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UNIVERSITY OF ALBERTA

MYOFIBRIL ALTERATIONS WITH EXHAUSTING ISOMETRIC CONTRACTIONS

BY

PHILIP CHARLES WILLIAMSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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THE UNDERSIGNED CERTIFY THAT THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED MYOFIBRIL ALTERATIONS WITH EXHAUSTING ISOMETRIC CONTRACTIONS SUBMITTED BY PHILIP CHARLES WILLIAMSON IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

H.A. Quinney

D.G. Syrotuik

V.E. Baracos

Date: 7.2.91

Dedication

To my parents, who taught me never to quit.

Abstract

The objective of the investigation was to examine any morphological and compositional changes in skeletal myofibrils as a result of exhausting isometric contractions. An in vitro electrical stimulation model was utilized with the rat epitrochlearis muscle preparation in order to isolate the influence of contractile activity on myofibril alterations. Muscles from 14 rats were each assigned to one of three different experimental conditions (freshly dissected, incubated-only or incubated-stimulated) and evaluated using electron microscopy and SDS-PAGE procedures. Incubated-only muscles demonstrated a selective loss of myofibrillar protein in the region of approximately 69 Kda, 37 Kda, and 34-35 Kda. Incubated-stimulated muscles showed a widespread, generalized loss of myofibrillar protein throughout the full molecular weight range. Electron microscopy study revealed an increased prevalence of mitochondrial swelling under both incubated conditions. Incubated-stimulated muscles demonstrated sarcomeric misalignment and dramatic vacuolation, likely of the T-tubule system. The data suggest that in the absence of anabolic hormonal factors incubated muscles undergo selective protein degradation, that proteolytic response becoming more widespread and generalized with fatiguing contractions. Furthermore, the extensive marked vacuolation observed in exhausted muscle may disrupt propagation of action potentials in the T-tubule system, and may play a role in the fatigue process.

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CHAPTER I - INTRODUCTION

There has been a great deal of research done to investigate the effect of exercise on muscle protein breakdown. Despite the abundance of related literature, different opinions still prevail so to whether or not exercise results in increased (Dohm et al. 1982, 1985, Belcastro et al. 1985), decreased (Rennie et al. 1980, 1981), or unchanged (Wolfe et al. 1984) rates of protein degradation. The disparities in the literature may be attributed to a wide variety of factors, including the methods and models used to determine protein breakdown rates, the nutritional status of the subjects, the type, intensity and duration of the exercise protocols utilized, and the fluctuations in protein turnover which may exist during different phases of exercise and recovery. Taken collectively, these factors make it extremely difficult to generalize a specific proteolytic response to exercise.

Even less well documented is the relationship between the overall rates of muscle protein synthesis and degradation and the turnover of myofibrillar protein specifically. This relationship provides an interesting basis for investigation of the regulation of muscle proteolysis, since it has been shown that myofibrillar and non-myofibrillar protein breakdown are regulated independently and by different mechanisms (Lowell et al. 1986, Goodman 1987, Kasperek and Snider 1989). In a broader context this represents an important problem, because an understanding of the assembly and replacement of the contractile apparatus may help to explain some disease states in muscle (certain endocrinopathies, polymyositis, and denervating diseases), as well as potentially providing some insight into the process of muscle fatigue (Dohm et al. 1980, Belcastro et al. 1988).

Previous research has demonstrated that muscle proteolysis is, to a large extent,

under hormonal control (Kettelhut et al. 1988, Balon et al. 1990). During single bouts of exercise, muscle metabolism is influenced greatly by exercise-induced multiple changes in hormone secretion (Richter 1986, Galbo 1986). Of these, insulin, glucocorticoids, and thyroid hormones have all been demonstrated to produce alterations in skeletal muscle protein turnover (Millward 1985, Kettelhut et al. 1988). Due to the existence of exercise-induced hormonal and nutritional changes, in vivo studies of muscle protein turnover fail to distinguish between the basal effects of muscle contractile activity and those effects which are systemically-induced (Belcastro et al. 1988, Kasperek and Snider, 1989). Thus, some of the previous work done in this area leaves some questions incompletely answered. For example, Belcastro et al. (1988) reported myofibrillar protein alterations in rats which were run to exhaustion on a treadmill, as observed by gel electrophoresis (SDS-PAGE) and electron microscopy. Whether or not these changes were induced by hormonal or nutritional alterations, or by the contractile activity itself, can only be hypothesized. Other in vivo studies have reported only non-myofibrillar protein breakdown in the period immediately following an exercise bout (Kasperek and Snider, 1989). As previously mentioned, these results may only demonstrate a synthesized picture, by failing to isolate the effects of the contractile activity from those induced by nutritional, neurogenic, or hormonal systemic influences associated with the exercise bout.

