Yanagida and Cooke and Thomas. Nonetheless a way to synchronize the cross-bridges during contraction was highly desirable. One way to synchronize the cross-bridges came from the experiments of Huxley and Simmons (1971). When a muscle fiber contracting isometrically was shortened by less than 0.4 % in 0.2 ms, force dropped to near zero and then partially recovered rapidly in the next 2–5 ms and then slowly thereafter (see Fig. 5.4 and associated text). Later Ford et al. (1974) determined that the stiffness of the muscle fiber was the same during the isometric contraction and during rapid partial recovery of force. From these results, they proposed that the step shortening did not dissociate cross-bridges but rather drove them into a low force generating state from which they rapidly recovered in a more or less synchronous fashion. These results implied that if one could monitor changes in cross-bridge structure under these conditions and on the same time scale, it might be possible observed cross-bridge rotation[3]. This was the goal of the X-ray diffraction study of Huxley and colleagues (1983). They utilized powerful synchrotron radiation as a high intensity X-ray source to obtain millisecond resolution of changes in the X-diagram with step shortening of a frog skeletal muscle. They found a major decrease in the intensity of the 14.3 nm meridional reflection (see Fig. 6.5) within 1 ms of the change in force which they attributed to "specific configurational changes in the cross-bridges during the working stroke". They proposed a model in which the 14.3 nm intensity in a contracting muscle arises mainly from the attached cross-bridges and is generated by the part of the myosin head near the S1–S2 junction. They speculated that one end of the cross-bridge remained at the same axial level during the working state and the other end moved axially with the actin. Thus the long "honeymoon" for the swinging-tilting cross-bridge model dating to 1969 was over. Even Hugh Huxley now believed that at least part of cross-bridge did not change its orientation with respect to actin during muscle contraction.

Other attempts to at least partially synchronize cross-bridges have been made. One approach is to place a muscle fiber in rigor and start the contraction in the presence of Ca^{2+} by releasing caged ATP. Dantzig et al. (1998) list structural techniques that have been coupled with caged ATP photolysis. Another approach is to use nucleotide analogs, the development of which was pioneered by Ralph G. Yount at Washington State University, to promote or prolong an intermediate position in the cross-bridge cycle (Yount et al. 1971; dos Remedios et al. 1972b). These approaches have been attempted with varying degrees of success. Looking back on this era, Simmons (1992) has commented: "The main complication is that the action of cross-bridges is never truly synchronized, so what is observed comes from the overlapping cycles of many cross-bridges working in parallel."

[3] It was not possible to record an EPR spectrum during a mechanical transient in the early 1980s. The EPR technique required data collection of 1 min or more and thus was limited to steady states: rigor, rest, steady contraction. Fluorescence polarization (Irving et al. 1995) and the intrinsic technique of birefringence changes (Irving 1993) (See Chap. 1, footnote number 2 and associated text) do have the required sensitivity and temporal resolution.

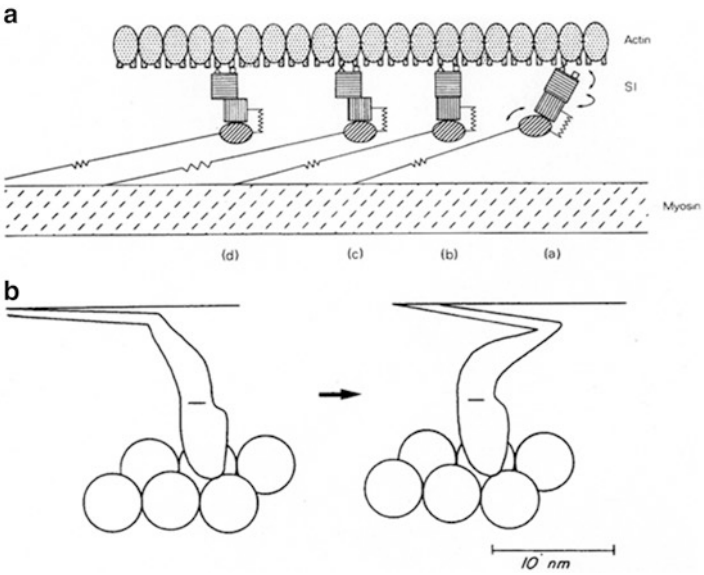


Fig. 9.8 Schematic representations of proposed cross-bridge configurations during muscle contraction. (**a**) Shows the cross-bridge in a weakly bound (*a*), then strongly bound (*b*), then force-generating (*c*) and then rigor (*d*) state (Huxley and Kress 1985. With permission Springer). (**b**) A hypothetical model for the changes in cross-bridge conformation that occur during the power stroke. The configuration shown on the left represents the beginning of the power stroke (Cooke 1986. With permission Informa Healthcare). In both models it is assumed that the massive end of S-1 attaches to actin and has an orientation that remains constant during the power stroke while the orientation of the narrow neck region changes

9.3.3 A Refined Cross-Bridge Model: Some Innocence of the Original Model Was Lost

Both Huxley and Kress (1985) (see Chap. 6, footnote 9) and Cooke (1986) proposed a refined cross-bridge model based on the results of the early 1980s (Fig. 9.8). The models are very similar. The key feature is that the cross-bridge is envisioned to be flexible with a portion attached to actin that does not move relative to actin during the working stroke but another portion of the head that rotates during contraction. But as stated above the data could not rule out the type of model proposed by Harrington (1971) where the cross-bridge does not move at all but the S-2 segment shortens reversibly.

9.3.4 More Complications for the Sliding Filament Model

In the 1980s things were getting complicated. Some of the solution biochemistry studies led to the conclusion that there might be a non-dissociating pathway for ATP hydrolysis by actomyosin (see Chap. 5) in contrast to the simple Lymn and Taylor

(1971) scheme. There was the problem of the unexplained energy, especially during rapid muscle shortening (Homsher et al. 1981) (see Chap. 5). Then a potentially major complication arose from experiments measuring the rate of ATP hydrolysis during rapid sliding of actin and myosin filaments under zero load. Yanagida, Toshiaki Arata and Fumio Oosawa (1985) devised an intriguing experiment. They treated isolated myofibrils from crab leg muscle in a way that digested the Z-band, leaving isolated single sarcomeres. Crab muscles have long sarcomeres (~7 μm) and long thin (~2.7 μm) and thick (~4.8 μm) filaments. They then labeled the thin filaments with the toxin phalloidin that had a fluorescent probe attached. [This was a technique that Yanagida et al. (1984) reported the year before that would become crucial in the development of in vitro motility assays (see later in this chapter).] The sarcomeres shortened slowly near 0 °C facilitating measurements of velocity of sliding in the fluorescence microscope. In parallel experiments they measured the rate of ATP hydrolysis. The average sliding distance of a thin filament induced by hydrolysis of 1 mol of ATP was calculated from the sliding velocity of the thin filaments and the ATPase activity during sliding. The answer was very surprising. The average sliding distance of the thin filaments during one ATP cycle was greater than 60 nm! How is this possible since it was thought that a cross-bridge moved about 10 nm during the cross-bridge cycle. Yanagida et al. (1985) proposed various interpretations of the data. One possibility was that there were multiple cross-bridge cycles per each ATP hydrolyzed (so-called loose coupling) during rapid filament sliding. Thus one could no longer be certain of one cross-bridge cycle per one ATP hydrolyzed (tight coupling). But they stated that a more straightforward interpretation of the results was that the range of movement over which a myosin cross-bridge can attach to the actin filament during one ATP hydrolysis cycle under an unloaded condition is at least 600 Å. This was a shocking conclusion. Thus the results and conclusion of Yanagida et al. (1985) were a direct threat to the straightforward sliding filament model of contraction. This conclusion caused great confusion in the muscle field before a plausible alternative interpretation was provided in the 1990s (see next section).

Then there was also the strange case of striated muscle from the *Limulus*, the horseshoe crab. Of course a core tenet of the sliding filament model of contraction is that the filaments slide rather than shorten during contraction. The *Limulus* telson muscle seemed to be an exception. Throughout the 1960s into the 1980s there was evidence that contraction in *Limulus* striated muscle involved both "sliding filaments and shortening thick filaments". The evidence for this conclusion was summarized by Dewey and his colleagues (Dewey et al. 1982). When examined in the electron microscope, thick filaments isolated from resting *Limulus* striated muscle were long. In contrast thick filaments isolated from muscle that shortened during stimulation were about 30 % shorter. At long sarcomere lengths, shortening seemed to occur via filament sliding but at shorter sarcomere lengths, the shortened filaments were observed. Unlike vertebrate striated muscle, the thick filaments of *Limulus* striated muscle had a paramyosin core and thus it was possible that the mechanism of contraction could be different in this "living fossil". In 1985 the idea of physiological shortening of thick filaments in *Limulus* muscle became less likely when it was shown that the shorter thick filaments exhibited the same diameter and same cross-bridge orientation as the longer thick filaments (Levine and Kensler 1985). The authors suggested that the thick filament shortening was due to a dissociation

of about 15 % of each end of the thick filament in a phosphorylation/dephosphorylation dependent process. Thus they proposed that the thick filaments were shorter because of reversible depolymerization of the ends. It was agreed that there was a natural variation of thick filament lengths in *Limulus* muscle. After assessing this evidence, Hugh Huxley concluded (Huxley 1985. With permission Company of Biologists Limited):

> It further supports the likelihood that a variety of filament lengths is always present in these muscles irrespective of their state of activity, and that the apparent predominance of shorter A bands and shorter filaments in muscles which had been caused to contract and shorten was a sampling artefact.

Further support for the natural variation of thick filament lengths came from a study that indicated that there are at least two different fiber types in Limulus striated muscle. One fiber type possessed short A bands and short sarcomeres and another exhibited long A bands and long sarcomeres (Levine et al. 1989). The authors still believed that all thick filaments shortened. This idea did not gain much support and after more than 20 years of investigation, interest in *Limulus* striated muscle contraction seemed to fade away. Nonetheless in the early 1980s this work, at a minimum, suggested an apparent exception to the universality of the sliding filament model.

It was these complications and the negative results relating to excepted changes in cross-bridge orientation during muscle contraction that caused the significant erosion of confidence in the straightforward sliding filament mechanism alluded to by Huxley (1996). But these issues also stimulated some naysayers to question the whole concept of the sliding filament model. Prominent among the naysayers was Gerald H. Pollack, a bioengineer from the University Washington. In Pollack 1983 he produced a critical review of the cross-bridge theory. Besides pointing out issues about the theory that were unresolved at the time, he produced a table citing about 40 publications in which it was claimed that either A band width or thick filament length varied under different conditions. Pre-eminent of these studies were the results from *Limulus* striated muscle (discussed above). When Pollack presented information like this at muscle meetings, Hugh Huxley would stand up and state: "There are a lot of ways of doing the experiment wrong." In the end, Pollack's ideas were not persuasive to the majority and he left the muscle field.

Cooke[4] (1986) and Irving (1987) have written excellent reviews summarizing the state of knowledge regarding the mechanism of muscle contraction through the early 1980s. Thomas 1987 has summarized the results from spectroscopic techniques to investigate cross-bridge rotation in Thomas 1987.

The complications, negative results and naysayers represented the mood at the time. What was needed were entirely new ways to examine the mechanism of cross-bridge action. Also there was a pressing need to elucidate the three dimensional structures of actin and myosin in order to understand their interaction at the molecular level. There was no question that the field was "bogged down". What happened next

[4] Roger Cooke 1997 has written highly influential reviews in 1986, 1997 and 2004 summarizing the state of knowledge regarding the mechanism of muscle contraction.

was absolutely transformational. First came the in vitro motility assays to study cross-bridge movement at a fundamental level, and then came the crystal structures of actin and myosin mixed in with the advent of molecular engineering of muscle proteins.

9.4 In Vitro Motility Assays: Myosin Generated Movement and Force Production In Vitro

Because of the complexity of muscle systems and the erosion of confidence in the straight forward model of muscle contraction emerging in the early 1980s, there was a pressing need to be able to study cross-bridge (i.e. myosin) function in the most fundamental manner. One could perform sophisticated in vitro assays of the kinetics of actomyosin ATPase but, of course, these assays did not assess myosin mechanics. The idea of studying the mechanical behavior of myosin in vitro dates back to the myosin threads generated by H. H. Weber in 1935. Albert Szent-Gyorgyi utilized this preparation to "produce life", i.e., motion, in a test tube in 1942. The experiment required only myosin, newly discovered actin, ATP and some ions. He later recalled that seeing the threads shorten was "perhaps the most thrilling moment of my life" (see Chap. 1 for details).

9.4.1 Myosin Generated Movement In Vitro

Thirty years after Szent-Gyorgyi's classic experiments, James A. Spudich (Fig. 9.9) established as a goal upon accepting his first faculty position at the University of California at San Francisco the development of a quantitative in vitro motility assay to assess myosin mechanics based on the accumulated knowledge of actin and myosin structure and function since the 1940s. His second goal was to try to unravel the mechanism of motility in nonmuscle cells (Spudich 2011). It would take a decade before the break through that he sought would occur. In 1982 Michael Sheetz[5] came from the University of Connecticut to do a sabbatical year with Spudich who was now at Stanford University. They were aware of the likelihood that an actin-myosin based system was involved in cytoplasmic streaming in plant cells like the algae *Nitella*. In fact Yolande Kersey in Norman Wessells's laboratory in the department of biological sciences at Stanford University showed oriented actin cables lying along chloroplast rows in *Nitella* (Kersey et al. 1976). Sheetz and Spudich (1983) cut open a *Nitella* cell longitudinally and pinned the cell wall flat onto an optically clear substratum, exposing the oriented actin cables that are attached to the cytoplasmic face of the cell surface (Fig. 9.10). In order to observe myosin movement,

[5] Michael P. Sheetz shared the 2012 Lasker award for Basic Medical Research with James A. Spudich and Ronald D. Vale "for discoveries concerning cytoskeletal motor proteins, machines that move cargoes within cells, contract muscles, and enable cell movements."

Fig. 9.9 James A. Spudich received a Ph.D. in biochemistry from Stanford University in 1968. After postdoctoral training at Stanford University in genetics and then in structural biology with Hugh E. Huxley at the MRC Laboratory of Molecular Biology in Cambridge, he joined the faculty at the University of California, San Francisco, in 1971. He then moved back to Stanford University as a professor in 1977. Spudich has received numerous honors and awards. Notably he was elected to the National Academy of Sciences in 1991, received the E. B. Wilson award from the American Society of Cell Biology in 2011 and most recently the Lasker Award for Basic Medical Research along with Michael P. Sheetz and Ronald D. Vale "for discoveries concerning cytoskeletal motor proteins, machines that move cargoes within cells, contract muscles and enable cell movements." For a video interview of the Lasker awardees, see: http://www.laskerfoundation.org/awards/2012_b_interview_sheetz.htm (Spudich 2012). Photo: courtesy J. Spudich

rabbit heavy meromyosin (HMM) was attached covalently to inert fluorescent beads that were about 1 μm in diameter and these beads were observed to move in an ATP dependent manner on the *Nitella* actin cables. Thus Sheetz and Spudich (1983) reported the first direct visualization of myosin movement on actin. They went on to speculate about the utility of their in vitro motility assay for understanding myosin function (Sheetz and Spudich 1983. With permission Nature Publishing Group):

> With our *in vitro* assay for myosin movement it is now possible to determine what regulatory factors influence myosin motility *per se*, irrespective of their effects on ATPase activity. Additionally regions of the myosin molecule necessary for motility can be mapped. Thus, the development of this assay provides the basis for the detailed analysis of the biochemical and structural features of myosin that allow it to act in the conversion of chemical energy into the mechanical force of movement.

Maybe not as thrilling as Szent-Gyorgyi's discovery but it was very exciting when Sheetz showed a video at a scientific meeting of the myosin coated beads moving along the actin cables. Now one started to talk about myosin motors running on actin tracks.

As exciting as these results were, Spudich sought a simpler in vitro motility assay system where all of the ingredients could be biochemically defined and controlled.

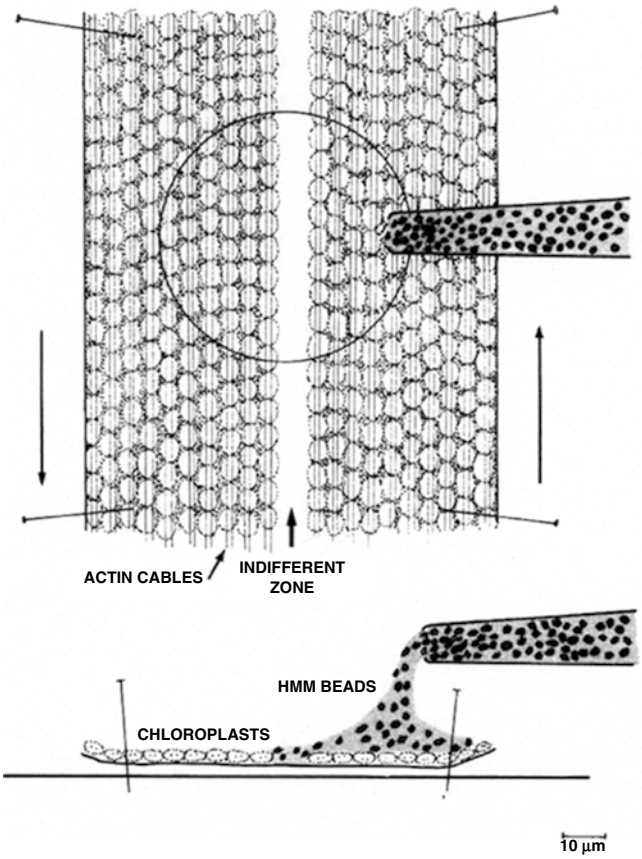


Fig. 9.10 Schematic drawing of the experimental procedure for the assay of myosin movement. Fluorescent beads coated with rabbit skeletal heavy meromyosin (HMM) were added to actin filaments of the alga *Nitella* which were exposed by direct dissection of the cell (~1 mm×4 cm). All the actin filaments have one polarity to the left of the indifferent zone and opposite polarity to the right as indicated by the *long thin arrows*. There are about five polar cables per role of chloroplasts and each cable contains hundreds of actin filaments all of the same polarity. The pipetted beads settled on the rows of actin cables and moved in the presence of ATP. Measured sliding velocities were comparable to sliding of filaments in unloaded contracting muscle (Sheetz and Spudich 1983. With permission Nature Publishing Group)

Stephen J. Kron, a M.D./Ph.D. student doing his graduate work in the Spudich laboratory, and Spudich and Sheetz oriented the actin filaments into parallel tracks to monitor myosin movement. They utilized severin, a protein that selectively binds to the "barbed" end of actin filaments, to attach the filaments to an optically clear substratum. In appropriate flow conditions, the bound actin filaments would orient with the free "pointed" end in the downstream direction. Myosin coated beads in the presence of ATP moved from the pointed end to the barbed end as predicted by Huxley's 1969 model of muscle contraction. The velocity of movement was comparable

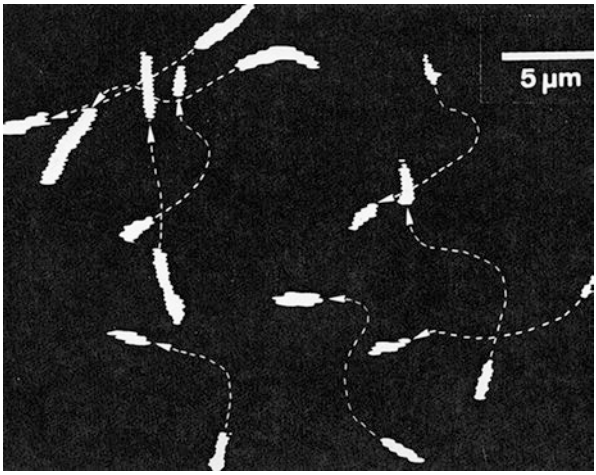


Fig. 9.11 Sliding movement of single filaments labeled with fluorescent phalloidin on a coverslip coated with single headed myosin filaments. To demonstrate the movement of the actin filaments, two video images taken 1.5 s apart were photographed on the same frame of a film by double exposure. Notice the somewhat haphazard movement of the actin filaments (Harada and Yanagida 1988. With permission John Wiley & Sons Inc)

to that observed in intact muscle under zero load and similar to that observed in the *Nitella* system (Spudich et al. 1985). Thus they published the first demonstration that purified actin and ATP are sufficient to support movement of myosin at rates consistent with the speeds of muscle contraction. Nevertheless the assay was difficult and a simpler assay system was sought. A crucial observation in this regard was made by Toshio Yanagida in Fumio Oosawa's laboratory in 1984 (Yanagida et al. 1984). Theodor Wieland and colleagues (Wulf et al. 1979) synthesized a fluorescent derivative of the mushroom toxin phalloidin, molecular weight 1,250 Da, that exhibited a high affinity for filamentous actin. Wieland supplied the phalloidin combined with the fluorescent dye rhodamine to Yanagida. Phalloidin-rhodamine did not alter the physiological function of purified F-actin but did allow direct visualization of the actin filaments. Kron and Spudich (1986) took advantage of this new discovery. They decided to invert their assay system by immobilizing myosin filaments on glass coverslips and allowing single actin filaments combined with fluorescent phalloidin to attach to the bound myosin. The fluorescent actin filaments exhibited ATP-dependent movement over the myosin-coated surface. Because the myosin filaments were not oriented on the coverslips, actin filaments followed winding paths and statistical analysis was nontrivial (for example, see Fig. 9.11 and to locate a movie see footnote[6]). Nonetheless this myosin-coated surface assay system was a real break through and is, with modifications, in routine use today. Kron and Spudich (1986) concluded with a brief description of their future plans. Their strategy would be to use this system along with methods of molecular genetics to identify those aspects of actin and myosin structure that are important for coupling ATP hydrolysis to movement and to localize the regions of mobility in the

[6] For a movie from the work of Kron and Spudich (1986) that shows fluorescently labeled actin filaments sliding over muscle myosin molecules that are attached to a glass microscope slide see: http://www.laskerfoundation.org/awards/2012_b_action02.htm.

myosin molecule responsible for movement and force generation. They planned to use site-directed mutagenesis of the cloned genes for *Dictyostelium* actin and myosin to generate modified proteins by expression in an appropriate host cell. These expressed proteins would then be characterized by a variety of techniques, including this in vitro movement assay. Spudich went on to accomplish these goals in spectacular fashion.

