MEASUREMENT OF PULSE PROPAGATION IN SINGLE PERMEABILIZED MUSCLE FIBERS BY OPTICAL DIFFRACTION

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Bioengineering)
in The University of Michigan
1996

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ABSTRACT

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The advent of tissue engineering has necessitated the characterization of the viscoelastic behavior of structural tissues of the body, including skeletal muscle. The purpose was to test the hypotheses that the viscoelastic behavior of relaxed and partially activated single muscle fibers is dependent upon strain pulse amplitude and strain rate. The instrumentation to test this hypothesis was designed and built. Chemically permeabilized single muscle fibers from soleus muscles of male F344 rats were tested in the relaxed and partially activated condition at 15 °C. The first order optical diffraction pattern was detected at two positions along single muscle fibers. A servo motor imposed single longitudinal strain pulses on the fibers. Data were analyzed to determine the pulse propagation velocity and attenuation coefficient. The pulse velocity and the attenuation coefficient were measured for strain pulse amplitudes from 0.5% to 10%, and pulse frequencies from 250 Hz to 2 kHz. During shortening pulses of both relaxed and partially activated fibers, buckling of fibers prevented analysis of the data. Under all other conditions, the attenuation coefficient was negligible. For both relaxed and partially activated muscle fibers, the maximum pulse propagation velocity occurred at strain amplitudes of from 1% to 5%. The peak velocity represents a 9- to 25-fold increase in the elastic modulus when

compared with the elastic modulus for strain pulse amplitudes both below and above 1% to 5% strain. In both relaxed and partially activated muscle fibers, the repeated emergence and disappearance of a peak stiffness with increasing strain pulse amplitude suggested a phenomenon of *recoverable yield*. The recoverable yield was hypothesized to result from the attachment and detachment under strain of weakly-bound cross-bridges. The phenomenon of "steps and pauses" observed in every sarcomere length-time record was hypothesized to be caused by an optical artifact. The source of the artifact was identified as interference from scattering sources in the optical path. The instrumentation was modified by employing a diode laser module with reduced coherence length. The modification eliminated the artifact and permitted more accurate measurements of strain pulse amplitude.

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ACKNOWLEDGMENTS

Several individuals deserve specific mention and thanks for their assistance during the process of my doctoral work and the writing of this dissertation. Dr. Neil Cole for his inestimable help at all stages of the development of the instrumentation, hypotheses, and models used in this work as well as his enthusiasm for the research; Dr. Clyde Owings for his many and important contributions to both this dissertation and my understanding of medical and research instrumentation; Dr. Gordon Lynch for his assistance with all aspects of handling and storing skinned fiber preparations as well as all chemical solutions required for this research, and the special weekly lab meetings which were regularly held to discuss this research and other related topics (TYVM); Dr. Peter C. D. Macpherson for his assistance with the methods employed in the muscle mechanics laboratory for handling skinned fibers, preparing muscle bundles, and for generously supplying me with the necessary surgical equipment, as well as allowing me to make many of my mistakes on his equipment before beginning the design and assembly of my own setup; Rich Hinkle for efficiently providing general assistance with every logistical aspect of my research including the ordering and housing of animals, lab supplies, computing and surgical equipment and all of the other miscellaneous details without which scientific research is impossible; Dr. Fred Bookstein for his very important technical contributions and support; Dr. Loree Kalliainen who was most helpful and flexible in scheduling animal surgeries and providing the necessary tissues so that we could make maximum use of every animal; Dennis Kayner and Mark Stock of the Orthopedic Research Laboratory for providing unlimited machine shop access; Trudy Dennis for providing dozens of data books and free samples of electronic components, without which many of my instruments could not have

been realized; Dr. Karl Grosh and Dr. Noel Perkins of the Mechanical Engineering Department at the University of Michigan, who provided assistance with the theoretical and experimental aspects of the wave propagation experiments; Dr. Steve Goldstein for his very important contributions to the initial formulation of these experiments and his emphasis on the Big Picture; Dr. Duncan Steel for regularly helping me to remove the technical road blocks that are so often encountered in basic research, as well as his general enthusiasm for my work; and perhaps most importantly to Dr. John Faulkner, who generously provided the material, financial, and space resources and allowed me the freedom to pursue a new area of research in muscle mechanics. Without the unwavering support and guidance of John Faulkner, this work would not have been possible.

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CHAPTER I
INTRODUCTION

Structural and functional properties of the skeletal muscles and skeletal muscle fibers have been studied in great detail. The studies have focused largely on the traditional aspects of structure/function relationships, which include length-tension, cross-sectional area-force, mass-power, and length-velocity (Woledge et al., 1985). These relationships are useful in the explanation of the dependency of function on specific structural properties of muscle. Despite the wealth of knowledge regarding structure/function relationships, the advent of musculoskeletal tissue engineering requires a full understanding of the nontraditional mechanical properties of muscle tissue. Of these, the most important is *viscoelasticity* because viscoelasticity describes muscle as a material with a stress-strain relationship that is time and strain-rate dependent (Fung, 1993).

Early attempts to describe the stress-strain behavior of muscle tissue usually relied upon the assumption that the muscle tissue specimen under study exhibited primarily linear elastic deformation behavior (Schoenberg et al., 1974; Hasan and Mason, 1978). The assumption was that the viscous effects were negligible. This assumption is implicit in the large body of literature on muscle mechanics that deals with measurements of muscle *stiffness.* In the context of muscle physiology, stiffness is to be distinguished from the clinically recognized syndrome of "muscle stiffness", the latter being a sensation rather than a structural property. In physiological usage, the word stiffness refers to the elastic behavior of a solid object in which the stiffness is defined as the applied load divided by the resulting deformation.

Alternatively, muscle stiffness is defined as the ratio of the change in stress divided by the resulting change in strain of a skeletal muscle or fiber under otherwise fixed conditions. The use of stiffness measurements alone to describe the stress-strain relationship in muscle tissue involves two fundamental assumptions. First, that the viscous behavior of the muscle tissue is negligible, and second, that the stress/strain relationship is linear. With these two assumptions, stiffness measurements of muscle tissue are a simplification of the more complete description afforded by measurements of viscoelasticity and non-linear elastic behavior.

Several methods for measuring the viscoelasticity of muscle fibers have been reported (Ford, et al, 1981; Dortmans et al., 1984; Blange and Stienen, 1985; De Winkel et al, 1993; De Winkel et al., 1994). Each of the methods has limitations. For example, typical models of muscle tissue viscoelasticity frequently assume linear elastic and viscous behavior, with a stable, homogeneous microstructure of the muscle fiber. Although these models explain some of the viscoelastic properties of muscle, they lack the sophistication necessary to explain more complex behavior, such as structural instability during activation and mechanisms of focal injury to individual sarcomeres. One of the primary obstacles to the development of adequate models of viscoelasticity for muscle is the lack of detailed and accurate data which relate the stress to the time-resolved local strain state within a muscle fiber, without assumptions of linearity or longitudinal homogeneity. The purpose of the present work was to develop an apparatus and methodology for the measurement of the viscoelastic properties of single muscle fibers in both the relaxed and activated state that were free of the unrealistic assumptions of linear mechanical behavior and structural homogeneity. Such an apparatus could be used for measuring the viscoelasticity of single muscle fibers as well as for studying the dynamics of small groups of sarcomeres during stretches that result in contraction-induced injury.

The viscoelastic behavior of a whole muscle depends upon the diverse viscoelastic behaviors of the large numbers of individual muscle fibers of which the whole muscle is composed. Therefore, models of whole muscle viscoelasticity must be based upon the behavior of microstructural elements at the level of individual muscle fibers. For this reason, most recent investigations in muscle tissue viscoelasticity have focused on the viscoelasticity of single fibers. Consequently, throughout the remainder of this document, muscle tissue viscoelasticity will be discussed in the context of a single muscle fiber.

Structure/Function Relationships in Skeletal Muscle

General Structure and Function of Muscle

The traditional view of muscle function is of muscle fibers as generators of force. Although not incorrect, this view is incomplete. An alternative view is that the function of muscle fibers includes that of a structural material which, with activation, is capable of undergoing rapid and dramatic changes in its material properties. Chemical changes within muscle fibers lead to activation and force generation which is traditionally viewed as the primary function of muscle (Woledge et al., 1985). The structural changes at the level of the sarcomere, which are the direct result of activation, also have dramatic affects on the viscoelasticity of muscle tissue. The changes in the length and material properties of muscle fibers provide the musculoskeletal system with a mechanism whereby fibers may undergo changes in both their shape and their rigidity. To understand the changes in the material properties of muscle tissue, the structural level at which the changes in the material properties occur must be considered. The hierarchical structure of muscle tissue from the sarcomere to that of the level of the fiber, or individual muscle cell, is illustrated schematically in Figure 1.1.

The fundamental contractile unit of muscle, the sarcomere, is the repeating unit in each myofibril, delimited by the Z disks. Skeletal muscles are composed of bundles of multinucleated fibers which vary in diameter from about 25 μm to 100 μm. Within each muscle fiber, electron micrographs reveal bundles of filaments which run axially from one end of the fiber to the other which are termed myofibrils. Skeletal muscle fibers have a banding pattern, or striation pattern, which arises from the highly organized subcellular contractile proteins, actin or thin filaments and myosin or thick filaments. When a muscle fiber is viewed under polarized light, the wide dark striations are the regions containing the lattice of myosin and actin filaments which interdigitate with each other. These dark regions are termed the A band. The anisotropic optical properties of the A band cause the region to appear dark when viewed with polarized light. The light striations are areas of the myofibril which contain thin, but no thick, filaments and are termed the I band. This region is optically isotropic and thus appears light when viewed under polarized light. Near the center of each I band is the Z disk or Z line. When viewed under polarized, light the Z line appears as a narrow dark line in the center of the I band (Berne and Levy, 1988).

The function of muscle is to transform chemical energy into mechanical force and work. The forces transmitted through tendons and bone provide support for the body parts. Concurrently, the work done by muscles produces torques about joints and movements of limbs and other body parts. Skeletal muscles exert force and do work on the external environment, but the external environment also exerts forces and performs work on the skeletal muscles. The interaction between muscle and its environment result in three types of contractions. When a muscle fiber is activated the tendency is for the fiber to shorten. What actually happens to the length of a muscle fiber when it is activated depends upon the interaction between the force developed by the muscle fiber and the load placed upon the fiber by the

environment. If the muscle fiber produces a force greater than that imposed upon it by the external environment, the fiber will shorten and this type of contraction is termed *miometric* . When an activated muscle fiber exerts a force equal to the tensile force exerted on the muscle by the environment, the muscle length remains the same and the contraction is termed *isometric* . In the case that the external force on the muscle is greater than the force generated by the muscle, the muscle will be lengthened, and the contraction is termed *pliometric* . With the same level of activation, miometric contractions produce forces less than those produced by isometric contractions, and pliometric contractions produce forces up to two-fold larger than the isometric force (Close, 1972).

The Contractile Proteins

The sliding filament, or cross-bridge theory is the generally accepted theory of muscle contraction (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954; Huxley, 1957). This theory relates the function to the protein structure of the sarcomere. The contractile proteins within a fiber consist of the thin filaments, actin, and the thick filaments, myosin. These filaments interdigitate with one another (Figure 1.1). During a miometric contraction, the sliding filament theory states that actin and myosin slide past one another to overlap more as the sarcomere shortens, or to overlap less during a pliometric contraction as the sarcomere lengthens. The sliding filament theory is based on the premise that during changes in the length of the sarcomere the length of the actin and myosin filaments remains constant.

With muscle activation, a change occurs in the chemical interaction between the actin and myosin filaments within sarcomeres. The S-1 or head portion of the myosin molecule protrudes laterally from the thick filament. Each myosin molecule has two globular heads which may, in the presence of

Ca^{++}, attach to binding sites along the actin thin filament, forming an actomyosin complex or cross-bridge. An ATP molecule binds to the S-1 fragment and is hydrolyzed within the actomyosin complex to form ADP and inorganic phosphate. The chemical energy from this hydrolysis is transformed into mechanical energy at the cross-bridge, through processes not completely understood. These processes involve a conformational change in the attached cross-bridge which results in a force between the thick and thin filaments that slides the filaments past each other to shorten the sarcomere. In the presence of ATP and free calcium ions, the cross-bridge then detaches and reattaches at another binding site on the thin filament. During activation a continuous cycling of cross-bridges occurs from unbound, to weakly bound, to strongly bound, through the driving stroke. The cycle finishes with a strongly bound cross-bridge detaching or returning to the weakly bound state (Goldman, 1987b). In living muscle, this process occurs rapidly, on the order of 150 or more cycles per second, and a sliding distance of about 10 to 20 nm per half sarcomere can be achieved for each interaction (Piazzesi, Linari & Lombardi, 1993; Toyoshima et al., 1990; Huxley and Simmons, 1971).

For a given level of activation, the maximum force that can be generated by a muscle fiber depends upon the degree of overlap of the thick and thin filaments. Optimal overlap is species dependent, but occurs at a sarcomere length of approximately 2.6 microns for skeletal muscles of rats (Stephenson & Williams, 1982). This optimal overlap is referred to as optimal length (L_o). In the absence of any external tensile load the activated muscle fiber will shorten until the thin filaments begin to interdigitate with each other. At a sarcomere length of about 1.6 microns, the thin filaments (Figure 1.1) from each half of the sarcomere will come into contact with each other at the M line, and the thick filaments will very nearly be in contact with the Z line at each end.

For both activated and relaxed fibers, if a single sarcomere is lengthened by approximately 55% beyond optimal length, the thick and thin filaments will be pulled beyond overlap, at least on one side of the sarcomere (Brooks and Faulkner, 1995; Woledge, Curtin & Homsher, 1985; Higuchi et al., 1988). At a sarcomere lengths greater than 3.9 microns no active force can be generated because the thick and thin filaments no longer interdigitate, so no cross-bridges can be formed. The optimal sarcomere length for the development of force is near the center of the length-tension curve and deviations from the plateau in either direction result in lower forces. Furthermore, the activated cross-bridges in each half of the sarcomere attempt to pull the thick filament toward their respective Z-lines. The symmetry of the sarcomere depends upon the stability of the thick filament in the center of the sarcomere.

Non-Contractile Structural Proteins in Muscle

In addition to the contractile proteins, actin and myosin, several filamentous proteins closely associated with the sarcomere are important during sarcomere development and for structural stabilization of the mature sarcomere. Notable among these are titin and nebulin, which have recently been incorporated into the existing two-filament sliding system of the sarcomere (Labeit and Kolmerer, 1995), and now are considered part of the four filamentous systems in the sarcomere (Granzier and Wang, 1993b).

Nebulin is one of a family of giant proteins ranging from 0.7 to 0.9 MDa in molecular mass. Nebulin is inextensible and is considered to be an integral part of the thin filaments of skeletal muscle fibers (Wang and Wright, 1988). Because nebulin is inextensible and is closely associated with the thin filament, nebulin is not thought to contribute to the passive elasticity of muscle, but is thought to act as a

template or scaffold for the assembly of the thin filament during muscle fiber development (Granzier and Wang, 1993b).

Titin is a very large (molecular mass ~ 3 MDa) single-peptide filamentous protein (Higuchi, Nakauchi, Maruyama and Fujime, 1993), greater than one micron in length. The structural role of titin (also called connectin) includes the maintenance of the longitudinal symmetry of the sarcomere, and the provision of passive elasticity to muscle tissue (Barinaga, 1995). The N-terminus of titin is connected to the Z-line and the C-terminus is connected to the thick filament at the M-line of the sarcomere (Wang, Sun and Jeng, 1991; Barinaga, 1995; Labeit and Kolmerer, 1995). Several isoforms of titin are known to exist, and these may be expressed selectively by muscle fibers to produce differing elastic properties and resting forces (Horowitz, 1992; Labeit and Kolmerer, 1995). Approximately one-third of the titin molecule functions as an elastic linkage between the Z-line and the distal end of the thick filament (Trinick, 1992; Labeit and Kolmerer, 1995). The elastic region of the titin molecule can return to its original shape after strains of up to 400% (Soteriou, Clarke, Martin and Trinnick, 1993). This region contains a newly-identified structural motif, known as the PEVK sequence, which may be responsible for the passive elastic properties of skeletal and cardiac muscle tissue. Labeit and Kolmerer (1995) postulate that the PEVK sequence can be differentially spliced into the I-band titin molecule to provide tissue-specific passive elasticity in skeletal muscle. Correlations between the length of the PEVK sequence and the elasticity of a variety of muscle tissues of differing elasticity support this hypothesis.

As the sarcomere is stretched to a length of 3.8 microns, near the point at which a loss of thick and thin filament overlap occurs, a portion of the titin molecule that lies along the thick filament may be released from its anchor point at the end of the thick filament, thus increasing the extensible length of the titin molecule, reducing the passive stiffness of the muscle fiber and shifting the yield point to a longer

sarcomere length (Wang, McCarter, Wright, Beverly and Ramirez-Mitchell, 1993 and 1991). In this way, titin may act as a non-linear (exponential), dual-stage molecular spring. Protein filaments outside the sarcomere apparently only begin to contribute significantly to passive force at sarcomere lengths greater then 4.5 microns (Wang, *et al.* 1993). Titin may have an inelastic domain near the Z-line, which interacts with the thin filaments (Trombitas, Pollack, Wright and Wang, 1993; Trombitas and Pollack, 1993).

Titin also plays an important role in the stability and function of muscle tissue in the active state. Disruption or chemical digestion of titin results in a reduction of maximum active force because of the resultant disordering of the sarcomere. Specifically, the thick filament will be pulled toward one Z-line or the other, thus losing overlap with the thin filaments in the other half of the sarcomere. In this way, the mechanical properties of titin can be seen to have importance in both the passive and the active states by contributing to the symmetry and stability of the sarcomere structure. (Labeit and Kolmerer, 1995; Higuchi, 1992; Horowitz & Podolsky, 1987).

Cross-bridge States

Although titin contributes to the elastic properties of the sarcomere by acting as a passive spring at each end of the thick filament, the viscoelasticity of a muscle fiber will depend predominantly upon the state of the cross-bridges. During activation, a continuous cycling of cross-bridges occurs from unbound, to weakly bound, to strongly bound, through the driving stroke, and finishing with a strongly bound cross-bridge detaching or returning to the weakly bound state (Goldman, 1987b).

Only strongly bound cross-bridges in the driving stroke can contribute to force development during isometric or miometric contractions. In contrast, during pliometric contractions force may also be

generated by the negative strain on weakly-bound cross-bridges and by strongly-bound cross-bridges which are not in the driving stroke (Huxley, 1980). The relatively stiff elastic properties of strongly-attached cross-bridges have been studied in detail (Ford et al., 1981). In the weakly-bound state, the cross-bridges form tenuous and non-active force producing interactions between the thick and thin filaments (Brenner, 1986). The contribution of weakly-bound cross-bridges to muscle viscoelasticity have not been investigated fully.

Although weakly-bound cross-bridges do not generate active force or perform mechanical work, weakly-bound cross-bridges may influence the mechanical properties of the sarcomere. The attachment of cross-bridges may be weak, but many weakly-bound cross-bridges may exist, acting in parallel and series. Thus, in the passive state, the effect of weakly-bound cross-bridges on the mechanical properties of muscle fibers may be significant. Under most conditions, weakly-bound cross-bridges are difficult to detect directly (Bagni, Cecchi, Colomo & Garzella, 1994 and 1995; Brenner & Eisenberg, 1987). The existence of weakly-bound cross-bridges is strongly suggested by the *in vitro* binding of actin to myosin in the absence of Ca++, and the observation that the stiffness of relaxed muscle fibers under high strain rate conditions depends in part on the thick and thin filament overlap (Brenner, Schoenberg, Chalovich, Greene & Eisenberg, 1982). In addition, the number of weakly-bound cross-bridges appears to increase with increased ionic strength (Granzier and Wang, 1993a and 1993b; Brenner, et al., 1982; Schoenberg, 1988). The relative contributions to muscle stiffness of titin and weak cross-bridges has been reported by Granzier and Wang (1993b) by the use of caldesmon to inhibit thick and thin filament interactions.

With varying levels of activation, muscle tissue has the ability to rapidly change its mechanical properties over a far greater magnitude and more rapidly than any other biological material. At any level of activation, the mechanical properties of a muscle fiber differ dramatically from the properties of the

same muscle fiber when it is fully relaxed. Consequently, a fully relaxed muscle fiber must be considered to be a material quite structurally distinct from a muscle fiber at any level of activation. Cross-bridge formation during activation of the muscle results in a large number of relatively stiff and inextensible connections between the thick filament and the adjacent thin filaments. This, in effect, provides a stiff mechanical force shunt which acts in parallel with the much less stiff and much more extensible titin molecule. In addition, weak interactions between thick and thin filaments may be supplanted by strong interactions during activation. This can be viewed as reversible chemical cross-linking within the muscle fiber which would be expected to strongly influence the mechanical properties of muscle when considered as a polymeric material. The change in the intrinsic material properties of muscle tissue, as opposed to the generation of force, has been postulated to contribute to the control of movement of the entire organism through control of antagonistic muscles by variation of the mechanical impedance of the muscles which interact during movement (Oguztoreli and Stein, 1991). Aside from the generation of force, the fact that the material properties of muscle tissue change with activation has importance in studies of all physical activities, changes in the musculoskeletal system with age, and contraction-induced injury.