Nie et al. (1989) have investigated myofibrillar and total protein breakdown in an isolated muscle preparation without the influence of hormones and other possible systemic influences. By using 3-methyl histidine and phenylalanine as indicators of myofibrillar and total protein breakdown respectively, these researchers found increases in myofibrillar protein degradation in heavily activated muscle, while finding no changes

in total protein breakdown.

The present study uses a similar in vitro muscle preparation in order to isolate the effects of contractile activity on myofibril alterations, however, investigates the problem in a qualitative fashion by using gel electrophoresis (SDS-PAGE) and electron microscopy procedures. This study is aimed at identifying any compositional or morphological change in the contractile apparatus of skeletal muscle as a result of fatiguing isometric contractions.

A. Purpose

The purpose of this study is to investigate morphological and compositional changes in skeletal myofibrils which may result from a fatiguing bout of isometric contractions.

B. Limitations

a) the incubation medium cannot take the place of true physiological plasma

b) the model relied solely on diffusion rather than a normal capillary network for oxygen supply

c) the time between the dissection and the freezing or incubation of the muscle averaged 140 ± 14.9 sec.

d) the resting clip for the incubated-only group and the resting tension which the incubated-stimulated group was placed under may not have been similar, and they may not have been representative of the resting tension in vivo.

e) the stimulus used to induce the muscle contractions was not physiological.

C. Delimitations

a) only male Sprague-Dawley rats between 115-127 gms. were used for this investigation

b) the muscles of only 14 rats were used for this study

c) only isometric-type contractions were used to fatigue the muscles

d) insulin, glucocorticoids, and thyroxine are not included in the incubation medium.

D. Definition of Terms

calpain (calcium-activated neutral protease) - a protease endogenous to skeletal muscle cells which requires calcium for its activation, and is known to degrade only certain components of the contractile apparatus (Baker et al. 1987).

epitrochlearis - a small thin muscle found in rats, arising from the tendon of insertion of the latissimus dorsi muscle and inserting into the medial epicondyle of the humerus (Wallberg-Henriksson 1987).

exhaustion - extreme weakness or fatigue (Random House College Dictionary 1982).

isometric contraction - muscle contraction without appreciable shortening or change in distance between its origin and insertion (Miller and Keane 1983).

muscle fatigue - the transient decrease in the performance capacity of muscles when they have been active for a certain period of time, evidenced by a failure to maintain or develop a certain expected force or power

(Asmussen 1979).

myofibril - bundles of myofilaments (within a muscle fibre) corresponding to the smallest structures within the contractile material which can be visualized using a light microscope (Schmalbruch 1985, p. 37).

protease - any enzyme that catalyzes the splitting of interior peptide bonds in a protein (Miller and Keane 1983).

proteolysis - the splitting of proteins by hydrolysis of the peptide bonds, with formation of smaller polypeptides (Miller and Keane 1983).

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis (Murakami and Uchida 1985).

CHAPTER II - REVIEW OF LITERATURE

A. Skeletal Muscle and the Protein Composition of Myofibrils

Vertebrate skeletal muscle fibres are single, multinucleate, membrane-bound cells, typically 10 μm in diameter and several centimetres or longer in length. They originate from smaller cells called myoblasts that merge before birth to form myotubes prior to their full differentiation (Lowey 1986).

Myofibrils are cellular organelles which are enveloped by sarcoplasmic reticulum and constitute almost 80% of the skeletal muscle fibre. Myofibrils are long cylindrical structures approximately 1 μm in diameter, possessing transverse striations. The sarcomere constitutes the fundamental unit of the myofibril, and is composed of filaments which comprise the different bands (I, H, and A) and disks (Z and M) found

of 20,000 daltons. Myosin can be fragmented by proteolytic enzymes such as trypsin which breaks the molecule into heavy meromyosin (HMM) and light meromyosin (LMM), and by papain, which breaks HMM into a globular subunit S_1, and a helical rod S_2. HMM- S_1 can be considered the most important part of the myosin molecule, as it contains the sites for the ATPase and for binding to actin.