The next important step occurred when postdoctoral fellow Yoko Y. Toyoshima, Spudich and colleagues showed that only myosin S1, the globular head of myosin, was required for movement of actin filaments (Toyoshima et al. 1987). This observation eliminated theories like the one proposed by Harrington (1971) where force generation was not in the myosin head but rather due to melting in the core of the thick filament (see above section). Thereafter research on how myosin transduced the chemical energy of ATP hydrolysis into mechanical motion focused on the S1 head of myosin.

Much progress had been made but there were still fundamental questions that needed resolution. One crucial issue related to the size of the step that the myosin molecule took for each molecule of ATP hydrolyzed. From studies of sliding in isolated single sarcomeres under zero load, Yanagida et al. (1985) had concluded that the myosin step size may be 60 nm or more (see section above). Using the myosin filament-coated surface in vitro assay, the Yanagida laboratory calculated the sliding distance of actin filaments during one ATP hydrolysis cycle to be greater than 100 nm (Harada et al. 1990). They now proposed "loose coupling" of ATP hydrolysis to myosin movement under zero load (Harada et al. 1990. With permission Elsevier):

> …the myosin head can translate an actin filament a long distance by undergoing many active cyclic interactions with it during one ATPase cycle near zero load and the translating distance is probably variable, depending on the load…What molecular mechanism is consistent with this conclusion? One possible mechanism is that the myosin heads are probably in the weak binding states, can fractionize the free energy produced by ATP hydrolysis and use the fractions for many positive attachment-detachment cycles.

In dramatic contrast the Spudich laboratory, using a very similar experimental technique, estimated values for filament sliding distance for one ATP hydrolysis cycle of 10–28 nm (Uyeda et al. 1990). They concluded that this range was within that of geometric constraint for conformational change imposed by the size of the myosin head, and therefore consistent with the swinging cross-bridge model tightly coupled with ATP hydrolysis.

The different conclusions rested strongly on the interpretation of the data. Howard (2001) has provided a lucid description of the possible resolution of this "step-size paradox" along the lines proposed by Uyeda et al. (1990). In essence the sliding distance per ATP hydrolyzed (Δ) is only near to the length of the working stroke (δ) of the cross-bridge when the duty ratio (r), the fraction of the time during the cycle that the cross-section is generating force (τ_{on}) divided by the total cycle time (τ_{total}), is near one. Thus:

$$\mathbf{r} = \tau_{on} / \tau_{total} = \delta / \Delta$$

For sliding at near a maximum velocity, the observed duty ratio might be about 0.05. Thus the distance shortened per ATP hydrolyzed would be about 20 times the length of the working stroke. For example applied to the results of Yanagida et al. (1985), this would lead to a working stroke of about 3 nm, well within the physical dimensions of the cross-bridge and consistent with a one-to-one coupling of the mechanical and chemical cycles of myosin. The crucial point is that each of the cross-bridges moving a thin filament may contribute only 3 nm or so movement while it hydrolyzes one molecule of ATP. While this cross-bridge is detached, the other cross-bridges move the filament along the rest of the way. Thus the distance moved per ATP is much greater than the working stroke of the cross-bridge because myosin has a low duty ratio. See Howard (2001: pages 219–222) for the detailed developed of the argument.

Nevertheless what was really needed was a direct measure of the size of the step taken by a single myosin molecule. That seemed like an impossible dream but in fact it became a reality by the mid-1990s.

9.4.2 Single Mechanoenzyme Mechanics: Laser Traps and Optical Tweezers

The progress that led to the measurement of the step size and force generation by a single myosin molecule dates back to around 1970. Arthur Ashkin[7], a physicist at Bell Laboratories in New Jersey, was fascinated by the possibility of utilizing laser light to capture or trap atoms. He interested his new supervisor, Steven Chu, in this problem. Chu, an outstanding experimentalist, eventually solved the problem and was able to trap sodium atoms using laser light. Chu would go on to share the Nobel Prize in 1997 for this achievement (Chu 1997). The technique of optical trapping and manipulation of small neutral particles by lasers is based on the forces of radiation pressure. These forces arise from the momentum of the light itself. With lasers one can make these forces large enough to accelerate, decelerate, deflect, guide, and even stably trap small particles. At first thought the idea of trapping particles with a single laser beam seems counter intuitive. A particle placed on the beam axis feels a forward scattering force trying to move it. However, if the beam is strongly focused on the particle, there is also a backward gradient force pulling the particle back toward the beam focus. The backward gradient force can readily equal or exceed the forward scattering force, making the laser trap stable (Ashkin et al. 1986). This trap was called "a single-beam gradient force optical trap" or later more colorfully "optical tweezers". Ashkin showed

[7] Arthur Ashkin (1922–) is considered by many to be the father of optical trapping using lasers. He received a Ph.D. in physics from Cornell University in 1952 and spent his forty year research career with Bell Laboratories, retiring in 1992. He has received numerous honors and awards and was elected to membership of the National Academy of Sciences in 1996. He has written an overview of the history of the whole field of optical trapping from physics to biology and has complied a compendium of historically significant reprints with commentaries (Ashkin 2006).

that laser manipulation techniques could be applied to particles as diverse as atoms, large molecules and small dielectric spheres in the size range of tens of nanometers to tens of micrometers. The use of laser traps in biology resulted from the Ashkin's accidental discovery of trapping of bacteria and the later demonstration of damage free trapping of cells using infrared lasers. Ashkin showed that biological particles as diverse as viruses, single living cells, and organelles within cells could be trapped and manipulated (for a historical perspective, see Ashkin 1997).

Steven M. Block, then at the Rowland Institute for Science, Cambridge, Massachusetts, and colleagues were the first to utilize optical tweezers to investigate the function of a molecular motor (Block et al. 1990). A new molecular motor, called kinesin, was discovered in the axoplasm of the squid giant axon by Vale et al. (1985a). In vitro, kinesin induced the movement of microtubules in an ATP dependent manner (Vale et al. 1985b)[8]. Block utilized optical tweezers to trap an individual bead coated with kinesin which was then moved to a single immobilized microtubule where upon the bead moved along the microtubule after being released from the trap. Karel Svoboda, Block and colleagues developed an optical trapping interferometer system and determined that beads carrying single kinesin molecules moved with 8 nm steps when placed on immobilized microtubules (Svoboda et al. 1993). In the same year, Scot C. Kuo and Sheetz (1993) measured the isometric force generated by a single kinesin molecule using optical tweezers. A bead in an optical trap was attached to a single translocating microtubule. In the kinesin force measurement, an immobilized kinesin molecule in the presence of GTP or ATP pulled on the microtubule that was balanced by an optical force applied to a microsphere some distance from the kinesin molecule. At high laser power holding the bead and microtubule, kinesin could not move the microtubule. Reduction of the laser power holding the bead eventually allowed the kinesin molecule to move the microtubule. After calibrating the system, it was determined that the stalled microtubule escaped an optical trapping force of about 2 piconewtons (pN). Thus a single kinesin molecule generated about 2 pN of force. This ability to measure force parameters of single macromolecules now allowed direct testing of molecular models for contractility. Therefore it was feasible to measure directly in a single molecular motor the step size in the nm range and the force generating capacity in the pN range.

[8] This work was greatly facilitated by the utilization of video-enhanced differential interference contrast microscopy (VE-DIC) to visualize the moving microtubules. VE-DIC was discovered independently by Robert Allen and colleagues (Allen, Allen, and Travis 1981) and Shinya Inoue (1981). An explanation of the development of the technique and its role in discovering the function of the kinesin motor has been reviewed by Edward D. Salmon (1995). With VE-DIC microscopy it is possible to visualize movement of organelles and macromolecular complexes like microtubules whose dimensions are smaller than the diffraction limit of resolution of the light microscope.

9.4.3 *Single Myosin Molecule Mechanics: Measurement of Force and Step Size*

Even though there was a great deal of satisfaction in reaching a 10 year goal in developing the myosin coated surface in vitro assay system, Spudich realized that this assay system was not sufficient to resolve fundamental aspects of the myosin motor behavior such as the step size produced for every ATP hydrolyzed and the maximum force that the molecular motor was capable of generating. These questions could only be answered at the single molecule level. Spudich was aware of the optical trap studies of Ashkin et al. (1986) and thought it feasible to position an actin filament over a single myosin molecule and so achieve the desired goal. But his laboratory needed the help of a physicist. Fortunately Steven Chu had just moved from Bell Labs to the physics department at Stanford University. Chu was interested in biological problems and was willing to collaborate on the project. In the late 1980s Robert M. Simmons, on sabbatical leave from Kings College London, joined the Spudich laboratory. Simmons was a collaborator with Andrew Huxley and Lincoln Ford in the classic mechanical experiments on single muscle fibers (see Chap. 5) and an expert in designing feedback systems utilized to maintain sarcomere length constant in the muscle experiments. Simmons and M.D./Ph.D. student Jeffrey T. Finer built the first dual beam, three bead laser trap for measuring displacement and force generation of single myosin molecules in Chu's laboratory with the help of Chu and members of his research group. After building an improved version of the system, Finer et al. (1994) published a classic paper on single myosin molecule mechanics wherein they measured the step size and force generating capacity of rabbit heavy meromyosin (HMM). A single actin filament was labeled with rhodamine phalloidin. Polystyrene beads coated with *N*-ethylmaleimide (NEM)-treated HMM were attached irreversibly near the each end of the actin filament using dual beam optical traps. The actin filament could be stretched taut and then readily moved in three dimensions. Latex beads fixed to a microscope coverslip were coated with HMM at a very low density (insufficient to cause actin filament sliding). The actin filament was moved over a latex bead and it interacted with a single myosin molecule in the presence of ATP (Fig. 9.12a). If the laser energy was low, the traps were relatively compliant. Under these low load conditions, rapid transient displacements were observed in saturating ATP (2 mM) (Fig. 9.12b). The observed average step size was 11 nm. If, on the other hand, the trap stiffness was increased by an electronic feedback system, the displacement of the trap position could be used as a measure of force on the trapped bead under approximately isometric conditions. The average force generated by a single myosin molecule was 3–4 pN (Fig. 9.12c).

These were spectacular results but there were some caveats. The orientation of the myosin molecule interacting with actin was unknown and thus the forces and displacements might well be underestimates. Also, since HMM was utilized they could not rule out the possibility that some interactions involved both heads of myosin with an actin filament. Nonetheless they felt that their results made unlikely models in which a myosin molecule made multiple interactions with an actin filament for each ATP hydrolysis cycle because the single displacements and single

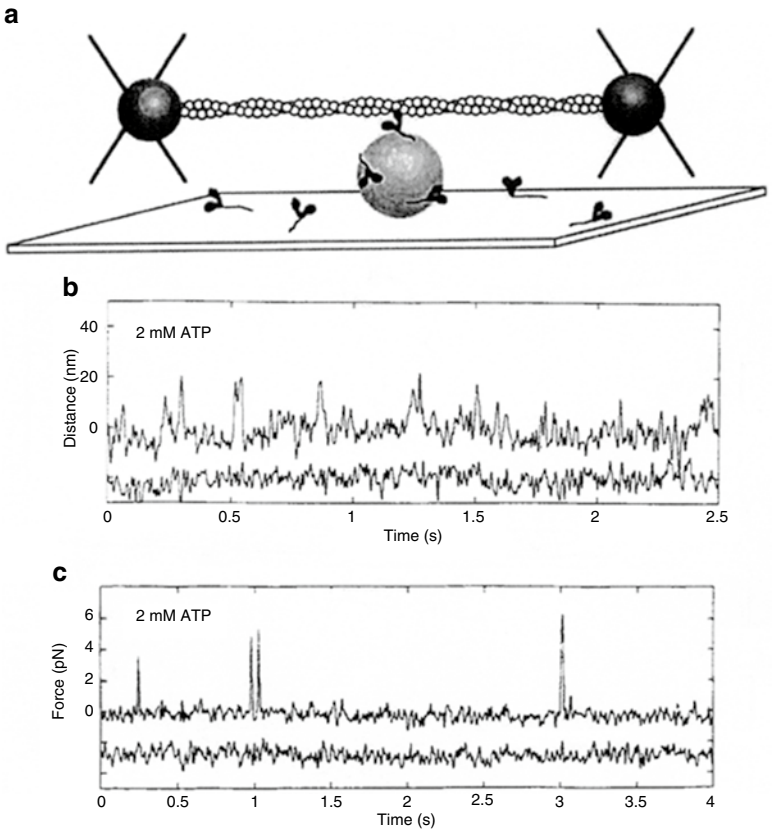


Fig. 9.12 Measurement of step size and force generation by a single myosin molecule. (**a**) Schematic diagram illustrating the use of two optical traps that are focused on beads attached to a single fluorescent actin filament. The actin filament is lowered onto a silica bead that is fixed to a microscope coverslip and sparsely coated with heavy meromyosin (HMM) so that the actin filament interacts with one HMM. (**b**) Single displacements at low load in the presence of ATP. The *upper trace* shows the movement of the trapped bead in the direction along the actin filament. The *lower trace* shows the movement of the bead in the perpendicular direction and represents brownian noise. Only isolated events greater than Brownian noise were counted. Displacements varied from 5 to 18 nm with an average displacement of 11 nm. (**c**) Single force transients near isometric conditions, i.e., when the bead position was held constant by feedback, in the presence of ATP. The *upper trace* shows the forces in the direction along the actin filament. The *lower trace* shows forces in the perpendicular direction. Force varied from 1 to 7 pN with an average of 3–4 pN (Finer, Simmons and Spudich 1994. With permission Nature Publishing Group)

forces that they observed at very low HMM density did not appear to be clustered. They concluded that the results could be explained by a simple cycle of force production followed by detachment in which the isometric force per interaction of a correctly oriented myosin head was 3–7 pN and the displacement at low load was 8–17 nm. Furthermore, they also concluded that these magnitudes of the single forces and displacements were consistent with predictions of the conventional swinging-crossbridge model of muscle contraction.

With refinement of techniques in the 1990s and more attention to statistical analysis, three research groups headed by Justin E. Molloy at the University of York, David M. Warshaw at the University of Vermont and Spudich independently found that the magnitude of the step size of a single myosin molecule was about 4–6 nm (Molloy et al. 1995; Guilford et al. 1997; Mehta et al. 1997). But there was a significant discrepancy with the results from Yanagida's group which found values of the myosin step size of 15–30 nm (Ishijima et al. 1996; Tanaka et al. 1998). This discrepancy was not simply one for esoteric debate because the crystal structure of myosin S1 (see section below) could accommodate a step size of 4–6 nm but not one of 15–30 nm. Furthermore the Yanagida group proposed that the myosin molecule undergoes multiple 5 nm substeps during one ATP hydrolysis cycle (Kitamura et al. 1999). In 2000 Yanagida reviewed the results of his laboratory and concluded that the myosin heads move along an actin filament with regular steps of 5.3 nm and undergo up to five steps to produce a maximum displacement of 30 nm during one biochemical cycle of ATP hydrolysis (Yanagida et al. 2000). Thus there was a significant controversy.

Perhaps Andrew Huxley spoke for many who attended the Frontiers in Molecular Motors Research symposium hosted by Yanagida in Japan in 2000 when he said, in his closing speech. "I came here confused about actin and myosin. Now I am still confused, but at a higher level." (Cyranoski 2000. With permission Nature Publishing Group). Yanagida's experiments were imaginative and elegant but the majority disagreed and still disagree.

9.5 Muscle Enters the Atomic Age: Atomic Structures of Actin and Myosin

With reliable in vitro motility assays being developed and the rapidly advancing genetic manipulation of myosin molecules feasible (Warrick and Spudich 1987), what was needed now was the three dimensional crystal structures of actin and myosin in order to guide rational design of experiments probing the mechanism of myosin function at a truly molecular level. The paths to the three-dimensional structures of the actin molecule, an actin filament and myosin molecule at the atomic level were long and arduous. But when these structures were achieved, they "supercharged" the muscle and motility fields. In a review of the advancement of knowledge in the muscle field from 1972 to 2004, Cooke (2004) has stated: "The structure of the myosin head, along with the fit to the actomyosin complex, represented an immense breakthrough in the field, which now can be subdivided into pre- and poststructural periods."

9.5.1 Atomic Structure of Actin

Actin was discovered by Straub in 1942 (see Chap. 1). In the absence of salt, actin can exist as a globular protein, G-actin. In the presence of trace amounts of salt, it becomes a highly symmetrical fibrous protein, F-actin. G-actin from rabbit skeletal

muscle consists of a single polypeptide chain of 375 residues, a nucleotide (ATP) and a divalent cation bound to a specific site. The major problem in determining the atomic structure of G-actin is its tendency to polymerize, thus preventing crystal formation and analysis by X-ray diffraction. The crystal structure of actin at the atomic level was finally achieved in 1990 but the path to that discovery can be traced back to before 1974. Uno Lindberg, in Sweden, had isolated an inhibitor of the enzyme pancreatic deoxyribonuclease I (DNase I). He visited the Cold Spring Harbor Laboratory in New York and worked with Elias Lazarides to determine if that inhibitor was actin. They proved that the inhibitor was indeed actin (Lazarides and Lindberg 1974). This observation stimulated the pursuit of the crystal structure of G-actin by Kenneth C. Holmes' research group (see Fig. 6.18) at the Max-Planck-Institut fur medizinische Forschung in Heidelberg. Hans Georg Mannherz and colleagues showed in 1975 that the 1:1 binding of G-actin to DNase I not only inhibited DNase I enzyme activity, it also prevented actin polymerization (Mannherz et al. 1975). These experiments provided the first hint that actin-binding proteins are important in controlling filament assembly. Mannherz et al. (1977) found that the actin:DNase I complex could be crystallized. They noted that although the physiological significance of the interaction between skeletal muscle actin and DNAase I was not known, the elucidation of the structure of the complex formed by them provided a route to the three-dimensional structure of actin.

Thus the quest for the atomic structure of actin was to go methodically through the determination of the atomic structure of DNase I and then the structure of its complex with G-actin. Dietrich Suck of the European Molecular Biology Laboratory in Heidelberg, Kabsch and Mannherz (1981) determined the three-dimensional structure of the actin:DNase I complex at 6 Å resolution. The next step was for Oefner and Suck (1986) to determine the crystal structure of DNase I at 2 Å resolution. Finally the long sought goal was reached when Kabsch et al. (1990) reported the atomic structure of actin at an effective resolution of 2.8 Å (with ATP bound) and 3 Å (with ADP bound). This work was greatly facilitated by the use of electronic area detectors instead of film to record the diffraction patterns and a computer program designed by Kabsch (Xray Detector Software, XDS).