Viscoelasticity of Muscle Tissue

General Description of Viscoelasticity

Viscoelasticity is the term used to denote the behavior of materials which when subjected to a stress undergo both elastic strain and viscous flow (Van Vlack, 1980). All materials, including biological tissues, demonstrate some degree of viscoelasticity, and therefore exhibit time-dependent stress-strain relationships, which include *stress relaxation, creep, and hysteresis* . *Stress relaxation* results if a tissue

is subjected to a constant strain and the stresses induced in the tissue decrease with time. *Creep* results if a tissue is subjected to a constant stress (or load) and the tissue deforms with time. *Hysteresis* occurs when the stress-strain relationship during loading is different from that during unloading (Fung, 1993). The consequence of viscoelastic behavior of a tissue is that the stress-strain relationship will depend upon strain rate and loading history.

An understanding of muscle tissue viscoelasticity is required in order to understand how muscle tissue will respond to the application of mechanical stress. The importance of understanding the viscoelasticity of muscle is demonstrated by the usefulness of one specific aspect of the viscoelasticity, muscle stiffness, in muscle mechanics research. Stiffness measurements of muscle have been used to determine the number of strongly attached cross-bridges during steady state contractions (Ford, et al., 1981; Ford, et al., 1986; Huxley and Simmons, 1971; Julian and Morgan, 1981; Tawada and Kimura, 1984; Metzger and Moss, 1990). Stiffness measurements were used because of the simplicity of both the experimental method and the analysis of the data, when compared with the measurement of the viscoelastic behavior. Viscoelasticity provides a more rigorous and complete description of the dynamic stress-strain behavior of a living tissue than stiffness. The advantage of viscoelasticity compared with stiffness assessment is that viscoelasticity includes the effects of strain rate (Fung, 1993). The measurement of stiffness of muscle without considering the effects of strain rate results in an incomplete picture because the viscoelasticity is described at only one point within a continuum of mechanical strain frequency and amplitude.

Models of Viscoelasticity

For mathematical convenience, the viscoelastic behavior of materials is based on one of the three general models (Figure 1.2) or on variations of these three models (Fung, 1993). These models provide a schematic representation of a material system which may be modeled conveniently, yet which incorporates many of the real behaviors of viscoelastic solids, *hysteresis, stress relaxation,* and *creep* (Fung, 1993, pg. 41). These three models are widely employed in the modeling of biological tissues, yet they suffer from severe limitations when applied to muscle tissue. The most obvious limitation is the assumption of linear elasticity either in parallel or in series with a linear damper. The nonlinearity of the elastic components in muscle tissue has been demonstrated repeatedly (Labeit and Kolmerer, 1995; Granzier and Wang, 1993a and 1993b). The existence of elastic nonlinearity of tissues is generally accepted, though often neglected because of the intractability of non-linear mathematical models. This is only the most obvious limitation of the simple models of viscoelastic solids. The most serious limitation, suggested by the microstructure of muscle tissue, is that the elastic and viscous behavior originates at the level of the sarcomere. Substantial evidence supports the premise that all sarcomeres within a fiber are not equivalent in structure or function (Huxley and Peachey, 1961; Julian and Morgan, 1979; Julian and Morgan, 1981; Burton et al., 1989; Morgan, 1990; Macpherson and Faulkner, 1996). In fact, some of the important mechanical responses of muscle are related directly to the fact that sarcomeres are different in their viscoelastic properties (Macpherson and Faulkner, 1996). Thus, to represent even a few millimeters of a muscle fiber length adequately the elements in each of the viscoelasticity models (Figure 1.2) must be repeated in series many times with varying quantities assigned to each of the parameters.

In addition to the viscoelastic properties intrinsic to each sarcomere, each sarcomere has built-in mechanical limits, such as the limited throw of a cross-bridge during its driving stroke which becomes evident during rapid length steps, and non-linear elastic regions which are evident during lengthening of

relaxed fibers (Granzier and Wang, 1993b). This poses a considerable challenge to those who would use such simplified models to characterize the viscoelastic behavior of muscle tissue. The problem becomes even more clear when one attempts to use experimental data on the viscoelasticity of muscle tissue to quantify the parameters in each of the viscoelasticity models. Nonetheless, muscle tissue is a physical material, and an accurate description of the stress-strain relationship for the full physiological range of muscle states is critical. Therefore, attempts to measure muscle tissue viscoelasticity must meet two stringent criteria. First, the experimental method must not be based upon assumptions, implicit or explicit, which undermine the real behavior of muscle tissue as a viscoelastic material. For example, the strain state cannot be assumed to be stable and homogeneous when it is not, nor can all sarcomeres be assumed to be equivalent mechanically. Second, non-linear behavior must be expected, and though such behavior is difficult to deal with in mathematical models, the non-linear viscoelastic behavior of muscle fibers must be characterized adequately. Therefore, any measurement of muscle tissue viscoelasticity must allow for the detection and quantification of nonlinear behavior. Experimental methods which do not meet these two criteria are of limited value for the measurement of muscle tissue viscoelasticity. Several methods for measuring muscle tissue viscoelasticity have been employed, and it is necessary to discuss each method in detail to assess the relative merits and limitations of each in light of the two criteria stated above.

Experimental Determination of the Viscoelasticity of Muscle Tissue

Overview

The experimental methods that have attempted to measure the viscoelasticity of muscle tissue have implicitly included certain simplifying assumptions about the behavior of muscle tissue. These

simplifying assumptions are the assumption of linear elastic and viscous elements, infinitely rapid and uniform strain propagation along a fiber, and longitudinal homogeneity of sarcomere lengths along the length of the muscle fiber. The experiments that one performs to measure viscoelasticity will depend directly upon which general model of viscoelasticity is used, the complexity of the model, that is, how many additional parameters have been added, and consequently the assumptions which accompany that model. In general, the viscoelasticity of muscle has been studied directly or indirectly by one of three different methods; by the application of continuous sine wave oscillations, by the stress relaxation following step length changes, and by the application of single longitudinal strain pulses to measure pulse propagation. Each method has advantages and disadvantages. In some cases to use one or more of these methods is inappropriate because the simplifying assumptions neglect one or more of the important aspects of the real viscoelastic behavior of muscle tissue. Each of the different experimental methods are presented in detail to assess their limitations, relative benefits and applicability.

Continuous Sine Wave Stiffness Method

The stiffness of a whole muscle or muscle fiber, both in the active and relaxed state, may be measured by applying high-frequency, low-amplitude length oscillations. The length oscillations result in force oscillations, which are recorded. The instantaneous stiffness is then calculated by the ratio of instantaneous stress/ instantaneous strain (dS/de). Muscle stiffness is thought to be a direct consequence of both the number of attached cross-bridges per half sarcomere and the mechanical strain state of each cross-bridge (Huxley and Simmons, 1971). The number of attached cross-bridges depends upon the level of activation as well as the amount of thick and thin filament overlap (Ford, et al., 1981; Julian and Morgan, 1981). In living muscle, this relationship is not simple. The rate at which cross-bridges are

being formed and broken is related to the strain state of each individual cross-bridge. Consequently, the steady-state stiffness of cross-bridges, or populations of cross-bridges, cannot be measured directly. During these types of experiments, the frequency of the length oscillations is supposed to be sufficiently rapid that the cycling of cross-bridges will no longer influence the stiffness. This frequency is generally taken to be approximately 1 or 2 kHz, above which the 'instantaneous' or 'dynamic' stiffness is assumed to be constant with increasing frequency (Halpern and Moss, 1976; Moss and Halpern, 1977; Ford, 1981; Cecchi, 1984). The high speed step and pulse data of De Winkel et al. (1993 and 1994) directly refute this assumption. The conclusion is that the complex Young's modulus, which includes terms to describe the strain rate as well as strain amplitude, increases nearly linearly with the log of increasing frequency from 50 Hz to at least 40 kHz. For experiments of this type, 40 kHz constitutes the present upper limit of experimental frequency spectral content.

Under certain circumstances, stiffness data are useful. The requirements are that the frequency and amplitude of the oscillations be recorded and the resulting stiffness values be reported for a range of frequencies. Applications include the use of stiffness data to determine the relative number of attached cross-bridges at various times during activation of a muscle. With the present method of measuring muscle stiffness, an additional error is the erroneous assumption that the strain state is constant along the length of the fiber. This error is implicit in the formula for instantaneous strain given above. The assumption of constant strain state along the length of a muscle fiber during a strain pulse is invalidated by the results of the pulse propagation studies in whole muscle (Truong, et al., 1978a and 1978b; Truong, 1971, 1972, and 1974). At any given time the local strain state at different locations along the specimen cannot be determined. Local strain state can only be determined by direct measurement at each location.

The continuous sine wave method has the advantage of simplicity, but does not account for attenuation due to specimen length or the inhomogeneous distribution of strain along the length of the specimen. These factors may have been overlooked because of the statistical effect of limitation of range of specimen lengths in the preparation of the muscle tissue specimens. The value of this method is limited to relative stiffness comparisons of muscle tissue specimens of similar geometry for length oscillations of a fixed single frequency. The bandwidth of existing force transducers also limits this method.

Step Length Change Method

The step length change method is based upon the idea of measuring the stress relaxation following a step length change. The force after a step length change will exhibit a log decay to a value near the initial force before the step, as indicated on the inset graphs of the relaxation function in Figure 1.2 (b) and 1.2(c). The relaxation time constant during stress relaxation may be measured by subjecting muscle tissue to a very rapid lengthening or shortening step and recording the force. The viscous stress relaxation of the tissue is fitted to the model proposed by Fung (1972 and 1993), or a similar model to determine the complex elastic modulus (Dortmans, 1984; De Winkel, 1993; De Winkel 1994). Typically, a series of relaxation time constants and weighting coefficients are calculated. One problem with this method is that in the mathematical derivation, the step length change is assumed to occur instantaneously. The model further assumes tacitly that the instantaneous length change is accompanied by an instantaneous and longitudinally uniform change in the strain state of cross-bridges throughout the muscle fiber (see Dortmans 1984, figure 1, Fung 1993, page 45 ff.). None of the models account for or make allowance for the fact that the fiber is stretched from one end, and for an unknown period of time

immediately after the step length change the strain is concentrated near the mechanical effector which caused the strain. The concentration of strain near one end is particularly true for very high velocity length changes, for long fibers, or particularly both conditions. The strain near the mechanical effector is transmitted along the length of the fiber at a finite velocity. For viscoelastic materials the finite velocity will be both frequency and amplitude dependent. The measurement of force must be highly time-resolved in order to determine the time course of stress development and relaxation. Generation of accurate force data for fibers is difficult with high frequency components in the frequency spectrum because of the bandwidth limitation of most force transducers, and the subsequent phase shifts and amplitude distortions within the bandwidth. The forces generated by most single muscle fibers are below 1 mN, and force transducers with enough resolution to be of value in this range tend to have low natural frequencies. The attachment of the fiber can easily change the natural frequency and damping coefficient of the force transducing system. Under these circumstances further errors in amplitude and phase are introduced when highly time-resolved force data are collected. Finally, the assumption of instantaneous and uniform strain change along the fiber resulting from the length step is not valid. During the critical time course of the force measurements which occur immediately after the step length change, the strain distribution along the fiber is both inhomogeneous and dynamic. Consequently, for some period of time immediately after a rapid length change of a fiber, force (or stress) cannot be related to strain unless strain is measured directly at the point of attachment of the force transducer. Such measurements of force have never been performed.

Recent attempts to improve this general class of experiments has resulted in the development of faster data collection systems with broader bandwidth force transducers, and faster servo mechanisms to impose more rapid steps in an attempt to more accurately meet the assumptions made in the

mathematical modeling of viscoelastic behavior by the step length change method. Improved transducer bandwidths and more rapid steps exacerbate the problems that result from the non-instantaneous and non-uniform response of the muscle tissue itself to increasingly rapid length changes.

The step length change method has the advantage of mathematical elegance but suffers from the grievous flaw of assuming unrealistic conditions such as instantaneous length steps and uniform longitudinal strain distribution in the specimen at all times. Thus, it is impossible to relate the strain state at any point along the muscle specimen to the force. Recent attempts to increase the accuracy of this method by increasing the bandwidth of the force transducer and the speed of the step have actually magnified the problem because the analysis of the resulting data focuses on force data with higher time resolution during and immediately after the application of the step, which is the time period during which the implicit physical assumptions of this method are least accurate.

Pulse Propagation Method

The viscoelastic properties of a material can be measured by applying single lengthening or shortening pulses to the material and measuring the pulse propagation velocity and attenuation as the pulse travels along the specimen. Therefore, the complex elastic modulus of a whole muscle, muscle fiber bundle, or single fiber may be measured by subjecting the specimen to single longitudinal pulses. The phase of the sinusoid or the arrival time of the pulse is measured by detecting strain or force at two positions along the specimen, and the resulting data are used to measure the longitudinal pulse propagation velocity and attenuation coefficient of the muscle specimen (Hasan, 1977; Blange and Stienen, 1985; Stienen and Blange, 1985; Schoenberg, 1974; Truong, 1971; Truong, 1972; Truong, 1974; Truong, 1978). These parameters are measured over a range of frequencies, and are then used to

determine the real component of the complex Young's modulus as a function of frequency (Truong, 1974). De Winkel et al. (1993 and 1994) have used rapid pulses and measured the time required for the resulting force change to reach the opposite end of the fiber. The propagation time is typically defined as the time delay between the initiation of a length change and the initial rise or fall or the resulting force at the opposite end of the specimen. Pulse velocity is simply calculated as $v = \Delta x/\Delta t$, where 'v' is the pulse velocity, 'Δx' is the distance between the mechanical effector and the position along the fiber at which the strain pulse is detected, and 'Δt' is the time delay. For these experiments, the pulse velocity is often taken to represent the propagation velocity of the highest frequency components within the pulse. In many cases, the values of the frequency components are not specified or estimated (Hasan, 1977; Schoenberg, 1974). The experiments which use sinusoidal pulses with one frequency component avoid this pitfall, but often require long periods of time to complete due to the large number of experiments that are required to cover the range of frequencies of interest (Truong, 1974). To reduce the time required to perform these experiments, methods have been developed to allow the calculation of the complex modulus from single pulse propagation data using Fourier analysis of the shape of a single pulse, which usually contains a wide range of frequencies (Truong, 1978).

The single pulse propagation method has the disadvantage of requiring much more sophisticated analysis, in the case of Fourier analysis of the single pulse shapes, or the requirement of a large series of experiments at various single frequencies of oscillation. The advantage of this method is that the analysis of the resulting data yield the real component of the complex elastic modulus without requiring unrealistic assumptions about the pulse characteristics or strain state of the specimen. Additional information arises from the fact that strain may be measured by many different methods, such as by optical diffraction, the use of strain markers, by the use of acoustic sensors. The strain may also be

measured at many points along the specimen, if desired. The use of a force transducer at the end of the specimen in these experiments is misleading, because it is functioning in this case as a displacement transducer, simply to detect the arrival time of the pulse. The placement of mechanical markers along the specimen allows the strain within a region to be measured (as an average), but the markers themselves may cause an impedance change which could interfere with the normal strain wave propagation along the specimen. This is a considerable problem for extremely thin specimens, such as individual muscle fibers. For example, acoustical pickups have been used with whole muscle specimens with some success (Truong 1974). Such an approach is not possible when single muscle fibers are to be used. The fact that muscle tissue has natural strain markers, namely the repeating protein structure of the sarcomere, and the fact that the local spacing of these markers can be readily detected using laser light diffraction, suggests that this method may be employed if at least two positions along the fiber can be sampled, and if the data acquisition is rapid enough to provide a highly time-resolved record of the local sarcomere strain at these two regions during the application of strain pulses.

One additional potential weakness of the pulse propagation method is that it assumes that, prior to the application of the pulse, the strain state of the fiber is homogeneous and stable between the points at which the strain is measured. However, the pulse propagation method does not assume that the strain between the two measurement points remains homogeneous and stable during the application of a pulse, unlike the previous two methods. Therefore the pulse propagation method is a valid means for measurement of viscoelasticity of muscle if it can be demonstrated that the strain between the two measurement points is stable and homogeneous before the application of a pulse. The stability and uniformity of the sarcomere lengths between the two measurement positions can be inspected visually by the method of optical diffraction during experimental setup. The process of inspecting the sarcomere

lengths for the region between the two sampling positions is discussed in detail in Chapter II and Chapter III.

If the strain state between the two measurement positions is known to be stable and reasonably homogeneous, then the pulse propagation method will allow the measurement of viscoelasticity for the fiber segment between the two positions. Optical detection of local strain at the two positions will also allow the detection of pulse spreading, which is not possible in the continuous sine wave and the step length change methods. The use of single pulses of fixed frequency content will permit nonlinear responses to be detected easily by varying the amplitude of the strain pulse, and therefore the method does not assume linear elastic behavior.

In summary, a properly implemented experiment which employs the method of pulse propagation can be used to accurately measure the viscoelasticity of single muscle fibers. A tabulated summary of the published results of viscoelasticity and stiffness measurements resulting from the application of the above three methods is included in Appendix A.

Aims of the Present Study

Based on the foregoing assessments, the measurements of the viscoelasticity of single muscle fibers will be based on the method of pulse propagation. Longitudinal strain wave propagation velocity and attenuation coefficient will be measured to quantify the viscoelasticity of single permeabilized muscle fibers, using laser light diffraction to directly measure the local sarcomere strain at two points along the single fiber specimen. Adequate instrumentation for optically measuring longitudinal strain pulse propagation in single muscle fibers has not been described. The aims of the present study are:

Aim #1 - Define the functional specifications for an optical system for the measurement of pulse propagation in single muscle fibers.

Aim #2 - Design and build a system to meet or exceed the functional specifications from commercially available optical, electronic, and mechanical components, that can be interfaced easily with a microcomputer.

Aim #3 - Under various experimental conditions, determine the complex elastic modulus of muscle by measuring the pulse propagation velocity and attenuation coefficient of longitudinal strain pulses in passive and partially activated single permeabilized skeletal muscle fibers. The experimental conditions will include different pulse amplitudes, from about 0.2% strain to about 10% strain, lengthening and shortening pulses, different pulse frequencies, level of activation of the fiber, initial sarcomere length, and age of the animal from which the fiber was taken. The instrumentation will be used to measure the viscoelasticity of relaxed, chemically permeabilized muscle fibers from the soleus muscles of rats.

Aim #4 - Optimize the functional specifications and design of the system based on the results of these experiments.

CHAPTER II
INSTRUMENTATION

Adequate instrumentation has not been developed for the measurement of longitudinal strain pulse propagation in single muscle fibers. In this series of experiments, instrumentation was developed for the measurement of muscle fiber viscoelasticity by means of optical diffraction. The first aim of the present study was to define the functional specifications for such an optical system. The second aim was to design and build a system to meet or exceed the functional specifications from commercially available optical, electronic, and mechanical components, that could be interfaced easily with a microcomputer. The third aim of the present study was to assess the system performance by using the system to measure longitudinal pulse propagation in single skinned muscle fibers.

Background

Ranvier (1874) made the original observation that the striation pattern of skeletal muscle could be used as an optical diffraction grating. The ability to use skeletal muscle as a transmission diffraction grating arises from the fact that the A and I bands of the sarcomere, the basic contractile unit of muscle, have different protein concentrations and thus have different optical refractive indices (Judy, et al., 1986). For nearly sixty years the optical diffraction method has been used to monitor sarcomere length

in muscle during experiments on muscle mechanics (Sandow, 1936). With the advent of low-cost lasers, the method has gained even more widespread application in muscle research (Sundell, et al., 1986).

Although the optical diffraction method is a convenient tool for monitoring sarcomere length in single muscle fibers, the technique has not yet been used to measure muscle viscoelasticity. For many years, the measurement of the viscoelastic properties of single muscle fibers by the use of optical diffraction methods has been feasible. The lack of any attempt to apply this technique for this purpose is probably due to performance limitations in the optical diffraction systems existing in muscle mechanics laboratories.

In most muscle mechanics laboratories, four performance limitations are evident in the instrumentation for optical diffraction measurements. The most important limitation is an inadequate bandwidth, or sampling rate for the detection of the first order diffraction peak. The second limitation is conceptual. Most systems presently in use in muscle research use a long segment of the muscle fiber as a diffraction grating, to give the best possible representation of the average sarcomere length along the fiber. The sarcomere length in a region of the fiber may be used to quantify the strain, because sarcomere length is defined by physical markers along the length of a muscle fiber, namely the Z-Lines (Figure 1.1). The optical diffraction method is most often used to measure the spatial average of sarcomere length along a large section of the fiber, not the strain in a small region along the length of the fiber. The third limitation is that most systems do not provide a means to accurately position a small, focused laser spot to at least two precise positions along the length of the muscle fiber, separated by a distance of at least one millimeter. The fourth limitation is that the highly time-resolved, dynamic measurement of sarcomere length at two positions along a fiber generates a large amount of data, which requires data storage, analysis, and handling techniques unlike those required for general sarcomere

length measurement systems. In order to use optical diffraction to measure longitudinal strain pulse propagation in single muscle fibers, instrumentation must be developed that will allow a laser to be focused to a small spot on the fiber, to position that spot to a precise location along the length of the fiber, and to collect, store, and analyze large numbers of diffraction spectra with a sampling rate in the range of hundreds of thousands of diffraction spectra per second.