Actin is the second major muscle protein, comprising approximately 25% of total myofibrillar protein. It is the most strongly conserved muscle protein, being present in both fast and slow skeletal muscles in identical forms (Perry 1985). Actin has a molecular weight of 42,000 daltons, and is present in muscle cells predominantly in the form of fibrous or F-actin, the polymerized form of G-actin.

Tropomyosin comprises between 5-10% of the total myofibrillar protein, and has a molecular weight of about 64,000 daltons. The molecule is a dimer, composed of two α-helical subunits which stretch over seven actin monomers. It lies within the grooves of the actin double helix. Tropomyosin plays a regulatory role in muscle contraction, being essential for Ca^{2+}-sensitivity of the MgATPase of actomyosin in the presence of troponin. Different forms of tropomyosin are associated with fast and slow muscle fibres (Perry 1985).

The components of the troponin complex comprise a complex of about 80,000 daltons. Troponin -C has a molecular weight of about 17,000-18,000 daltons, and functions to specifically bind Ca^{2+}. Troponin -I has a molecular weight of 20,000-24,000 daltons, binds to both actin and troponin-C, and inhibits the ATPase of myosin. Troponin-T has a molecular weight of 30,000-35,000 daltons. The protein binds to tropomyosin and interacts with the other proteins of the troponin complex. All three troponin proteins exist as isoforms in different types of skeletal muscle (Perry 1985).

C-protein is present along the middle portion of the myosin filament. It is a monomer containing a single non-coiled polypeptide chain of molecular weight 140,000 daltons. Antibody labelling has shown this protein to be located in the middle one third of each half of the A band, in seven narrow stripes approximately 43 nm apart. The function of C-protein, although unknown, has been postulated to be purely structural. C-protein may stabilize the organization of the myosin molecules by wrapping around the backbone of the thick filament (Schmalbruch 1985, Craig 1986).

Several M-line proteins have also been described. The M-line is a narrow transverse band located at the centre of the A band. It has been suggested that the M-line maintains the orientation of the thick filaments in relation to each other (Schmalbruch 1985, Craig 1986). M-creatine kinase has been shown to be a structural component of the M-line. This protein is a dimeric globular protein which consists of two subunits of 43,000 daltons. Two other M-line components have been identified, including a 165,000 dalton protein (M-protein) and a 185,000 dalton protein (myomesin) (Grove et al. 1984). Myomesin may act as a specific marker during the differentiation of myoblasts into myotubes and muscle fibres (Eppenberger et al. 1981).

Connectin, or titin, is an extensible and flexible protein with a molecular weight over 2 million daltons. This protein occurs as a doublet, and interacts with both actin and myosin, forming a net which is concentrated at the A-I junction of the myofibril. Its elastic properties contribute to the passive tension generated upon stretch of the muscle. It is thought that connectin connects the thick filaments to the Z-disks, thus keeping the thick filaments centred between the Z-disks (Alberts et al. 1989). It constitutes 8-12% of the myofibrillar mass.

Nebulin is another myofibrillar protein, and has been identified as a component

of the N-line. The N-line can be observed as an ill-defined transverse stripe within the I band. Nebulin has a molecular weight of about 600,000 daltons (Schmalbruch 1985).

The Z-disk of the skeletal muscle myofibrils plays a vital role in the contractile process by transmitting the tension developed by the interaction of the thick and thin filaments. The Z-disk functions as a point of attachment for thin filaments of adjacent sarcomeres, and serves to organize the filaments laterally into a regular pattern for proper interdigitation with the thick filaments. Schmalbruch (1985) provides a good account of the complex structure of this region. Despite extensive study of Z-disk ultrastructure, the composition and structural significance of the Z-line remains poorly understood. There have been two proposed models of the configuration of the Z-filaments (Takahashi and Hattori 1989). In addition, two distinct lattice forms have been described within the Z-disk (Edwards et al. 1989).