The information provided by the known structure of DNase I (which represented 40 % of the atoms in the actin:DNase I complex) was utilized to help deduce the actin structure. Figure 9.13 shows the secondary structure of the actin molecule with the first and last amino acid residues in the helices and sheet strands specified. The molecule consists of two domains dividing the molecule in half vertically. Because of their location within the actin filament, the two major domains of actin in Fig. 9.13 are known as the outer (right hand side) and inner domains, and because of their apparently different sizes in electron microscopy images, they have also been called the small and large domains, respectively (though they are about the same size). There is relatively little contact between the two major domains of actin. As a result two clefts are formed between the domains. The upper cleft contains the nucleotide and divalent cation. Traditionally a four-subdomain nomenclature has been adopted. Subdomains 1 and 2 are in the outer domain with the N and C terminals of the molecule located in subdomain 1 (lower right had side of Fig. 9.13) and a DNase I binding region in subdomain 2 (upper right had side). Also they speculated that subdomain 1 contained the actin binding sites for myosin and tropomyosin. Subdomains 3 (lower left hand side

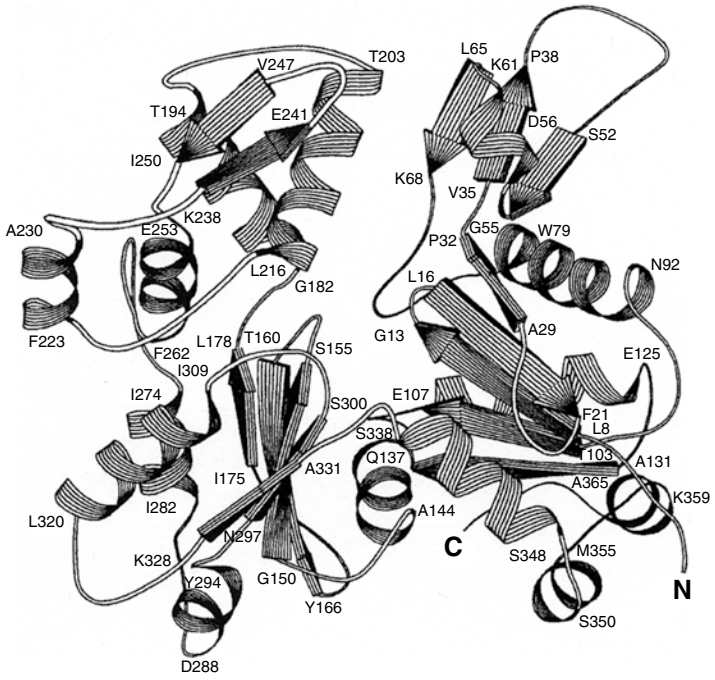


Fig. 9.13 Schematic representation of the secondary structure of ATP-actin. The actin molecule consists of two domains and ATP binds in the cleft between domains. See text for details (Kabsch et al. 1990. With permission Nature Publishing Group)

of Fig. 9.13) and 4 are located in the inner domain of the molecule. The actin monomer is rather flat, fitting into a rectangular prism with dimensions 55 Å × 55 Å × 35 Å. Thus, nearly 50 years after the discovery of the actin molecule, its atomic structure finally was elucidated. It is a historical curiosity that the physiological function, if any, of the highly specific actin:DNase I interaction is still unknown (Dominguez and Holmes 2011). (DNase I is regarded as a digestive secretory protein which acts enzymatically in the extracellular space to digest DNA.) Since this first structure of actin was reported in 1990, over 80 structures of actin have been reported. The majority of these structures have been obtained as complexes with actin-binding proteins and/or small molecules or by chemically modifying or mutating actin in order to prevent polymerization (Dominguez and Holmes 2011).

9.5.2 Atomic Model of an Actin Filament

From the viewpoint of muscle contraction, the atomic structure of the actin filament is of great interest. After all, G-actin does not effectively activate the ATPase of myosin but F-actin does and it is the F-actin which interacts with cross-bridges

during muscle contraction. Holmes et al. (1990) described the first atomic model of the actin filament in a paper that followed the G-actin crystal structure paper in Nature. Since it was not possible to crystallize the actin filament, the model of F-actin was constructed by fitting the atomic structure of the actin monomer to the observed X-ray fiber diagram derived from oriented gels of F-actin. The diffraction diagram of F-actin in gels had a resolution of about 8 Å, lower than that of G-actin. This first model was built by treating the G-actin subdomains as independent rigid bodies and adjusting their positions during the fitting. The procedure that they utilized was to build a model of F-actin from G-actin as found in the actin-DNase I complex, then check the model by calculating its diffraction pattern and comparing it with the observed fiber diffraction pattern. An optimization was achieved by trial and error or, when the model was good enough, by iterative least squares. Thus the accuracy of the model was limited by the fiber diffraction data. Even though they found only one orientation of the actin monomer derived from the actin-DNase I complex which accounted for the fiber diffraction pattern, they acknowledged that some changes of structure between monomeric and F-actin were likely. However they speculated that these changes likely would be small.

Their model is shown in Fig. 9.14 (for comparison with earlier models of F-actin, see Fig. 6.10). All four subdomains of actin are involved in contacts with neighboring monomers. Myosin is expected to bind the outer domain of a actin monomer since all known myosin binding residues of actin are located in outer domain. The maximum diameter of the F-actin model is 90–95 Å.

The generation of this first atomic model of F-actin was a major break through but over the years, the model would be refined facilitated by advancements in technology. Oda et al. (2009) in Japan increased the resolution of the X-ray diffraction data from oriented F-actin to 3.3–5.6 Å. In order to fit the fiber X-ray data, the G- to F-actin transition had to involve a 20° rotation of the outer domain of G-actin with respect to the inner domain about a rotation axis roughly at right angles to the helix axis. They called this rotation a "propeller-like twist". The net effect of the twist is to flatten the actin molecule. The major difference between this model and the Holmes model was that the F-actin structure was flatter and had a smaller diameter than in the original Holmes model. Thus the F-actin monomer is similar to but not identical to the G-actin monomer in conformation. With regard to the new structure, Holmes (2009) has speculated that the rotation of actin may be the switch for turning on the ATP hydrolyzing activity of F-actin. In addition, the flattening of monomers within F-actin substantially alters the site on this protein to which the myosin binds and this could explain why myosin binds with high affinity to F-actin but not at all to G-actin.

Takashi Fujii, Atsuko H. Iwane, Toshio Yanagida and Keiichi Namba at Osaka University (2010) have made improvements in cryo-electron microscopy that have resulted in direct visualization of the actin filament at a resolution of 6.6 Å. This technique shows structures in their natural hydrated environment (see Chap. 6). The density map clearly resolved all the secondary structures of G-actin, such as α-helices, β-structures and loops, and made unambiguous modeling and refinement possible. Their results are in general agreement with those of Oda et al. (2009). Two movies included in the supplementary material of their paper show dramatic visualization of the actin filament and the conformational changes of G-actin that occur when it is incorporated into the actin filament (Fujii et al. 2010).

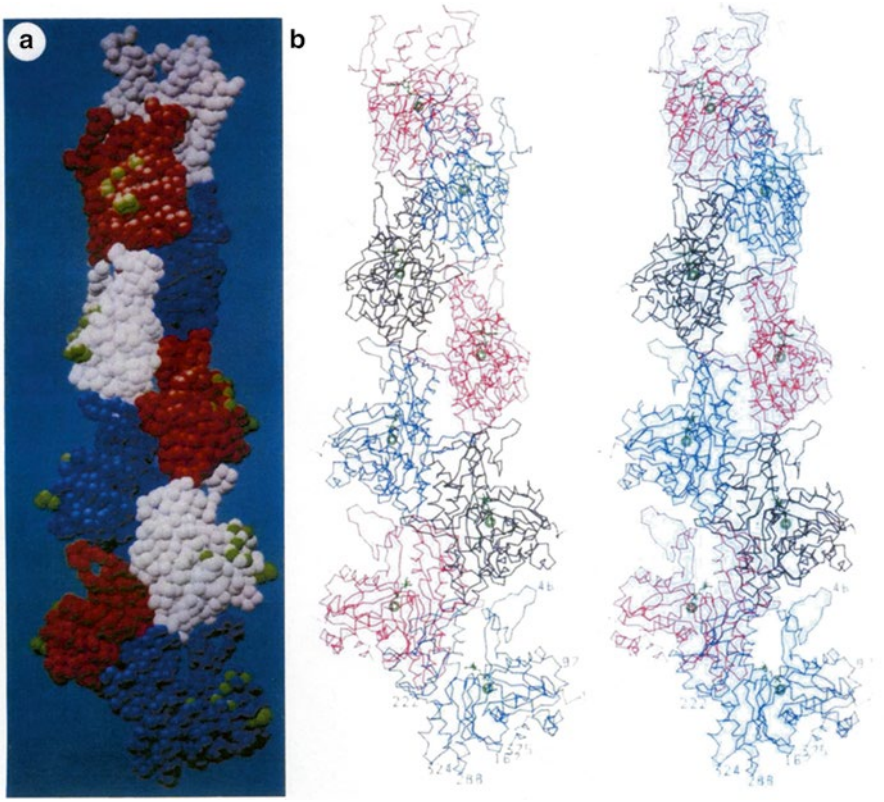


Fig. 9.14 Three dimensional representation of the F-actin helix. (**a**) Eight monomers of the F-actin helix shown as a space filling model. Each amino acid is represented by a sphere of radius 2.7 Å. (**b**) A stereo pair of the alpha carbon backbone positions in the corresponding eight monomers. The actin monomer at the bottom of the picture is at the "barbed" end of the filament (Holmes et al. 1990. With permission Nature Publishing Group)

Thus the structure of the actin filament has evolved over the years since 1963 when it was first described in the classic electron microscopic studies of Hanson and Lowy (1963). In the past decade the changes to the models of the actin filament have been mostly "fine tuning" in nature. Nonetheless, Holmes and colleagues (Splettstoesser et al. 2011) recently have commented that even though the present F-actin model is unlikely to be the last word on the subject, intense recent experimental and simulation experiments will likely lead to definitive atomic-detail descriptions of the filament in biologically functional states.

Fig. 9.15 Ivan Rayment received a Ph.D. in chemistry from Durham University in England in 1975. After postdoctoral training at Purdue University and Brandeis University, he accepted a faculty position at the University of Arizona in 1984. He moved to the University of Wisconsin in 1988 where he is professor of biochemistry. He has generated over 180 publications since 1974 on various aspects of protein structure and function. His website lists 45 different protein structures solved in his laboratory. Photo: courtesy of I. Rayment

9.5.3 Atomic Structure of Myosin and the Actin-Myosin Complex: Introduction

The atomic structure of myosin followed in 1993 soon after the publication of the actin structure in 1990. Like the pathway to the crystal structure of actin, the road to the structure of myosin was a decade long struggle. Lead author Ivan Rayment (Fig. 9.15) has commented that he never would have received tenure as a faculty member at the University of Wisconsin if he had worked only on the structure of myosin. That statement is no surprise since the first announcement of the crystallization of myosin appeared in 1984 and the definitive structure not until 1993. It all started "over beers" in 1983 with Donald A. Winkelmann, another postdoctoral fellow, at the Rosenstiel Basic Medical Research Center, Brandeis University (Taylor 1993). Rayment was looking for a project that would lead to a job and career in science. He decided that solving the crystal structure of myosin would be the goal. The problem at the time was that no laboratory had been successful in growing crystals that would diffract X-rays with the required resolution. Rayment had the idea that changing the surface of the protein might facilitate crystallization. Winkelmann

suggested that Rayment try the technique of reductive methylation to change the protein surface properties. In this technique, which is considered to be a gentle way of modifying proteins, the surface lysines of a protein are methylated. This mild modification preserves the net positive charge of lysine and simply increases the bulk and hydrophobicity of the ε-amino group (Yount 1993). Rayment tried it and it worked, eventually. But there were hurdles to overcome. The first problem to be solved was developing a myosin preparation that was pure and reproducible. He decided on attempting to crystallize myosin subfragment 1 (S1) from chicken skeletal muscle since S1 was a fully functional myosin that contained the actin binding site, ATP hydrolysis site and light chains of myosin. Myosin S1 consists of the heavy chain and the two light chains (see Fig. 3.11). The crystals that Rayment and Winkelmann (1984) described did not diffract X-rays particularly well and did not give reproducible results. But it was a start. The main problems were the variable purity of myosin S1 and the variable extent of methylation of the approximately 100 surface lysines of myosin S1. Some 6 years were spent optimizing the methylation procedure and refining the preparation and purification of S1. Then it took another 3 years for Rayment and his collaborator and wife Hazel M. Holden and their colleagues to collect and analyze the data from the best crystals. It was a team effort that included postdoctoral researchers, Wojciech Rypniewski, Karen Schmidt-Base, Diana Tomchick, and Matthew Benning along with research assistant Robert Smith and research scientist Gary Wesenberg at Wisconsin and Winkelmann, by then at the Robert Wood Johnson Medical School in New Jersey. The paper was received at the journal Science in April, accepted in June, and published with cover art in July of 1993 (Rayment et al. 1993b). According to Yount (1993) the long gestation period reflected the difficulty and challenge of solving the structure of one of the largest asymmetric single proteins and the "fierce determination" of Rayment to get the structure right the first time. And he did get it right the first time.

In a candid interview later that same year (Taylor 1993), Rayment described that he kept secret the means of crystallizing myosin for nearly a decade. In an acknowledgement of scientific realism, he stated (Taylor 1993. Journal no longer exists. Permission from quoted author, I. Rayment):

> I've taken a lot of heat over the years for not telling people how I grew the crystals…It's not something I'm proud of, but I'm not ashamed of it, either. Science is competitive. The rate-limiting step in getting the structure of the myosin head was growing the crystals, and I didn't want to wind up as a footnote in somebody else's paper [describing the myosin structure].

He certainly was not a footnote as the myosin crystal structure sent a shockwave through the muscle and motility fields. It is not an exaggeration to state that solving the myosin crystal structure at the atomic level was one of the major accomplishments in the muscle field in the twentieth century. It wasn't that solving the myosin structure provided the answer to the mechanism of muscle contraction but rather it provided a framework for design of experiments employing molecular biology, in vitro motility assays and chemical and kinetic techniques. Rayment understood this when he said (Taylor 1993): "The structure doesn't give us all the answers…It just resets the questions, and gives us a framework to hang the answers on. It's really just the end of the beginning." (Taylor 1993. Journal no longer exists. Permission from quoted author, I. Rayment)

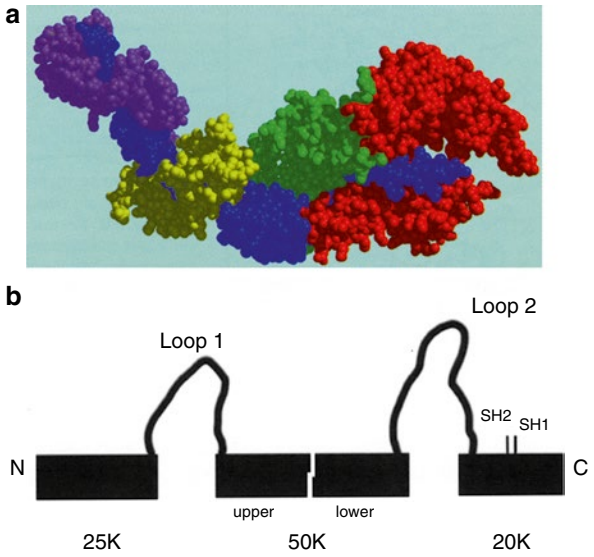


Fig. 9.16 Domains of myosin S1 molecule defined by proteolytic digestion. (**a**) Space-filing representation of all of the atoms in the model of myosin S1. The model is oriented such that the actin binding surface is located at the lower right-hand corner. The horizontal cleft that divides the central 50-kDa segment of the heavy chain into two domains (upper and lower defined by this orientation) is clearly visible (Rayment et al. 1993a. With permission The American Association for the Advancement of Science). (**b**) This depiction emphasizes that the domains of the myosin head are connected by surface loops 1 and 2 that are susceptible to proteolysis. The heavy chain is shown from the N terminal (N, *left*) to the C terminal (C, *right*). The 50-kDa domain is split into two sub-domains by a deep cleft extending from the actin binding site to the ATP binding site. The 20-kDa domain contains the two reactive sulfhydryl groups (SH1 and SH2) (Holmes 2008. With permission Springer)

9.5.4 Three-Dimensional Structure of Myosin Subfragment-1: A Molecular Motor

The myosin molecule, 520 kDa, contains two 220 kDa heavy chains and two pairs of light chains between 15 and 22 kDa. The molecule is highly asymmetric, consisting of two globular heads attached to a long tail. Myosin subfragment-1 (S1) contains the ATP, actin and two light chain binding sites. The globular head is sufficient to generate movement of actin in the in vitro motility assay system. Each globular head consists of a heavy chain fragment having a molecular size of 95 kDa and 2 light chains yielding a combined molecular size of ~130 kDa. Both light chains share considerable sequence similarity with calmodulin and troponin C although most of the divalent cation binding sites have been lost during evolution. Measurements from electron microscopy have suggested that the myosin head is pear-shaped, about 190 Å long and 50 Å wide at its thickest point.

Rayment et al. (1993b) solved the crystal structure of myosin S1, derived from papain digestion, at a resolution of 2.8 Å. They were able to build 1,072 amino acid

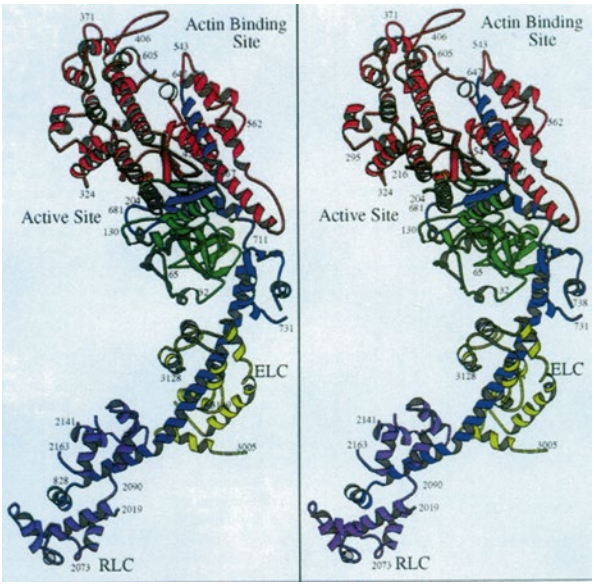


Fig. 9.17 A stereo view of the ribbon representation of myosin S1. In this figure, 2000 and 3000 have been added to the residue numbers of the regulatory and essential light chains, respectively, to distinguish them from the heavy chain. Heavy chain residues 4–204, 216–626, and 647–843 are separated by disordered loops for which no density is evident in the map. The actin binding surface has been defined by the location of the 50–20-kDa junction (residues 626 and 647) and by its interaction with actin (Rayment et al. 1993a. With permission The American Association for the Advancement of Science)

residues (of a total of 1,157) into the electron density map. Figure 9.16a shows a space-filling representation of the atoms in the myosin S1 model. The myosin head is asymmetric, with a length of 165 Å, a width of 65 Å, and a thickness of approximately 40 Å. Previous knowledge of the organization of the heavy chain in the myosin head was based on limited proteolytic digestion by trypsin. The head, called the motor domain, contains three major regions: 25-kDa amino-terminal nucleotide binding region, 50-kDa segment which binds to actin and 20-kDa carboxyl-terminal segment which also binds to actin. The light chains abut one another and are wrapped around a very long 85 Å α-helix of the heavy chain but do not overlap to any significant extent[9]. Figure 9.16b emphasizes that these regions are all part of a single polypeptide chain and also shows that these regions are connected by trypsin sensitive flexible loops. Some of the missing residues in the S1 structure are in these flexible loops.

Figure 9.17 shows the ribbon representation of the myosin S1 structure in stereo view. Myosin S1 contains 48 % α-helices centered around a large seven stranded β-sheet motif. The 50-kDa region has a complex topology that can be described as two major domains separated by a long narrow cleft. The cleft divides the distal

[9] The neck of the myosin S1 should not be confused with the S2 segment of myosin. See Figs. 3.11 and 3.23.

one-third of the myosin head into two regions, referred to as the upper and lower domains. There is no observed electron density corresponding to amino acid residues 627–646, representing loop 2 connecting the 50- and 20-kDa fragments. This 50–20 kDa junction is of great interest because previously it was implicated in actin binding from crosslinking and kinetic studies. They speculated that the positively charged unseen segment, loop 2, could readily interact with the negatively charged amino acids at the amino-terminus of actin.

Even though the crystal structure of myosin S1 was determined in the absence of nucleotide, it still was possible to locate the catalytic site. The catalytic site of the myosin head was identified by analogy to the phosphate binding loop in both the Ras protein and adenylate kinase and by the position of the amino acid residues previously identified by chemical studies with ATP analogues. The nucleotide binding pocket is located on the opposite side of the head from the proposed actin binding site and is in an open conformation (Fig. 9.17). The width of the nucleotide binding pocket at its surface is ~15 Å as measured between α carbons. They speculated that it was likely that the pocket closes when nucleotides bind in the active site. If the binding face to actin remains essentially stationary, this closure of the nucleotide binding cleft could produce a movement at the carboxyl-terminus of the myosin head of ~60 Å.

In a preview of the accompanying paper in Science, they commented that a better fit to the image reconstructions of S1-decorated actin would be obtained if the long narrow cleft between the upper and lower 50-kDa domains were to close. Also they noted that it appeared that a major function of the light chains was to create a longer molecule and hence amplify the conformational changes associated with the active site. These three features: opening/closing of the nucleotide binding pocket, opening/closing of the actin binding cleft and amplifying myosin movement by the light chain portion of the molecule would become intense areas of research in attempts to elucidate the molecular mechanism of muscle contraction.