Aim #1: System Specification

The following system specification was used in the design of the instrumentation for the measurement of longitudinal strain pulse propagation in passive and partially activated single skeletal muscle fibers. In addition to providing general performance requirements, this specification serves as an outline of the function of many separate components of the total system. The details of the design of each of the system components is given in the System Design section.

Principle of Operation

A system is required which allows the longitudinal strain propagation to be measured at two points along the length of a single permeabilized skeletal muscle fiber by means of laser diffraction. The repeating protein pattern of the fiber acts as a transmission diffraction grating. The grating spacing is defined by the sarcomere length. Therefore, the sarcomere length within the area of the fiber onto which the laser beam has been focused can be determined by measuring the diffraction angle of laser light. A data acquisition system is required that can simultaneously control a servo motor to apply rapid longitudinal strain pulses to the single fiber while collecting data on the position of the first order diffraction pattern to allow the sarcomere length to be calculated.

Selection of the Detector

Typical high-speed laser diffraction systems used in muscle mechanics experiments employ a linear optical position detector, from which two output currents are generated. The difference between the currents is divided by the total output current of the device, yielding the location of the centroid of the incident light on the detector surface. This device is generally referred to as a 'spot follower', and is often employed in length clamping systems in which the output is used as a feedback signal to a servo motor which controls the muscle fiber length. These devices yield information only on total diffraction peak intensity and the location of the centroid of the peak, but information about the peak width, or the existence of multiple peaks which are known to result from regions of differing sarcomere length within the sampled region, is lost. An array of fast photosensors permits not only the determination of the location of the first order peak, but also the shape and width of the peak and the presence of multiple peaks, if they exist. The location of the first order peak allows calculation of the sarcomere length, the width of the peak can be used to estimate the distribution of sarcomere lengths within the sampled region by deconvolving the peak from the Gaussian intensity profile of the laser spot on the fiber (Judy, et al., 1982). Multiple peaks would be present if there were several distinct populations of sarcomere lengths within the sampled region. In addition, the system must be able to detect sarcomere length changes of less than 10 nm to provide adequate resolution for strain amplitudes in the displacement range for attached cross-bridges, approximately 10 to 20 nm per half sarcomere (Piazzesi et al., 1993; Higuchi and Goldman, 1991; Toyoshima et al., 1990; Huxley and Simmons, 1971).

Estimate of Sampling Rate Requirement

Previously published data were analyzed to estimate the sampling rate requirements of the proposed instrumentation. The published range of pulse propagation velocities begins at approximately 30 m/s for fully relaxed fibers, and rises up to about 140 m/s for fibers at less than 50% activation (Appendix A). The relationship between the level of activation and the pulse propagation velocity for very low levels of activation is unknown. The pulse propagation velocity for fully activated fibers is in the range of 125 to 270 m/s. The maximum distance between sampling positions along a fiber is approximately 3 mm, because it is difficult to obtain fibers with longer sections in which the sarcomere length is consistent throughout. The fibers must be at least 5 mm long between mounting points at each end of the fiber to avoid mechanical interference between the attachment points and the 1 to 3 mm section of the fiber under study. Therefore the time delay between pulse arrival at the two sampled regions might be as low as 21 μs. Thus, to achieve a ±10% resolution for the propagation velocity the system must be capable of sampling at least every 2.0 μs, that is, 500,000 samples per second. This value for sampling rate is a reasonable upper limit for design purposes for the initial system design. This sampling rate requirement greatly exceeds the capability of the fastest systems presently available for use in muscle research, and is in fact five orders of magnitude faster than the previous system in use in our laboratory (Princeton Instruments, model ST-120). Serial analog-to-digital conversion of intensity signals in the array is not acceptable because the time delay between the sampling of each successive element in the detector array would permit strain conditions to change during the sampling sweep, and in any event would be precluded by the sampling rate requirement of 500,000 samples per second. Therefore, the system is required to employ a totally parallel architecture for all data conversions and storage. Additional analog-to-digital channels will be required for collecting servo motor position and force data for each time point.

A servomotor is required that can be controlled digitally or by an analog signal that is generated by the data acquisition system. The servo must be able to provide linear displacements of 12 microns to 600 microns, to allow a range of longitudinal pulse amplitudes from 0.2% to 10% to be applied to a 6 mm long muscle fiber. The servo must also have a bandwidth of DC to at least 1 kHz over the full range of displacements to allow rapid sinusoidal pulses to be applied to the muscle fiber.

A laser light source is to be focused to a small spot along the length of the fiber. The spot size is to be adjustable from a diameter of 50 to 300 microns, to allow different sized regions to be sampled if desired. The laser must be positioned to within 1% accuracy over a displacement of 2 mm, thus it is required that the position of the laser spot on the fiber be known to an accuracy of at least 0.02 mm. A calibrated target is to be provided which will allow the sarcomere length to be set by lengthening or shortening the fiber prior to the pulse propagation experiments.

Additional Fixturing and Equipment

The fiber is suspended in a fluid bath during all experiments. A flow-through bath is required to allow activating and relaxing solutions (Chapter III) to be introduced into the bath without subjecting the fiber to large force transients by pulling the fiber through a fluid surface during bath changes. An isovolumetric fluid exchanger is required for exchanging the bath fluids without causing leakage or air entrapment in the system. The bath must also allow access for the servo motor and force transducer, to which the single fiber is attached. The bottom of the bath must be transparent and free of optical obstructions because the diffraction pattern is projected through the bottom of the bath chamber onto the detector system below. The bath must also allow a glass cover slip to be placed on the free surface of the bath fluid during the experiments so that the meniscus formed by the bath fluid will not introduce a

concave or convex optical surface, which would distort the incident laser beam. A means for controlling the bath temperature must also be provided. The bath is to be maintained at 15 °C ± 0.2 °C.

A force transducer is required which is robust, small, and linear and sensitive to force changes as small as 1 μN. The output of the force transducer is to be displayed in real time on a four-digit LED display to allow the level of activation of the fiber to be easily adjusted between experiments. The force transducer must also include a means for baseline offset adjustment to correct for the surface tension of the fluid in the bath. The transducer displacement must not exceed 30 μm over the force range of 0.0 to 1000 μN to prevent unacceptable changes in muscle fiber length which would otherwise result from the use of a high-compliance force transducer.

The fiber must be aligned coaxially with the linear servo and force transducer. The fiber must be held horizontal and attached in such a way that no transverse or off-axis movements will be induced by the servo motor during pulses. The length of the fiber must also be adjustable to within 5 μm to permit the sarcomere length to be precisely set prior to experiments. Therefore the system must include a set of micromanipulators to position both the force transducer and the servo motor. The entire system must be isolated from mechanical vibrations from the external environment.

During the strain pulses, all data must be collected and stored for subsequent downloading to a computer for analysis. The exact method of analysis of the data will not be known until some data have been collected, thus all raw data must be stored to a large capacity data storage medium that is easily accessed for processing. Because a large amount of data will be collected, in excess of 2 GB, it will be necessary to provide software to process and display the results in a format that can be easily viewed.

Aim #2: System Design

A small, low-profile precision X-Y table was designed and built to hold the servomotor, both hydraulic micromanipulators, the flow-through bath, and the force transducer. The table was made of acrylic sheet and is shown schematically in Figure 2.1. The table allows precision positioning of the mounted single fiber under the microscope for length and diameter measurements and for laser spot positioning. The plates slide on flanged instrument bearings which are mounted to the X-Y table with hardened and ground shoulder screws and are guided along 2024 aircraft aluminum rails.

The table can be manually positioned to within ± 2 microns of the desired position by the use of triple nested-thread actuators for each axis (Appendix B). The external 3/4" - 16 threads allow rapid coarse positioning adjustments to be made over a range of 1". Once positioned, the coarse adjustment is locked in place with a 3/4" 16 brass jam nut. Fine adjustment is provided by the inner two threads which counteract each other over a range of 0.40". A single rotation of the 1/2"-20 brass shaft is counteracted by a counter-rotation of the 1/4"-28 stainless-steel inner shaft, resulting in a net linear motion of 0.0143" (363 microns) per revolution of the 1" diameter knurled brass knob. All threads were thoroughly coated with lithium axle grease to provide smooth, low-hysteresis motion. This general mechanism can be easily adapted to provide a wide range of very low cost, high precision positioning mechanisms for research purposes.

In the Y-axis (forward and back motion), the table is aligned optically using the microscope reticule for each experiment. Displacement in the X direction (right and left motion) is measured using Mitutoyo MyCAL calipers which are firmly mounted to the antivibration table surface (Fig. 2.1). The depth gauge rod of the calipers is preloaded against the right side of the X-axis plate with a compression spring in the caliper jaws. The table is traversed to the point where the cross hairs in the eyepiece reticule of the stereomicroscope are aligned with the tip of the force transducer trough, and the calipers

are set to zero. This arrangement allows positions along the fiber to be measured from the end of the fiber at the force transducer mounting point. Thus, the fiber length and the distance between the laser spot positions can be determined to within ± 0.01 mm.

The performance of these experiments requires precise control of the positions of the mounting points of the single fiber. The fiber must be held level, the servo actuator must be in line with the fiber, and the baseline sarcomere length must be adjusted with precision. Fiber mounting, immersion in the bath, centering of the specimen, and adjustment of the sarcomere length, as well as retracting the fiber mounting troughs for removal of the flow-through bath for regular cleaning and maintenance requires that the micromanipulators have a movement range of 3.0 cm right and left, 1.0 cm fore and aft, and 2.0 cm vertical. No commercially available hydraulic micromanipulators could meet the movement range requirements, so it was necessary to build a set. The micromanipulators were machined as a mirror-image pair of three-axis dovetail slides from 70-30 brass. The slides are actuated by single-sided 0.425" diameter brass pistons with single O-ring seals, and the slides are pre-loaded against the pistons with compression springs on each axis. Each assembly is driven by a remotely located master cylinder manifold machined from 2024 aluminum plate, with 0.4990" diameter pistons and dual o-ring seals. The master cylinders are internally preloaded with compression springs and are driven by 3/8"-16 power screws with a knurled brass knob for each axis of each positioner. The hydraulic fluid is standard mineral oil, degassed and vacuum bled with a syringe during assembly. The total fluid volume was minimized during the design to reduce thermal drift. High-density polyethylene instrumentation tubing was used for the hydraulic lines to minimize hydraulic compliance. The micromanipulators were tested for positional stability and it was determined that each axis would maintain position to within 3 microns for 8 hours under steady load. This performance is comparable to commercially available units.

A high-speed moving-magnet scanner (Cambridge Technologies, Model 308) of the type used in laser light shows, was used for applying high-frequency longitudinal pulses to the fiber. The scanner arm produces angular displacements of ±20° with a 0.479" lever arm. The scanner arm was modified with a clevis and rod arrangement as a mechanical linearizing mechanism (Appendix C). These modifications transformed the rotary motion of the scanner actuator arm into linear motion with minimal off-axis movement. Without this modification the rotating servo arm would impart a transverse vertical displacement to the end of the muscle fiber each time a longitudinal strain pulse is applied. This condition is unacceptable because transverse vibrations of the fiber would be induced during the longitudinal pulses. Therefore the design criteria include minimizing vertical and lateral excursion during the linear motion, keeping the mass of the mechanism to an absolute minimum, and keeping the sliding resistance below 0.1 mN. The negligible sliding resistance minimized the mechanical hysteresis and maximized the mechanical bandwidth. The scanner and linearizer mechanism assembly are referred to as the servo, or servo motor.

A 29 ga. stainless tube is used as a trough for mounting the muscle fibers to the servo (Fig. 2.2). After assembly, the linear servo mechanism was tested to check for the presence of lateral and vertical vibrations at the end of the trough during the application of rapid pulses (Appendix C). Lateral and vertical vibrations were detected by an optical method identical to that used for the precision optical force transducer (Appendix D). For all servo excursions at all pulse amplitudes and speeds, the maximum lateral and vertical excursion of the end of the trough was less than ± 7 μm. This was judged to be acceptable for this series of experiments.

The scanner control electronics were specially modified for this series of experiments (Appendix C). These modifications were necessary for several reasons. The scanner position resolver

signal is not available to the user, so it was necessary to tap the position demodulator signal, and buffer it through a high-speed Bi-FET op-amp voltage follower. This produced a ± 5 VDC signal corresponding to the scanner angular position. In addition, the control voltage input for the scanner is ± 10 VDC for full scale movement. The digital waveform synthesizer, which is used to generate the control voltages for the servo position, only has a resolution of 8-bits. The analog input for the array controller, which detects the servo position at each time point, also has only 8-bit resolution. The array controller was built with 8-bit analog-to-digital and digital-to-analog converters because these were the maximum number of bits available for hybrid IC's that were sufficiently fast for this series of experiments at the time of the design of the system. The limited resolution was compensated for through modification of the servomotor control circuits. Three operational ranges were used to cover the full range of the 8-bit input and output resolution of the array controller (Appendix C).

During an experiment, frequent switching of the ranges of the voltage for motor control required the suppression of transient servo motor surges. The surges resulted from transients in the control voltage. The suppression was accomplished by the provision of a toggle switch to interrupt the scanner coil current through the initiation of an open circuit at junction J1-1. Between experiments and during servomotor range changes this switch was left in the OPEN position. In addition to these changes, the ± 15 V power supplies for the scanner were upgraded to ±24 V, as recommended by the manufacturer, to improve the high-speed performance of the system. Because of this modification, a close-fitting aluminum compression-style vertical-vaned heat sink was added directly to the scanner housing to maximize the heat transfer by free convection. During high-speed operation this modification prevented overheating of the servo and subsequent damage to the rare-earth element magnet in the rotor.

A precision optical force transducer was designed and constructed for this series of experiments. Several high-sensitivity, high-precision force transducers were available on the market, but they were costly and had several other limitations which made them undesirable for experiments of this type. The bonded resistance strain gauge-based systems have too much full scale mechanical compliance, typically 0.010" (254 microns) or more. During muscle fiber experiments this compliance allows changes of fiber length. In addition, this family of force transducers is not sensitive enough to detect the small forces generated by a single muscle cell when fully activated, which is typically less than 1.0 mN. The most commonly used type of force transducer for single fiber muscle mechanics experiments is based upon a variable capacitance. The force transducer is essentially a thin quartz cantilever, coated on one side with aluminum by vacuum deposition. These force transducers have the disadvantage of baseline drift due to humidity because air is used as the dielectric, they are expensive, and the thin quartz cantilever is prone to breakage. Damage to these transducers is often not remediable without sending the device back to the manufacturer. For these reasons, a highly sensitive and robust optical force transducer for general laboratory use was developed. The principle of operation is that an LED acts as a light source for two very small phototransistors which are separated by a distance of 0.100 inches. A load element is fashioned to deflect approximately 0.002" (50.8 μm) full scale, and a 0.100" wide vane of stainless steel foil is attached to the load element and is interposed directly between the LED and the center of the two phototransistors (Appendix D). A sensitivity to deflections of nearly 1/3000 of full scale is possible. The phototransistors are connected to load resistors in emitter-follower configuration. The outputs are conditioned by means of a low gain differential amplifier (Appendix D). Using a 0.0005" thick stainless steel foil load element, the force transducer has a sensitivity of 0.001 mN , with a full linear range of 1.20 mN. The accuracy is ± 0.002 mN over the full range and the undamped natural frequency is 350 Hz.

The output signal from the phototransistors is large, so the support electronics for the transducer are simple (Appendix D). This type of transducer was developed specifically for this series of experiments, but may be used in a broad range of muscle mechanics experiments, and is notable because it allows any laboratory with access to a machine shop to produce very robust force transducers with performance equal or superior to the best laboratory-grade transducers on the market, in any force range desired, for a fraction of the cost.

A flow-through bath was designed to allow the bath fluid to be changed without passing the muscle fiber through a fluid surface. The bath fluids must be changed because the level of activation of permeabilized muscle fibers is controlled by varying the calcium concentration in the bath. The details of the use of activating and relaxing bath solutions are given in Chapter III. By passing the fiber through a fluid surface, the surface tension causes the muscle fiber to be subjected to a transient force which is often greater than the maximum force that the fiber can produce when fully activated. The muscle fiber may be damaged unless the fluid can be exchanged without subjecting the fiber to these transient forces.

The bath was machined from 304 stainless steel and is designed to accommodate muscle fibers up to 10 mm long. The design facilitated the placement of standard 18 mm square cover slips both above and below the muscle fiber. The bottom cover slip was glued into place permanently with cyanoacrylate adhesive, though it can be periodically changed if necessary. Fluid was introduced into and withdrawn from the bath chamber by means of 18 gauge stainless steel tubing placed at each end of the bath (Fig. 2.2). The fluid flow into and out of the bath was perpendicular to the axis of the bath to maximize mixing during fluid exchange (Appendix E).

An isovolumetric bath fluid exchanger was built to allow the fluid in the bath to be changed without forcing leaks or introducing air into the system. The fluid exchanger holds two 5 cc disposable

plastic syringes anti-parallel to one another. One of the syringes is clamped into place while the other is allowed to slide axially. The syringes are coupled such that the body of the sliding syringe moves with the plunger of the stationary syringe. Thus, by actuating the fluid exchanger, a volume of fluid is expelled from the stationary syringe that is equal to the volume of fluid drawn into the sliding syringe. Each syringe is connected to the flow-through bath by 0.040" I.D. thin wall Tygon tubing. When the fluid has been exchanged, the Tygon tubing is disconnected from the flow-through bath, and the fluid remains in the bath by means of its surface tension. The transfer volume of the fluid exchanger was calculated based upon a first order differential equation which assumes constant bath volume, good fluid mixing during fluid exchange, and a 99.9% purity of new fluid after the exchange is complete. A safety factor of two was then added, and the final value of approximately 5 cc for fluid exchanges was established (Appendix E).

The bath temperature was monitored by a surface mount linear CMOS temperature sensor (LM35D, National Semiconductor). The sensor is located in a cavity in the stainless steel bath close to the single fiber (Fig. 2.2). The output of the sensor is converted to degrees centigrade, and is displayed in real time on a 4-digit LCD display. The output from the sensor is used to control a solid state relay which controls a submersible pump (Fig. 2.1). The pump is immersed in an ice water bath and, when turned on, forces cold water through a flow-splitting heat exchanger manifold that was machined to tightly fit to the bottom of the stainless steel flow-through bath assembly. The system response time is controlled by an adjustable valve (Fig. 2.1) to eliminate excessive thermal fluctuations by limiting the flow rate through the heat exchanger. The bath temperature was controlled to 15.0 ± 0.2 °C for all experiments.

An optical field illuminator prism was constructed to provide a bright field of white light for photographing and measuring the fibers when they were mounted on the apparatus (Fig 2.1). The prism could be retracted to clear the optical path during the pulse propagation experiments. A small Michelson interferometer was constructed (Appendix F) for the measurement of the coherence length of the laser diode module (Applied Laser Systems P/N: VLM 2-5C(L)). Laser light of different coherence lengths was required for the experiments described in Chapter IV: Steps and Pauses. A variable output, low-noise power supply was built to supply current to the laser diode module, both during experiments and while measuring the coherence length of the laser light as a function of supply voltage.

The system optics are shown in schematic form in Figure 2.3. During mounting of the fiber a standard dissecting microscope (Leica WILD M3Z Kombistereo microscope) was used for the measurement fiber dimensions and for the placement of the laser spot on the fiber (Fig. 2.2). The laser is mounted behind the setup and the beam enters the system horizontally and above the level of the single fiber through a slot in the microscope mounting post. The laser beam is focused by a 58 mm focal length meniscus lens. The laser beam is then directed downward through a 90° prism to strike the fiber. The lens and prism are mounted on a sliding mechanism with adjustable stops. The lens and prism are retracted during fiber mounting and setup, and are slipped into position during the final adjustment of the muscle fiber length and for the duration of the experiment. The laser beam passes through an 18 mm square, 0.13 mm thick glass cover slip, through a 4.67 mm deep fluid bath in which the fiber is suspended, and out through a 0.13 mm thick cover slip glued to the bottom of the bath. The transmitted laser beam and the diffraction pattern are projected through the bottom cover slip, through a cylinder lens and onto a first surface mirror below the anti-vibration table. A small calibrated target slides into position below the muscle fiber and bath (Fig. 2.3). The sarcomere length is read directly from the

diffraction pattern on the target, and is adjusted prior to execution of the experiments. The target is removed prior to data collection. The cylinder lens focuses the diffraction pattern onto the sensitive area of the detector array. The first surface mirror directs the diffraction pattern toward the detector array. The optical path length from the muscle fiber to the zero-order peak on the detector array is adjustable. For most experiments the optical path length was set to 290.5 mm. The phototransistors in the array are less sensitive to light from oblique angles, so the detector array was tilted to maximize the signal strength over the full range of the array by minimizing the incidence angle near the center of the array. This resulted in a two-fold increase in detected signal strength.

A function generator was built to provide control signals for the servo to generate maintenance ramps. The purpose of the maintenance ramps is to subject the muscle fiber to periodic shortening ramps at maximum shortening velocity. The periodic shortening ramps stabilize the sarcomere lengths throughout the fiber and allow the fiber to remain viable for a much longer period of time (Brenner, 1983). The ramp function generator allows the ramps to be adjusted on the basis of fiber length, ramp amplitude relative to fiber length, and time delay between ramps (Appendix G).