It is generally accepted that the Z-disk is composed of two phases, Z-filaments and an unstructured matrix component (Chowrashi and Pepe 1982, Ohashi and Maruyama 1989). The Z-filament phase forms the structural backbone of the Z-disk. It is located in the interior of the Z-disk and is made up of the protein α-actinin. α-actinin is a rod-like protein dimer consisting of two 95,000-dalton peptide chains, and is thought to connect the thin filaments of adjacent sarcomeres. Another protein, named Z-protein, has been localized in the filamentous phase by some (Ohashi and Maruyama 1989) and in the amorphous phase by others (Takahashi and Hattori 1989). It is a 55,000-dalton polypeptide chain which tends to migrate as a dimer in SDS-PAGE experiments.

Several other proteins have been identified in the periphery of the Z-disk, constituting the unstructured matrix component. Amorphin is a 85,000-dalton protein which immunolocalizes at the Z-disk and is believed to account for the amorphous

component of the Z-disk (Chowrashi and Pepe 1982). Eu-actinin is another Z-line protein, having a molecular weight of 42,000 daltons. It has been reported to bind to both actin filaments and α-actinin (Kuroda et al. 1981), and may aid in reinforcement of the binding of α-actinin to the thin filaments.

Intermediate filament proteins such as desmin, vimentin, and synemin have also been observed in the periphery of the Z-disk, and are believed to mechanically integrate the contractile actions of the muscle fibre. It is postulated that these filaments link adjacent myofibrils laterally at their Z-disks and also link the Z-disks to the plasma membrane and other membranous organelles (Lazarides et al. 1981). Desmin has a molecular weight of 55,000 daltons; vimentin, approximately 52-55,000 daltons; and synemin, 230,000 daltons (Schmalbruch 1985). Filamin, another protein affiliated with the outer circumference of the Z-band of mature myofibrils, has a molecular weight of 250,000 daltons. It is also believed to play a role in the maintenance of myofibrillar organization during contraction stresses. Table 1 presents the major proteins of the myofibril.

The proteins constituting the myofibril can be separated by the technique of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). In basic terms, isolated myofibrils are solubilized in a solution containing a powerful negatively-charged detergent (sodium dodecyl sulphate) and a reducing agent (mercaptoethanol). The detergent binds to hydrophobic regions of the protein molecules and causes them to unfold, releasing them from their associations with other protein or lipid molecules. The reducing agent serves to break any disulphide linkages in the proteins, allowing for the separate analysis of individual polypeptides (subunits) which constitute multisubunit molecules. The solubilized myofibrils are then electrophoresed through a slab of

Table 1 Major Proteins of the Myofibril

Protein	Subunit Structure	Approx. Molecular Weight (daltons)	Approx. % of Total Myofibrillar Protein
Actin	nG-actin	42,000	22%
α-Actinin	2 chains	95,000(2)	1%
Amorphin	single chain	85,000	
C-Protein	single chain	140,000	1%
Connectin (Titin)	2 chains	1,400,000 1,200,000	8-12%
Desmin	single chain	55,000	
Eu-Actinin	single chain	42,000	
Filamin	2 chains	250,000	
M-Creatine Kinase	2 chains	43,000(2)	
M-Protein	single chain	165,000	
Myomesin	single chain	185,000	1%
Myosin	2 heavy chains	200,000(2)	44%
	4 light chains	16,000-27,000(4)	
Nebulin	single chain	600,000	3%
Synemin	single chain	230,000	
Tropomyosin	2 chains	32,000(2)	5%
Troponin-C	single subunit	17,965	
Troponin-I	single subunit	20,864	5%
Troponin-T	single subunit	30,000-35,000	
Vimentin	single chain	52,000-55,000	
Z-Protein	single chain	55,000	

Adapted from: Schmalbruch (1985), Craig (1986), Lowey (1986), Alberts et al. (1989), and Ohashi and Maruyama (1989).

polyacrylamide gel, which serves to separate the proteins on the basis of size, or molecular weight. The major proteins can subsequently be detected by staining with a dye such as Coomassie Blue. For a more detailed description of the SDS-PAGE procedure, the reader is referred to Gordon (1973).

A large number of investigations have utilized SDS-PAGE in the study of myofibrillar proteins. Belcastro et al. (1988) and Snyder et al. (1984) have looked at myofibrillar protein loss with exercise using this technique. In addition, numerous other researchers have utilized SDS-PAGE in the study of skeletal myofibrils, including Mikami et al. (1990), Murakami and Uchida (1985), Baumann et al. (1984), Sender et al. (1971), Gutierrez et al. (1990), and Busconi et al. (1987).