It was soon discovered that the structure of the kinesin motor domain was very similar to that of the myosin motor domain suggesting the possibility that all motor molecules may operate in a similar manner (Kull et al. 1996).

9.5.5 Structure of the Actin-Myosin Complex and Implications for Muscle Contraction

The myosin S1 structure was solved not only in the absence of nucleotide but also in the absence of actin. Nonetheless an attempt was made to model myosin binding to F-actin by combining the molecular structures of the myosin and actin with the low-resolution, ~30 Å, electron density maps of the actin-myosin complex derived by cryo-electron microscopy and image analysis. This model resulted from a collaboration where the myosin structure was contributed by Rayment and Holden, the actin and F-actin structure by Kenneth Holmes and Michael Lorenz and the cryo-electron microscopy of F-actin decorated with myosin S1 by Ronald A. Milligan and his collaborators Michael Whittaker and Christopher B. Yohn. The model was

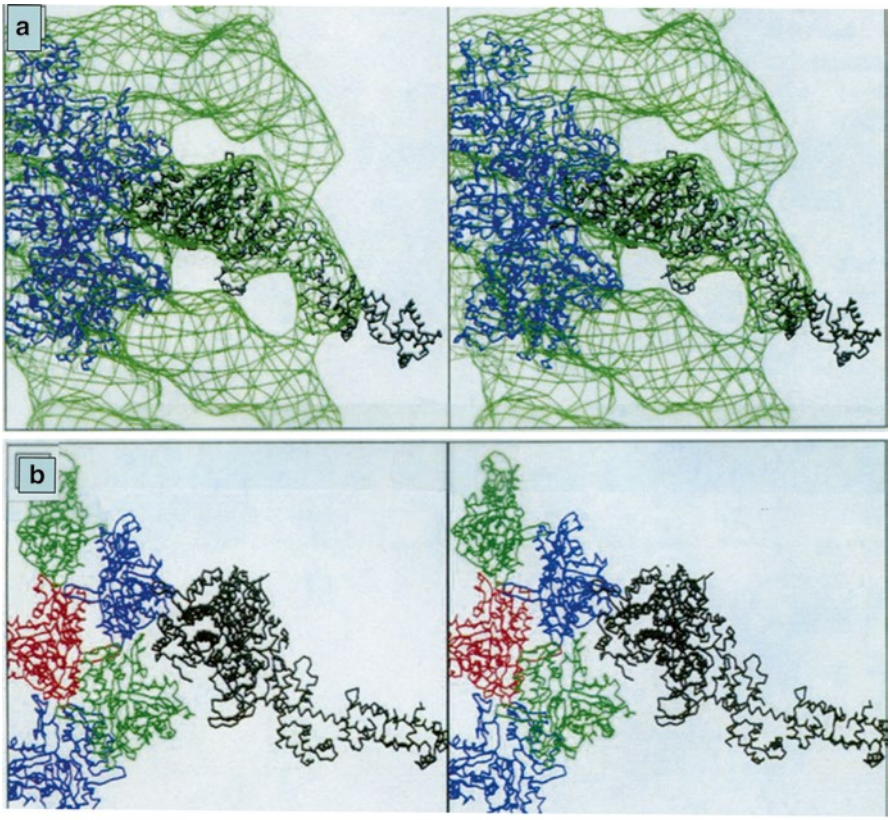


Fig. 9.18 Stereo images of the actin-myosin complex. (**a**) The best fit of the F actin model and the myosin S1 X-ray structure in the molecular envelope of the S1-decorated F actin obtained by cryo-electron microscopy. (**b**) An **α**-carbon plot of five actin monomers and one molecule of myosin S1. The S1-decorated F actin reconstruction was obtained with a myosin subfragment that lacked the regulatory light chain. Thus the neck of the myosin S1 X-ray structure which includes the regulatory light chain protrudes out of the S1-decorated F actin envelope as shown in (**a**) (Rayment et al. 1993b. With permission The American Association for the Advancement of Science)

published in a paper that accompanied the myosin S1 structure paper in Science (Rayment et al. 1993a). In docking myosin onto F-actin, they assumed that the myosin structure was most likely that of a rigor conformation. The model was built in stages: first, the F-actin model was positioned in the F-actin electron microscope envelope, the actin envelope was replaced by that of S1-decorated actin and finally myosin S1 X-ray structure was rotated and translated into place. Since the myosin head is highly asymmetric, it was straightforward to position the molecule unambiguously into the envelope. Even with low resolution of the electron micrographs, myosin could be placed with an accuracy of ~5 Å in the model. Thus it was immediately clear that the large motor domain of the myosin head must be close to actin, whereas the segment that contained the light chains must be at a high radius in the filament. The end result gave a good fit between the X-ray structure of the myosin head and the molecular envelope (Fig. 9.18). But there was an apparent problem.

There was a collision of actin and myosin at the site of the actin-S1 interaction involving regions close to the carboxyl-terminus of actin and the lower 50-kDa domain of S1. Although this might appear unacceptable, they viewed this collision in a different way. They speculated that this collision suggested that there may be a conformational change induced in the myosin head when it binds to actin. A better fit to the image reconstructions of S1-decorated actin could be obtained if the long narrow cleft between the upper and lower 50-kDa domains were to close, thus implying that this is an important structural feature of the molecule. They speculated that opening and closure of this cleft was the most likely mechanism for communication between the nucleotide binding site and the actin binding site. They noted that this actin-myosin interface could be potentially composed of three types of interactions: (a) an ionic interaction involving a flexible loop ("weak binding"), (b) a stereospecific interaction involving hydrophobic residues and (c) a strengthening of this interaction by the recruitment of additional loops from the upper 50-kDa domain as the cleft closes.

They proposed a structural hypothesis for the contractile cycle that was compatible with previous work on the cross-bridge cycle (Fig. 9.19). This hypothesis was based on the spatial relationship between the ATP binding pocket on myosin and the major contact area of myosin on actin. Starting at the rigor complex (Fig. 9.19a), the narrow cleft between the upper and lower domains of the 50-kDa segment was assumed to be in a closed conformation. The binding of nucleotide was suggested to be a two-step process. In the first step of ATP binding, the narrow cleft between the upper and lower domains of the 50-kDa segment opened, thereby disrupting the strong binding interaction between myosin and actin but still allowing the weak binding state (Fig. 9.19b). In the second step of ATP binding, closure of the nucleotide binding pocket around ATP caused a net change in the curvature of the molecule such that the carboxyl-terminus of the heavy chain moved at least 50 Å relative to the actin binding site leading to a repriming or recovery of the power stroke. Hydrolysis of ATP followed and resulted in a metastable state with bound products (Fig. 9.19c). Rebinding of myosin to actin led to the formation of the weak ionic interaction followed by a stronger stereospecific interaction with actin involving the lower domain of the 50-kDa segment. Incorporation of components from the upper domain of the 50-kDa segment of myosin S1 allowed the gap between the upper and lower domains to close to produce strong binding. Closure of the cleft lowered the affinity of the molecule for phosphate, which was then released. Loss of the phosphate triggered the start of the power stroke and allowed the myosin molecule to reverse the conformational change induced by binding of ATP (Fig. 9.19d). This resulted in reopening of the active site pocket after which myosin returned to its rigor state (Fig. 9.19e). During this process, ADP was released, and ATP could then rapidly rebind.

This model was highly speculative. After all only one structural state of myosin S1 was determined and that state did not contain either bound actin or nucleotide. Nonetheless, the model made several specific predictions that were testable. Most important was the reciprocal relationship between the opening and closing of the actin binding site and the nucleotide binding site. It was important to test these predictions but first, a more convenient way to generate the crystals of myosin S1 in different states was needed.

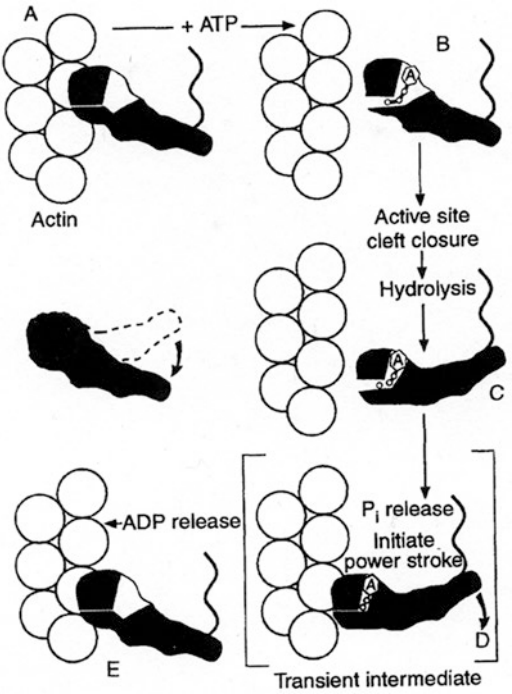


Fig. 9.19 A proposed model of the contractile cycle incorporating structural features of the myosin head and their proposed involvement in the cycle. Actin is represented as a sphere. In the near axial third of the myosin head, the narrow cleft that splits the 50-kDa segment of the myosin heavy chain sequence into two subdomains is represented as a horizontal gap perpendicular to the filament axis. Note that during the power stroke the 50-kDa cleft closes and the nucleotide binding pocket opens. Also note the proposed changes in the orientation of the neck to the motor domain throughout the cycle. See text for details (Rayment et al. 1993b. With permission The American Association for the Advancement of Science)

9.5.6 Structure of the Myosin Motor Domain in the Presence of Nucleotide Analogs: Post Rigor and Pre-powerstroke States

Molecular genetics provided a much needed break through in the production of suitable crystals of the myosin motor domain. With the use of homologous recombination in the slime mold *Dictyostelium discoideum* (see section on mutagenesis of myosin below), the myosin II motor domain (which does not contain the light chain binding region of the molecule) was expressed and purified by Andrew J. Fisher, Rayment and their colleagues (Fisher et al. 1995). The molecule was truncated at the start of the light chain binding region and thus contained only the motor domain. It could be easily crystallized without the need for chemical modification. The structure of the chemically unmodified *Dictyostelium* motor domain was essentially

the same as the chemically modified skeletal muscle S1 motor domain. This result thus alleviated any concern about the effects of chemical modification on the skeletal S1 structure. This was an important result since White and Rayment (1993) showed that methylated skeletal myosin S1 while retaining most of the fundamental features of the actomyosin ATP hydrolysis nonetheless exhibited altered the transient kinetic properties.

The goal of these experiments was to use nucleotide analogues to trap the motor domain in an ATP like state (Fig. 9.19b) or in a post hydrolysis ADP.P_i like state (Fig. 9.19c) and then explore the predicted conformational changes in the nucleotide binding pocket associated with each state. The Rayment group expressed the motor domain in the presence of MgADP-beryllium fluoride (ADP.BeF_x) (Fisher et al. 1995) and MgADP.vanadate (Smith and Rayment 1996). Because the coordination geometry of beryllium fluoride was close to tetrahedral, ADP.BeF_x was expected to be an analog of the ATP state (Fig. 9.19b) whereas vanadate ion was bipyramidal and thus ADP.V_i complexes were used as analogs of the transition state (Fig. 9.19c). The ADP.BeF_x structure was similar to chicken muscle S1 structure without nucleotide (Fig. 9.19b). This state would become known as the post rigor state. But the ADP.V_i structure showed large changes in the S1 structure at the carboxyl end of the molecule. This state represented the pre-powerstroke state (Fig. 9.19c). The carboxyl-terminal part of the molecule in the ADP.Vi structure rotated by about 70° compared to the ADP.BeF_x or chicken S1 structure. This region of the molecule containing ~70 amino acid residues was called the converter domain by postdoctoral fellow Anne Houdusse and Carolyn Cohen (1996) at Brandeis University. It would normally connect to the light-chain binding region of myosin. Using the coordinates of chicken S1 to model the missing light-chain-binding region, Holmes (1996) concluded that the rotation of the converter domain would move the carboxyl-terminus of the tail by about 12 nm from the position found in the chicken S1 in the rigor state. Another main conclusion from these studies was that the anticipated large closing of the nucleotide binding pocket upon binding nucleotide did not occur. Thus it appeared that small changes in the motor domain of myosin were amplified by the light-chain binding region. The 1969 "swinging cross-bridge" model of Hugh Huxley (see Fig. 3.23) now was refined to a "swinging lever arm" hypothesis of cross-bridge action.

9.5.7 Swinging Lever Arm Hypothesis of Cross-Bridge Action: Integration of Structural Biology, Molecular Genetics and In Vitro Motility Assays

Even though the Holmes modeling was highly suggestive, it was not direct structural evidence of a swinging lever arm action during the cross-bridge cycle. The first direct visual evidence of lever arm movement came from a study by Ronald A. Milligan at The Scripps Research Institute in California and H. Lee Sweeney at the University of Pennsylvania and their collaborators (Whittaker et al. 1995). They examined three-dimensional maps of F-actin decorated with recombinant smooth

muscle myosin S1 in the presence and absence of MgADP employing cryo-electron microscopy. The idea was that addition of MgADP to myosin might result in a partial reversal of the power stroke compared to the rigor state. The motor domain was similar in both states but there were major orientational differences in the light chain binding domain. This domain acted as a rigid lever arm pivoting about the end of the motor domain and swinging ~23° which resulted in a ~35 Å step.

Further structural evidence supporting lever arm movement came from a study by Dominguez et al. (1998). They examined the crystal structure of the expressed smooth muscle myosin motor domain-essential light chain complex in the presence of MgADP.BeF_x. In this state the converter domain was rotated by about 70° compared to the post rigor state represented by the structure of the nucleotide free skeletal muscle S1. Thus, unlike that observed in *Dictyostelium* myosin, the smooth muscle MgADP.BeF_x structure corresponded to the pre-powerstroke state. Since this motor domain-essential light chain complex lacked the more distal regulatory light chain, its position was estimated by superimposing the lever arm from the skeletal S1 structure onto the smooth muscle myosin structure. The resulting distance between the ends of the lever arm in the two conformations was ~12 nm (Fig. 9.20). Thus structural evidence supported the lever arm hypothesis.

Spudich (1994) also was thinking in terms of the lever arm hypothesis but from a functional perspective. He reasoned that the step size that a myosin molecule would take should be proportional to the length of the neck domain or lever arm. If that was true and the actomyosin ATPase rate was unaltered, then the velocity of movement of F-actin in the in vitro motility assay should also be proportional to the length of the neck domain. Uyeda et al. (1996) addressed this issue using a recombinant myosin II from *Dictyostelium* with either shorter or longer neck domains. They found that these versions of myosin moved F-actin at a velocity in the in vitro motility assay that was linearly related to the length of the neck domain without a corresponding change in the rate of actin-activated ATPase. David M. Warshaw and Kathleen M. Trybus and their collaborators were thinking along the same lines (Warshaw et al. 2000). They expressed smooth muscle heavy meromyosin (HMM) mutants with either shorter or longer necks by deleting or adding light chain binding sites. The mutant HMMs were characterized with emphasis on measurements of unitary displacements, using the laser trap technique, and velocity of F-actin sliding using in vitro motility assay. The two shorter necked constructs had smaller unitary step sizes and moved F-actin more slowly than wild type HMM in the motility assay. A longer necked construct that contained an additional essential light chain binding site exhibited a 1.4-fold increase in the unitary step size compared with its control. These results supported the hypothesis that the neck functions as a rigid lever, stabilized by the light chains, with the fulcrum for movement located at a point within the motor domain. These conclusions were reminiscent of the proposed models of Cooke (1986) and Huxley and Kress (1985) (see Fig. 9.8). Thus there was general agreement that the swinging movement of the lever arm while the motor domain remained bound to actin in a relatively fixed orientation was the basis by which actin movement was powered by myosin.

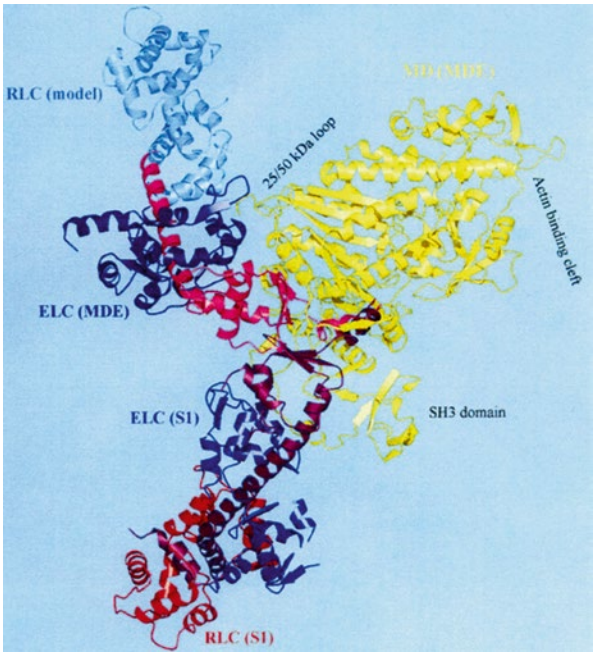


Fig. 9.20 Ribbon diagram representing the two different orientations of the myosin lever arm. Shown are the beginning of the power stroke (structure of the smooth muscle motor domain-essential light chain complex [MDE]) and the end of the power stroke (nucleotide-free chicken skeletal S1). The motor domains from the two structures have been superimposed. The motor domain is shown only for the MDE structure. The two structures diverge at an angle of about 70°. The regulatory light chain (RLC) in the lever arm of nucleotide-free chicken skeletal S1 is shown. Since the MDE construct lacks the RLC, shown here is a model obtained by superimposing the essential light chain (ELC) and the C-terminal helix of the heavy chain from nucleotide-free chicken skeletal S1 onto the corresponding amino acids of the MDE structure. The distance between the ends of the two lever arm conformations is 12 nm (Dominguez et al. 1998. With permission Elsevier)

9.5.8 Nucleotide Free Myosin V as a Model of the Rigor State

One of the problems in establishing a structural mechanism of cross-bridge action was that there were no crystal structures of the states where actin was bound to myosin. At first it was thought that the nucleotide free structure of chicken S1 represented the rigor state but later it was considered to be a post rigor state. Anne Houdusse, now at the Institut Curie CNRS in Paris, Lee Sweeney, Pierre-Damien Coureux and their collaborators solved the structure of an unconventional myosin, myosin V, without bound nucleotide (Coureux et al. 2003). Myosin V is an unconventional myosin involved in transporting vesicles along actin cables in the cell. Myosin V is a double headed processive motor which means that it exhibits many

mechanochemical cycles before dissociating from its filamentous track (myosin II is a non-processive motor, taking only one step per mechanochemical cycle). To achieve processive movement, the rates of key kinetic steps of myosin V are very different from myosin II which results in each head spending most of its ATPase cycle strongly bound to actin. The X-ray crystallographic structure of the motor domain of nucleotide-free myosin V showed that the cleft between the upper and lower 50-kDa domains was shut. This cleft closure was in contrast to the post-rigor and pre-powerstroke states where the 50-kDa cleft was open. Thus the nucleotide free myosin V appeared to be structurally in the strong binding state and therefore was taken as a model of myosin in the rigor complex (it was therefore referred to as rigor-like).

9.5.9 What Causes the Lever Arm to Move? Communication Between the Nucleotide Binding Site, the Actin Binding Domain and the Lever Arm

With structural information for three of the four primary states of the cross-bridge cycle as shown in Fig. 9.19, it was possible to propose a detailed mechanism for communication within the myosin motor domain (Geeves and Holmes 2005; Sweeney and Houdusse 2010). These three states are: rigor-like, post-rigor and pre-powerstroke. The core of the motor domain consists of a 7-stranded β-sheet surrounded by numerous α-helices, and several short nucleotide-binding sequences that show a striking tertiary structural homology with the G-proteins. The ATP binding site lies close to the apex of the 50-kDa cleft and consists of a "P-loop" (phosphate binding loop) motif flanked by switch 1 (SW1) and switch 2 (SW2) segments, similar to those found in G-proteins (Smith and Rayment 1996). The P-loop, SW1 and SW2 each contain 6–8 amino acid residues. The SW2 segment, which is structurally part of the lower 50K domain, connects with the "relay helix" and SH1 helix which in turn connect with the converter domain which connects to the lever arm.

Internal coupled rearrangements within the myosin motor domain allow direct communication between the nucleotide-binding site, the actin-binding interface, and the lever arm. After the binding of ATP in the post-rigor state, the SW2 segment moves about 5 Å and contacts the γ-phosphate of ATP and ATP hydrolysis occurs. This movement traps P_i and explains the stability of the pre-powerstroke conformation which underlies the low intrinsic ATPase activity of myosin in the absence of actin. The movement of SW2 also produces a "kink" in the relay helix and the converter domain rotates about 60° to cause lever arm repriming or the recovery stroke. Thus a motor domain movement of 0.5 nm leads to an approximate 10 nm movement at the end of the lever arm.