For high-speed studies of sarcomere dynamics a detector/buffer system was built to drive the servo motor and to collect and store the diffraction data. This system is referred to throughout this document as the *array controller*. A linear array of 64 fast phototransistors (Panasonic PN127) was constructed. The phototransistors are each connected in emitter-follower configuration to a load resistor (Appendix H), and the resulting voltage for each is converted to an 8 bit digital value. The analog-to-digital conversion for all 64 phototransistors is performed in parallel, using sixty-four 8-bit analog-to-digital converters on a common clock. Each analog-to-digital converter is capable of 50 million conversions/second, with an aperture time of 0.8 ns for each conversion. Each converter has a dedicated

static ram (SRAM) memory chip (25 ns access time), 128K x 8 (1 megabyte) for each channel (Appendix I), thus 131,072 consecutive diffraction spectra can be collected in parallel by this system before downloading the data to computer is necessary. The system is capable of collecting analog signals into all 64 channels simultaneously at 10 MHz without detectable distortion at 8 bits of resolution. The practical sampling rate limit for the phototransistor array is about 500,000 samples per second, based upon the small-signal rise and fall times of the phototransistors with 470 Ohm load resistors (Appendix H).

To take full advantage of the speed potential of the paralleled data acquisition system, the detector head may be changed to include an array of 64 high-speed photodiodes with preamplifiers. The change may be accomplished quite easily if greater sensitivity or increased temporal resolution is found to be necessary in future experiments. The detector/buffer system also includes a 64-to-1 multiplexer-demultiplexer for the 8-bit data bus (Appendix I) to facilitate data downloading into any computer equipped with a 24-bit digital I/O board. The system has four additional analog input channels which are dedicated to other analog signals of interest, such as temperature, force, laser position, and servo motor position. The system is also equipped with four built-in 8-bit digital waveform synthesizers (Appendix I) which are used for providing command positions for the servo motor. The digital waveform synthesizers may also be used to provide stimulus pulses for electrodes during experiments with single intact fibers, control signals for driving a laser deflector, or for triggering a laser for use in studies involving caged molecules. The digital waveform synthesizers are programmed immediately prior to each experiment. A summation input is included for the servomotor command driver to allow maintenance ramps to be imposed upon the skinned fibers so that the sarcomeres will remain in register. These ramps maintain the

sarcomere pattern in activated permeabilized fibers for the period between experiments, and during experimental setup.

Once the data is downloaded from the microcomputer into the digital waveform synthesizer memory, the experiments are executed from the control panel on the data acquisition system (Appendix I). Alternatively execution may be done remotely from the computer keyboard. All functions of the data acquisition system are clocked in parallel by a precision quartz oscillator. Consequently, no corrections are required for time delays that would result from serial detection of individual phototransistor outputs, motor position commands, and servo position feedback voltage values. Based on the bandwidth limit of the phototransistor array, the system can provide a temporal resolution of 2 microseconds between complete spectra. The entire diffraction spectrum is stored at each sampling interval, as are all other analog signals such as servo motor position and force. This allows both the location of the first order diffraction peak and the distribution of sarcomere lengths within the sampled region to be calculated (Judy, et al., 1982).

The array controller design includes several additional controllable parameters for maximum flexibility in the execution of experiments. The entire system is timed by precision quartz oscillators, which could be selected on the control panel. The clock could also be driven by an external source, or by a tunable, adjustable oscillator (Appendix I). The oscillator signal is then divided by a selectable value of 2, 4, 8, 16, 32, 64, 128, or 256, to allow a wide range of clock frequencies to be selected by combining different oscillators and dividers. The memory of the data acquisition system could be divided into 1, 2, 4, 8, 16, 32, 64, or 128 separate experiments to allow the execution of a series of different experiments either in rapid succession, or with a delay between experiments. When necessary, adjustments such as changing the bath temperature, bath solutions, or laser position along the fiber, are

made between experiments. This feature was used extensively to impose a large number of different strain pulse amplitudes on each fiber within a short period of time by dividing the memory into discrete sectors for each longitudinal strain pulse.

The array controller has several modes to simplify the experimental set up and execution. A *real time output mode* causes the array controller memory output multiplexer to clock through each analog input channel sequentially and output the value to the databus DAC. A multiplexer reset trigger pulse is also provided. The diffraction spectrum intensity profile is then viewed in real time on an oscilloscope for troubleshooting and optical alignment of the system. *Input mode* is used for the collection of actual spectral data and any suffix data, such as servo motor position. This mode also enables each digital waveform synthesizer to generate output signals to BNC lugs on the front panel of the data acquisition system. During the actual experiment these signals are used to drive or control any external devices that are used. *Load DAC mode* permits the computer to download values into the digital waveform synthesizer memory. *Output data mode* is used for downloading all stored spectral data from the data acquisition system into data files on the computer hard drive. This mode disables the analog signal generators and enables the ADC/M board multiplexer to serially download each 8-bit value from the memory chip in each of the 64 channels (Appendix I). In addition to these features, several additional features were added which simplified system calibration and functional diagnostics, but these are excluded from further comment as they are not germane to the functional capability of the system or the viscoelasticity experiments.

To complete the system, several commercially available fixtures and instruments were used. In some cases, significant modifications were made for this series of experiments. The entire experimental apparatus is mounted on an anti-vibration table, Newport P/N XSN-34** and VX-3060-SP, with a 3 inch

diameter hole specially machined through the table to allow the diffracted laser beam to pass through directly beneath the muscle fiber specimen. A Leica WILD M3Z Kombistereo microscope is bolted to the anti-vibration table and is used for fiber mounting and fiber length measurements using the dissecting objective. This microscope has sufficient focal depth to view the fiber and forceps during the mounting and tying procedure. Diameter measurements are made using the high-power objective (Plan L 25X/.040) with a 100-division eyepiece reticule (Leica # 394-771). Reticule resolutions of 3.106, 1.984, and 1.306 microns per division are obtained at the three highest magnification settings (16x, 25x and 40x).

A Dell 60 MHz Pentium OptiPlex 560/L computer is used for generating and downloading digital wave forms, remotely triggering the experiment, and for collecting and storing the 8-bit digital data once the experiment has been completed (Fig. 2.4). The computer is also used for processing all data. The raw data is backed up on 3.5", 128 MB rewritable optical disks, which permit easy access to the raw data for further processing if desired. The computer interfaces with the array controller via a standard 24-bit digital I/O card (National Instruments NB-DIO-24 ISA).

A Gould 200 MHz digital oscilloscope (DSO 475) is used for monitoring servomotor and digital waveform synthesizer function during the experiments. Due to the speed at which the experiments were performed, a real-time display of the sarcomere length calculated from the diffraction pattern was not possible during pulse propagation experiments.

A 10 mW He-Ne laser (Uniphase Model 1105P) is mounted behind the setup. The beam is directed through a slot machined in the stereomicroscope post, is focused through a 58 mm f.l. meniscus lens, and is reflected at 90° by a prism to strike the single fiber (Fig. 2.3). The beam is focused to a diameter of approximately 250 microns, and the laser spot may be positioned at any desired point along

the fiber by traversing the X-Y table right or left and reading the position change from the digital depth meter (Figs. 2.1 and 2.2). The prism and focusing lens is retracted and the fiber is viewed under the stereomicroscope using the high-power objective. For experiments involving the use of reduced coherence length laser light, a visible laser diode module was used (Applied Laser Systems P/N# VLM 2-5C(L))

Software

To execute this series of experiments, several programs were written. All programs were written in VisualBASIC for DOS and were executed by a 60 MHz DELL Pentium OptiPlex 560/L IBM-Compatible personal computer. Because these experiments generated very large amounts of data (the diffraction spectrum and servo motor position data for 1280 longitudinal strain pulses on a single fiber occupies approximately 110 MB, uncompressed) every effort was made to optimize the code for efficient calculation and data handling. For example, superfluous data were eliminated early in the process, all calculations were made on integer values where possible, and methods such as Horners method for rearranging polynomial expressions to reduce calculation steps and thus reduce calculation time, were employed whenever possible. Nonetheless, each fiber required approximately 30 minutes total computer data setup time (To load the DAC memory); 80 minutes data downloading time to retrieve data from the Diode Array Controller; 90 minutes to convert all diffraction spectra into sarcomere length vs. time records by calculating the location of the intensity centroid of the 1° diffraction peak and applying the grating equation at each time point; 150 minutes to back up the raw data and the transformed spectral data onto 128 MB optical disks; 90 minutes to apply median filters of rank 1 to all sarcomere length vs. time data to remove occasional noise spikes; 250 minutes to apply cross-correlation algorithms to all data

sets to calculate and record pulse propagation delay, and to calculate and record pulse amplitudes for each experiment. Thus, a total of approximately 600 minutes (10 hours) of processing time is required for each single fiber. A description of the function of each program, the filtering and data manipulation algorithms, and flow charts are provided (Appendix J).

Analysis of Data

The measurement of the complex Young's modulus, or viscoelastic modulus, by means of pulse propagation requires a system that will allow the measurement of both pulse propagation velocity (v) and attenuation coefficient (α). The measurement of v and α require that two positions along the fiber be sampled. To accomplish this, a laser is focused at one position along the fiber near its attachment to a linear servo motor to produce a diffraction pattern. The fiber is then be subjected to a number of lengthening and shortening pulses by the linear servo motor, each of which induce longitudinal strain waves that propagate down the length of the fiber to the opposite end, which is attached to a force transducer. The diffraction spectra of one of the first order diffraction patterns is collected, as is the servo motor position data, and both are stored continuously as the longitudinal strain pulse passes through the region of the fiber that is being sampled by the focused laser spot. The focused laser spot is then moved to a new position along the fiber, further from the servo by a distance of 1 to 3 mm. The fiber is then subjected to a battery of lengthening and shortening pulses identical to those at the first position, and the diffraction spectra and servo position data are again collected as the longitudinal strain waves pass through the second sampled region along the length of the fiber.

The raw data are downloaded to computer and the diffraction data are used to locate the position of the centroid, X, of the first-order diffraction peak for each time point:

$$x = w \frac{\sum_{p=m-3}^{p=m+3} V_p (p-1)}{\sum_{p=m-3}^{p=m+3} V_p} \qquad \text{Eq. 2.1}$$

in which m is the phototransistor number with the maximum voltage, V_p is the voltage at phototransistor number 'p', W is the width of each phototransistor (2.21 mm), and X is the distance of the 1° peak centroid from the center of phototransistor #1, in mm. The exception to this calculation was when the peak value occurred within three photo-transistors of either end of the array. In this event, bias of the centroid toward the center of the array was avoided by modification of the calculation of the centroid, using m ± n as the limits of the summations, where n is the number of phototransistors between the location of the maximum voltage and the first (or last) phototransistor in the array. If the peak value occurred at either the first or last phototransistor in the array, an out-of-range flag is recorded.

The diffraction angle, θ_m, is calculated from the position of the centroid of the first order diffraction peak and the known geometry of the experimental setup by applying simple trigonometry. For each time point in each experimental record, the sarcomere length is calculated from the diffraction angle by applying the grating equation (Hecht, 1990):

$$L(\sin\theta_m - \sin\theta_i) = m\lambda \qquad \text{Eq. 2.2}$$

in which L is the sarcomere length, m is the order of the diffraction peak, which is set equal to 1 because only the first order diffraction peak (one of the two that are available) is recorded, θ_m is the diffraction angle of the first order peak, θ_i is the incident angle of the laser light as it strikes the muscle fiber, and λ is the wavelength of the laser, 632.8 nm in the case of a helium-neon (He-Ne) laser, and 670 nm in the case of a laser diode module.

The resulting sarcomere length data allow the calculation of the propagation delay time, Δt, for the longitudinal strain wave to pass through the two sampled regions, which are separated by a distance Δx. The propagation velocity, v, is then calculated as:

$$v = \Delta x/\Delta t \qquad \text{Eq. 2.3}$$

In addition to the time delay between strain pulse arrival at the two points, the absolute strain state of the fiber is known for the entire time record, because the diffraction data allow the sarcomere lengths to be recorded directly. The baseline sarcomere length, L_1, is the sarcomere length before the arrival of the longitudinal strain pulse. The peak strain is the maximum sarcomere length resulting from a lengthening pulse, or the minimum sarcomere length, in the case of a shortening pulse, and this value is denoted as L_2. The amplitude of the strain wave is calculated as:

$$A = (L_2 - L_1)/L_1 \qquad \text{Eq. 2.4}$$

The strain pulse amplitude at the first point is denoted A_1, and the strain pulse amplitude at the second point, further from the servo, is denoted A_2. The attenuation coefficient is then calculated as:

$$\alpha = \Delta x^{-1} * \ln (A_1/A_2) \qquad \text{Eq 2.5}$$

Assuming that the attenuation coefficient of the muscle fibers is not equal to zero, the viscoelastic modulus, E*, is complex and is composed of a real elastic component, E_1, and an imaginary viscous component E_2 (Truong, 1974):

$$E^* = E_1 + iE_2 \qquad \text{Eq. 2.6}$$

The real component of the complex modulus can be calculated directly if the density of the fiber, ρ, the cyclic frequency of the pulse, ω, the pulse velocity, v, and the attenuation coefficient, α, are known (Truong, 1974):

$$E_1 = \rho v^2\omega^2(\omega^2 - \alpha^2v^2)/(\omega^2 + \alpha^2v^2)^2 \qquad \text{Eq 2.7}$$

If the attenuation coefficient is found to be equivalent to zero, which would be the case in which there is no viscous damping within the fiber, Equation 2.7 reduces to:

$$E = \rho v^2 \qquad \text{Eq. 2.8}$$

in which the Young's modulus is simply denoted as E, because the modulus no longer has a complex component. Note that in this case, the Young's modulus is no longer dependent upon the cyclic frequency of the strain pulse.

Ideally the fiber would be sampled simultaneously at two points by the laser, with the resulting diffraction spectrum collected for each position simultaneously as well. This was not possible because of the need for much more complicated optics and the duplication of the entire data acquisition system to collect the additional diffraction data. The next best solution is to first sample at the first position, then the second, then return to the first position, and so on, alternating rapidly between the two positions to allow each to be sampled at the specified 500,000 samples per second. An attempt was made to sample by this method, but it was not possible to alternate positions on the fiber quickly enough due to limitations in the bandwidth of existing laser deflectors. The method used in this series of experiments was to sample a number of pulses at the one position on the fiber near the servomotor, then to traverse the laser to the second position and repeat the pulses that were applied when sampling at the first position. The sarcomere length vs. time records for each pulse were cross correlated with the servo position data, and the time lag between the servo pulse application and the arrival of the strain wave at the sampling spot for each individual pulse was determined. Data were then grouped on the basis of servo pulse amplitude and frequency. For a given pulse amplitude and frequency, the time lag and sarcomere length pulse amplitude were calculated. The resulting data from the first and second sampling points for any given pulse amplitude and frequency were then used to determine the pulse velocity and

attenuation coefficient (Equations 2.3, 2.4, and 2.5). The error was estimated by adding the standard error for each data group under each of the pulse conditions in quadrature. This method biases the data, but is necessary until the instrumentation is modified to allow the sarcomere length to be sampled at two points along the fiber simultaneously.

Aim #3: System Evaluation

The system was tested by measurement of the pulse propagation velocity and attenuation coefficient obtained on twenty-four single permeabilized fibers from the soleus muscles of adult and aged F344 rats. The fibers were tested in the fully relaxed state and in a condition of submaximal activation. A total of 14,592 pulses were generated and a total of 14,942,208 diffraction spectra were collected. Along with each diffraction spectrum, the servo motor position was recorded.

Experimental Procedure

A single muscle fiber which had been permeabilized by incubation in glycerin-bearing skinning solution was placed in the experimental apparatus (Figure 2.2). The experimental sequence of pulses was downloaded into the digital waveform synthesizer memory and the desired settings were selected for the clock speed and number of experiments to be performed. A small target was placed under the fiber and the laser was turned on and focused to a 250 μm diameter spot near the center of the fiber. The resulting diffraction pattern was projected directly onto the target and the sarcomere length of the muscle fiber was adjusted by lengthening or shortening the fiber by means of the hydraulic micromanipulators until the desired sarcomere length was achieved, typically 2.55 μm.

The digital waveform memory was downloaded from the computer. Experiments were initiated remotely by the computer keyboard. A battery of 128 separate lengthening and shortening pulses were executed on the fiber with a delay of 2 to 3 seconds between pulses to allow the fiber striation pattern to stabilize. Each fiber was periodically checked to assure that the diffraction pattern was still present. After the fiber had been subjected to 64 pulses, the position of the laser spot on the fiber was adjusted using the X-Y table traverse (Fig 2.1) to a new position, 1 to 3 mm from the original position, and the final 64 pulses were applied. When a total of 128 pulses had been executed, the diode array controller memory was downloaded and temporarily stored to hard disk on the computer. The digital waveform synthesizer memory was reprogrammed at this time, and a different series of pulse amplitudes was performed on the fiber. The downloading and reprogramming process required approximately 8 minutes, during which the fiber was maintained in relaxing solution at 15°C and the servo coil was de-energized to prevent transient pulses from occurring during reprogramming (Appendix C). In this manner, data were collected for the sarcomere length at two points along the fiber for a large number of lengthening and shortening pulses under conditions of varying activation level, initial sarcomere length, pulse amplitude and frequency, etc. At the end of each experiment, the fiber was fully activated and the maximum force was recorded on the data sheet to determine if the fiber had been damaged during the experimental sequence.

The raw data were converted from the diffraction spectrum values into sarcomere length as a function of time (Eq 2.1 and 2.2) before any further analysis. The servo position data were also transformed using the servo calibration data, and the transformed data set was stored as comma-delimited ASCII values of sarcomere length and servo position for each time point. The total data set of 131,072 time points was then divided into separate files for each of the pulses (typically 128 files, each containing

1024 time points). This allowed easy access to the data in the form of sarcomere length vs. time for each fiber for any given pulse to which it was subjected. Each series of 128 pulses generated a raw data file in comma-delimited ASCII format of approximately 11 MB. Up to 10 such series of experiments were executed on each fiber, so it was impossible to view each spectrum individually. A set of criteria were defined which were implemented in software to discriminate between acceptable spectral data and meaningless noise. The array controller was designed specifically to return an identifiable value when the diffraction signal was too weak to be detected or flagged as out-of-range. In addition, several signal quality criteria were defined, such as minimum acceptable signal intensity and detector saturation, to be implemented in software to distinguish between acceptable and unacceptable diffraction spectra (Appendix J). All diffraction patterns were automatically processed in this way, and typically over 98% of all diffraction spectra for each fiber were found to be acceptable. Unacceptable spectra were flagged in the data set to indicate that they were not to be considered for further analysis.

A very small amount of 'salt-and-pepper' type noise (occasional, single time point spikes) was evident in some of the sarcomere length vs. time traces, so a median filter of rank 1 was applied uniformly to each data set, which totally eliminated this noise (Appendix J). No further filtering was necessary, although additional filters were made available in the software. The raw diffraction spectrum data and the converted data were then stored to rewritable optical disk.

To determine the pulse propagation velocity, the data files for each of the fibers under a given set of experimental conditions were grouped according to pulse amplitude and frequency. Typically, for each fiber 6 or 7 pulse data files were grouped for any given pulse amplitude and frequency. Sometimes fewer files were included, as in cases where some of the files contained large amounts of missing data due to loss of signal. These files were excluded from further analysis. The sarcomere length vs. time

data were cross correlated with the servo position vs. time data for each of the pulses in the group, and the time delay between pulse application by the servo and pulse arrival at the sampled position on the fiber was calculated as the lag, determined by cross correlation, multiplied by the sampling interval. This method of determining the pulse propagation delay to each point was deemed very acceptable because the maximum cross correlation values for each pulse were always very well defined and had r^2 values typically greater than 0.99. The average delay was calculated for each group of files, which represented the pulse delay for a pulse of fixed amplitude and frequency to one of the two points along the fiber. The process was repeated for the group of pulses at the second point along the fiber for the same pulse conditions, and the time difference of the delays (t) was taken to be the time delay between the pulse arrival at the two points along the fiber separated by a known distance of x. The error of the delay was estimated by adding in quadrature the standard errors of the time delays to each of the two points.

To determine the attenuation coefficient, the baseline sarcomere length prior to the pulse was determined by averaging the sarcomere lengths over a short time interval immediately prior to the pulse. The peak amplitude of the sarcomere length pulse was then determined for each pulse in each group. The peak minus the baseline sarcomere length was taken to be the absolute strain amplitude of each pulse. The absolute strain values were averaged for each of the groups and recorded. The attenuation coefficient was calculated as defined in Eq. 2.5 (Truong, 1974). Data were tabulated as necessary for each hypothesis test, and files were generated from the converted data in a compressed format that was usable by graphics programs (Appendix J).

Analysis of Results

A total of nearly 15 million diffraction spectra were collected, each with a corresponding servo position value. Due to the large amount of data collected, only a small fraction of the spectra could be inspected visually. Therefore a number of automated tests were run to determine the quality of the diffraction data. Only the first 32 phototransistors in the array of 64 were used because they provided adequate resolution for system evaluation. The benefit of using only the first 32 elements in the array is that the total volume of collected data and the total analysis time for each fiber is reduced by 50%. This is significant, because the resulting data handling and analysis time savings is about ten hours for each of the 24 fibers. In addition, the raw data storage space was reduced by 50%, which is significant because this amounts to over 2 GB.