It is important to note, however, that the SDS-PAGE procedure is not without problems. The precise identification of certain proteins by one-dimensional discontinuous SDS-PAGE may be subject to inadequacies for several reasons. Baumann et al. (1984) suggest that the classical Laemmli method results in incomplete resolution. These authors suggest that modifications be made to this standard method such as substituting L-isoleucine for the conventional glycine in the electrophoretic buffer, and utilizing a modified silver stain method. Another possible complication in the precise identification of specific myofibrillar proteins is the apparent variability in published molecular weights found within the literature. This may be explained by species-specific differences, and by the existence of various protein isoforms found within different muscle types (Murakami and Uchida 1985), fibre types (Baumann et al. 1984, Schachat et al. 1985, 1988), and even within the same muscle fibre (Schiaffino et al. 1989). Furthermore, the heterogeneous nature of the myofibrillar proteins makes it difficult to discriminate between degradation products and the presence of additional proteins.

Problems in this regard may be circumvented by the generation of monoclonal antibodies against certain protein fractions.

Electron microscopy is another frequently used technique which has been used to identify alterations in skeletal myofibrils. This technique has been utilized to assess morphological alterations resulting from a number of experimental conditions, including exercise (George et al. 1987, Belcastro et al. 1988, Ogilvie et al. 1988), incubation (Casademont et al. 1988), electrical stimulation (Gonzalez-Serratos et al. 1978), and acute muscle trauma (Fisher et al. 1990). Some of the significant morphological alterations which have been observed in skeletal muscle under these conditions include the following: fragmentation of myofibrils, disorganization of myofibrils, appearance of clear vacuoles, dilatation of internal membrane systems, loss of Z-lines, Z-line streaming, mitochondrial loss, presence of autophagic vacuoles, appearance of lipofuscin bodies, disordered sarcomeric alignment, presence of membranous whorls, presence of polymorphonuclear leucocytes, and disruptions of the A-band region. The presence of such alterations in skeletal muscle morphology will be investigated in the present study.

B. Fatigue

The many factors which are currently thought to be involved in the process of muscle fatigue will not be considered here in detail, but only mentioned in a cursory manner. Several recent reviews on this topic may be referred to for more detailed presentation (Fitts and Metzger 1988, Vollestad and Sejersted 1988, Maclaren et al. 1989, Roberts and Smith 1989).

Muscle fatigue may be defined as the transient decrease in performance capacity of muscles when they have been active for a certain period of time, evidenced by a

failure to maintain or develop a certain expected force or power (Asmussen 1979). Extreme fatigue is termed exhaustion. Muscle fatigue may result from detrimental alterations in the muscle itself, termed peripheral fatigue, or from alterations in the properties of the central nervous system proximal to the motoneurons, termed central fatigue. Briefly, central fatigue involves afferent impulses arising from muscle receptors, which may inhibit at various sites along the motor pathway. Peripheral fatigue, on the other hand, may be involved with impaired neuromuscular transmission, excitation-contraction uncoupling, or contractile element failure (Belcastro et al. 1985). The specific etiology of muscle fatigue is dependent on a number of important variables, including the intensity and duration of exercise, the muscle fibre type composition, the individual's level of fitness, and to a number of environmental factors (Fitts and Metzger 1988). It has also been postulated that the onset of muscle fatigue may be due to an accumulation of metabolites causing impairment of force generation, or from a depletion of certain metabolites necessary for the contractile process (Maclaren et al. 1989). A build up of a number of metabolites has been implicated in the failure of muscle to generate force, including sodium, hydrogen ions, ammonia, inorganic phosphate and extracellular potassium. Calcium and sodium accumulation in the transverse tubules have also been implicated in the fatigue process (Gonzalez-Serratos et al. 1978, Bianchi and Narayan 1982). Depletion of the energy substrates ATP, PCr and glycogen, as well as phosphate depletion, may also impair muscle contraction (Vollestad and Sejersted 1988).