As far as the steps that take place on actin, there are only structures that represent the beginning (pre-powerstroke state, which is a weak binding state) and end (rigor-like state of myosin V, which is a strong binding state) of the cycle. Nonetheless one can generate an approximate model of the power stroke by imagining the recovery

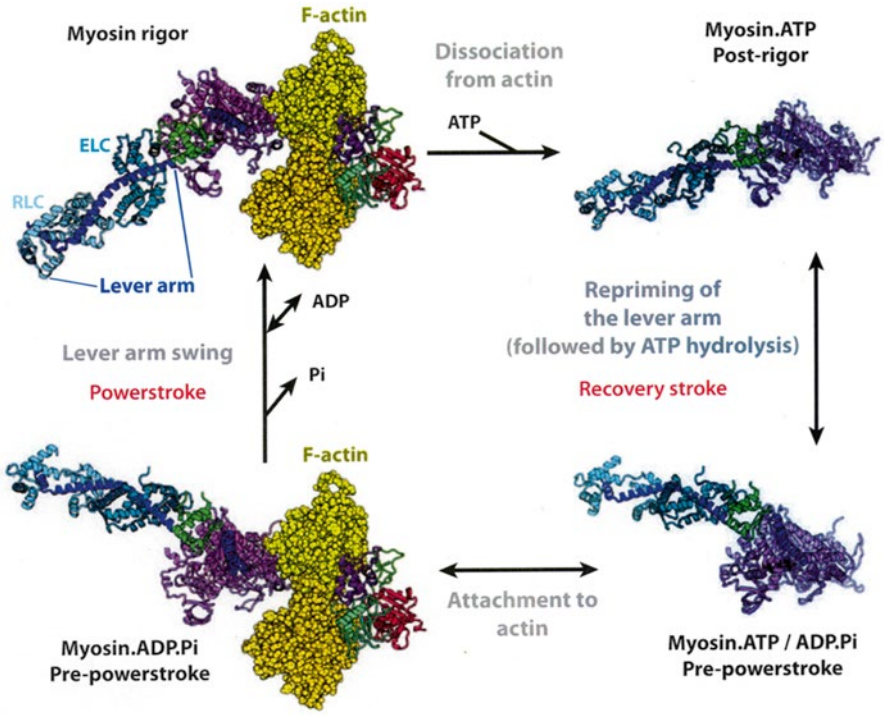


Fig. 9.21 Myosin structural states in the force-generating cycle. The scallop striated muscle myosin II S1 fragment is depicted in three structural states: rigor (nucleotide-free, docked to F actin), post rigor (detached from F actin, bound to an ATP analog), and pre-powerstroke (bound to ADP. P_i analog representing post hydrolysis of ATP with P_i trapped at the active site). The lever arm position is controlled by the position of the converter which swings relative to the rest of the motor domain. The distance measured at the distal end of the lever arm in the pre-powerstroke versus the rigor-like state is ~12 nm. Three actin monomers are indicated to represent the F actin filament. Two of the actin monomers are shown space-filled and one is shown as a ribbon diagram with the four subdomains (Sweeney and Houdusse 2010. With permission Annual Reviews)

stroke running backwards. Movement from the pre-powerstroke state to the rigor-like state (the power stroke) occurs upon myosin binding to actin. There is first a weak binding followed by strong binding and closure of the 50-kDa cleft which causes twisting of the β-sheet, movement of SW1 away from the γ-phosphate which opens the nucleotide binding pocket and leads to Pi release. This movement provides a structural explanation for the reciprocal relationship of ATP and actin binding to myosin. The closure of the of the 50-kDa domain and β-sheet distortion causes straightening of the relay helix, rotation of the converter domain and movement of the lever arm about 10 nm (the power stroke). A contemporary, though still somewhat speculative, view of structural aspects of the cross-bridge cycle is shown in Fig. 9.21. For more details, see reviews by Holmes (2008) and Sweeney and Houdusse (2010).

There are a number of interesting sidelights to the evolution of research on myosin structure determination since the first crystal structure was published in 1993. First, the heroic efforts of Ivan Rayment and his colleagues to produce suitable crystals for X-ray diffraction of myosin S1 have been superseded by recombinant techniques that allow production of truncated forms of myosin, without the lever arm, that crystallize easily. Protein methylation has not been utilized to generate crystals of the myosin motor domain since 1993. Also crystals of myosin II from vertebrate skeletal muscle have not been investigated since 1993. Rather the crystal structures of *Dictyostelium*, vertebrate smooth muscle and scallop muscle myosin II and myosin V (and myosin VI) have been studied extensively. There are two main reasons for this approach. First is the reasonable expectation of the universality of the mechanism of motility at the molecular level. Second, the expressed forms of the myosin motors allow controlled sequence manipulation via mutagenesis which has opened up a wide range of experimental possibilities (see the section below).

Despite the spectacular progress in elucidating myosin structure and the structural aspects of actin-myosin interaction in the past 20 years, Geeves and Holmes have emphasized the limitations of the structural results and the importance of considering kinetic and physiological information in understanding the cross-bridge cycle (Geeves and Holmes 2005. With permission Elsevier):

> Structural data can only yield the stable end states of a dynamic process. Moreover, so far there are no X-ray crystallographic data on the actin-myosin complex, meaning that electron microscopy data must by used to study the actin-myosin interaction. These data are of limited resolution and can only be interpreted by combining them with high-resolution structural data derived from myosin crossbridges not bound to actin. Therefore, we depend on kinetic and physiological measurements to correlate the crystallographic data with the crossbridge cycle.

There is still much more to be learned about the structural mechanism of the cross-bridge cycle.

9.6 Engineering the Myosin Motor Domain: Structure/Function Relationships

Along with the development of in vitro motility assays and the elucidation of the crystal structure of myosin II, the advent of molecular genetic approaches to mutating the myosin molecule stimulated the reductionist strategy to understanding myosin function at the molecular level. By the 1980s techniques were well developed to produce site-directed mutations into proteins, truncate the proteins or to generate chimeric proteins of a desired structure. An important starting point was development of expression systems that would allow the production of amounts of functional proteins sufficient for biochemical studies. In the motility field, the first successful expression system was developed in James Spudich's laboratory. Graduate student Arturo De Lozanne disrupted the myosin II heavy chain gene in the slime mould *Dictyostelium discoideum* (De Lozanne and Spudich 1987) and

Manstein et al. (1989) reported its complete knockout. With this null myosin II strain, it became possible by homologous recombination to incorporate specific mutations into the *Dictyostelium* myosin II heavy chain gene in order to investigate how this molecular motor transduces the chemical energy of ATP hydrolysis into mechanical movement. This expression system proved to be very useful but expression of proteins from higher eukaryotic organisms in *Dictyostelium* generally has not been successful. There was a need to develop heterologous systems in which active recombinant expression of myosin was possible. In the motility field this was first accomplished in 1994 by Sweeney and collaborators (1994) and then later that same year by Trybus (1994). They expressed enzymatically active heavy meromyosin-like fragments along with their respective light chains using baculovirus driven expression in an insect cell (*Spodoptera frugiperda*, Sf9) system. Other expression systems have been developed, particularly a bacterial system using *E. coli*. Sweeney and Holzbaur (1996) have reviewed the early work on mutational analysis of motor proteins and Sellers (1999) has listed the myosin mutations that were generated and studied through 1998 in his monumental monograph on the myosins.

9.6.1 Structure/Function Studies of Myosin: Kinetic Tuning of Myosin via Surface Loops

In December of 1994, Spudich put forward a provocative hypothesis in the journal Nature in an article entitled: "How molecular motors work". This swinging lever arm hypothesis integrated information from in vitro motility assays, molecular genetics and structural biology. As described above, he proposed that the myosin S1 neck acted like a lever arm. This aspect of the hypothesis is now generally accepted. He also proposed that the isoform diversity of kinetic properties, actomyosin ATPase and actin filament sliding velocity, within a myosin class was attributed mainly to two myosin surface loops, loop 1 and loop 2. These loops are flexible and thus were unresolved in the myosin crystal structure. The core of the myosin motor domain is well conserved in various myosin isoforms but the flexible loops are poorly conserved. Spudich suggested that it was this heterogeneity of the loops that determined isoform variations. Loop 1 (residues 204–216 in Fig. 9.17) connects the 25-kDa to the 50-kDa domain and is near the ATP binding pocket (Fig. 9.16b). Loop 2 (residues 627–646 in Fig. 9.17), lying between the upper and lower 50-kDa subdomains, connects the 50-kDa domain to the 20-kDa domain (Fig. 9.16b) and is located at the myosin binding interface with actin. Structural studies indicated that loop 2 interacted with the negatively charged amino-terminal part of actin.

Spudich suggested that there were two rate limiting steps in the actomyosin cycle, one that controlled the ATPase rate and the other that controlled the speed of actin filament movement. To understand this argument, first consider the movement of an actin filament generated by a single myosin head. The sliding velocity and ATPase activity are intimately linked since a single head can move the actin filament only at a velocity limited by the total time of the actin-activated ATPase cycle (t_c).

Thus the velocity of movement equals d/t_c, where d is the stroke size. In contrast, two myosin heads working asynchronously can produce twice the velocity because one head is generating movement during the time in which it is strongly bound while the other head is getting ready to generate movement. Since an actin filament cannot be moved any faster than the myosin heads can detach, the maximum velocity of movement by an ensemble of myosin heads working asynchronously is determined by the length of time that myosin is strongly bound to actin, t_s. The maximum velocity of filament movement therefore is d/t_s. The two different rate constants of the actomyosin cycle are $1/t_c$ and $1/t_s$. They would only be the same if the duty ratio, t_s/t_c, of myosin approached one. The duty ratio of myosin II is about 0.05 and thus the rate constants must be different (see Sect. 9.4.1).

Spudich proposed that the maximum actin activated ATPase activity ($1/t_c$), possibility limited by the rate of conversion of the weakly bound complex to the strongly bound complex, was determined primarily by loop 2. Whereas the maximum velocity of actin filament movement (d/t_s), likely limited by the ADP release rate, was determined primarily by loop 1. He did emphasize that probably this was not an all-or-none effect, as changing the myosin structure in any way was likely to have some effect on both the velocity and the rate of actin-activated ATPase. Nevertheless, he proposed that the trend would be strongly in the predicted direction.

Loop 2 myosin chimeras This proposal with regard to the function of the surface loops was derived from observations in the Spudich laboratory that were also published in Nature in 1994. Uyeda et al. (1994) generated chimeras of *Dictyostelium* myosin combined with loop 2 from different myosin molecules, including *Dictyostelium*, α- and β-isoforms of rat cardiac myosin, rabbit skeletal myosin and chicken smooth muscle myosin. Loop 2 from these different myosins varied in length from 9 to 27 amino acid residues. They then determined the actomyosin ATPase activity of each expressed chimeric myosin and the speed of movement of actin filaments in the in vitro motility assay. They found that the actin-activated myosin ATPase activity varied by about tenfold and correlated well with the activity of the myosin from which loop 2 was derived, i.e., skeletal muscle > α-cardiac > β-cardiac > smooth muscle myosin. Thus the results suggested that loop 2 was important in determining the enzymatic activity of myosin. In contrast, all the mutant myosins moved actin filaments more slowly than the wild-type *Dictyostelium* myosin. Furthermore the sliding velocity varied by less than a factor of two and no correlation was observed between the speed of the chimeric myosins and that of the donor of loop 2. Thus the actin-activated ATPase activity and the sliding velocity were disproportionately altered by these mutations. These results were contrary to the expectation based on Barany's (1967) observation of a strong correlation between actomyosin ATPase activity and maximum velocity of muscle shortening in various muscles. Those results suggested the same rate limiting step for actomyosin ATPase and maximum shortening velocity but the current results supported two rate limiting steps that could be uncoupled from each other under certain conditions. Uyeda, Ruppel and Spudich explained the discrepancy this way (Uyeda et al. 1994. With permission Nature Publishing Group):

> Previously, a comparison of the enzymatic and motile activities of skeletal muscle myosins from several animal species demonstrated a good correlation between ATPase activity and contraction speed[20]. In terms of kinetics, however, it is not clear why these two parameters should be tightly coupled. In generally accepted models, the myosin ATPase cycle consists of alternating weak- and strong-affinity states for actin [21,22]. The weak-affinity state is initiated by the binding of ATP to the myosin head, which is then followed by hydrolysis of ATP and release of inorganic phosphate (P_i) which leads to the strong-affinity state for actin. It is believed that the force-producing portion of the cycle follows or coincides with P_i release. The rate-limiting step of this cycle is proposed to be somewhere in the weakly bound state[22,23]. Conversely, the contraction speed should be related to the force-generating strongly bound state[24,25] and thus be relatively unaffected by the weakly bound state. Therefore, the observed correlation between the ATPase and motile activities of the skeletal muscle myosins is probably a consequence of functional optimization of these proteins for particular purposes, rather than being causal or obligatory. It is reasonable that different structural features of the motor limit each of these activities and that mutagenesis could alter one activity without affecting the other.

Thus they concluded that the Barany correlation was generally valid but not a mechanistic description of how molecular motors work.

Based on these results, Spudich (1994) made his proposal that a divergent surface loop, loop 2, at the actin-binding interface "tunes" the rate of phosphate release and thus sets the maximum actomyosin ATPase activity. Rovner et al. (1995) engineered loop 2 from skeletal or β-cardiac myosin into smooth muscle heavy meromyosin (HMM). Unlike wild-type HMM whose activity was regulated by light chain phosphorylation, these chimeric HMMs became unregulated like the myosin from which the loop was derived. They found that the maximum actomyosin ATPase activity was unchanged in these chimeras but that the K_m for actin was altered with the loop 2 substitutions. Thus the apparent actin affinity was highest for the skeletal loop, intermediate for the cardiac loop, and lowest for the wild-type smooth muscle loop. Since Uyeda et al. (1994) performed the actomyosin ATPase measurements at only one sub-saturating actin concentration, their results could not distinguish changes in maximum actomyosin ATPase activity from changes in K_m. The differences in K_m data supported a model in which loop 2 provides weak electrostatic binding to actin which positioned the myosin head on the actin surface so that strong interactions leading to force generation could proceed. Subsequently it was observed that the charge of loop 2, not the length of loop 2, determined the strength of interaction between mutant motor domains and actin (Furch et al. 1998), i.e., the greater the positive charge of loop 2, the greater the affinity of myosin for actin. Whether loop 2 affects only K_m or the maximum actomyosin ATPase activity or both, it clearly does alter the steady state ATPase activity of myosin. Thus there was strong evidence that loop 2 tunes the actin-myosin interaction.

Loop 1 myosin chimeras In a comprehensive study, Sweeney and his collaborators examined the role of loop 1 by inserting loops from different myosin II isoforms that displayed a range of actin filament sliding velocities into a smooth muscle myosin backbone (Sweeney et al. 1998). They generated and characterized a battery of ten chimeras. Although the nature of loop 1 affected the rate of ADP release (up to ninefold), the in vitro motility (2.7-fold), and the maximum actin-activated ATPase

activity (up to twofold), the properties of each chimera did not correlate with the relative speed of actin filament sliding generated by the myosin from which the loop was derived. The greatest effect of loop 1 was on the affinity of actomyosin for ADP. The rate of ADP release was a function of loop size/flexibility with the larger loops giving faster rates of ADP release. They found loop 1 could alter all of these kinetic properties of myosin to a degree that suggested that it has a role in determining isoform diversity. However, it did not appear to be the major determinant of any of the kinetic properties of myosin. Thus, loop 1 appeared to be a modulator of kinetic properties which are primarily determined by other structural elements within the myosin motor. Like Uyeda et al. (1994) they also observed that the rate of in vitro motility and the maximum actomyosin ATPase could be dissociated from each other suggesting that there was no fundamental mechanistic link between the two parameters. In agreement with Uyeda et al. (1994), they suggested that Barany's observed correlation may reflect evolutionary pressure to maintain proportionality between these parameters. Finally, Sweeney et al. (1998) concluded that although loop 1 is not the primary determinant of shortening speed of myosin, it is an important modulatory element of myosin kinetics. Furthermore they concluded that it is likely that the kinetic properties of myosin will be the sum total of numerous modulatory regions that must be altered in concert to achieve the observed functional range of the myosin motors.

It appears that the Spudich's (1994) provocative hypothesis of how molecular motors work is too simplistic from the viewpoint of the proposed separation of the roles of the surface loops 1 and 2. The surface loops are important and each of the loops affects both in vitro motility and actomyosin ATPase though the connection between the two parameters can be disrupted. Even though the correlation of maximum velocity of muscle shortening and maximum actomyosin ATPase observed by Barany in 1967 may not provide molecular insight into cross-bridge action, it is still a valuable predictor of cross-bridge behavior. Muscles that hydrolyze ATP rapidly, shorten rapidly. Murphy and Spudich (2000) reviewed the literature and summarized their perspective on the roles of the surface loops of myosin. They concluded that changes at the loops of myosin may be a general mechanism for motors and other enzymes to modify their activities without altering their core functions.

Other myosin surface loops Besides the major surface loops 1 and 2, there are other myosin surface loops that have been shown to affect myosin function. One of these surface loops has been called the hypertrophic cardiomyopathy loop (containing amino acid residues 403–416 in Fig. 9.17)[10] because a point mutation, arginine-403 to glutamine, in this loop was found to result in hypertrophic cardiomyopathy in humans. Sweeney and collaborators engineered this mutation into rat α-cardiac heavy meromyosin (truncated heavy chain with rat ventricular essential and regulatory light chains) and examined the kinetic properties of the expressed HMM (Sweeney et al. 1994). They found that that the actomyosin ATPase activity was

[10] The numbering of some of the myosin surface loops is approximate since they vary in position slightly and length amongst the various myosin molecules.

greatly reduced with a decrease in maximum ATPase activity and an increase in K_m. In vitro motility was reduced nearly fivefold by this single amino acid mutation. Thus, Arg-403 likely contributes to an important interaction at the actin interface of myosin affecting both maximum actomyosin ATPase activity and motility. Yet another surface loop, loop 3 (containing amino acid residues 565–579 in Fig. 9.17) is a secondary actin binding site of myosin II. The amino acid sequence of this loop preferentially affects the interaction of skeletal muscle myosin with actin without altering the rate of ATP binding or that of ATP hydrolysis by the actin-myosin complex (Van Dijk et al. 1999). There is also another loop, loop 4 (containing amino acids 364–380) that lies near to the HCM loop. It also has been implicated in actin binding (Gyimesi et al. 2008). Thus it appears that four myosin surface loops (loops 2, 3 and 4 and the HCM loop) are involved in binding to actin and stabilizing the interaction of myosin with actin. To resolve these interactions at the atomic level will ultimately require crystal structures of actin-myosin interaction at various points in the cross-bridge cycle.

9.7 Cross-Bridge Structural Changes During Contraction In Situ

9.7.1 Fluorescence Probes Re-visited

The advances in elucidation of the atomic structures of actin and myosin, in myosin engineering and in assessments of myosin motor function with in vitro motility assays were spectacular in the 1990s and led to the revision of Hugh Huxley's "swinging cross-bridge" theory of contraction to the "swinging lever arm" hypothesis of muscle contraction. Nonetheless it was still a major goal to test this hypothesis within the confines of the muscle sarcomere. Malcolm Irving (Fig. 9.22) at King's College London has investigated structural changes of cross-bridges during muscle contraction for more than 20 years by employing various biophysical techniques. In the late 1980s he settled on the use of extrinsic fluorescent probes attached to cross-bridges. This technique was first employed by Aronson and Morales (1969) 20 years previously. But now there were ways to assure specificity of probe location. Based on the work in the early 1980s that indicated that the catalytic domain of the myosin molecule did not tilt during contraction (Cooke et al. 1982), Cooke (1986) and Huxley and Kress (1985) proposed the revised cross-bridge model shown in Fig. 9.8. In this model, it was the neck of myosin S1 that moved during the power stroke. The light chains of myosin S1 were known to be located in the neck region even before the crystal structure of myosin S1 was resolved. Irving reasoned that a probe attached specifically to a myosin light chain might sense cross-bridge movement during contraction. Richard L. Moss at the University of Wisconsin had developed a protocol to reversibly remove the regulatory light chain from single skinned psoas muscle fibers isolated from rabbits (Moss et al. 1982). This procedure

Fig. 9.22 Malcolm Irving received a Ph.D. in physiology from the University of London working with Roger C. Woledge in 1979. He did postdoctoral training with Earl Homsher at UCLA and W. Knox Chandler at Yale University. He is currently a professor of biophysics at King's College London. Besides his long standing interest in the structural mechanism of cross-bridge action, he is also investigating changes in protein structure associated with muscle activation. His research has focused on dynamic changes of protein structure within muscle cells using polarized fluorescence and, in collaboration with the research group of Vincenzo Lombardi at the University of Florence, X-ray diffraction techniques. He was elected as a fellow of the Royal Society in 2003. Photo: courtesy M. Irving

was based on a protocol originally developed for scallop muscle by Chantler and Szent-Gyorgyi (1980). Irving developed a long term collaboration with John Kendrick-Jones at the MRC Laboratory of Molecular Biology in Cambridge, England. They purified the regulatory light chain from chicken gizzard and covalently attached a rhodamine derivative to its single cysteine residue (cys108). They then exchanged this light chain into rabbit skinned psoas muscle fibers. In a preliminary report (Irving et al. 1989), they estimated that about 50 % of the myosin heads contained the labeled regulatory light chain and that this exchange resulted in only a modest decrease (about 10 %) in Ca^{2+} activated isometric force. Thus they knew that the fluorophore was located on the myosin lever arm and no where else. They measured the fluorescence intensity polarization ratios when the fiber was excited with polarized light oriented parallel or transverse to the fiber axis and collected the light emitted parallel or transverse to the fiber axis.[11] These preliminary steady state results suggested that there was a change in orientation of the region

[11] Fluorescence polarization ratios (Q or sometimes P) are defined as $Q_{\perp} = ({}_{\perp}I_{\perp} - {}_{\parallel}I_{\perp})/({}_{\perp}I_{\perp} + {}_{\parallel}I_{\perp})$ and $Q_{\parallel} = ({}_{\parallel}I_{\parallel} - {}_{\perp}I_{\parallel})/({}_{\parallel}I_{\parallel} + {}_{\perp}I_{\parallel})$. The pre- and post-subscripts in the fluorescence intensities (I) indicate excitation (pre) and emission (post) polarization relative to the fiber axis, either perpendicular ($\perp$) or parallel ($\parallel$).