Signal Strength:

A representative sample of the raw spectral data are shown in Figure 2.5., in which a series of eight spectra are shown (lettered **a** through **h**). The spectra are from fiber # 19. The fiber was fully relaxed and maintained at 15°C. The sarcomere length of the fiber had been previously set to 2.57 microns. A He-Ne laser was focused to a 250 micron diameter spot on the fiber near the end attached to the servo. These spectra were collected near the beginning of the set of 1024 time points, just prior to the application of a lengthening pulse of 0.5% L_f. The sampling interval was 4 micro seconds.

The spectra shown in Figure 2.5 are representative of the actual spectra collected by the system. Each phototransistor in the array produced a voltage across a load resistor which was converted to the 8-bit digital values in the range of 0 to 255. For the remainder of this discussion, the term signal strength will refer to the digital value of the voltage for each of the phototransistors in the array. The actual voltage is calculated as:

$$V = 0.007813\ S \qquad \text{Eq. 2.9}$$

in which V is the voltage, and S is the digital signal strength, which is an integer value in the range 0 to 255, inclusive. In Figure 2.5, the vertical axis represents the digital signal strength, in integer values from 0 to 255, of the diffraction pattern on each of the 32 phototransistors in the array. In this figure, the peak signal strength ranges from 36 to 38. A short program was written in VisualBASIC (SPECANAL.BAS) to characterize all of the raw diffraction spectra. The maximum signal strength from any of the spectra was 147, and the minimum signal strength was zero (no signal detected). All spectra with a peak signal strength below 10 were excluded from further analysis. More than 98% of the spectra had signal strengths equal to or greater than 10. The average peak signal strength was around 40. For any given fiber, the peak signal strength generally dropped as the level of activation of the fiber was increased for all of the spectra in any series of experiments. As the level of activation was reduced, the peak signal strength increased. The strongest signals were collected from fibers under the fully relaxed condition. It was not possible to detect diffraction peaks for fibers activated more than 30%, due to the reduction in the intensity of the first-order diffraction peak with activation of the fiber.

Background Noise:

A close examination of Figure 2.5 reveals that the eight diffraction spectra are all very nearly identical. In seven of the eight plates no baseline noise is evident, but plate **h** shows evidence of baseline noise. This problem was detected very early in this series of experiments, and several steps were taken to determine the source of the baseline noise. First, when the sampling rate was set to 250 kS/sec, the noise was only present in about one of every 6 or 7 spectra. Increases or decreases in the sampling rate resulted in a commensurate increase or decrease in the frequency of occurrence of the noise. Initially the

source of the noise appeared to be ground bounce resulting from current transients due to digital switching or ADC and DAC conversions. This seemed reasonable, as these operations were occurring in parallel for all 32 analog input channels, as well as for the digital waveform synthesizer channels. A 0.1 μF ceramic capacitor was added across the power supply pins of each integrated circuit chip with the shortest lead length possible, and larger tantalum capacitors were added to the power traces on each board. Voltage references were powered locally by linear regulators, and liberally decoupled with capacitors both on their inputs and outputs. Ground loops had been minimized in the initial design of the system. The baseline noise problem was not mitigated by these efforts.

Some form of high-frequency optical noise was a possibility, so the phototransistor array was tightly covered with a sheet of 1/16" aluminum plate. The baseline noise problem persisted. The system was then operated with all other devices, such as the servo motor and He-Ne laser unplugged. The He-Ne laser was identified as the cause of some of the occasional noise spikes. In addition, the He-Ne was suspect because it was producing a slightly barbell-shaped spot, indicating that it was no longer operating in TEM_{00} mode (a single round spot), but was in fact operating in TEM_{01} or TEM_{10} mode (Hecht, 1990, page 583), suggesting that the laser had been damaged. The original He-Ne laser was replaced with a new 10 mW He-Ne (632.8 nm wavelength), which was found to be operating in TEM_{00} mode. The occasional baseline noise problem was improved, except that a periodic baseline noise persisted, every 24 to 28 microseconds, with a peak digital value of 1 or 2. No amount of shielding reduced the problem, and the noise was eventually attributed to the fact that switching power supplies were used to power the array controller system. This hypothesis was further supported by the fact that the noise occurred at a frequency of 36 kHz to 42 kHz, and the switching power supply in the system switches at approximately 40 kHz. A linear power supply with sufficient current output was not available to test this

hypothesis, and due to the current draw of the system, the use of batteries was out of the question. The solution, which will be implemented in the next design, will be to use CMOS logic instead of LS (low-power Schottky), to reduce the power supply requirements sufficiently to allow battery or linear power supply operation.

The baseline noise was measured by the program SPECANAL.BAS for all diffraction spectra, and in no case did it exceed a digital signal strength of 2. This was considered acceptable, because a minimum peak amplitude of 10 was required for further analysis of each spectrum, and the calculation of the centroid by inclusion of only values within three positions of the peak (Eq. 2.1) effectively excluded most of the baseline noise from analysis. Nonetheless, the centroid was occasionally influenced by this source of noise, but was eliminated by the application of a median filter of rank 1 to the data, after calculation of sarcomere length for all spectra in each of the data sets.

System resolution:

Due to the 8-bit intensity resolution of the detector array, the nature of the grating equation and the system geometry, the resolution (minimal detectable change in sarcomere length) will be a function of signal strength and diffraction angle. The system resolution was calculated as follows. A diffraction peak digital signal strength of intensity S is assumed. The full width half maximum value of the peak is approximately one phototransistor width, as suggested by the raw diffraction spectra in Figure 2.5, which are very typical. By definition, then, the signal strength on either side of the peak value is S/2. A conservative estimate of the sensitivity of the system to a shift in the centroid of the first order peak is to assume that one of the half maxima signals increases by a value of 1, whereas the other half maxima

decreases by a value of 1. The shift of the centroid of the first order peak on the detector array can be readily shown to be:

$$d = 2W/S \qquad \text{Eq. 2.10}$$

in which d is the centroid shift in mm, W is the width of the phototransistors in mm, and S is the digital value of the peak signal strength, in this case from 10 (the acceptable minimum) to 255. The result is that stronger signals and shorter sarcomere lengths result in increased resolution, whereas lower signal strengths and longer sarcomere lengths result in lower resolution, as shown in Figure 2.6.

The range of resolvable sarcomere lengths is defined by the length of the phototransistor array, the wavelength of the laser light used, the optical path length from the muscle fiber to the array, and the placement of the zero order peak with respect to the sensitive area of the array. A program was written in Visual BASIC to include all of these parameters (TILTNORM.BAS), and display the resulting system resolution and range. The geometry of the experimental setup was adjusted to accommodate sarcomere lengths of 1.794 to 4.270 µm.

The system was calibrated photographically by using an optical standard (Bausch and Lomb) to measure the average sarcomere length of a region of sarcomeres. The laser was turned on and focused on this region, and the resulting diffraction spectrum was collected. The fiber was lengthened slightly, the sarcomere length was again measured photographically, and the process was repeated for the full range of sarcomere lengths detectable by the system. The absolute error of the system is estimated to be less than 1% over the full range of sarcomere lengths, with larger errors for longer sarcomere lengths and smaller errors for shorter sarcomere lengths.

Sarcomere length vs. time records:

Typical converted data files are shown in Figure 2.7. These are the data files which are used for the calculation of attenuation coefficient and pulse propagation velocity (Eq 2.3, 2.4, and 2.5). Each data file contains 1024 time points for which both the sarcomere length and servo position are recorded. The top trace is the servo position record for a typical lengthening pulse, with the corresponding sarcomere length trace shown directly below. The bottom two traces are the servo position and sarcomere length traces for a typical shortening contraction. There were over 7000 lengthening and 7000 shortening pulses, of varying amplitude and frequency, applied to the 24 fibers in the study, and these traces are typical. In many cases the step and pause pattern of the lengthening pulses was more pronounced, and shortening pulses often had plateaus at their minimum length, probably due to fiber buckling.

Detection of Multiple Peaks:

A careful analysis of the spectra was required to detect the presence of multiple peaks. The presence of multiple peaks or subpeaks would indicate the existence of discrete sarcomere length distributions within the sampled region of the single fiber (Judy, et al., 1982). The presence of discrete regions within the sampled area of the fiber with different sarcomere lengths would require a much more detailed analysis than the case in which only one peak was detected. The VisualBASIC program SPECANAL.BAS also included an algorithm for the detection of multiple peaks. The dominant peak was defined as the maximum signal on the 32-element array for each time point, provided that the signal strength was at least 10. A second peak was defined as a local maximum digital value of 10 or greater, separated from the dominant peak by at least one phototransistor position. Due to the finite width of the phototransistors (2.21 mm), some spatial averaging occurred, making it impossible to detect multiple diffraction peaks that were very close together. To be distinguishable from the dominant peak, the

second peak must be separated from the dominant peak by a signal of lower strength than either of the peaks, resulting in a saddle shape (a local minima). It was necessary to select a threshold value for the local minima. Because of the presence of background noise (Figure 2.5, plate **h**), a threshold value of 2 was selected. Therefore, the presence of multiple peaks would be indicated if two local peaks could be detected in any given spectrum, each with a signal strength of at least 10, and if they were separated by a local minima with a signal strength at least 2 points lower than the lowest peak. This algorithm was implemented in SPECANAL.BAS, and it was determined that none of the nearly 15 million diffraction spectra had multiple peaks. This greatly simplified the subsequent analysis, by supporting the assumption that the location of the centroid of the diffraction peak could be used to calculate a representative average sarcomere length for the region sampled by the laser spot.

Although no cases of multiple peaks were detected in the total data set of all spectra, there is still the possibility that multiple peaks exist because of the finite width of the phototransistors, resulting in some amount of spatial averaging. For example, it is possible that two distinct peaks exist, but that they are both projected onto the same phototransistor because they are very closely spaced. It is impossible to speculate about the probability of multiple peaks which can not be detected by the system because of how closely they are spaced, but it is possible to determine the sensitivity of the system in terms of how far apart the peaks must be to be clearly detected. Based on worst-case assumptions, that is, minimum signal strength and minimum detectable peak separation of two phototransistor widths, the masking limit for secondary and lesser peaks is illustrated in Figure 2.8. In this figure, the primary first order diffraction peak value is located on the abscissa. The range of secondary or lesser first order diffraction peaks that will be masked by the primary peak is defined by the range of sarcomere lengths on the ordinate that fall between the two lines on the graph. For example, if a primary first order diffraction

peak is detected with a calculated sarcomere length of 2.6 μm, secondary or lesser peaks with sarcomere lengths in the range of 2.45 to 2.75 μm would not be detected.

Discussion and Conclusions

The performance of the present system supports the feasibility of measuring the longitudinal pulse propagation in single skeletal muscle fibers by means of optical diffraction at two points along the length of the fiber. The results also allow the system specification and design to be modified to enhance system performance. To measure the pulse propagation in fully activated fibers the system will require design modifications. The diffraction pattern intensity from activated muscle fibers dropped dramatically below the signal intensity for the same fiber when fully relaxed. The consequence of this is that an 8-bit conversion of diffraction intensity is not adequate to resolve the first order diffraction signal from both fully relaxed and fully activated fibers. A phototransistor array with 14-bit intensity resolution can detect the diffraction pattern from both fully activated and relaxed fibers, but the system is five orders of magnitude too slow to collect the data necessary for pulse propagation measurements. At the time the present system was designed (1992 to 1993), no analog to digital converters above 8 bits resolution and conversion rates of 1 MS/sec were available. A parallel architecture system requires one converter per channel, and there are a minimum of 32 channels in the present system, therefore the price of the analog to digital converters is also an important design consideration. Recent developments in pipeline architecture for hybrid converter integrated circuits has resulted in low-cost, 12-bit analog to digital converters with conversion times of about 1 μs, with higher resolution converters (14 and 16 bit) also reduced in cost. Therefore, to measure pulse propagation in fibers at full activation, the system will be redesigned to employ higher resolution analog to digital converters.

Multiple first order diffraction peaks were not detected in the diffraction data. Therefore the system architecture may be modified to use a Schottky barrier diode linear position detector (spot follower) instead of a detector array. This reduces the complexity of the system and the total amount of data that would need to be collected by about 97%. The reduction of system complexity has several very significant advantages. First, the total power consumption will be reduced, thereby allowing linear power supplies or batteries to be used, reducing the noise. The use of one converter per sampled region instead of 32 greatly reduces the total cost of the system. The possibility of adding another laser to the system to sample at two positions simultaneously then becomes feasible. The lasers would need to strike the fiber along skew diameters, increasing the complexity of the optics somewhat, but the resulting decrease in total system cost, total volume of data, and software analysis routines makes this an attractive option. In addition, the pulses can be cross correlated with each other to determine the propagation velocity, instead of cross correlating each pulse at two different positions with the servo motor position signal and taking the lag time difference, as was required in the present study. This would improve the accuracy of the propagation delay measurements by reducing bias.

CHAPTER III
VISCOELASTICITY

Introduction

The explicit study of muscle tissue as a viscoelastic material can be traced to Levin and Wyman (1927). Since that time, the viscoelasticity of muscle tissue has been studied with little attention given to the effect of activation levels other than full activation and full relaxation (Sandow, 1947; Schoenberg, 1974). All of the studies of muscle tissue viscoelasticity that have used pulse propagation have arbitrarily selected a single pulse amplitude (Appendix A). Furthermore, some of the methods employed in the study of muscle viscoelasticity were flawed due to unrealistic assumptions. For example, studies of muscle viscoelasticity by the application of a step-length change have assumed that the sarcomere length is uniform and homogeneous during rapid length changes. To avoid the pitfalls of unrealistic assumptions about the strain behavior of muscle tissue, the method of strain pulse propagation will be used to measure the viscoelasticity of muscle fibers.

No studies have addressed the effect of strain pulse amplitude on the resulting viscoelastic behavior of muscle tissue. A consideration of the effect of strain pulse amplitude on the viscoelasticity of muscle fibers is important for several reasons. First, in activated muscle, cross-bridges contribute significantly to muscle fiber stiffness (Ford et al., 1977; Ford et al., 1981). Cross-bridges cycle continuously, on the order of 150 times per second, and therefore remain attached for only a short period

of time (Piazzesi, Linari and Lombardi, 1993). Also, cross-bridges have a limited range over which they may remain attached (Higuchi and Goldman, 1991), which is much shorter than the range over which muscle changes its length. The logical conclusion, in the case of activated muscle, is that the viscoelastic properties of activated muscle that arise from cross-bridges are limited to strain amplitudes less than or equal to the maximum cross-bridge strain, which is approximately 10 to 20 nm per sarcomere. Second, for relaxed fibers the relative contribution of weakly-bound cross-bridges to muscle viscoelasticity has not been established definitively, but has been estimated to be as high as 80% (Chalovich et al., 1991). Furthermore, the strain range over which weakly-bound cross-bridges may remain attached is not known. Consequently, whether weakly-bound cross-bridges remain attached over the same strain range as strongly-bound cross-bridges remains unclear. At low levels of activation, some of the weakly-bound cross-bridges are transformed into strongly-bound cross-bridges. Therefore, activation not only changes the viscoelasticity of muscle fibers, but also likely changes the range over which the viscoelasticity is dominated by thick and thin filament interactions. In addition, the stiffness of passive elastic filaments such as titin in the sarcomere increases non-linearly, but smoothly, with increasing sarcomere length (Granzier and Wang, 1993b). For these reasons, the effect of strain pulse amplitude on the viscoelastic properties of relaxed muscle fibers must be studied.

The problem of the effect of strain pulse amplitude on muscle viscoelasticity has generally been avoided by muscle mechanists. Usually, when muscle tissue stiffness or viscoelasticity is measured a single strain amplitude is selected which is based on previous work, with little or no justification (Chichi, et al., 1991; Granzier and Wang, 1993a and 1993b; Ford et al., 1981; Truong 1971, 1972 and 1974; Truong et al, 1978b; Blange and Steinen, 1985). The tacit assumption is that the viscoelasticity of muscle can be considered as quasi-linear over a small range of strain amplitude. Though this may be

true, the effect of strain amplitude on muscle stiffness and viscoelasticity has not been reported. Therefore, the range of strains over which the viscoelasticity of muscle may be assumed to behave quasi-linearly is not known with any degree of certainty.

The primary goal of this study was to determine the effect of strain pulse amplitude on the viscoelasticity of relaxed and partially activated single permeabilized muscle fibers. The working hypothesis is that *the viscoelasticity of single permeabilized muscle fibers is dependent upon the strain pulse amplitude, strain pulse frequency, and the level of activation of the fiber.* Therefore, the method which will be employed to measure the viscoelasticity must not be based on the assumption that viscoelasticity is independent of strain pulse amplitude. Much of the published literature on muscle fiber viscoelasticity includes the tacit assumption that the strain pulse amplitude does not affect the viscoelasticity of muscle fibers. The pulse propagation method of determining viscoelasticity does not assume that the strain along the length of the fiber is homogeneous, nor that the viscoelasticity is independent of strain pulse amplitude. The viscoelasticity of single permeabilized skeletal muscle fibers will be calculated from the propagation velocity and attenuation coefficient of longitudinal strain pulses, which will be measured by means of optical diffraction at two positions along the single fiber. The method of optical diffraction at two positions along the muscle fiber was selected as the optimal method for measuring the strain at each position because it allows rapid and accurate detection of strain in a small region along the fiber length but does not require that markers be placed on the fiber.

Methods

This series of experiments were performed on single chemically permeabilized skeletal muscle fibers harvested from the soleus muscle of the rat. A total of 19 fibers were subjected to lengthening and

shortening pulses. Adult (4 to 12 month old) and aged (27 month old) male Fisher rats (F344) were dissected to remove the soleus muscle of the right hind leg by carefully dissecting away the fascia and transecting the proximal and distal soleus tendons. The rats were anesthetized with pentobarbital sodium. The tissue was harvested from the animals after *in vivo* sustained power experiments on the left leg, which were unrelated to this series of experiments, to maximally utilize the tissue from each animal. Because of multiple use of each animal by several investigators, no animals were sacrificed specifically for this series of experiments.

The soleus muscle was placed in mammalian Ringer's solution (137 mM NaCl, 24 mM NaHCO, 11 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, and 0.025 mM tubocurarine chloride) immediately after removal from the animal. The muscle was pinned at each end to hold it slightly longer than its free length (under no tension) and all external fat and loose connective tissue was carefully removed. Using Vannas spring scissors, the muscle was cut longitudinally, parallel to the muscle fibers, into six or seven thin bundles, depending upon muscle size. Each bundle was approximately 12 to 15 mm long and approximately 1 mm in diameter. The bundles were tied to short pieces of glass capillary tube using 5-0 braided silk suture with a double overhand knot at each end. Each bundle length was adjusted by sliding the knots along the glass capillary tube so that the bundle was straight and very slightly extended beyond the free length.

The tied bundles were placed into 5 cc plastic vials filled with skinning solution (125 mM K-propionate, 5 mM EGTA, 2 mM ATP, 2 mM $MgCl_{20}$, 20 mM imidazole, and glycerol 50% vol/vol, adjusted to pH 7.1 with 4 M KOH), were capped securely, and were placed into a freezer at -20°C. The bundles were allowed to remain in the skinning solution at -20°C for a minimum of four days, and within four months to assure viability.

In addition, relaxing and maximum activating solutions were required for the execution of the experiments. Both the relaxing and maximum activating solution contain 1 mM free Mg^{++}, 4.4 mM ATP, 7.00 mM ethyleneglycol-bis(B-aminoethyl ether) tetra-acetic acid (EGTA), 20.0 mM imidazole, and 14.5 mM creatine phosphate. Both solutions were adjusted to pH 7.0 with KOH. Maximum activating solution was adjusted to pCa of 4.5, and relaxing solution was adjusted to pCa of 9.0. The final concentrations of each metal, ligand, and metal-ligand complex were calculated based on published computer algorithms (Fabiato and Fabiato, 1979) and stability constants (Godt and Lindley, 1982) for a solution temperature of 15° C.

Immediately prior to an experiment, the tied fiber bundles were removed from the skinning solution and placed into a small dissecting dish filled with chilled relaxing solution. The bundles were allowed to thaw for ten minutes to avoid damaging the tissue. The 5-0 silk suture knots were removed and the fiber bundle was released from the glass capillary tube. The bundle was pinned to the bottom of a small dissecting dish using 0.10 mm diameter insect pins (Ianni Butterfly Enterprises). The ends of the bundle, which were compressed by the 5-0 suture, were removed by cutting with Vannas spring scissors. Sharpened #5 forceps were used to slowly draw out a small group of fibers from the bundle, which typically contained four to ten fibers. By drawing out a group rather than a single fiber, the shear load on each fiber in the group was significantly reduced. Several of the fibers in each group showed evidence of excessive strain; they tended to curl into a spiral immediately after withdrawal, often were kinked, and typically had an irregular, pale peach color at a magnification of 6.0 X when viewed under a dark field microscope. The fibers that were near the center of the group remained nearly straight after withdrawal, showed no evidence of damage or kinking, and had a regular iridescent blue-green appearance when viewed at 6.0 X magnification with a dark field microscope.