Muscle fatigue resulting from eccentric-type work may be due to factors which differ from those influencing concentric and isometric contractions. Some researchers believe that the muscular fatigue observed in eccentric work is a result of mechanical

damage rather than the metabolic processes associated with muscular contraction (Roberts and Smith 1989). Eccentric contractions result in greater forces per active cross-sectional area than concentric contractions, since tension produced in eccentric-type work is distributed over a smaller number of active fibres (Armstrong et al. 1983, McCully and Faulkner, 1985). This tension is believed to be great enough to cause disruption of the Z-disk, causing a disorganization of the contractile proteins. The concomitant loss of force is larger than is seen with an equal amount of concentric work (Roberts and Smith 1989). Eccentric muscle actions have been shown to produce a greater amount of heat than concentric contractions at similar workloads, and the resultant high temperatures may damage certain proteinaceous elements within the muscle cell (Ebbeling and Clarkson 1989).

The action of proteolytic enzymes has also been hypothesized to be a contributing factor to the process of muscular fatigue. Belcastro et al (1985) have suggested that a leaky sarcoplasmic reticulum (S.R.) membrane and the subsequent uncoupling of the S.R.-ATPase activity and Ca^{2+} transport may be due to the action of proteolytic enzymes. Furthermore, it has been postulated that proteolytic disruption of the Z-band region concomitant with increased intracellular calcium levels may be responsible for the observed decrements in force transmission seen in exhaustively-exercised rats (Belcastro et al. 1988). These findings provide an interesting basis for further investigation.

C. The Process of Protein Turnover

All cells in a living system have the capability to degrade the proteins which reside within. The process of protein turnover within all living cells is continuous and ongoing (Beynon and Bond 1986). Two distinct systems exist within cells to degrade

proteins, and the relative contribution of each system to overall protein degradation is open to debate. The lysosomal system is a well characterized locus for proteolytic activity (Jahoor and Wolfe 1987, Gerard et al. 1988). Literature suggests that it is responsible for degradation of extracellular proteins entering the cell by endocytosis, and also for breakdown of long-lived proteins which have been denatured or marked for breakdown (Gerard et al. 1988). The lysosome possesses an array of hydrolytic enzymes which are termed cathepsins (Jahoor and Wolfe 1987). Nine cathepsins have been identified to date; cathepsins B, H, I, L, N, S, and T have cysteine residues at their active sites and are referred to as cysteine proteases. Cathepsins D and E have aspartic residues at their active sites and are thus termed aspartic proteases (Beynon and Bond 1986). Despite the existence of relatively few lysosomes within skeletal muscle, the cathepsins have been consistently observed in muscle preparations. It has been shown that purified cathepsins B, D, H, and L are capable of degrading purified actin and myosin in vitro to a limited degree, however, research suggests that lysosomes are not directly involved in the breakdown of myofibrillar proteins (Lowell et al. 1986).

The lysosome may function by two distinct mechanisms (Jahoor and Wolfe 1987). By fusion of an autophagic vacuole with the lysosome surface, the sequestration and subsequent digestion of matter may occur. This process is dependent upon the formation of autophagic vacuoles (autophagosomes), which are formed by the process of autophagy, the internalization of cytosolic material into a membranous structure. The process of autophagy is believed to be mediated by extracellular signals which may be hormonal in nature, and thus may potentially be influenced by exercise. Another mechanism by which material may enter the lysosomal compartment is through the process of microautophagy. By this process, proteins may be directly internalized in the

absence of autophagic vacuoles (Beynon and Bond 1986, Parkhouse 1988).

Both lysosomal processes may require that proteins possess specific structural characteristics, or undergo specific conformational changes in order to facilitate uptake. The exposure of hydrophobic regions of a protein to autophagosomal or lysosomal membranes may be a necessary prerequisite for such processes to take place (Jahoor and Wolfe 1987). Changing metabolic states such as occurs with exercise (Takekura and Yoshioka 1988) may influence conformational changes in protein structure and may serve to regulate lysosomal proteolysis.