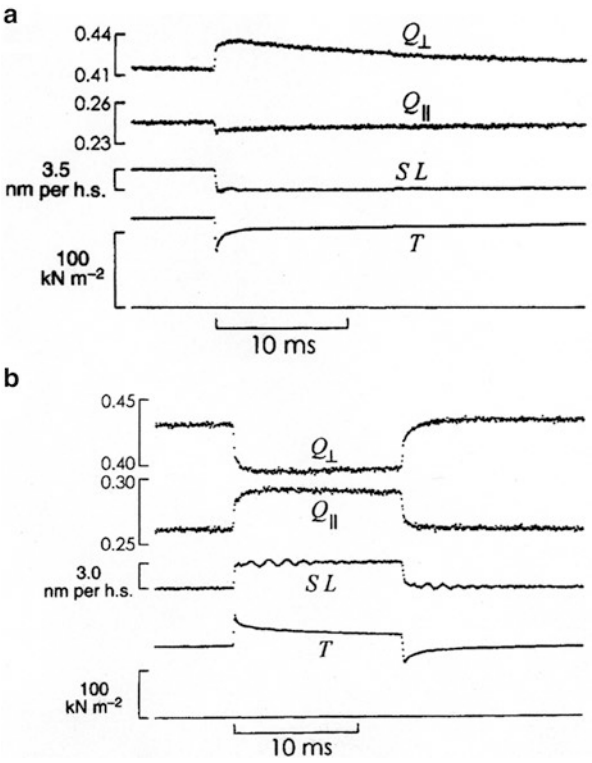


Fig. 9.23 Fluorescence polarization ratios (Q) (see footnote 11) from rhodamine probes on myosin regulatory light chains, sarcomere length change (SL), and tension (T) in response to rapid length steps during active contraction of single skinned fibers from rabbit psoas muscle (12 °C). (**a**) Release step. (**b**) Stretch/release cycle. Note that the fluorescence polarization ratios change without delay during and after a step and change in opposite directions from release to stretch. See text for interpretation of ratio changes (Irving et al. 1995. With permission Nature Publishing Group)

of the myosin head near cys108 in the regulatory light chain when myosin binds to actin in rigor.

In order to test the swinging lever arm hypothesis with extrinsic fluorescent probes, it was necessary to measure fluorescence changes during the power stroke itself. This was a vexing challenge because cross-bridges were known to work asynchronously. Hugh Huxley and Robert Simmons and collaborators (Huxley et al. 1983) attempted to synchronize cross-bridge movement with rapid (sub-millisecond), small changes in muscle length. According to the work of Huxley and Simmons (1971), during the rapid step and for the first few milliseconds of force recovery after the length change, no cross-bridges detached and no new cross-bridges attached. Thus one was examining only the effects of the changes of length on the orientation of attached force generating cross-bridges. This interpretation meant that the fluorescence changes had to be monitored with submillisecond time resolution during a release and initial force recovery. To accomplish this goal, Irving collaborated with Yale E. Goldman at the University of Pennsylvania. Together they were able to produce 150 μs length steps in a single contracting muscle fiber and simultaneously measure the changes in polarized fluorescence with 50 μs temporal resolution. The results of this technical *tour de force* were published in Nature in 1995 (Irving et al. 1995). Figure 9.23 shows examples of the results when rapid

length steps were imposed on a muscle fiber. They monitored the tension change (T), the sarcomere length (SL) and the fluorescence polarization ratios ($Q_{\parallel}$, parallel to the fiber axis, and $Q_{\perp}$, perpendicular to the fiber axis). The increase in $Q_{\perp}$ and decrease in $Q_{\parallel}$, as observed on a rapid shortening (Fig. 9.23a), indicated that the rhodamine dipole had become more perpendicular to the fiber axis. When a quick stretch was applied to the active muscle fiber (Fig. 9.23b), $Q_{\perp}$ decreased and $Q_{\parallel}$ increased, which indicated that the rhodamine dipole became more parallel to the fiber axis. These tilting motions of the light chain domain of the myosin head took place both during the length step itself and during the rapid force recovery associated with the working stroke. They concluded that: "…the results provide strong evidence for tilting of the light-chain region during length changes and during force generation, two central tenets of the tilting crossbridge model." (Irving et al 1995. With permission Nature Publishing group).

As interesting as these results were, there was a problem. The amplitude of the fluorescence polarization signals was small compared with the predictions of the working stroke model. The calculated lever arm movement during the power stroke was only about 1 nm, much less than the expected 5–10 nm step that other experiments suggested. This discrepancy suggested that not all the myosin heads in a muscle fiber responded to the applied length step. The results could be made compatible with other experiments if only 10–20 % of the cross-bridges generated force during contraction. The fluorescence polarization signals represented the average motion of all the heads in the fiber, whether strongly bound to actin, weakly bound in a wide range of conformations, or detached. If the weakly bound and detached heads do not respond to the length step, the amplitude of the fluorescence polarization signals would be relatively small, as they observed.

In a commentary on these experiments, Andrew Huxley (1995) praised the work as a "most encouraging start" of a new approach to the central problem of muscle contraction. But he also emphasized uncertainties in the interpretation of the measurements. One of the problems was that the orientation of the fluorophore dipole(s) relative to the long axis of the myosin head was unknown and furthermore the orientation of the reporter fluorophore relative to the protein might change during the contractile cycle. In order for the measured tilting of the regulatory light chain to be in the expected direction during and after a shortening or lengthening step, it was necessary for Irving et al. (1995) to assume a particular fixed orientation of the probe with respect to the regulatory light chain. To alleviate some of this uncertainty, organic chemist John E. T. Corrie working with colleagues James S. Craik and V. Ranjit N. Munasinghe (1998) in David R. Trentham's research group at the National Institute for Medical Research, Mill Hill, London, synthesized a bifunctional rhodamine for labeling proteins with defined orientations of the fluorophore. The bifunctional rhodamine was designed for two-site, 1:1 labeling of proteins that contain two suitably disposed cysteine residues and was intended to constrain the orientation of the rhodamine absorption and emission dipoles in a predictable relationship to the protein structure. With the probe dipole fixed in the coordinate frame of the protein, it should be possible to measure molecular coordinates in real time in

a cell and thus to bridge the gap between static crystal structures and cellular function.

With regard to muscle, a set of labeled regulatory light chains was generated with differing three-dimensional orientations of the fluorophore and fluorescence changes were measured during rest, contraction and rigor (Corrie et al. 1999). The crystal structure of the regulatory light chain bound to myosin S1 was used to select four pairs of solvent-accessible locations for the cysteines. New cysteines were introduced into the regulatory light chain (RLC) by site-directed mutagenesis and the proteins expressed in an *E. coli* expression system. These modifications of the regulatory light chains did not alter light chain function and did not significantly decrease force production in psoas fibers. The fluorescence polarization results confirmed and extended the earlier observations but now without the uncertainty of probe orientation. They concluded (Corrie et al 1999. With permission Nature Publishing Group): "Thus, our results show that the motions of the RLC region predicted by the crystallographic models do take place in working muscle fibres, and that filament sliding involves both tilting and twisting of this domain." These experiments were extended in 2002 with a detailed quantitative analysis of the results (Hopkins et al. 2002). Even though the results are convincing, not all of the issues have been solved and these experiments are ongoing.

9.7.2 X-Ray Diffraction and Mechanical Studies of Cross-Bridge Conformation and Motion

Despite the strong supporting evidence for the swinging lever arm hypothesis of cross-bridge action from in vitro and from skinned muscle studies, it was still important to test this hypothesis in an intact muscle preparation. Realistically the only way to monitor possible cross-bridge structural changes and motions with submillisecond resolution in an intact muscle is with X-ray diffraction techniques. The obvious disadvantage of this approach is that the interpretation of the results require a model of the cross-bridge. By the late 1990s crystallography had provided an atomic model of the cross-bridge in two proposed states of the cross-bridge cycle (see Fig. 9.24) that could be tested by X-ray diffraction techniques. Also by the turn of the century a new approach to determining the extent of cross-bridge motion under different loading conditions, X-ray interferometry, had revitalized the field.

Despite the enigmatic nature of X-ray diffraction, the technique has important advantages. One can examine structural changes simultaneously with changes in the generation of force or shortening in intact muscle fibers with high temporal resolution in a nondestructive manner. Effort has concentrated on measuring and interpreting the meridional or axial X-ray reflections because these reflections are produced by the regular array of myosin heads along the myosin filaments. In particular the M3 reflection corresponds to the 14.5 nm (earlier believed to be 14.3 nm) axial repeat of the myosin heads and as such is sensitive to axial motions of the

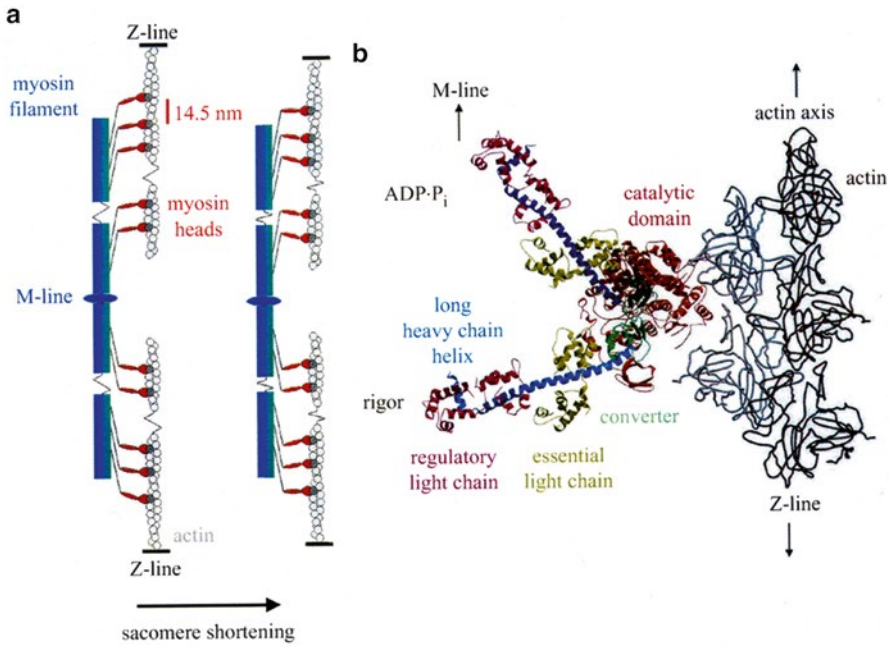


Fig. 9.24 Structural models for the mechanism of the myosin motor. (**a**) Arrangement of the actin and myosin molecules in the muscle sarcomere. Sarcomere shortening (*left to right panel*) is associated with tilting of myosin heads so that their mass moves towards the center of the sarcomere (the M-line). [The "springs" in the thick and thin filaments represent the fact the filaments are compliant.] (**b**) Atomic model for the working stroke in the myosin head. Actin filament (Holmes et al. 1990) axis vertical. The lower conformation of the myosin head shows the ATP-free crystal structure of a head fragment bound to the actin filament (Rayment et al 1993a, b); the upper one (ADP.P_i) is based on a crystal structure of a smooth muscle myosin head fragment with an ADP.P_i analogue bound at the active site (Dominguez et al. 1998), with its catalytic domain superimposed on that of the rigor conformation, and assuming that the light-chain domain of the head moves as a rigid body (see Fig. 9.20) (Lombardi et al. 2004. With permission The Royal Society)

heads (see Fig. 6.5). Furthermore this reflection remains strong and readily measurable during muscle contraction. The meridional X-ray reflections provide three independent sources of information: (a) intensity (or amplitude) due to the conformation of the myosin heads, (b) interference fine structure attributed to the extent of axial motions of the myosin heads and (c) spacing due to periodicity and compliance of the myosin filaments.

9.7.3 Myosin Head Conformations During and After Rapid Changes in Muscle Length

In a pioneering study by Hugh Huxley and colleagues (Huxley et al. 1983), cross-bridge action in whole muscles was synchronized by rapid changes in muscle length during contraction and the changes in meridional reflections and mechanical

responses monitored during the first few milliseconds after the length perturbation. Irving et al. 1992 extended these experiments to single muscle fibers where it was possible to obtain higher temporal resolution of structural and mechanical changes[12]. They monitored the changes in the intensity of the M3 reflection during and immediately after a rapid, 150 μs, change in fiber length. Based on a simple structural model of the working stroke, they concluded that the myosin heads moved by about 10 nm with the same time course as the elementary force-generating process.

After the elucidation of the myosin S1 crystal structures in what were proposed to be the beginning and end of the working stroke (Rayment et al. 1993a, b; Dominguez et al. 1998) (Fig. 9.24), it was possible to construct a more realistic model of the expected changes in the intensity of the M3 reflection when the cross-bridges were in different orientations during the working stroke. This model reflected the interdomain bending of the lever arm during the working stroke. Irving, Lombardi and their collaborators measured the changes in the intensity of the M3 reflection (I_{M3}) during and after 150 μs changes in length of a contracting muscle fiber (Fig. 9.25a) (Irving et al. 2000). I_{M3} increased slightly during the elastic phase 1 of the force transient and then decreased substantially during the rapid force recovery presumed to be due to the working stroke, phase 2. This decrease suggested that a large change in the conformation of the myosin heads accompanied the rapid force recovery. They then compared these results to the expected changes in I_{M3} calculated from the mass projections of the crystallographic structures onto the filament axis (Fig. 9.25b). The I_{M3} versus displacement of the myosin head-rod junction (Δz) in Fig. 9.25b was calculated assuming that all the myosin heads in the fiber have the same orientation but the relationship was almost identical for a constant Δz dispersion of a few nanometers. The critical assumption was that the dispersion did not change during the length step, i.e. that the myosin heads that were attached to actin before the length step was applied remained attached during the rapid mechanical responses. Δz was taken to be zero for the rigor conformation and was +10.6 nm for the ADP•P_i conformation. The I_{M3} reflection is relatively weak in both these conformations because the axial mass projection of the light-chain domain is displaced from that of the catalytic domain (Fig. 9.24). The maximum value of I_{M3} is obtained for an intermediate tilt of the light-chain domain, $\Delta z = 5$ nm (Fig. 9.25b), when both domains have the same axial position. When a length step is applied, it was proposed that the light chain domain of the myosin heads tilted during both the elastic phase 1 response and the active phase 2 response. The changes in I_{M3} produced by shortening and stretch were accounted for quantitatively if Δz during isometric contraction was +7 nm (Fig. 9.25b). The main conclusions

[12] In X-ray diffraction experiments with whole muscles, an X-ray detector collects information from about 100 muscle fibers (Huxley et al. 2006). Thus single fiber experiments result in an approximate 100 fold lower X-ray detection for the same beam intensity and require extensive signal averaging of repeated contractions. (Reconditi et al. 2004). Nonetheless experiments with single fibers allow almost an order of magnitude higher temporal resolution of mechanical and structural changes than possible with whole muscle. Because muscle fibers diffract X-rays very weakly, it is necessary to use a very bright X-ray source and this means that the experiments must be performed at a synchrotron or electron storage ring facility such as the European Synchrotron Radiation Facility (ESRF), Grenoble, France or the Advanced Photon Source (APS), Argonne National Laboratory (ANL), outside of Chicago, Illinois. (Reconditi et al. 2004; Huxley et al. 2006).

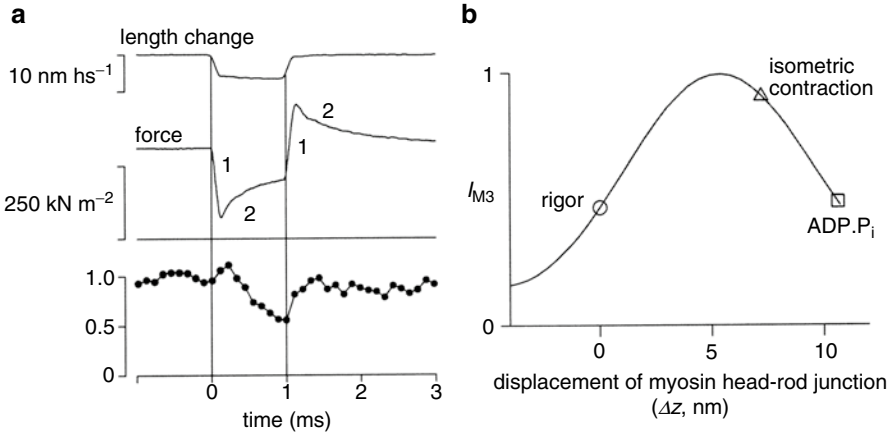


Fig. 9.25 Changes in the intensity of the M3 reflection (I_{M3}) produced by length steps in a single muscle fiber. (**a**) Changes in force and I_{M3} in response to a shortening step followed 1 ms later by a re-stretch to the original length. (**b**) Relationship between I_{M3} and the displacement of the myosin head rod junction ($\mathbf{\Delta}z$), calculated from the structural model in Fig. 9.24b. $\mathbf{\Delta}z=0$ corresponds to the ATP-free or rigor structure (*circle*) (Rayment et al. 1993a, b). The square corresponds to the ADP.P_i structure (Dominguez et al. 1998). The *triangle* represents the cross-bridge orientation during the isometric contraction. This structural model can quantitatively explain the changes in I_{M3} in terms of cross-bridge orientation during and after the step change in length (Lombardi et al. 2004. With permission The Royal Society)

from these experiments on the changes in I_{M3} induced by length steps were that that myosin heads tilted during both the elastic response and during the working stroke and that, in terms of the in vitro structural model for the working stroke (Fig. 9.24), the conformation of the light-chain domain of the myosin head was tilted up by 7 nm during isometric contraction from that observed in the rigor (ATP-free) complex. Thus the modeling of the observed changes in the I_{M3} reflections supported the proposed lever arm movement of the cross-bridge during contraction.