The blue-green iridescence was caused by the regular sarcomere spacing. The fibers act as a diffraction grating for visible light, and the blue-green color was an important indication that the fiber was undamaged and the sarcomere pattern was highly regular. The undamaged fibers were shielded mechanically as the group of fibers was withdrawn by the fibers that were on the outside of the group, the latter being subjected to higher shear gradients during withdrawal and thus sustaining damage. After withdrawal, the damaged fibers were simply pulled away using sharpened forceps, leaving one or two undamaged fibers which may be up to 9 mm in length. This technique allowed very long skinned fibers to be removed without damage and was developed specifically for use in this series of experiments because fibers up to 8 mm in length were necessary for the pulse propagation experiments.

The flow through bath (Appendix E) was filled with relaxing solution until slightly over full, and was chilled to 15°C prior to the removal of the single fiber from the fiber bundle. The fiber was connected at one end to a force transducer and at the other end to a linear servo motor by means of 29 ga. stainless steel thin-wall tubing. The stainless steel tubing has an inner diameter of 0.007", and was filed down using a fine ceramic sharpening stone so that both tubes that protrude into the flow-through bath had a spoon-like trough into which the single fiber ends were placed (Figure 2.2). The troughs were wetted with relaxing solution and were then raised slightly above the surface of the relaxing solution in the bath so that a meniscus formed between each trough and the bath. Single nylon strands of waxed dental floss, approximately 21 microns in diameter, were tied into loose double overhand knots and were placed over the ends of each of the stainless steel tubes past the troughs before the single fiber was transferred into the bath.

The single fiber was transferred to the flow-through bath and was held at one end by sharpened forceps. The small dissecting dish containing the remaining bundle was placed in a styrofoam box

containing ice for short-term storage. Due to the time required to complete a battery of experiments on each fiber, up to about 2.5 hours, only two or three fibers were used from each bundle. Approximately 1 mm of one end of the fiber was lifted out of the solution and laid carefully into the one of the troughs. The surface tension of the relaxing solution pulled the fiber flat into the trough and held it in position while it was secured in place. A very short piece (plug) of 5-0 nylon monofilament suture (0.6 to 0.7 mm long) was placed in the trough directly over the end of the single fiber. The trough was then raised slightly to increase the downward force on the monofilament plug from the surface tension of the relaxing solution. This held the muscle fiber and nylon plug in place while one of the waxed nylon strand knots was pulled over the plug and muscle fiber and pulled tight. Once secured, the trough was lowered slightly to reduce the surface tension force and the second nylon strand knot was pulled over the plug and was pulled tight. The use of two knots on each plug eliminated the tendency of the muscle fiber to pull out from beneath the plug during an experiment. The tying procedure was repeated on the other end of the single fiber, the result being that the single fiber was fastened at each end to a stainless-steel trough and was suspended in a bath of relaxing solution. The loose ends of the waxed nylon filaments were snipped off with microsurgical scissors and were removed from the bath to prevent force shunting between the fiber, the troughs, and the sides of the flow-through bath.

Both stainless steel tubes, one of which was attached to the linear servo motor and the other of which was attached to the optical force transducer, were lowered using the hydraulic micromanipulators so that they were centered in the slots at each end of the flow-through bath and the single fiber was suspended in the relaxing solution. The single fiber was centered in the flow-through bath and was pulled until it is just straightened out (Figure 2.2). A small amount of relaxing solution was added to the bath to form a slight positive meniscus. An 18 mm square cover slip was placed over the top of the bath

and was pulled into place by the surface tension of the relaxing solution. The entire process, from placing the fiber into the flow-through bath until the placement of the cover slip on top of the bath required less than ten minutes, and frequently as little as five minutes. This procedure was developed to minimize the handling of the fiber to reduce the risk of damage during mounting.

The laser prism holder was slipped into position and the X-Y table of the apparatus was adjusted so that the He-Ne laser beam was focused to a 250 μm diameter spot near the center of the fiber (Figure 2.3). A small calibration target mounted on an aluminum beam was slid into place 75 mm directly beneath the fiber, and the diffraction pattern was projected onto the target. The sarcomere length was read directly from the position of the first order diffraction peak on the target, and the position of the left trough (connected to the servo motor) was adjusted until the sarcomere length had been set to 2.55 microns. The X-Y table was then translated right and left to inspect the diffraction pattern along the entire length of the fiber. If the pattern was clearly visible and stable along the length of the fiber, the fiber was accepted for further use. If the diffraction pattern proved to be weak or irregular, it was assumed that the fiber was damaged during dissection or placement in the apparatus and was removed and replaced by a new fiber. The process was repeated until a suitable fiber was identified. Approximately 20% of the fibers were rejected at this point in the experiment. The practical limit for the distance between the laser spot positions on the fiber was about 2 mm, because this was the maximum distance over which the typical fiber had a striation pattern which was highly consistent and stable when inspected by axially translating the fiber through the laser beam.

The relaxing solution in the flow-through bath was slowly replaced (over about 30 seconds) by activating solution, and the force generated by the fiber during this process was displayed by the optical force transducer system in μN (Appendix D). During this process, an isovolumetric fluid exchanger

(Appendix E) was used to assure that equal volumes of fluid were simultaneously injected and removed. A flow-through bath was used rather than a multiple bath system because the force imposed on the fiber when drawing it through a fluid surface is substantially more than the single fiber can generate when fully activated. Isovolumetric fluid exchange in the bath therefore reduces the potential for damage to the fiber. The force generated by the fully activated fiber was allowed to stabilize after the fluid exchange was complete, and the peak force was recorded. Using the fluid exchanger, the activating solution in the flow-through bath was replaced by relaxing solution.

The fiber length, L_f, was measured between the nylon monofilament plugs and was determined by traversing the X-axis of the X-Y table along the entire length of the fiber. The total distance traversed was measured using a digital depth gauge (Figure 2.1) which was clamped to the surface of the vibration-isolation table, allowing fiber length to be measured accurately to within 0.01 mm. The X-Y table traverse indicator was set to zero at the force transducer end, and all further location measurements were referenced to this position. To measure the diameter, the fiber was viewed by a Leica WILD M3Z Kombistereo microscope at 40X magnification with a 25X/0.40 high-power objective and a finely divided eyepiece graticule. The resolution of the 100-division eyepiece graticule at 40X magnification was determined, using a Bausch and Lomb engraved optical standard, to be 1.306 microns per division. The fiber length was recorded, and the diameter was measured at six equally spaced positions along the fiber, to be used later for the calculation of fiber cross-sectional area.

The experimental sequence of pulses was downloaded into the digital waveform synthesizer memory and the desired settings were selected for the clock speed and number of experiments to be performed (Chapter II). For this series of experiments, the digital waveform synthesizers were loaded with profiles for lengthening and shortening pulses. Pulse amplitudes of 20%, 40%, 60%, 80%, and

100% of full range for both shortening and lengthening pulses were used. A total of 128 pulses were programmed for each experimental battery. The lengthening and shortening pulses of different amplitudes were alternately mixed together throughout the battery to eliminate any systematic effect of the ordering of the pulse amplitudes on the outcome of the experiment. Once the digital waveform memory had been loaded, the entire experiment was controlled and all data are collected by the array controller. Each pulse was initiated remotely by the computer keyboard. A two to three second delay between the pulses allowed the fiber striation pattern to stabilize. Each fiber was periodically checked to assure that the diffraction pattern was still present.

The first 64 pulses were applied to the fiber with the laser at the first sampling position along the muscle fiber. The fiber was then translated axially by 1 to 2 mm using the X-Y table, and the new laser spot position on the fiber was recorded to the nearest 0.01 mm. The final 64 pulses were applied to the fiber with the laser spot at the second position. When a total of 128 pulses had been executed, the array controller memory was downloaded and temporarily stored to hard disk on the microcomputer. The digital waveform synthesizer memory was reprogrammed at this time. The downloading and reprogramming process required approximately 8 minutes, during which the fiber was maintained in relaxing solution at 15°C and the servo coil was de-energized to prevent transient pulses from occurring during reprogramming (Appendix C). Up to 10 batteries of 128 pulses were applied to each of the fibers. In this manner, data were collected for the sarcomere length at two points along the fiber for a large number of lengthening and shortening pulses under conditions of varying activation level, initial sarcomere length, pulse amplitude and pulse frequency. At the end of each experiment the fiber was fully activated and the force was recorded on the data sheet. The analysis and storage of the data is discussed in detail in Chapter II. The raw data was processed after all experiments were completed to

yield a data set for each pulse, which includes servo position and sarcomere length as a function of time. There are 1024 time points of data for each pulse.

The cross-sectional area was determined by two methods, one assuming a circular cross-section and the other assuming an elliptical cross-section. For the assumption of circular cross-section, the average of the six diameters was used and the fiber cross-section was calculated as $A = \pi D^2/4$ (left side of Fig 3.1). For the elliptical assumption the cross-sectional area was calculated as $A = \pi Dd/4$, where D is the largest of the six diameters, assumed to be the major axis of the ellipse, and d is the smallest of the six diameters, assumed to be the minor axis of the ellipse (right side of Fig 3.1). *Specific force* is defined as the force generated by the muscle fiber divided by the cross-sectional area. The maximum specific force (P_O) of each fiber was measured to ensure that the fiber was viable. Several of the fibers broke during activation and were excluded from further analysis.

The pulse velocity was calculated by cross-correlating the servo position pulse with the resulting sarcomere length pulses at two separate points along the length of the fiber. The difference in the lag between the two cross correlations was multiplied by the sampling interval to determine the time delay between the pulse arrival at the two points.

Results

A total of 19 fibers, 5 from aged rats and 11 from adult rats, remained viable throughout the duration of the experiment without breaking. The muscle fibers ranged in length from 3.06 mm to 7.35 mm (for sarcomere length set to 2.55 microns/sarcomere). No significant differences were observed between the P_Os of the two age groups or between the two methods of measuring the cross-sectional areas (Figure 3.1). The data are consistent with published data (Macpherson, 1995).

As the level of activation was increased, the intensity of the first order diffraction peak decreased markedly. This phenomenon is probably due to an increase in the heterogeneity of the sarcomere lengths within the fiber as the fiber is activated (Macpherson, 1995). For this reason, the 8-bit detector system was unable to collect data on fibers above 35% activation.

During shortening pulses, the measurement of pulse propagation velocity and attenuation coefficient was not possible because the fibers showed evidence of buckling (Fig 3.2). Buckling was evident in both relaxed fibers and fibers at low levels of activation. Further evidence of buckling during shortening pulses was clear from the sarcomere length-time records (Fig. 3.3), nearly all of which had a plateau after a small amount of shortening, with no further reduction of sarcomere length during the shortening pulse. Because of the strong evidence for buckling of the fibers when shortening pulses were applied, shortening pulses cannot be considered further. As would be expected, no evidence of buckling occurred during lengthening pulses.

The two sarcomere length-time traces during a lengthening pulse represent the strain pulse as it passed through each of the two sampling positions along the fiber (Figure 3.4). The pulses occurred at slightly different times due to the distance between the positions at which the sarcomere length was sampled. Position 2 is located 1.34 mm further from the servo motor than position 1, therefore the strain pulse arrived at position 1 first. The strain pulse amplitude at position 2 is larger than that at position 1, indicating that the amplitude increased as the strain pulse traveled along the fiber. The increase in amplitude results in a slightly negative attenuation coefficient.

Steps and pauses were evident in most of the sarcomere length-time records. The sarcomere length-time records (Figure 3.4) had the least evidence of steps and pauses in the entire data set, and yet the distortion of the peak amplitude of the strain pulse is evident, especially for position 2. Slight

changes of the laser spot position, as small as 0.04 mm, visibly changed the appearance of the steps and pauses. To test the sensitivity of the pulse velocity and attenuation coefficient to changes in the steps and pauses caused by repositioning the laser spot along the fiber, several fibers were subjected to additional pulses in which the distance between the laser spots was changed slightly, and the full battery of pulses was repeated (fibers 11, 19, and 21). In each case, pulses of the same amplitude did not show a change in the pulse velocity, whereas the attenuation coefficient varied randomly, frequently changing sign. The issue of steps and pauses is treated in greater detail in Chapter IV.

The calculation of pulse propagation velocity is relatively insensitive to noise or distortion of the peak strain amplitude in the sarcomere length-time traces because it is determined by cross-correlation, which is essentially an averaging process. The calculation of the attenuation coefficient was highly sensitive to very small fluctuations in amplitude because its calculation required accurate knowledge of the peak amplitude of the sarcomere length pulse at both points along the fiber. This is evident from Equation 2.5. The steps and pauses caused distortion of the peak amplitude of the sarcomere length-time record. As a consequence, the attenuation of the strain pulses was too small to be detected.

The attenuation coefficient was small for all cases and was not statistically significantly different from zero. This is convenient, because it simplifies the viscoelastic model by supporting the assumption that the single fiber acts as a purely elastic material for strain amplitudes up to at least 8% L_f. To test whether or not the attenuation coefficient was different from zero, all pulse amplitudes for each fiber were grouped by strain amplitude, and the mean value was compared with zero using a two-tailed t-test (Figure 3.5). The only condition in which the attenuation coefficient was found to be statistically different from zero was for relaxed fibers with strain pulse amplitudes of 3% or greater. Although

statistical significance at the $p < 0.05$ level was demonstrated in this case, the difference was probably due to the systematic error resulting from the amplitude distortion by the steps and pauses in the sarcomere length-time records. In this case, the value for the attenuation coefficient was $-0.068\ m^{-1}$ (Figure 3.5). This value represents an average difference in strain pulse amplitudes of less than one part per thousand over a distance of 3 mm. Furthermore, the amplitude actually increased with distance from the servo instead of decreasing, as would normally be expected in linear viscoelastic materials. Based on these data, the attenuation coefficient in single permeabilized skeletal muscle fibers during lengthening pulses will be assumed to be negligibly small for pulse frequencies in the range of 250 Hz to 2000 Hz.

Because the average attenuation coefficient for any strain pulse amplitude was approximately zero, a muscle fiber may be approximated as an undamped (non-viscous) homogeneous material. In a thin rod, the elastic modulus (E) can be determined by:

$$E = v^2 \rho \qquad \text{Eq 2.8}$$

where v is the pulse velocity (m/s), and ρ is the density of the muscle fiber (kg/m^3). There is no frequency dependence in this equation, nor is any damping assumed. For longer fibers (3 to 10 mm), it is important to correct for the increase in the apparent density of the fiber (Steinen and Blange, 1985). The apparent fiber density is the sum of the actual fiber density and extra density, which results from the surrounding fluid adhering to the fiber, which increases the inertia. Taking the actual fiber density as 1,060 kg/m^3, the apparent fiber density was approximated by Ford, et al. (1977) and Blange and Steinen (1985) as:

$$\rho = \rho_f + \rho' \sim 1060 + (2\rho_m\eta / \omega R^2)^{0.5} \qquad \text{Eq 3.1}$$

where ρ is the apparent fiber density (kg/m^3), ρ_f is the actual fiber density (1,060 kg/m^3), ρ' is the extra density due to adhering fluid in the bath, ρ_m is density of the fluid in the surrounding medium (1000 kg/m^3), η is the dynamic viscosity of the surrounding fluid (1.65 x 10^{-3} Ns/m^2, or Pa·s), ω is the frequency of the pulse (s^{-1}), and R is the fiber radius (meters). The use of Equation 3.1 to calculate the apparent fiber density modifies Equation 2.8 for elastic modulus, making it a function of both fiber radius and pulse frequency. At very high frequencies, the added inertia from the surrounding fluid becomes insignificant, but at lower frequencies, the extra density can be significant. For example, a muscle fiber of radius 40 μm would have an apparent density of 2206 kg/m^3 at 250 Hz, but only 1465 kg/m^3 at 2000 Hz. Based on the observation that the attenuation coefficient is negligible, the elastic modulus will be calculated based upon Equations 2.8 and 3.1.

Based on published data (Granzier and Wang, 1993b), the relationship between strain pulse amplitude and pulse propagation velocity was expected to be non-linear for relaxed fibers (Figure 3.6). The expected non-linearity was based on the observed increase in stiffness of the titin molecule with increased sarcomere length above 2.5 μm per sarcomere. The propagation velocity is highly non-linear with increasing strain pulse amplitude, though not as expected (Figure 3.7). The non-linearity occurred at the low-amplitude end of the range of strain amplitudes. Of 19 fibers, insufficient data were collected on 4 to detect non-linear behavior in the range of 1% to 5% L_f. Of the remaining 15 fibers, 11 fibers show clear evidence of non-linear behavior in the lengthening strain pulse amplitude range of 1% to 5% L_f, either when relaxed, at low levels of activation, or both (Table 3.1). Using the complete set of all plots of pulse velocity as a function of pulse amplitude, as in Figure 3.7, a schematic representation of the

non-linear behavior was developed (Figure 3.8). The rapid increase in pulse velocity with increasing strain in the range of 1% to 5% strain range indicates an increasing stiffness up to a certain strain amplitude, at which the stiffness rapidly falls off, which is characteristic of a non-linear stress-strain relationship taken to the yield point. If the process of subjecting the fiber to increasing strain pulse amplitudes is repeated, the yield is observed to occur repeatedly. Therefore, the term *recoverable yield* will be used because the yield occurs many times within the same fiber as the strain pulse amplitude is increased over the full experimental range. In some cases, more than 1000 pulses were applied to the fiber during an experiment. After more than 1000 pulses, the recoverable yield was evident each time the pulse amplitudes increased beyond the yield strain. Nearly 75% of the fibers show very strong evidence of the recoverable yield, which occurs for lengthening pulse strains of typically 1 to 5% L_f.

The baseline pulse propagation velocity for relaxed fibers was approximately 23 ± 3 m/s, and was regularly measured at less than 10 m/s for some fibers. The baseline was determined in the strain amplitude range of 4% to 8% L_f, because this was the range of strain in which relaxed fibers exhibited the least non-linear behavior (Fig 3.7). In the non-linear range, the peak velocity was typically four to five times greater than baseline velocity. Based on Equations 2.8 and 3.1, the peak velocity represents a sixteen to twenty-five-fold increase in the elastic modulus at the recoverable yield point when compared with baseline velocity. For strain pulse amplitudes below 1% L_f , the viscoelastic behavior is not well described. Of the 15 fibers with full data sets in the low strain amplitude range, the data sets from 4 fibers showed no evidence of the recoverable yield (Table 3.1), which suggests that the phenomenon is not a systematic artifact.

The strain pulse amplitude at which the propagation velocity is a maximum indicates the strain at the point of recoverable yield (Fig. 3.8). For fibers which exhibit a recoverable yield, the maximum

velocity of pulse propagation occurs in the strain pulse amplitude range of 1% to 4% L_f for relaxed fibers, and in the strain pulse amplitude range of 1% to 5% L_f for muscle fibers at low levels of activation (Figure 3.9). The exact strain cannot be determined because of the limited amount of data in the low strain amplitude range for each fiber. To determine the exact strain value for the recoverable yield for each fiber would require many more pulses to be applied to the fiber in the strain pulse amplitude range of 1% to 5% L_f.

For some fibers, the recoverable yield appears to occur over a very narrow range, thus the standard error of the velocity at strains near the yield point increases sharply due to small variations in the pulse amplitude. This is evident, particularly in the top plate of Figure 3.7. The narrow range over which the recoverable yield occurs may explain why some fibers did not demonstrate recoverable yield, particularly those fibers for whom only a few strain values below 2% L_f were recorded. In general, the strain range below 2% was initially under-sampled because non-linear behavior was not expected at low strain pulse amplitudes. As a consequence, it is difficult to compare the pulse propagation velocities of different fibers unless a range of pulse amplitudes is selected in which the pulse velocity is relatively stable (Figure 3.7). The pulse propagation velocity for strain pulse amplitudes in the range of 1% to 4% L_f is highly non-linear and cannot be used for comparisons among relaxed fibers. The same is true for partially activated fibers in the strain pulse amplitude range of 1% to 5% L_f. The data indicate that strain amplitudes in the range of 4% to 8% L_f appear to be reasonably consistent and have relatively small standard errors. To test the effect of activation level on the viscoelastic modulus, the propagation velocity for all fibers in the range of 4% to 8% was used (Figure 3.10). Because of these relatively large strain amplitudes, cross-bridges in any state must dissociate during the pulse. There is no clear relationship between the elastic modulus and the level of activation for strain pulse amplitudes above 4%.

Similarly, for pulse amplitudes in the range of 4% to 8% L_f, the elastic modulus for each relaxed fiber is unrelated to the pulse frequency, sometimes rising with increasing pulse frequency, sometimes falling, and sometimes remaining the same (Figure 3.11).

Discussion

The highly non-linear nature of the propagation velocity with respect to strain pulse amplitude was unexpected at low amplitudes. The non-linearity was observed in the range of lengthening pulse amplitudes of approximately 1% to 4% L_f for relaxed fibers, and 1% to 5% L_f for fibers at low levels of activation. The elastic modulus is proportional to the square of the pulse propagation velocity because the attenuation coefficient is negligible. Therefore, the sharp increase in propagation velocity represents a recoverable yield, because the yield is repeated each time the strain pulse amplitude increases beyond the range of 1% to 5% L_f. I hypothesize that the recoverable yield is due to the stretching and ultimate detachment of weakly-bound cross-bridges in relaxed and partially activated muscle fibers. Based on previous reports (Granzier and Wang, 1993a and 1993b) the pulse velocity was expected to increase non-linearly, but smoothly, for increasing pulse amplitudes. Although this previous study involved slow stretches of fibers with the actin filament selectively removed, Granzier and Wang (1993b) showed that the stiffness of the passive elastic elements in the fiber, subjected to continuous 0.1% L_f sinusoids, increased in an exponential manner with increased sarcomere length as the fiber was slowly stretched. The non-linearity described by Granzier and Wang can therefore be attributed to the passive elastic elements in the sarcomere. In the present series of experiments, a large non-linearity in pulse propagation velocity for small amplitude pulses was not expected.