Non-lysosomal proteolysis also plays a role in the degradation of cellular proteins (Pontremoli and Melloni 1986). Literature suggests that the functionality of this system lies in the breakdown of short-lived and abnormal proteins, and is representative of a specialized system of protein removal (Beynon and Bond 1986). Cytosolic proteases are poorly characterized relative to the lysosomal cathepsins, however, they have been the focus of much research in recent years and many new cytosolic proteases and peptidases have been discovered over the past decade (Hershko 1988, Rivett 1989). Cytosolic proteases tend to demonstrate greater specificity in their proteolytic actions, and are often accompanied intracellulary by inhibitors and activators which may provide additional means for regulation. The best characterized of the cytosolic proteases are calcium-activated neutral proteases (calpains) (Murachi 1985, Suzuki et al. 1987), and an ATP-dependent protease (Pontremoli and Melloni 1986, Hershko 1988). More recently, a high molecular weight protease has also been characterized within the cytoplasm (Rivett 1989).

Much heterogeneity exists in the turnover rates of proteins. This great heterogeneity suggests that a system must exist to impart a component of selectivity to

the proteolytic process. Literature suggests that the structural properties of the protein substrates may be a major determinant of the turnover rate (Beynon and Bond 1986). Certain structural parameters have been postulated to increase a cell's vulnerability to the degradative process, the result being a higher rate of turnover. In general, long-lived proteins tend to be molecules with a basic isoelectric point, small subunits, low surface hydrophobicity, and low carbohydrate content, whereas short-lived cytoplasmic proteins have the tendency to be acidic, have large subunits, considerable surface hydrophobicity and high carbohydrate content (Dahlmann et al. 1984, Jahoor and Wolfe 1987). The specific functions of the protein have also been postulated to be a factor in determining turnover rate. For example, enzymes that have a regulatory role in controlling flux through a metabolic pathway tend to have higher turnover rates. This allows for precise control over metabolic flux (Beynon and Bond 1986). In addition, dark (slow-twitch) muscle fibres have been found to possess higher rates of protein turnover than pale (fast-twitch) fibres (Millward 1985).

D. Exercise and Protein Turnover

The degradation of whole body protein during exercise has been consistently found by a number of researchers (Dohm et al. 1985, Kasperek and Snider 1985). The net protein breakdown observed with endurance exercise is predominantly due to increases in liver protein degradation. Recent work suggests that the proteolytic activity accompanying this type of exercise is a result of autophagy, as revealed by increased numbers of autophagic vacuoles in liver (Dohm et al. 1987). The liver protein degraded during endurance exercise is made available for increased rates of oxidation and gluconeogensis to help meet the energy demands of sustained activity (Viru 1987,

Poortmans 1988).

Exercise has been shown to induce dramatic change in the metabolic profile of rat skeletal muscle (Takekura and Yoshioka 1988) and it is likely that such lability in metabolic status such as exists with exercise may be associated with strict regulation of protein turnover rates within muscle. The precise regulation of proteolysis in skeletal muscle is extremely vital, as this component comprises the body's major reservoir of amino acids. Metabolic control of muscle turnover is essential for the maintenance of overall energy homeostasis, control of muscle mass and body growth, and adaptations to a variety of environmental and physiological conditions (Viru 1987).

Despite the fact that protein metabolism in exercise has been investigated for many years, a firm consensus on whether or not exercise induces an increased rate of skeletal muscle protein breakdown has not been reached (Booth and Watson 1985). Inconsistencies within the literature may be due in part to specific variables within the protocol such as degree of training, previous diet, age of subjects, in vitro versus in vivo models, as well as the indices used for the determination of protein turnover. Urinary 3-methyl histidine is often used as a quantitative measure of myofibrillar protein degradation in vivo (Forsberg and Liu 1989), but the use of this as a valid measure has been questioned by some due to contributions of this amino acid from non-muscle sources (Rennie and Millward 1983). In addition, the type, duration, and intensity of exercise, all important variables, are not standardized methodologically throughout the literature. Thus, difficulties arise in comparing experimental results derived from variable exercise conditions. However, it has been generally accepted that changes in the relative amounts and types of protein within muscle occur in response to external stimuli (Booth and Watson 1985, Kasparek and Snider 1989). Whether or not this is a result of

changes in synthesis or degradation or a combination of both has yet to be firmly established, but it appears evident that the type and frequency of stimulus is determinative (Booth and Watson 1985, Dohm et al. 1987).