9.7.4 X-Ray Interference and Axial Motions of Myosin Heads

The X-ray intensity profiles of the thick and thin filament-based axial reflections from skeletal muscle do not always have the simple shape expected for diffraction from a uniformly spaced array of structures but are often split into two or more subpeaks. This is the result of X-ray interference between the two arrays of structures in the bipolar filaments. This result of X-ray interference in skeletal muscles was first recognized many years ago by Ed J. O'Brien, a member of Jean Hanson's research group at King's College London. In a series of interesting experiments, Elizabeth Rome utilized X-ray diffraction to determine the location on the thick filament of antibodies to C-protein in skinned muscle fibers (Rome 1973; Rome et al. 1973a). Based on electron micrographic studies, she expected the antibody to

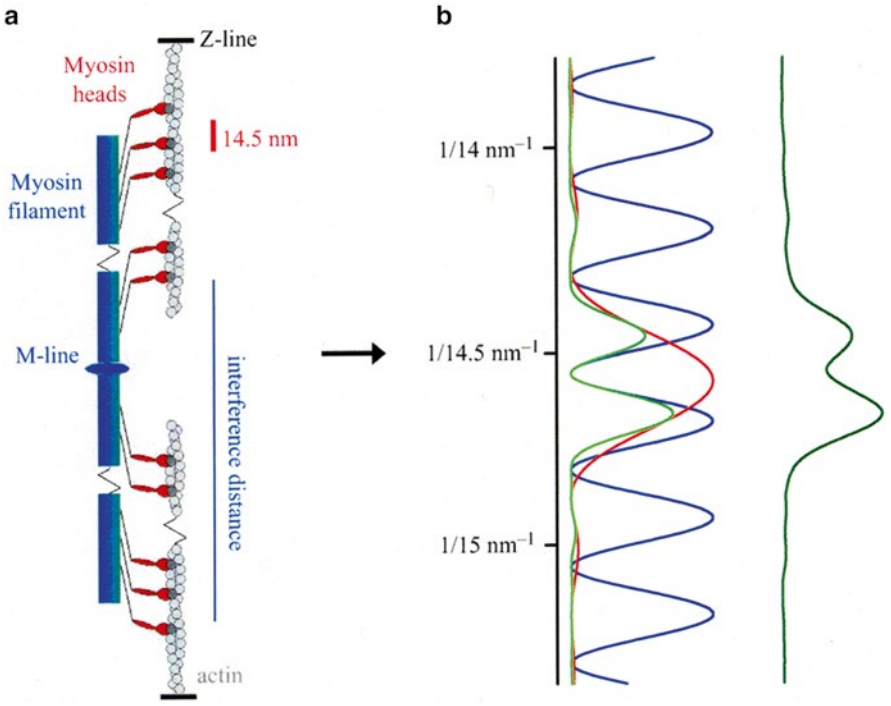


Fig. 9.26 Origin of the interference fine structure of the M3 X-ray reflection in muscle fibers. (**a**) Sarcomere structure and (**b**) calculated and observed intensity profiles. Each myosin filament in the sarcomere contains two arrays of myosin heads. A single array would give the diffracted intensity profile shown as a single broad peak (**b**) near 1/14.5 nm^{-1}. Interference between the two arrays multiplies the diffraction pattern by a sinusoidal function (multiple peaks in **b**) with spacing corresponding to the interference distance between pairs of myosin heads in the filament. The resulting intensity profile due to interference (smaller split peaks) is similar to that observed from a single muscle fiber during isometric contraction (*right hand side*) (Lombardi et al. 2004. With permission The Royal Society)

exhibit a single peak on the meridian with a 44 nm periodicity but what she saw was a doublet where the single peak was expected. In 1968 O'Brien suggested to Rome that the doublet might result from X-ray interference effects in the bipolar thick filament. The same interpretation was applied to the doublet associated with troponin on the thin filament (O'Brien et al. 1971; Rome et al. 1973b).

X-ray interference would also be expected to occur between the two arrays of myosin heads in each half of the myosin filament (Fig. 9.26)[13]. Each half-myosin filament has 49 layers of myosin heads with a regular axial periodicity of 14.5 nm (Fig. 9.26a). The calculated intensity distribution in the M3 region of the axial dif-

[13] One would not expect to see interference effects in the myosin pattern between one A-band in a myofibril and the next A-band in the same myofibril since the actin filaments between them have a subunit repeat different from that of the myosin, and are unlikely to maintain exact registration (Huxley et al. 2006).

fraction pattern corresponding to a spacing of 14.5 nm is shown in reciprocal space as the single broad peak in Fig. 9.26b. However, for every level of heads in one half of each myosin filament there is another level in the other half about 860 nm further along the filament (Fig. 9.26a). In general, the two half filaments do not diffract in phase with each other, but form an interference pattern. Interference between the diffracted X-rays from the two arrays has the effect of multiplying the diffraction pattern from a single array by a finely spaced sinusoidal function (multiple peak curve in Fig. 9.26b), with a periodicity corresponding to the 860 nm interference distance. Due to interference the resulting M3 reflection is split into two smaller peaks (Fig. 9.26b). The M3 profile observed during isometric contraction of a single muscle fiber (Fig. 9.26b, far right) is similar to the calculated curve. That this interpretation is correct was confirmed by Linari et al. (2000). They measured the intensity of the M3 reflection during active contraction at different stretched sarcomere lengths. The total I_{M3} decreased in proportion to the decrease in myofilament overlap which suggested that the reflection came from the myosin heads and that myosin heads that cannot bind to actin during active contraction were disordered and thus made little contribution to the M3 reflection. Furthermore the separation between the two M3 peaks was smaller at longer sarcomere lengths. This decrease in separation was explained quantitatively by the increase in interference distance at the longer sarcomere lengths, i.e., the M3 peaks were closer together in reciprocal space because the interference distance was greater. Finally the relative intensity of the M3 peaks during contraction was independent of sarcomere length which indicated that the axial periodicities of the nonoverlap and overlap regions of the myosin filament had the same 14.5 nm value. They concluded that the results established x-ray interferometry as a new tool for studying the motions of myosin heads during muscle contraction with unprecedented spatial resolution.

The beauty of this technique as recognized by Vincenzo Lombardi and his colleagues was that the X-ray interference fine structure of the M3 reflection provided an extremely sensitive measure of the average axial position of the center of scattering mass of the cross-bridges and thus the motion of the myosin heads toward the M-line of the sarcomere. In fact movements in the subnanometer range could be readily measured. For example, as pointed out by Linari et al. (2000), a change in shape of the heads that produced a centroid motion of only 0.5 nm in each half-sarcomere would change the relative intensity of the M3 peaks from 1:0.93 to 1:0.69, which would be easily resolved. This sensitivity, combined with the association of the M3 reflection with the force-generating population of myosin heads, makes the fine structure of the M3 reflection a powerful tool for studies of the mechanism of muscle contraction. Another important point is that the structural information obtained from the ratio of interference peaks (R_{M3}) is essentially independent of that from the total intensity of the reflection, I_{M3}. As discussed above, the total intensity of the M3 reflection (I_{M3}) depends on the conformation of the myosin heads. In contrast the effect of interference on I_{M3} is less than 2 % at any interference distance above 800 nm (Linari et al. 2000). Finally Lombardi et al. (2004) noted that R_{M3} depends only on the axial displacement of the center of mass of the myosin heads from the M-line and does not depend on the conformation of the myosin

heads. Moreover R_{M3} is also independent of changes in the disorder of the myosin heads. These properties imply that changes in R_{M3} can be interpreted without a detailed structural model which increases the generality of the conclusions. Thus changes in the ratio of the M3 interference peaks can be an extremely sensitive measure of extent of cross-bridge motion without the burden of developing a detailed structural model. Nonetheless there were complications that had to be overcome.

The Lombardi and Irving research groups first examined the effects of step changes in muscle fiber length on R_{M3} with high temporal resolution. But the interpretation of the results was made difficult by the fact the thick filaments, thin filaments and cross-bridge all have about equal compliance (Huxley et al. 1994; Wakabayashi et al. 1994). This fact complicates the determination of cross-bridge movement during the initial fast recovery of force from a quick release. As the cross-bridges move and go through their working stroke, force increases which simultaneously causes increases in thick and thin filament lengths. Thus to interpret the results in terms of cross-bridge movement alone, one must know accurately the compliance of the thick and thin filaments and make a correction for compliance effects. A conceptually simpler approach would be to generate an abrupt change in fiber force and monitor the subsequent cross-bridge movements at constant force. In fact the first study of mechanical transients by Podolsky (1960) did utilize force steps rather than length steps. Andrew Huxley determined that the transient phases (phases 1, 2, 3 and 4) in the length and force steps had the same interpretation but he preferred length steps because length steps could be made faster than force steps (see Chap. 5). Lombardi and his research group are experts at performing transient mechanical experiments and they succeeded in generating 150 μs force steps during an isometric contraction (Piazzesi et al. 2002).

These so called load clamp experiments, as opposed to length clamp experiments, allowed a determination of the changes in R_{M3} uncontaminated by changes in force and thus without simultaneous changes in thick and thin filament lengths (Reconditi et al. 2004). An example of their mechanical results is shown in Fig. 9.27e. Force was rapidly decreased to 50 % of the isometric value and the transient changes in fiber length recorded at high resolution. Four phases were observed. Phase 1 represented the instantaneous elasticity of the fiber. During phase 2 the sarcomeres continued to shorten rapidly for a few milliseconds after the load step. The shortening velocity then transiently decreased (phase 3) before increasing again to the value associated with the steady state force–velocity relationship of the fiber. As in the length clamp experiments, the myosin heads remain attached to actin and their center of mass moves toward the M-line of the sarcomere during both phase 1 and phase 2 of the response to a load step. This interpretation is shown schematically in Fig. 9.27a–c. Because the force and the strain in the filaments are constant during phase 2 of the velocity transient, the axial motions of the myosin heads during this period provide a *direct measure* of the working stroke of the heads. The axial motions of the myosin heads are reflected in variation of the ratio of the intensities of the higher and lower angle peaks of the M3 reflection (R_{M3}) during the velocity transient as shown in Fig. 9.27f. When they examined the results at three

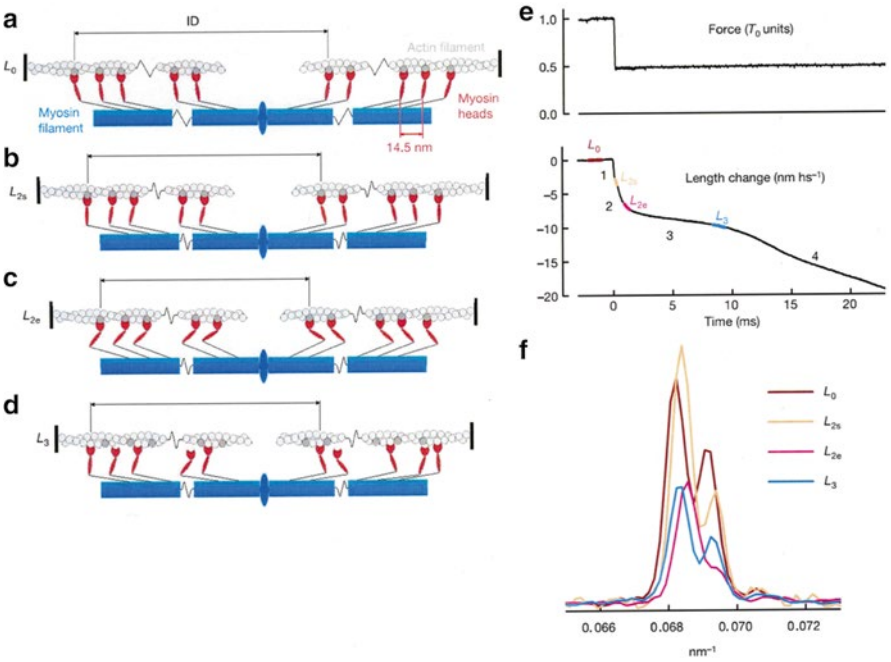


Fig. 9.27 Changes in the interference fine structure of the M3 reflection following a load step in a single muscle fiber and interpretation in terms of the axial motions of the myosin heads. (**a**–**d**) Motions of the myosin heads at the four stages of the velocity transient shown in (**e**): (**a**) isometric contraction (L_0); (**b**) start of phase 2 (L_{2s}); (**c**) end of phase 2 (L_{2e}); (**d**) end of phase 3 (L_3). (**f**) Axial intensity distribution in the region of the M3 X-ray reflection at the periods corresponding to the X-ray exposure times shown in (**e**). Note that the ratio of the two M3 peaks (R_{M3}) changes dramatically during the various phases of the load clamp (Reconditi et al. 2004. With permission Nature Publishing Group)

different loads, they observed that both the working stroke and the subsequent detachment from actin were markedly slower at higher loads. Furthermore a plot of the R_{M3} versus the extent of filament sliding in each half sarcomere, gave the size of the working stroke. These results showed directly that the working stroke of the myosin motor is smaller at a higher load. At a low load the working stroke is about 12 nm. At higher loads the myosin head detaches from actin before reaching the structural limit that defines the low load stroke. The 12 nm stroke size at low load compared well with the predicted stroke size based on the atomic structures of myosin but was about twice as large as that observed in the in vitro motility assay by Molloy et al. (1995). Lombardi et al. (2004) speculated that the in vitro assays underestimated the performance of the motor under physiological conditions, presumably because they do not preserve the native structural and geometrical relationship between the myosin and actin filaments.

During the same period of time that Lombardi and Irving and colleagues were performing their X-ray interference experiments on single fibers, Hugh Huxley, Massimo Reconditi, Alex Stewart and Tom Irving (no relation to Malcolm Irving) (2006) were performing similar experiments using length clamps on whole muscles. They concluded that the results on whole muscles in quick releases were similar to those obtained on single fibers by Reconditi et al. (2004).

Excellent reviews of the single fiber X-ray experiments and their interpretation in terms of cross-bridge conformation and motion have been written by Lombardi et al. (2004) and Reconditi (2006). Also a clear description of the concept of X-ray interference related to the cross-bridges is provided by Huxley et al. (2006).

9.8 Conclusion

When the 80 year old Huxley (2004) reviewed 50 years of investigation of the sliding filament hypothesis of muscle contraction during which time he was a pivotal contributor, he was finally able to state with confidence: "…I really do believe that, altogether, there is now incontrovertible evidence for the correctness of the tilting lever-arm model…" (Huxley 2004. With permission John Wiley & Sons Inc) In the true spirit of scientific investigation, Huxley quickly added: "…although of course many important details still remain to be worked out." Thus in many ways there is now a very satisfactory answer to the question: "what is the mechanism of muscle contraction"? But Ken Holmes at about the same time issued this note of realism (Holmes 2004. With permission The Royal Society): "We expect that well within the next 50 years a complete understanding of the structural events behind the power stroke will emerge." What is the difference between the two statements? Huxley's view might be considered a molecular mechanism and Holmes' view an atomic mechanism. Either way much has been discovered with still more mysteries to unravel.

References

Allen RD, Allen NS, Travis JL (1981) Video-enhanced contrast, differential interference contrast (AVEC-DIC) microscopy: a new method capable of analyzing microtubule-related motility in the reticulopodial network of allogromia laticollaris. Cell Motil 1:291–302

Aronson JF, Morales MF (1969) Polarization of tryptophan fluorescence in muscle. Biochemistry 8:4517–4522

Ashkin A (1997) Optical trapping and manipulation of neutral particles using lasers. Proc Natl Acad Sci U S A 94:4853–4860

Ashkin A (2006) Optical trapping and manipulation of neutral particles using lasers. World Scientific Publishing Co., Hackensack

Ashkin A, Dziedzic JM, Bjorkholm JE, Chu S (1986) Observation of a single-beam gradient force optical trap for dielectric particles. Opt Lett 11:288–290

Barany M (1967) ATPase activity of myosin correlated with speed of muscle shortening. J Gen Physiol 50(Pt. 2):197–216
Block SM, Goldstein LSB, Schnapp BJ (1990) Bead movement by single kinesin molecules studied with optical tweezers. Nature 348:348–352
Borejdo J, Putnam S (1977) Polarization of fluorescence from single skinned glycerinated rabbit psoas fibers in rigor and relaxation. Biochim Biophys Acta 459:578–595
Bremel RD, Weber A (1972) Cooperation within actin filament in vertebrate skeletal muscle. Nat New Biol 238:97–101
Brune M, Hunter JL, Corrie JET, Webb MR (1994) Direct, real-time measurement of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment 1 ATPase. Biochemistry 33:8262–8271
Chantler PD, Szent-Gyorgyi AG (1980) Regulatory light-chains and scallop myosin: full dissociation, reversibility and co-operative effects. J Mol Biol 138:473–492
Chu S (1997) Nobel lecture: the manipulation of neutral particles. Nobelprize.org. pp 122–158
Cooke R (1986) The mechanism of muscle contraction. CRC Crit Rev Biochem 21:53–118
Cooke R (1997) Actomyosin interaction in striated muscle. Physiol Rev 77:671–697
Cooke R (2004) The sliding filament model: 1972–2004. J Gen Physiol 123:643–656
Cooke R, Highsmith S (2011) Manuel Francisco Morales, 1919–2009. Biogr Mem: Nat Acad Sci 3–16
Cooke R, Morales MF (1969) Spin-label studies of glycerinated muscle fibers. Biochemistry 8:3188–3194
Cooke R, Pate E (1985) The effects of ADP and phosphate on the contraction of muscle fibers. Biophys J 48:789–798
Cooke R, Crowder MS, Thomas DD (1982) Orientation of spin labels attached to cross-bridges in contracting muscle fibres. Nature 300:776–778
Corrie JET, Craik JS, Munasinghe VRN (1998) A homobifunctional rhodamine for labeling proteins with defined orientations of a fluorophore. Bioconjug Chem 9:160–167
Corrie JET, Brandmeier BD, Ferguson RE, Trentham DR, Kendrick-Jones J, Hopkins SC, van der Heide UA, Goldman YE, Sabido-David C, Dale RE, Criddle S, Irving M (1999) Dynamic measurement of myosin light-chain-domain tilt and twist in muscle contraction. Nature 400:425–430
Coureux P-D, Wells AL, Menetrey J, Yengo CM, Morris CA, Sweeney HL, Houdusse A (2003) A structural state of the myosin V motor without bound nucleotide. Nature 425:419–423
Cyranoski D (2000) Swimming against the tide. Nature 408:764–766
Dantzig JA, Hibberd MG, Trentham DR, Goldman YE (1991) Cross-bridge kinetics in the presence of MgADP investigated by photolysis of caged ATP in rabbit psoas muscle fibres. J Physiol 432:639–680
Dantzig JA, Goldman YE, Millar NC, Lacktis J, Homsher E (1992) Reversal of the cross-bridge force-generating transition by photogeneration of phosphate in rabbit psoas muscle fibres. J Physiol 451:247–278
Dantzig JA, Higuchi H, Goldman YE (1998) Studies of molecular motors using caged compounds. In: Marriott G (ed) Methods in enzymology, vol 291, Caged compounds. Academic, New York, pp 307–348
De Lozanne A, Spudich JA (1987) Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. Science 236:1086–1091
Dewey MM, Colflesh D, Brink P, S-f F, Gaylinn B, Gural N (1982) Structural, functional, and chemical changes in the contractile apparatus of *Limulus* striated muscle as a function of sarcomere shortening and tension development. In: Twarog BM, Levine RJC, Dewey MM (eds) Basic biology of muscles—a comparative approach. Raven Press, New York, pp 53–72
Dominguez R, Holmes KC (2011) Actin structure and function. Annu Rev Biophys 40:169–186
Dominguez R, Freyzon Y, Trybus KM, Cohen C (1998) Crystal structure of a vertebrate smooth muscle myosin motor domain and its complex with the essential light chain: visualization of the pre-power stroke state. Cell 94:559–571

dos Remedios CG, Millikan RGC, Morales MF (1972a) Polarization of tryptophan fluorescence from single striated muscle fibers. J Gen Physiol 59:103–120
dos Remedios CG, Yount RG, Morales MF (1972b) Individual states in the cycle of muscle contraction. Proc Natl Acad Sci U S A 69:2542–2546
Ferenczi MA (1986) Phosphate burst in permeable muscle fibers of the rabbit. Biophys J 50:471–477
Ferenczi MA, Homsher E, Trentham DR (1984) The kinetics of magnesium adenosine triphosphate cleavage in skinned muscle fibres of the rabbit. J Physiol 353:575–599
Finer JT, Simmons RM, Spudich JA (1994) Single myosin molecule mechanics: piconewton forces and nanometer steps. Nature 368:113–119
Fisher AJ, Smith CA, Thoden J, Smith R, Sutoh K, Holden HM, Rayment I (1995) X-ray structures of the myosin motor domain of *Dictyostelium discoideum* complexed with MgADP·BeF_x and MgADP·AlF_4^-. Biochemistry 34:8960–8972
Ford LE, Huxley AF, Simmons RM (1974) Mechanism of early tension recovery after a quick release in tetanized muscle fibres. J Physiol 240:42–43P
Ford LE, Huxley AF, Simmons RM (1985) Tension transients during steady shortening of frog muscle fibres. J Physiol 361:131–150
Fujii T, Iwane AH, Yanagida T, Namba K (2010) Direct visualization of secondary structures of F-actin by electron cryomicroscopy. Nature 467:724–729
Furch M, Geeves MA, Manstein DJ (1998) Modulation of actin affinity and actomyosin adenosine triphosphatase by charge changes in the myosin motor domain. Biochemistry 37:6317–6326
Geeves MA, Holmes KC (2005) The molecular mechanism of muscle contraction. In: Squire JM, Parry DAD (eds) Advances in protein chemistry: fibrous proteins—muscle and molecular motors. Elsevier Academic Press, San Diego, pp 161–193
Goldman YE, Brenner B (1987) Special topic: molecular mechanism of muscle contraction. Annu Rev Physiol 49:629–636
Goldman YE, Hibberd MG, McCray JA, Trentham DR (1982) Relaxation of muscle fibres by photolysis of caged ATP. Nature 300:701–705
Goldman YE, Hibberd MG, McCray JA, Trentham DR (1984a) Relaxation of rabbit psoas muscle fibres from rigor by photochemical generation of adenosine-5′-triphosphate. J Physiol 354:577–604
Goldman YE, Hibberd MG, McCray JA, Trentham DR (1984b) Initiation of active contraction by photogeneration of adenosine-5′-triphosphate in rabbit psoas muscle fibres. J Physiol 354:605–624
Gordon AM, Homsher E, Regnier M (2000) Regulation of contraction in striated muscle. Physiol Rev 80:853–924
Guilford WH, Dupuis DE, Kennedy G, Wu J, Patlak JB, Warshaw DM (1997) Smooth muscle and skeletal muscle myosins produce similar unitary forces and displacements in the laser trap. Biophys J 72:1006–1021
Gyimesi M, Tsaturyan AK, Kellermayer MSZ, Malnasi-Csizmadia A (2008) Kinetic characterization of the function of myosin loop 4 in the actin-myosin interaction. Biochemistry 47:283–291
Hanson J, Lowy J (1963) The structure of F-actin and actin filaments isolated from muscle. J Mol Biol 6:46–60
Harada Y, Yanagida T (1988) Direct observation of molecular motility by light microscopy. Cell Motil Cytoskeleton 10:71–76
Harada Y, Sakurada K, Aoki T, Thomas DD, Yanagida T (1990) Mechanochemical coupling in actomyosin energy transduction studied by *in vitro* movement assay. J Mol Biol 216:49–68
Harrington WF (1971) A mechanochemical mechanism for muscle contraction. Proc Natl Acad Sci U S A 68:685–689
He Z-H, Chillingworth RK, Brune M, Corrie JET, Trentham DR, Webb MR, Ferenczi MA (1997) ATPase kinetics on activation of rabbit and frog permeabilized isometric muscle fibres: a real time phosphate assay. J Physiol 501:125–148