The contribution of weakly-bound cross-bridges to the elastic modulus of permeabilized muscle fibers in the relaxed state has been estimated to be as much as 80% (Chalovich et al., 1991), and that when the cross-bridge state is changed, such as when the fiber is put into rigor, the cross-bridges are in a different configuration and no longer contribute in the same way to the elastic modulus (De Winkel et al., 1994; Granzier and Wang, 1993).

In relaxed fibers, pulse propagation velocities slower than 10 m/s were regularly observed. This is too slow for the step length assumption of instantaneous and homogeneous strain to be applicable. The finite pulse propagation velocity of both lengthening and shortening strain waves in skinned fiber preparations will significantly influence research in the area of muscle stiffness measurements involving rapid "step" length change experiments in which the viscoelastic relaxation time constants are determined. Step length change studies of relaxed muscle fibers are invalid because the strain state along the length of the fiber will vary as a function of both time and position due to finite strain pulse propagation velocity.

Above 4% strain, no clear relationship was observed between the elastic modulus and the level of activation for strain pulse amplitudes. This observation does not agree with earlier findings (Ford, 1977; Ford, 1981). Similarly, for pulse amplitudes in the range of 4% to 8% L_f, the elastic modulus for each relaxed fiber is unrelated to the pulse frequency, sometimes rising with increasing pulse frequency, sometimes falling, and sometimes remaining the same. This observation is also apparently contradicted by earlier investigations (Truong, 1974; Truong, 1978b; De Winkel, 1994). The contradictions result from differences in the strain pulse amplitudes used. Truong, Ford, and De Winkel each used pulse amplitudes at or below 1% Lf, and the data given in the Results is from strain pulse amplitudes in the range of 4% to 8% Lf, which was the only strain pulse amplitude range for which the data were free from

the effects of the recoverable yield. At these much larger strain pulse amplitudes, the cross-bridges, in any state, must detach and reattach, and therefore any comparison between this data and the earlier studies is inappropriate.

The effect of strain pulse amplitude on the measured viscoelasticity is significant, and influences how the viscoelasticity of muscle may be studied. Strain pulse amplitudes in the range of 4% and larger are too large for comparing viscoelasticity among fibers. The viscoelasticity of weakly-bound cross-bridges may be investigated in the range of 1% to 4% strain amplitude to determine the strain amplitude at which the recoverable yield occurs. Viscoelastic comparisons among fibers must be conducted at strain pulse amplitudes well below 1% L_f, even for relaxed muscle fibers, because of the non-linear viscoelastic behavior of the weakly-bound cross-bridges in the strain range of 1% to 4% L_f. This result supports the use of strain pulse amplitudes below 1% L_f for the measurement of muscle fiber stiffness, which is common in the literature.

The most significant technical barrier to the use of laser diffraction to measure muscle fiber viscoelasticity by pulse propagation is the presence of steps and pauses in the sarcomere length-time records. The presence of steps and pauses in sarcomere length-time records has been reported frequently (Burton and Huxley, 1995; Burton and Baskin, 1986; Burton and Huxley, 1991; Burton et al, 1989; Goldman and Simmons, 1984; Goldman, 1987; Granzier and Pollack, 1985; Pollack et al, 1977; Pollack et al., 1988; Rüdel and Zite-Ferenczy, 1979a and 1979b). Steps and pauses are usually attributed to an artifact. Despite extensive studies of steps and pauses, the cause of the artifact was not discovered until this series of experiments had been completed (Burton and Huxley, 1995). The step and pause artifact is covered in more detail in the following chapter.

Table 3.1. Summary of data for recoverable yielding in relaxed and partially activated single muscle fibers. Data is reported as mean ± SE, and are for lengthening pulse frequencies of 500 to 2000 Hz. Baseline data were determined by averaging the pulse propagation velocity for each fiber in the range of 5% to 8% L_f. The data in which no recoverable yield was observed are reported for fibers in the relaxed state only.

	n	Peak V (m/s)	Yield Strain (%L_f)	Baseline V (m/s)
Relaxed	**8**	**106.1 ± 25.3**	**1.94 ± 0.15**	**22.7 ± 2.9**
Partially Activated	**6**	**142.2 ± 20.3**	**2.78 ± 0.37**	**27.8 ± 7.2**
No Yield	**4**	**-**	**-**	**25.5 ± 5.3**

For both the relaxed and partially activated fibers, the peak velocity was significantly different from the baseline pulse propagation velocity at the $p < 0.01$ level, using a two-tailed t-test.

CHAPTER IV
STEPS AND PAUSES

Introduction

The study of high-speed sarcomere dynamics depends on the ability to measure the average sarcomere lengths of small groups of sarcomeres within striated muscle fibers. The average sarcomere length is determined by using the fiber as a transmission diffraction grating, and focusing laser light to a small region on the fiber. One major technical obstacle to the successful use of this technique is the presence of *steps and pauses* in the traces of the time dependent changes in sarcomere length. The presence of steps and pauses in the sarcomere length-time records causes a distortion of the amplitude of the strain pulse during pulse propagation experiments. As a consequence, steps and pauses present a major problem in the measurement of the high-speed sarcomere dynamics of single muscle fibers. In addition, steps and pauses have had a broader impact on the field of muscle mechanics in general, because the presence of steps and pauses has led some investigators to question our fundamental understanding of the molecular mechanisms of muscular contraction.

Steps and pauses in sarcomere length-time records were first reported by muscle researchers in the Soviet Union (Emel'yanov et al., 1966). A "sawtooth-like" shortening pattern in the diffraction records of contracting muscle was described, but due to the poor resolution of the primitive optics employed in the study, the observation had little impact. With improved optics and the use of laser

diffraction methods, steps and pauses have been reported widely during experiments on muscle fiber mechanics (Huxley, 1990; Zite-Ferenczy et al., 1986; Baskin et al., 1981; Yeh et al., 1980; Rüdel and Zite-Ferenczy, 1979b; Pollack et al., 1977). Steps and pauses have been observed in active and passive fibers, and during both lengthening and shortening of fibers (Burton and Huxley, 1995; Pollack et al., 1988).

Steps and pauses became a critical issue in the field of muscle mechanics in 1977, when Gerald Pollack and his colleagues published a paper in Nature in which the phenomenon was referred to as "stepwise shortening", although the phenomenon also occurred during lengthening of muscle fibers. The "stepwise shortening", later referred to as *steps and pauses,* was observed in both skeletal and cardiac muscle. The steps and pauses were detected using three ostensibly different techniques, though each actually just detected the position of the first order diffraction pattern. The location of the first order diffraction peak was measured by a 128-element photodiode array, by projection through a slit onto film in a kymographic camera, and by a Schottky barrier photodiode position sensor, also known as a "spot follower". Based on these results Pollack challenged the generally accepted sliding-filament theory of contraction (Huxley, 1957). The rationale for the challenge was that the theory did not predict steps and pauses, and did not account for the mechanism by which a large number of contiguous contractile units could act synchronously as implied by the steps and pauses (Pollack et al., 1988; Granzier and Pollack, 1985; Pollack et al., 1977).

The phenomenon of the steps and pauses was immediately challenged as an instrumentation artifact (Rüdel and Zite-Ferenczy, 1979b; Goldman and Simmons, 1984; Altringham et al., 1984). Some investigators suggested that the source of the steps and pauses might be the tilting of striation planes (Z lines) within the thickness of the fiber during length changes, because the fiber thickness, which is much

greater than the wavelength of the illuminating light, would be expected to give rise to Bragg angle effects (Huxley, 1986; Rüdel and Zite-Ferenczy, 1979a). Several successful attempts were made to modify the instrumentation used in the laser diffraction experiments to minimize the occurrence of steps and pauses. These attempts included the use of white light (Goldman, 1987), a wide range of angles of incidence (Burton and Baskin, 1986; Brenner, 1985; Lieber et al, 1984; Goldman and Simmons, 1984; Rüdel and Zite-Ferenczy, 1979a), and several of the diffraction orders, with averaging of the results (Burton et al., 1989, Rüdel and Zite-Ferenczy, 1979b). All of these methods tended to reduce the presence of the steps and pauses, but added considerable complexity and computational burden to the general method of laser diffraction. In general, these solutions negate the benefits, primarily simplicity, of using lasers as the light source.

Following the publication of the phenomenon of steps and pauses in Nature, Pollack marshaled a battery of evidence to support his claim that the observed steps and pauses were not artifacts. Using thin black hairs as transverse strain markers on single muscle fibers, Granzier and Pollack (1985) tracked segment lengths optically and noted steps and pauses in 16 out of 21 fibers. Using a similar hair marker technique, Edman et al. (1981 and 1982) observed that several of the segment length-time records showed distinct pauses . Steps and pauses were also evident when Housmans (1984) employed glass microelectrode tips as strain markers. Tameyasu (1985), reported that high-speed video of cardiac muscle cells from the frog also exhibited the steps and pauses, though to a lesser degree than previous data from laser diffraction studies on cardiac cells.

Working on the assumption that the steps and pauses represented the real behavior of sarcomeres in muscle, Pollack devised new hypotheses which were at odds with the generally accepted sliding filament theory. Pollack hypothesized that, "By elimination, we have arrived at the hypothesis

that stepwise length changes are mediated by length changes of two elements, connecting filaments [now known as titin] and thick filaments [myosin]." Pollack summarizes the body of supporting evidence and concludes by stating, "Stepwise shortening has now been confirmed in several laboratories using four different classes of methods. The phenomenon, unlike the mechanism proposed to account for it, is out of the speculative arena," (Pollack, 1986).

A. F. Huxley (1986) responded to these statements by pointing out several major methodological deficiencies in the studies of Pollack and the other investigators whom Pollack cites. The first deficiency was the use of laser light for the cine-micrography, so the photographic images would be subject to the same optical artifacts as any other method of detecting the first order diffraction pattern from a laser. In the hair-marker method employed by Granzier and Pollack (1985), no controls were run, for example, by translating the fiber axially through the laser spot without actually changing the length. Also, the apparatus used by Granzier and Pollack was not described in adequate detail to allow an estimate of the magnitude of errors which may be introduced. The final deficiency pointed out by Huxley was the fact that the electronic method of detecting the hair markers may be sensitive to the presence of the striation pattern of the muscle fiber as well, which would give rise to a step-like pattern as sarcomeres come into the area sampled by the electronic hair-marker detector system. Edman cautioned in comments to Pollack (1988) that, a rubber band showed the same stepwise shortening as demonstrated by a fiber. Consequently, the stepwise shortening was an artifact arising from the discreteness of the photo detection elements in his data collection system.

The debate about the adequacy of the sliding filament theory as a general model for the fundamental mechanism to account for muscle contraction continues to the present. The unexplained phenomenon of steps and pauses is still used as the basis for models of muscle contraction which include,

as their central idea, the shortening of the thick filaments during contraction (Pollack, 1988; Pollack, 1995). The response of the majority of muscle mechanists has been generally to assume that the steps and pauses are artifactual in nature.

Steps and pauses present a significant problem in the sarcomere length-time records that must be resolved to permit accurate measurements to be made of sarcomere dynamics during muscle fiber length changes. Consequently, the cause of the steps and pauses and a method to resolve them was the highest priority before further experiments could be undertaken in the measurement of muscle fiber viscoelasticity by means of optical diffraction at two positions along a fiber. The working hypothesis was that *the steps and pauses are an artifact and do not represent the actual strain state within the sampled region of the fiber*. To test the working hypothesis, the classical method of multiple alternative hypotheses (Chamberlin, 1897; Platt, 1964) was employed. A series of experiments were executed to exclude each possible source of artifact which could give rise to the observed steps and pauses. A list of the alternative testable hypotheses were formulated that could explain the steps and pauses in the order of decreasing likelihood. *The steps and pauses in the sarcomere length-time records are an artifact resulting from: (1) forced mechanical transverse vibration, (2) free mechanical transverse vibration, (3) optical interference or scattering unrelated to changes in the length of sarcomeres, (4) Bragg angle artifact due to the thickness of the muscle fiber, or (5) artifact resulting from the discrete width of the phototransistor array elements.*

The alternative hypotheses were tested in order, and alternative hypothesis 3 was supported. Two additional experiments were conducted subsequently to verify the result that the steps and pauses were the result of an optical artifact and could be eliminated by using a laser light source with short

coherence length. The practical solution to the steps and pauses artifact is in agreement with the experimental and theoretical findings of Burton and Huxley (1995).

General Methods

Each of the alternative hypotheses tested for a different source of the presumed artifact of steps and pauses. In some cases, the instrumentation was tested, with no biological sample required. In other cases, the raw spectral data from earlier experimental work, in which the steps and pauses were evident, was reanalyzed without requiring additional data to be collected. The final tests and verifications required additional data to be collected from single muscle fibers. In the case where additional biological data was required, the muscle fibers were harvested from the soleus muscle of 4 to 5 months old F344 rats. The rats were dissected to remove the soleus muscle from the right hind leg by carefully dissecting away the fascia and transecting the proximal and distal soleus tendons. The rats that were used in these studies were anesthetized with pentobarbital sodium. The tissue was harvested from the animals after *in vivo* sustained power experiments on the left leg, which were unrelated to this series of experiments, to maximally utilize the tissue from each animal. Because of multiple use of each animal by several investigators, no animals were sacrificed specifically for this series of experiments.

To collect the tissue, the soleus muscle was placed in mammalian Ringer's solution (137 mM NaCl, 24 mM NaHCO, 11 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, and 0.025 mM tubocurarine chloride) immediately after removal from the animal. The muscle was pinned at each end to hold it slightly longer than its free length (under no tension) and all external fat and loose connective tissue was carefully removed. The muscle was cut longitudinally parallel to the muscle fibers into six or seven (depending upon muscle size) thin bundles. Each bundle was approximately 12 to 15

mm long and approximately 1 mm in diameter. The bundles were tied to short pieces of glass capillary tube using 5-0 braided silk suture. Each bundle length was adjusted by sliding the knots along the glass capillary tube so that the bundle was straight and very slightly extended beyond the free length.

The tied bundles were placed into 5 cc plastic vials filled with skinning solution, were capped securely, and are placed into a freezer at -20°C. The bundles are allowed to remain in the skinning solution at -20°C for a minimum of four days. All fibers in this study were used within four months of storage in skinning solution to assure viability. The skinning solution, as well as the relaxing and activating solutions, are described in detail in the previous chapter. Immediately prior to an experiment, the tied fiber bundles were removed from the skinning solution and placed into a small dissecting dish filled with chilled relaxing solution. The bundles were allowed to thaw for ten minutes to avoid damaging the tissue. The 5-0 silk suture knots were removed and the fiber bundle was released from the glass capillary tube. The bundle was pinned to the bottom of a small dissecting dish using 0.10 mm diameter insect pins (Ianni Butterfly Enterprises). The ends of the bundle, which were compressed by the 5-0 suture, were removed by cutting with Vannas spring scissors, and sharpened #5 forceps were used to slowly draw out a small group of fibers from the bundle. This small group typically contained four to ten fibers. After withdrawal, the damaged fibers were simply pulled away using sharpened forceps, leaving one or two undamaged fibers, up to 9 mm in length.

The flow through bath (Appendix E) was filled with relaxing solution until slightly over full, and was chilled to 15°C prior to the removal of the single fiber from the fiber bundle. A single fiber was transferred to the flow-through bath and was held at one end by sharpened forceps. Approximately 1 mm of one end of the fiber was lifted out of the solution and laid carefully into the one of the troughs. The surface tension of the relaxing solution was used to pull the fiber flat into the trough and help to hold it in

position while it was secured in place. A very short piece (plug) of 5-0 nylon monofilament suture (0.6 to 0.7 mm long) was placed in the trough directly over the end of the single fiber. The trough was then raised slightly to increase the downward force on the monofilament plug from the surface tension of the relaxing solution. This held the muscle fiber and nylon plug in place while one of the waxed nylon strand knots was pulled over the plug and muscle fiber and pulled tight. Once secured, the trough was lowered slightly to reduce the surface tension force and the second nylon strand knot was pulled over the plug and is pulled tight. The tying procedure was repeated on the other end of the single fiber, the result being that the single fiber was fastened at each end to a stainless-steel trough and was suspended in a bath of relaxing solution. The loose ends of the waxed nylon filaments were snipped off with microsurgical scissors and removed from the bath to prevent force shunting between the fiber, the troughs, and the sides of the flow-through bath.

Both stainless steel tubes, one of which was attached to the linear servo motor and the other of which was attached to the optical force transducer, were lowered so that they were centered in the slots at each end of the flow-through bath and the single fiber was suspended in the relaxing solution. The stainless steel tubes to which the single fiber was tied were carefully positioned so that the single fiber was centered in the flow-through bath and was pulled until it was just straightened out (Figure 2.2). An 18 mm square cover slip was placed over the top of the bath and was pulled into place by the surface tension of the relaxing solution.

The laser prism holder was slipped into position and the X-Y table of the apparatus was adjusted so that the focused He-Ne laser beam spot was near the center of the fiber (Figure 2.3). A small calibration target mounted on an aluminum beam was slipped into place 75 mm directly beneath the fiber, and the diffraction pattern was projected onto the target. The sarcomere length was read directly

from the position of the diffraction pattern on the target, and the position of the left trough (connected to the servo motor) was adjusted to achieve a sarcomere length of 2.55 microns. The X-Y table was then translated right and left to inspect the diffraction pattern for all points along the fiber. If the pattern was clearly visible and stable along the length of the fiber, the fiber was accepted for further use. If the diffraction pattern proved to be weak or irregular it was assumed that the fiber was damaged during dissection or placement in the apparatus and was removed and replaced by a new fiber. The process was repeated until a suitable fiber was identified. Approximately 20% of the fibers were rejected at this point in the experiment. The practical limit for the distance between the laser spot positions on the fiber was about 2 mm, because this was the maximum distance over which the typical fiber had a striation pattern which was highly consistent and stable when inspected by translating the laser along the fiber.

The fluid exchanger was charged with 5 cc of chilled activating solution. The relaxing solution in the flow-through bath was slowly replaced (over about 30 seconds) by activating solution, and the force generated by the fiber during this process was displayed on the precision optical force transducer display (Appendix D) in μN. The force generated by the fully activated fiber was allowed to stabilize after the fluid exchange was complete, and the peak force was recorded. The fluid exchanger was charged with 5 cc of relaxing solution, and the activating solution in the flow-through bath was replaced by relaxing solution.

The fiber length was measured between the nylon monofilament plugs and was determined by traversing the X-axis of the X-Y table along the entire length of the fiber. The total distance traversed was measured using a digital depth gauge (Figure 2.1) which was clamped to the surface of the vibration-isolation table. The X-Y table traverse indicator was set to zero at the force transducer end, and all further location measurements were referenced to this position. To measure the diameter, the fiber was

viewed by a Leica WILD M3Z Kombistereo microscope at 40X magnification with a 25X/0.40 high-power objective and a finely divided eyepiece graticule. The fiber length was recorded, and the diameter was measured at six equally spaced positions along the fiber.

The experimental sequence of pulses was downloaded into the digital waveform synthesizer memory and the desired settings are selected for the clock speed and number of experiments to be performed (Chapter II). For this series of experiments, the digital waveform synthesizers were loaded with profiles for lengthening and shortening pulses. Pulse amplitudes of 20%, 40%, 60%, 80%, and 100% of full range for both shortening and lengthening pulses were used. A total of 128 pulses were programmed for each experimental battery. The lengthening and shortening pulses of different amplitudes were alternately mixed together throughout the battery to eliminate any systematic effect of the ordering of the pulse amplitudes on the outcome of the experiment. Each pulse was initiated remotely by the computer keyboard. A two to three second delay between the pulses allowed the fiber striation pattern to stabilize. Each fiber was periodically checked to assure that the diffraction pattern was still present. The first 64 pulses were applied to the fiber with the laser at the first sampling position along the muscle fiber. The fiber was then translated axially by 1 to 2 mm using the X-Y table, and the new laser spot position on the fiber was recorded to the nearest 0.01 mm. The final 64 pulses were applied to the fiber with the laser spot at the second position. When a total of 128 pulses had been executed, the array controller memory was downloaded and temporarily stored to hard disk on the microcomputer. The digital waveform synthesizer memory was reprogrammed at this time. The downloading and reprogramming process required approximately 8 minutes, during which the fiber was maintained in relaxing solution at 15°C and the servo coil was de-energized to prevent transient pulses from occurring during reprogramming (Appendix C). Up to 10 batteries of 128 pulses were applied to

each of the fibers. In this manner, data were collected for the sarcomere length at two points along the fiber for lengthening and shortening pulses under conditions of varying pulse amplitude. Pulse frequency and activation level were not modified for this series of hypothesis tests. All tests were performed with single 1000 Hz pulses on fully relaxed fibers.

Testing of Hypotheses

The alternative hypotheses were tested in the following sequence, with the methods and results for each hypothesis test given where appropriate. Beginning with hypothesis 3, the formulation of hypotheses depended upon the results of the previous test.

Hypothesis 1 - The steps and pauses are an artifact caused by forced lateral vibrations of the muscle fiber due to transverse vibration of the servo trough during a pulse.