Hibberd MG, Dantzig JA, Trentham DR, Goldman YE (1985) Phosphate release and force generation in skeletal muscle fibers. Science 228:1317–1319
Hill TL (1974) Theoretical formalism for the sliding filament model of muscle contraction of striated muscle. Part 1. Prog Biophys Mol Biol 28:267–340
Holmes KC (1996) Muscle proteins—their actions and interactions. Curr Biol 6:781–789
Holmes KC (2004) Introduction. Philos Trans R Soc Lond B Biol Sci 359:1813–1818
Holmes KC (2008) Myosin structure. In: Coluccio LM (ed) Myosin: a superfamily of molecular motors. Springer, Dordrecht, pp 35–54
Holmes KC (2009) Actin in a twist. Nature 457:389–390
Holmes KC, Popp D, Gebhard W, Kabsch W (1990) Atomic model of the actin filament. Nature 347:44–49
Homsher E, Millar NC (1990) Caged compounds and striated muscle contraction. Annu Rev Physiol 52:875–896
Homsher E, Irving M, Wallner A (1981) High-energy phosphate metabolism and energy liberation associated with rapid shortening in frog skeletal muscle. J Physiol 321:423–436
Homsher E, Lacktis J, Regnier M (1997) Strain-dependent modulation of phosphate transients in rabbit skeletal muscle fibers. Biophys J 72:1780–1791
Hopkins SC, Sabido-David C, van der Heide UA, Ferguson RE, Brandmeier BD, Dale RE, Kendrick-Jones J, Corrie JET, Trentham DR, Irving M, Goldman YE (2002) Orientation changes of the myosin light chain domain during filament sliding in active and rigor muscle. J Mol Biol 318:1275–1291
Houdusse A, Cohen C (1996) Structure of the regulatory domain of scallop myosin at 2 Å resolution: implications for regulation. Structure 4:21–32
Howard J (2001) Mechanics of motor proteins and the cytoskeleton. Sinauer Associates, Inc., Sunderland
Huxley AF (1995) Crossbridge tilting confirmed. Nature 375:631–632
Huxley AF, Simmons RM (1971) Proposed mechanism of force generation in striated muscle. Nature 233:533–538
Huxley HE (1969) The mechanism of muscular contraction. Science 164:1356–1366
Huxley HE (1973) Muscle 1972: progress and problems. Cold Spring Harb Symp Quant Biol 37:689–693
Huxley HE (1985) The crossbridge mechanism of muscular contraction and its implications. J Exp Biol 115:17–30
Huxley HE (1996) A personal view of muscle and motility mechanisms. Annu Rev Physiol 58:1–29
Huxley HE (2004) Fifty years of muscle and the sliding filament hypothesis. Eur J Biochem 271:1403–1415
Huxley HE, Kress M (1985) Crossbridge behaviour during muscle contraction. J Muscle Res Cell Motil 6:153–161
Huxley H, Reconditi M, Stewart A, Irving T (2006) X-ray interference studies of crossbridge actin in muscle contraction: evidence from quick releases. J Mol Biol 363:743–761
Huxley HE, Simmons RM, Faruqi AR, Kress M, Bordas J, Koch MHJ (1983) Changes in the x-ray reflections from contracting muscle during rapid mechanical transients and their structural implications. J Mol Biol 169:469–506
Huxley HE, Stewart A, Sosa H, Irving T (1994) X-ray diffraction measurements of the extensibility of actin and myosin filaments in contracting muscle. Biophys J 67:2411–2421
Inoue S (1981) Video image processing greatly enhances contrast, quality, and speed in polarization-based microscopy. J Cell Biol 89:346–356
Irving M (1987) Muscle mechanics and probes of the crossbridge cycle. In: Squire JM, Vibert P (eds) Fibrous protein structure. Academic Press, New York, pp 495–528
Irving M (1993) Birefringence changes associated with isometric contraction and rapid shortening steps in frog skeletal muscle fibres. J Physiol 472:127–156

Irving M, Kendrick-Jones J, Shrimpton C, Sleep J (1989) The orientation of rhodamine probes attached to myosin light chain-2 (LC-2) in relaxing and rigor conditions in single fibres isolated from rabbit psoas muscle. J Physiol 418:57P

Irving M, Lombardi V, Piazzesi G, Ferenczi MA (1992) Myosin head movements are synchronous with the elementary force-generating process in muscle. Nature 357:156–158

Irving M, Allen TSC, Sabido-David C, Cralk JS, Brandmeier B, Kendrick-Jones J, Corrie JET, Trentham DR, Goldman YE (1995) Tilting of the light-chain region of myosin during step length changes and active force generation in skeletal muscle. Nature 375:688–691

Irving M, Piazzesi G, Lucii L, Sun Y-B, Harford JJ, Dobbie IM, Ferenczi MA, Reconditi M, Lombardi V (2000) Conformation of the myosin motor during force generation in skeletal muscle. Nat Struct Biol 7:482–485

Ishijima A, Kojima H, Higuchi H, Harada Y, Funatsu T, Yanagida T (1996) Multiple- and single-molecule analysis of the actomyosin motor by nanometer-piconewton manipulation with a microneedle: unitary steps and force. Biophys J 70:383–400

Kabsch W, Mannherz HG, Suck D, Pai EF, Holmes KC (1990) Atomic structure of the actin: DNase I complex. Nature 347:37–44

Kaplan JH, Forbush BIII, Hoffman JF (1978) Rapid photolytic release of adenosine 5′-triphosphate from a protected analogue: utilization by the Na:K pump of human red blood cell ghosts. Biochemistry 17:1929–1935

Kawai M, Halvorson HR (1991) Two step mechanism of phosphate release and the mechanism of force generation in chemically skinned rabbit psoas muscle. Biophys J 59:329–342

Kersey YM, Hepler PK, Palevitz BA, Wessells NK (1976) Polarity of actin filaments in *Characean* algae. Proc Natl Acad Sci 73:165–167

Kitamura K, Tokunaga M, Iwane A, Yanagida T (1999) A single myosin head moves along an actin filament with regular steps of 5.3 nanometres. Nature 397:129–134

Kron SJ, Spudich JA (1986) Fluorescent actin filaments move myosin fixed to a glass surface. Proc Natl Acad Sci U S A 83:6272–6276

Kull FJ, Sablin EP, Lau R, Fletterick RJ, Vale RD (1996) Crystal structure of the kinesin motor domain reveals a structural similarity to myosin. Nature 380:550–555

Kuo SC, Sheetz MP (1993) Force of single kinesin molecules measured with optical tweezers. Science 260:232–234

Lazarides E, Lindberg U (1974) Actin is the naturally occurring inhibitor of deoxyribonuclease I. Proc Natl Acad Sci U S A 71:4742–4746

Levine RJC, Kensler RW (1985) Structure of short thick filaments from *Limulus* muscle. J Mol Biol 182:347–352

Levine RJC, Davidheiser S, Kelly AM, Kensler RW, Leferovich J, Davies RE (1989) Fibre types in Limulus telson muscles: morphology and histochemistry. J Muscle Res Cell Motil 10:53–66

Linari M, Piazzesi G, Dobbie I, Koubassova N, Reconditi M, Narayanan T, Diat O, Irving M, Lombardi V (2000) Interference fine structure and sarcomere length dependence of the axial x-ray pattern from active single muscle fibers. Proc Natl Acad Sci U S A 97:7226–7231

Lombardi V, Piazzesi G, Reconditi M, Linari M, Lucii L, Stewart A, Sun Y-B, Boesecke P, Narayanan T, Irving T, Irving M (2004) X-ray diffraction studies of the contractile mechanism in single muscle fibres. Philos Trans R Soc Lond B Biol Sci 359:1883–1893

Lymn RW, Taylor EW (1971) Mechanisms of adenosine triphosphate hydrolysis by actomyosin. Biochemistry 10:4617–4624

Mannherz HG, Leigh JB, Leberman R, Pfrang H (1975) A specific 1:1 G-actin:DNAase I complex formed by the action of DNAase I on F-actin. FEBS Lett 60:34–38

Mannherz HG, Kabsch W, Leberman R (1977) Crystals of skeletal muscle actin: pancreatic DNAase I complex. FEBS Lett 73:141–143

Manstein DJ, Titus MA, De Lozanne A, Spudich JA (1989) Gene replacement in *Dictyostelium*: generation of myosin null mutants. EMBO J 8:923–932

McConnell HM, McFarland BG (1970) Physics and chemistry of spin labels. Q Rev Biophys 3:91–136

McCray JA, Herbette L, Kihara T, Trentham DR (1980) A new approach to time-resolved studies of ATP-requiring biological systems: laser flash photolysis of caged ATP. Proc Natl Acad Sci U S A 77:7237–7241

Mehta AD, Finer JT, Spudich JA (1997) Detection of single-molecule interactions using correlated thermal diffusion. Proc Natl Acad Sci 94:7927–7931

Molloy JE, Burns JE, Kendrick-Jones J, Tregear RT, White DCS (1995) Movement and force produced by a single myosin head. Nature 378:209–212

Moss RL, Giulian GG, Greaser ML (1982) Physiological effects accompanying the removal of myosin LC_2 from skinned skeletal muscle fibers. J Biol Chem 267:8588–8591

Murphy CT, Spudich JA (2000) Variable surface loops and myosin activity: accessories to a motor. J Muscle Res Cell Motil 21:139–151

O'Brien EJ, Bennett PM, Hanson J (1971) Optical diffraction studies of myofibrillar structure. Philos Trans R Soc Lond B Biol Sci 261:201–208

Oda T, Iwasa M, Aihara T, Maeda Y, Narita A (2009) The nature of the globular- to fibrous-actin transition. Nature 457:441–445

Oefner C, Suck D (1986) Crystallographic refinement and structure of DNase I at 2 A resolution. J Mol Biol 192:605–632

Piazzesi G, Lucii L, Lombardi V (2002) The size and the speed of the working stroke of muscle myosin and its dependence on the force. J Physiol 545:145–151

Podolsky RJ (1960) Kinetics of muscular contraction: the approach to the steady state. Nature 188:666–668

Pollack GH (1983) The cross-bridge theory. Physiol Rev 62:1049–1113

Rayment I, Winkelmann DA (1984) Crystallization of myosin subfragment 1. Proc Natl Acad Sci U S A 81:4378–4380

Rayment I, Holden M, Whittaker M, Yohn CB, Lorenz M, Holmes KC, Milligan RA (1993a) Structure of the actin-myosin complex and its implications for muscle contraction. Science 261:58–65

Rayment I, Rypniewski WR, Schmidt-Base K, Smith R, Tomchick DR, Benning MM, Winkelmann DA, Wesenberg G, Holden HM (1993b) Three-dimensional structure of myosin subfragment-1: a molecular motor. Science 261:50–58

Reconditi M (2006) Recent improvements in small angle x-ray diffraction for the study of muscle physiology. Rep Prog Phys 69:2709–2759

Reconditi M, Linari M, Lucii L, Stewart A, Sun Y-B, Boesecke P, Narayanan T, Fischetti RF, Irving T, Piazzesi G, Irving M, Lombardi V (2004) The myosin motor in muscle generates a smaller and slower working stroke at higher load. Nature 428:578–581

Rome E (1973) Structural studies by X-ray diffraction of striated muscle permeated with certain ions and proteins. Cold Spring Harb Symp Quant Biol 37:331–339

Rome E, Offer G, Pepe FA (1973a) X-ray diffraction of muscle labelled with antibody to C-protein. Nature 244:152–154

Rome E, Hirabayashi T, Perry SV (1973b) X-ray diffraction of muscle labeled with antibody to troponin-C. Nature 244:154–155

Rovner AS, Freyzon Y, Trybus KM (1995) Chimeric substitutions of the actin-binding loop activate dephosphorylated but not phosphorylated smooth muscle heavy meromyosin. J Biol Chem 270:30260–30263

Salmon ED (1995) VE-DIC light microscopy and the discovery of kinesin. Trends Cell Biol 5:154–158

Sellers JR (1999) Myosins, 2nd edn. Oxford University Press, Oxford

Sheetz MP, Spudich JA (1983) Movement of myosin-coated fluorescent beads on actin cables *in vitro*. Nature 303:31–35

Siemankowski RF, Wiseman MO, White HD (1985) ADP dissociation from actomyosin in subfragment 1 is sufficiently slow to limit the unloaded shortening velocity in vertebrate muscle. Proc Natl Acad Sci U S A 82:658–662

Simmons RM (1992) Testing time for muscle. Curr Biol 2:373–375

Sleep JA, Hutton RL (1980) Exchange between inorganic phosphate and adenosine 5′-triphosphate in the medium by actomyosin subfragment 1. Biochemistry 19:1276–1283
Smith CA, Rayment I (1996) X-ray structure of the magnesium(II)·ADP·vanadate complex of the *Dictyostelium discoideum* myosin motor domain to 1.9 Å resolution. Biochemistry 35:5404–5417
Splettstoesser T, Holmes KC, Noe F, Smith JC (2011) Structural modeling and molecular dynamics simulation of the actin filament. Proteins 79:2033–2043
Spudich JA (1994) How molecular motors work. Nature 372:515–518
Spudich JA (2011) Molecular motors: forty years of interdisciplinary research. Mol Biol Cell 22:3936–3939
Spudich JA (2012) One path to understanding energy transduction in biological systems. Nat Med 18:8–12
Spudich JA, Kron SJ, Sheetz MP (1985) Movement of myosin-coated beads on oriented filaments reconstituted from purified actin. Nature 315:584–586
Suck D, Kabsch W, Mannherz HG (1981) Three-dimensional structure of the complex of skeletal muscle actin and bovine pancreatic DNase I at 6-Å resolution. Proc Natl Acad Sci 78:4319–4323
Svoboda K, Schmidt CF, Schnapp BJ, Block SM (1993) Direct observation of kinesin stepping by optical trapping interferometry. Nature 365:721–727
Sweeney HL, Holzbaur ELF (1996) Mutational analysis of motor proteins. Annu Rev Physiol 58:751–792
Sweeney HL, Houdusse A (2010) Structural and functional insights into the myosin motor mechanism. Annu Rev Biophys 39:539–557
Sweeney HL, Straceski AJ, Leinwand LA, Tikunov BA, Faust L (1994) Heterologous expression of a cardiomyopathic myosin that is defective in its actin interaction. J Biol Chem 269:1603–1605
Sweeney HL, Rosenfeld SS, Brown F, Faust L, Smith J, Xing J, Stein LA, Sellers JR (1998) Kinetic tuning of myosin via a flexible loop adjacent to the nucleotide binding pocket. J Biol Chem 273:6262–6270
Takagi Y, Shuman H, Goldman YE (2004) Coupling between phosphate release and force generation in muscle actomyosin. Philos Trans R Soc Lond B Biol Sci 359:1913–1920
Tanaka H, Ishijima A, Honda M, Saito K, Yanagida T (1998) Orientation dependent displacements by single one-headed myosin molecules in a synthetic myosin filament. Biophys J 75:1886–1894
Taylor R (1993) Crystal structure of the myosin head turbocharges molecular-motor research. J NIH Res 5:58–62
Thomas DD (1987) Spectroscopic probes of muscle cross-bridge rotation. Annu Rev Physiol 49:691–709
Thomas DD, Cooke R (1980) Orientation of spin-labeled myosin heads in glycerinated muscle fibers. Biophys J 32:891–906
Toyoshima YY, Kron SJ, McNally EM, Niebling KR, Toyoshima C, Spudich JA (1987) Myosin subfragment-1 is sufficient to move actin filaments *in vitro*. Nature 328:536–539
Trybus KM (1994) Regulation of expressed truncated smooth muscle myosins: role of the essential light chain and tail length. J Biol Chem 269:20819–20822
Uyeda TQP, Kron SJ, Spudich JA (1990) Myosin step size: estimation from slow sliding movement of actin over low densities of heavy meromyosin. J Mol Biol 214:699–710
Uyeda TQP, Ruppel KM, Spudich JA (1994) Enzymatic activities correlate with chimaeric substitutions at the actin-binding face of myosin. Nature 368:567–569
Uyeda TQP, Abramson PD, Spudich JA (1996) The neck region of the myosin motor domain acts as a lever arm to generate movement. Proc Natl Acad Sci U S A 93:4459–4464
Vale RD, Reese TS, Sheetz MP (1985a) Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. Cell 42:39–60
Vale RD, Schnapp BJ, Reese TS, Sheetz MP (1985b) Organelle, bead, and microtubule translocations promoted by soluble factors from the squid giant axon. Cell 40:559–569

Van Dijk J, Furch M, Lafont C, Manstein DJ, Chaussepied P (1999) Functional characterization of the secondary actin binding site of myosin II. Biochemistry 38:15078–15085
Wakabayashi K, Sugimoto Y, Tanaka H, Ueno Y, Takezawa Y, Amemiya Y (1994) X-ray diffraction evidence for the extensibility of actin and myosin filaments during muscle contraction. Biophys J 67:2422–2435
Warrick HM, Spudich JA (1987) Myosin structure and function in cell motility. Annu Rev Cell Biol 3:379–421
Warshaw DM, Guilford WH, Freyzon Y, Krementsova E, Palmiter KA, Tyska MJ, Baker JE, Trybus KM (2000) The light chain binding domain of expressed smooth muscle heavy meromyosin acts as a mechanical lever. J Biol Chem 275:37167–37172
White HD, Rayment I (1993) Kinetic characterization of reductively methylated myosin subfragment I. Biochemistry 32:9859–9865
White HD, Taylor EW (1976) Energetics and mechanism of actomyosin adenosine triphosphatase. Biochemistry 15:5818–5826
Whittaker M, Wilson-Kubalek EM, Smith JE, Faust L, Milligan RA, Sweeney HL (1995) A 35-Å movement of smooth muscle myosin on ADP release. Nature 378:748–751
Wulf E, Deboben A, Bautz FA, Faulstich H, Wieland T (1979) Fluorescent phallotoxin, a tool for the visualization of cellular actin. Proc Natl Acad Sci U S A 76:4498–4502
Yanagida T (1981) Angles of nucleotides bound to cross-bridges in glycerinated muscle fiber at various concentrations of ε-ATP, ε-ADP and ε-AMPPNP detected by polarized fluorescence. J Mol Biol 146:539–5650
Yanagida T, Nakase M, Nishiyama K, Oosawa F (1984) Direct observation of motion of single F-actin filaments in the presence of myosin. Nature 307:58–60
Yanagida T, Arata T, Oosawa F (1985) Sliding distance of actin filament induced by a myosin crossbridge during one ATP hydrolysis cycle. Nature 316:366–369
Yanagida T, Esaki S, Iwane AH, Inoue Y, Ishijima A, Kitamura K, Tanaka H, Tokunaga M (2000) Single-motor mechanics and models of the myosin motor. Philos Trans R Soc Lond B Biol Sci 355:441–447
Yount RG (1993) Subfragment 1: the first crystalline motor. J Muscle Res Cell Motil 14:547–551
Yount RG, Babcock D, Ballantyne W, Ojala D (1971) Adenylyl imidodiphosphate, an adenosine triphosphate analog containing a P-N-P linkage. Biochemistry 10:2484–2489

ERRATUM TO

Mechanism of Muscular Contraction

Jack A. Rall

J.A. Rall, *Mechanism of Muscular Contraction*, Perspectives in Physiology,
DOI 10.1007/978-1-4939-2007-5

DOI 10.1007/978-1-4939-2007-5_10

The online version of the original book can be found at
http://dx.doi.org/10.1007/978-1-4939-2007-5

Index

J.A. Rall, *Mechanism of Muscular Contraction*, Perspectives in Physiology,
DOI 10.1007/978-1-4939-2007-5