Methods

The servo motor (Figure 2.2) was instrumented to test if lateral or vertical vibrations resulted from the application of rapid pulses (Appendix C). A very thin vane of aluminum foil was attached to the end of the trough, and an optical detector was used to detect off-axis movement of the trough during the pulse. The optical detector was identical in design to the optical detector used in the precision optical force transducer (Appendix D). When properly implemented, this arrangement allows movements as small as 1 micron to be detected. The servo was first moved very slowly through the full range of lengthening and shortening pulses used in this series of experiments. These data were recorded and plotted on a digital oscilloscope to establish a baseline. The servo was then driven at full speed, resulting in a single sinusoidal pulse for each amplitude and direction used in this series of experiments. The data

were also recorded and compared with the baseline values. This process was repeated to detect both lateral and vertical vibrations of the servomotor trough.

Results

No measurable lateral or vertical vibrations of the trough were detected.

Discussion and Conclusions

This hypothesis represents the most likely explanation from a mechanical point of view. No lateral or vertical vibrations were detected, which demonstrates that the servo motor did not directly cause forced lateral or vertical vibrations of the fiber. Therefore, the steps and pauses can not be attributed to forced transverse vibrations of the muscle fiber resulting from servo trough vibrations.

Hypothesis 2 - The steps and pauses are an artifact caused by free lateral vibrations of the single fiber, which are induced by the servo pulse.

Methods

A large amount of data which exhibit steps and pauses are available from the earlier studies (Chapter II and Chapter III). Data from 10 sarcomere length-time records were selected at random for analysis. An FFT of each of the sarcomere length-time records indicated a very strong frequency component at approximately 4 kHz. This is easy to verify by estimating the cyclic interval of the steps from Figure 4.1(a), in which the first four full steps occur in slightly less than 1 ms, suggesting a frequency of slightly more than 4 kHz. The subject of free vibrations of muscle tissue has been treated in detail (Cole, 1992), which allows the results of the FFT analysis to be compared with theoretical values for transverse free vibrations of muscle under tension. The details of the model are available in the cited publication, in which the Rayleigh method is used to predict the muscle resonant frequency based upon

the known geometry and material properties of the muscle. In the case of a single muscle fiber under tension, equation 4.7 (Cole, 1992, page 58) reduces to the case of a cylindrical, homogeneous string under tension. This results in the following standard equation:

$$\nu = \frac{n}{2l}\sqrt{\frac{F}{\mu}} \qquad \text{(Eq. 4.1)}$$

in which ν is the frequency of vibration, n is the mode number, l is the length of the string (m), F is the string tension (N) and μ is the linear density (kg/m). Taking the worst case values to maximize the transverse free vibration frequency; l = 6 mm, F = 10 micro Newtons (the fiber is fully relaxed), μ = 8 x10-6 kg/m, and neglect the effect of the surrounding water medium, which would tend to reduce the resonant frequency because of the viscosity of the water and the increase in the linear density of the muscle fiber due to water molecule adhesion, the resulting frequency for free lateral vibrations in the first mode is 93 Hz.

Results

An FFT analysis of 10 sarcomere length-time records indicates that, if the steps and pauses are due to transverse free vibrations of the muscle fiber, the vibrations are occurring at a frequency of approximately 4 kHz. A mathematical model of free transverse vibrations of muscle under tension indicates that the absolute maximum acceptable value for the frequency of transverse free vibration of a single muscle fiber is 93 Hz.

Discussion and Conclusions

Although lateral and vertical trough vibrations were excluded, it was possible that the lateral or vertical vibrations may have been caused by transverse acoustic coupling within the fiber, such that the non-longitudinal vibrations were induced by the single longitudinal pulse itself, resulting in transverse free vibrations of the muscle fiber. The sensitivity to artifacts caused by higher modes of vibration is limited by the width of the fiber and laser spot diameter, so it is unlikely that the artifact is due to a high mode of vibration. This is further supported by the fact that no lower modes were indicated by the FFT. This procedure was repeated on ten fibers with similar results. The steps and pauses occur at a frequency that is two orders of magnitude larger than can be explained by free lateral vibrations, and therefore this possibility is excluded from consideration in explaining the steps and pauses artifact.

Hypothesis 3 - The steps and pauses result from an optical artifact which is unrelated to actual changes in sarcomere length within the muscle fiber.

Methods

A muscle fiber was mounted on the apparatus and all other preparations were made exactly as for the previous pulse propagation experiments (Chapter III). The sarcomere length of the fiber was set to approximately 2.6 microns. The fiber was maintained in relaxing solution at 15 °C throughout this experiment to maintain the most stable sarcomere pattern possible. No pulses were applied to the fiber. In this experiment, as in all previous experiments, a He-Ne laser was used as the light source. The fiber was axially translated through the laser spot very slowly by means of the X-Y table (Chapter II). Instead of programming the array controller to induce pulses and automatically record the data, the array controller was set to real-time output mode, which had originally been designed for calibration and alignment purposes (Chapter II). In this mode, the output from the array is serially multiplexed,

converted to analog, and displayed on a digital storage oscilloscope. The intensity profile of the first order diffraction pattern on the phototransistor array is visible in real time as a trace on the oscilloscope.

Results

The fiber length was not changed, but as the fiber was translated axially through the laser beam, a periodic right-and-left shifting of the first order diffraction pattern was evident. The intensity of the first order peak periodically dropped, and the centroid of the peak appeared to shift right and left slightly, with the same period as the peak intensity drop.

Discussion and Conclusions

The results are in agreement with the conclusions of Burton and Huxley (1991), that the steps and pauses could be reproduced simply by translating a plane grating through the laser beam. Burton and Huxley had also suggested that the same was true if a muscle fiber were translated through the beam. If the right-and-left shifting of the centroid were superimposed upon the otherwise smooth sarcomere length trace during a lengthening or shortening pulse, the effect would clearly be to produce a step-like pattern. Although this behavior could be viewed on the oscilloscope, the data could not be recorded and analyzed in this format. The results support the hypothesis that the steps and pauses are due to an optical artifact in which the centroid of the first order diffraction pattern is influenced by translation of the fiber through the laser beam without any changes in the actual sarcomere length. This has importance in pulse propagation experiments because the application of lengthening or shortening pulses not only changes the sarcomere lengths within the muscle fiber, but also causes the fiber to be translated axially with respect to the laser spot.

Hypothesis 3a - The steps and pauses in the pulse propagation data are due to the same optical artifact that causes centroid shifts in the first order diffraction pattern when a fiber is translated through the laser beam without a change in sarcomere length.

Methods

This hypothesis was tested by reviewing data from earlier experiments (Chapter III). Twenty-four sarcomere length-time records were selected at random from the total data set of lengthening pulses. The raw diffraction data for each of these data sets were also recovered for analysis of the peak intensity of the first order diffraction pattern as a function of time for each trace. The sarcomere length data were differentiated to amplify the steps and pauses, and plotted along with the values for the peak first order diffraction intensity from the raw spectral data for each spectrum in the record.

Results

In each of the 24 records, the maximum rate of change of sarcomere length correlates very well with sudden drops in diffraction peak intensity (Figure 4.2).

Discussion and Conclusions

The results suggest that the artifact may be due to the interfering light causing a shift in the centroid of the first order diffraction pattern that is due to light and dark bands moving across the 1° diffraction peak. Thus, the mechanism which resulted in the centroid shifts while translating a fiber through a laser beam without changing the sarcomere length (Hypothesis 3) may be the same mechanism which results in centroid shifts when applying pulses to an otherwise stationary fiber. The results further support the notion that the steps and pauses arise from the translation of the fiber through the laser beam that results from length changes, and not from the sarcomere length changes themselves. The maximum rate of change of sarcomere length correlates very well with sudden drops in diffraction peak intensity, as

would be expected if a dark interference band were moving across the 1° diffraction pattern with each step. This general result was repeated each time dL/dt and 1° peak intensity were plotted together, for 24 different traces. This strongly supports the hypothesis and is in agreement with the results of Burton and Huxley (1995), who were able to show a correlation between the peak intensity drops and steps for much slower length changes.

Hypothesis 3b - The appearance of steps and pauses can be significantly reduced or eliminated by the use of a light source for the diffraction experiments with a very short coherence length.

Methods

For optical diffraction, a desirable light source will be quasi-monochromatic, collimated, and have a short coherence length. During the development of an optical interferometric force transducer it became apparent that the laser light produced by a diode laser is of much shorter coherence length than that produced by a He-Ne laser. A suitable laser diode module was selected, and a Michelson interferometer was constructed to test the coherence length of the laser light as a function of the supply voltage (Appendix F). The coherence length of the diode laser light could be reduced to about 300 microns (approximately three fiber diameters) by powering the diode laser at +4.90 VDC. This is a much shorter coherence length than the light produced by a He-Ne laser, which is typically reported to be 10 to 30 cm, or longer. Lengthening pulses (1 kHz single sinusoids) were imposed on each fiber, first using a He-Ne laser as the light source as in previous experiments. The He-Ne was then replaced by the diode laser and the fiber was again subjected to the same series lengthening pulses. Changing lasers in the experimental setup is a complex issue, and it is difficult to realign each of the lasers when they are first put into place. The operation is also time consuming, so only a very limited number of fibers could

be used in such a test. This test was performed on two fibers, number 22 and number 23. The fibers were maintained in a relaxing solution at 15 °C for the duration of the experiments.

Results

For both fibers, the steps and pauses evident when the He-Ne was used disappeared completely when the laser diode module was used (Figure 4.3).

Discussion and Conclusions

By using the laser diode operating such that the coherence length was reduced to 300 microns, interference with scattered light from sources more distant than 300 microns would be substantially reduced or eliminated. This included the most likely scattering sources in the light path, namely scratches and bubbles located on the top and bottom cover slips of the fluid bath, which are approximately 2 mm above and below the fiber. The use of a diode laser module operated at a short coherence length resulted in a total elimination of the steps and pauses, which are evident when a He-Ne laser is used (Figure 4.3).

General Discussion

Unexpectedly, the most serious problem encountered with the pulse propagation experiments was the pervasive occurrence of the steps and pauses in the sarcomere length-time records. Such steps and pauses were typical for all of the lengthening pulses in the total data set of nearly 15,000 pulses. The steps and pauses were less apparent for the shortening pulses of the relaxed fibers, probably because of fiber buckling. Similar steps and pauses have been observed by nearly all investigators who use light diffraction to measure sarcomere length in muscle fibers undergoing length changes, though typically for much slower length changes (Burton and Huxley, 1995; Burton and Baskin, 1986; Burton and Huxley,

1991; Burton et al, 1989; Goldman and Simmons, 1984; Goldman, 1987; Granzier and Pollack, 1985; Pollack et al, 1977; Pollack et al., 1988; Rüdel and Zite-Ferenczy, 1979a and 1979b), but the probable source of the steps and pauses has only been very recently discovered and published (Burton and Huxley, 1995).

The effect of the steps and pauses can be appreciated based on the technique and equations for the calculation of pulse velocity and the attenuation coefficient. The pulse velocity is calculated by cross-correlating the servo position with the resulting sarcomere length pulses at two separate points along the length of the fiber. The difference in the lag between the two cross correlations is taken as the time delay, Δt, between the pulse arrival at the two points. Pulse velocity is then simply calculated by dividing the distance between the two points, Δx, by the time difference, Δt (Eq 2.3). The process of cross-correlation essentially averages the entire sarcomere length-time record in the process of correlating. Noise, or any other superimposed signal with an expected value of zero over the duration of the time record of the experiment, on the sarcomere length pulse trace will tend to reduce the maximum correlation coefficient, but will not change the lag. Therefore the time delay calculation is fairly insensitive to the steps and pauses, so long as their amplitude is small compared with that of the actual sarcomere length pulse, which is most often the case. The calculation of the attenuation coefficient, on the other hand, is highly sensitive to very small fluctuations in amplitude. This is because calculation of the attenuation coefficient requires accurate knowledge of the peak amplitude of the sarcomere length pulse at both points along the fiber, as is evident from Equation 2.5 in Chapter II. The steps and pauses have a very significant and visually evident effect upon the peak sarcomere length value for each pulse (Figure 4.1).

Aside from the impact of steps and pauses on pulse propagation experiments, the existence of steps and pauses in dynamic sarcomere length data has been a major issue in muscle research for nearly 20 years. Burton and Huxley (1995) provided evidence, both theoretical and experimental, that the steps and pauses are caused by scattered laser light which interferes with the first-order diffraction pattern. The scattering of the laser light is caused by small particles in or near the single fiber, and interference is possible because of the long coherence length of light from a He-Ne laser. This result is supported by the findings of Goldman (1987), who noted that the coherence length of the white light used in his studies had a coherence length of only a few wavelengths. The use of white light for diffraction experiments is not an elegant or practical solution because the diffraction angle is dependent in the wavelength of the light as well as the incident angle and the sarcomere spacing. Burton and Huxley, therefore, suggest the use of phase-randomized laser light, as is used in confocal microscopy, but do not demonstrate the effectiveness of the use of phase-randomized light as a practical solution. The method of multiple alternative hypotheses used in this series of experiments quickly yielded both an answer, that was found to be in agreement with the recently published findings of Huxley and Burton (1995), as well as a practical solution for dealing with the problem of steps and pauses.

The results of this study indicate that the use of a laser diode operated at low coherence length is a highly effective, practical, and low cost solution to a problem that has plagued the researchers in muscle mechanics for nearly 20 years. This result will greatly influence the design of future instrumentation for use in the measurement of high-speed sarcomere dynamics. The solution can also be employed by any other laboratory in which laser diffraction is used for measuring sarcomere length. The elimination of the step and pause artifact will allow the measurement of attenuation coefficient in pulse propagation experiments. Subtle features in the sarcomere length-time record, which were masked by

the presence of the step and pause artifact, may become evident. Although this important result happened too late to be of use in the reported experiments in pulse propagation (Chapter III), the findings will be of great significance when designing new instrumentation for use in pulse propagation studies of muscle fibers.

CHAPTER V
SUMMARY AND CONCLUSIONS

This series of experiments was designed to test the hypothesis that the viscoelastic behavior of relaxed and partially activated single muscle fibers is dependent upon strain pulse amplitude and strain rate. Instrumentation was developed to permit the measurement of the viscoelastic properties of single permeabilized skeletal muscle fibers by measuring the pulse propagation velocity and attenuation coefficient between two positions on a fiber separated by 1 to 3 mm. Chemically permeabilized single muscle fibers from soleus muscles of male F344 rats were tested in the relaxed and partially activated condition at 15 °C. A servomotor imposed single longitudinal strain pulses on the single fibers. The first order laser diffraction pattern was detected for the two positions along each single muscle fiber to measure the sarcomere length at each of the positions during the application of the strain pulses. Data were analyzed to calculate the pulse propagation velocity and attenuation coefficient of the strain pulse amplitude. The pulse velocity and attenuation coefficient were measured for strain pulse amplitudes from 0.5% to 10%, and pulse frequencies from 250 Hz to 2000 Hz.

During shortening pulses of both relaxed and partially activated fibers, buckling of fibers prevented data analysis. Due to the buckling of fibers at all strain pulse amplitudes, shortening pulses could not be used for the determination of the viscoelasticity of relaxed single permeabilized muscle fibers. Under all other conditions, the attenuation coefficient was not significantly different from zero.

The negligible magnitude of the attenuation was due, in part, to the short distance between the two sampling positions on the fiber (1 to 3 mm), over which distance little attenuation occurred. Furthermore, during rapid pulses the presence of steps and pauses in the sarcomere length-time records distorted the peak sarcomere length amplitude. Consequently, the steps and pauses masked any small attenuations in the strain pulse amplitude which may have been present. Because the attenuation coefficient was negligible, the single fibers were assumed to exhibit purely elastic behavior. The real component of the complex elastic modulus of a single fiber was therefore approximated as: $E = v^2\rho$. In the range of 250 Hz to 2000 Hz, the observation of nonviscous behavior for single permeabilized muscle fibers is in contrast to the significant attenuation observed for relaxed whole muscles during single lengthening pulses in the same frequency range (Truong, 1974). No other studies have reported attenuation coefficients for muscle tissue measured by the single pulse propagation method. The difference in the attenuation of strain pulses in single permeabilized fibers and whole muscles may be attributable to the greater amount of non-contractile and extra-cellular material contained in whole muscles compared with the single muscle fibers. During rapid deformations water, connective tissue, and other extra-cellular material of whole muscles may undergo viscous flow.

For both relaxed and partially activated muscle fibers, the maximum pulse propagation velocity occurred at strain amplitudes of from 1% to 5%. The peak velocity represents a 9- to 25-fold increase in the elastic modulus when compared with the elastic modulus for strain pulse amplitudes both below and above strains of 1% to 5%. In both relaxed and partially activated muscle fibers, the repeated emergence and disappearance of a peak stiffness with increasing strain pulse amplitude suggested a phenomenon of *recoverable yield.* This behavior is described as a recoverable yield, because the phenomenon occurred repeatedly each time the strain amplitudes were increased from below to beyond the apparent yield point.

The nature of the recoverable yield suggested a structure within the contractile apparatus of relaxed muscle fibers that became increasingly stiff until a strain amplitude of 1% to 4% was achieved. At this degree of strain the structure yielded temporarily, resulting in a dramatic reduction of stiffness. The recoverable yield was hypothesized to result from the attachment and subsequent detachment under increasing strain of weakly-bound cross-bridges. The phenomenon of recoverable yield is not likely to result from an instrumentation artifact, because the phenomenon was observed in only 75% of the fibers. An instrumentation artifact would be expected to be observed in all fibers. Based on a maximum 10 nm per half sarcomere strain prior to detachment of strongly-bound cross-bridges (Goldman, 1987b), the detachment of weakly-bound cross-bridges would be expected to occur at strains of approximately 0.78% for an initial sarcomere length of 2.55 μm/sarcomere if weakly-bound and strongly-bound cross-bridges are assumed to be structurally similar. Based on X-ray diffraction studies, weakly-bound cross-bridges are structurally dissimilar to strongly-bound cross-bridges (Brenner, 1987). The structural differences between the weakly- and strongly-bound cross-bridge states may explain the larger strain required to cause dissociation of the weakly-bound cross-bridges.

The measurement of pulse propagation in relaxed fibers for a range of pulse amplitudes may provide a means for studying directly the mechanical characteristics of weakly-bound cross-bridges. The method of pulse propagation may also be useful as a probe for the changes in cross-bridge populations at low levels of activation, although the data obtained from this series of experiments is insufficient to demonstrate conclusively that the recoverable yield occurred at a higher strain pulse amplitude for fibers at low levels of activation compared with relaxed fibers.

The "steps and pauses" observed in the sarcomere length-time records were hypothesized to be caused by an optical artifact. The occurrence of the steps was found to correlate strongly with sudden

drops in the intensity of the first order diffraction peak. The source of the artifact was identified as interference from scattering sources in the optical path. This observation was in agreement with the findings of Burton and Huxley (1995). The instrumentation was modified by employing a diode laser module with reduced coherence length (approximately 300 μm). The modification eliminated the artifact and permitted more accurate measurements of strain pulse amplitude at each of the two sampling positions.

Future Directions for Research

The hypothesis that the phenomenon of recoverable yield results from the straining and subsequent detachment of weakly-bound cross-bridges can be tested more rigorously by varying the ionic strength to manipulate the number of weak cross-bridges (Brenner et al., 1982; Schoenberg, 1988; Granzier and Wang, 1993a), or by the use of smooth muscle caldesmon, which has been shown to function as a competitive inhibitor of cross-bridge formation (Wang et al., 1991; Granzier and Wang, 1993b). In both mammalian skeletal muscle and insect flight muscles, ionic strength and caldesmon have been used effectively to distinguish passive tension and stiffness resulting from weakly-bound cross-bridges from passive tension and stiffness resulting from titin (Granzier and Wang, 1993b). The studies of Granzier and Wang were performed at very low speed, and employed measurements of stiffness which included only one value of strain amplitude, superimposed on a steady-state length change of the fiber.

To determine the relevance of the findings of this series of experiments to living systems, pulse propagation must be studied in intact muscle fibers and small muscles composed of parallel arrays of fibers. These experiments will allow the measurement of the contribution of the intact muscle cell, as well as that of the extra-cellular material, to the viscoelastic properties of muscle tissue. The

employment of higher levels of structural organization and rapid length pulses will enable the structural level from which the viscous behavior of muscle originates to be determined.

The method employed in this series of experiments can be improved by a number of significant modifications. Two laser spots will be focused on the fiber simultaneously and the diffraction data collected simultaneously. The spots will be maintained at a constant separation of 2 mm. This will allow direct cross correlation between each pulse at the two positions, thus reducing the experimental bias that is introduced by sampling at only one position for each pulse. This modification will reduce the computational burden by nearly 50%. For the same number of experiments, the total amount of stored spectral data will be reduced by 33%. The analog-to-digital converters for the system will be upgraded to 12-bit resolution. These analog-to-digital converters have only recently become available with conversion rates above 1 MHz. The increased resolution will allow the viscoelasticity of fully activated muscle fibers to be studied. The digital waveform synthesizers will also be upgraded to 12 bits, and an IEEE 488.2 GPIB will be used for communication between the array controller and the computer to increase the data transmission rate during downloading to the computer. These modifications to the equipment used in the present study will permit a whole new array of hypotheses to be formulated and tested for the viscoelastic behavior of muscle tissue.

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