Research has shown that protein synthesis is suppressed in muscle during activity (Millward et al. 1982, Dohm et al. 1985). Literature suggests that the intensity and duration of the exercise bout determines tne extent of the suppression in synthesis (Booth and Watson 1985). An energy shortage within fatigued muscle cells, especially within fast glycolytic fibers (Lieber and Friden 1988) is believed to be responsible for this observation (Viru 1987). Glucocorticoid levels, which rise concomitant with high intensity exercise (Galbo et al. 1977) have also been implicated in the inhibition of protein synthesis with exercise (Viru 1987).

Some research has suggested that myofibrillar degradation is decreased during an exercise bout (Rennie et al. 1980) and that the response during recovery is dependent upon exercise intensity. Following exhaustive exercise, rates of muscle protein breakdown have been found to increase, as measured by tyrosine release from incubated rat muscles (Dohm et al. 1980, 1987). Degradation of approximately 5% of the total number of muscle fibres has been shown to occur in skeletal muscle about 1-2 days post-exhaustive exercise in untrained rats (Booth and Watson 1985). The increased degradation of skeletal muscle protein during recovery is concomitant with increased rates of protein synthesis (Laurent et al. 1978). The observed increase in protein turnover rate may be considered instrumental in the renewal of structural integrity and improved functioning of the contractile elements which constitute the myofibrils. This process provides a basis for certain training regimens, in that it serves to eliminate compromised proteinaceous elements within the muscle cell, thereby permitting

improvements in contractile function (Viru 1987).

On the other hand, others have provided evidence of myofibrillar protein degradation during fatiguing exercise. Belcastro et al. (1988) found decreases in the relative proportions of 58 Kda and 95 Kda proteins as determined by gel electrophoresis following exhaustive treadmill running. These proteins were believed to be desmin and α-actinin, which constitute an integral part of the Z-line structure of muscle cells. It was hypothesized that the loss of these cytoskeletal proteins and the concomitant dissolution of the Z-line structure may contribute to the decreased force transmission observed with fatigue. Nie et al. (1989), observed an increase in 3-methyl histidine release from an isolated muscle preparation which was stimulated to contract. The authors concluded that myofibrillar protein degradation was increased in heavily activated muscle (Nie et al. 1989), however it is difficult to determine whether the observed increase in net degradation was a result of increased degradation or decreased synthesis or a combination of both.

E. Modulators of Muscle Protein Turnover

The regulation of protein degradation within skeletal muscle has been the focus of ongoing study. The observed heterogeneity in protein halflives within cells has led to postulation that there exists a system by which certain proteins are marked or branded for the degradative process (Beynon and Bond 1986, Parkhouse 1988). It has been suggested that post-translational covalent modifications may determine protein halflives by decreasing the stability of native protein molecules, thus increasing their susceptibility to proteolytic attack. Some examples of post-translational modifications which may serve as mediators in this regard are phosphorylation, deamination, ubiquitin conjugation,

formation of glutathione-protein mixed disulphides, and other forms of oxidation (Beynon and Bond 1986). It is conceivable that phosphorylation and oxidation (mixed disulphide formation) processes contribute a regulatory component to protein breakdown during exercise, since the potential for both of these processes changes with exercise (Parkhouse 1988). It is also probable that non-lysosomal proteolytic pathways mediate proteolytic action in response to exercise-induced metabolic changes; the ATP-dependent ubiquitin conjugate system may exert its regulatory influence in response to changing intracellular energy levels (Hershko 1988, Kettelhut 1988), and the calcium-activated proteolytic system may do likewise in accordance with calcium levels within the cytosol (Murachi 1985).

In eukaryotic cells, oxidation of cysteine residues may cause destabilization of proteins (McKay and Bond 1985). Cytosolic enzymes can be inactivated by thiol-disulphide exchange processes, such inactivation corresponding to rates of degradation in vivo (Beynon and Bond 1986). It is hypothesized that the formation of disulphides, such as glutathione disulphides, serve to destabilize protein structure and thus increase their susceptibility to proteases (McKay and Bond 1985, Tischler et al. 1985). Proteolytic rates have been found to be intimately related to NADH/NAD^+ ratios. Comparison of redox state and protein breakdown in incubated muscle shows that protein degradation is faster as muscle becomes more oxidized (Tischler et al. 1985). A thioltransferase has been found in muscle which catalyses the formation of protein-glutathione mixed disulphides (Isaacs and Blinkley 1977). Oxidized glutathione, formed under oxidized conditions, may oxidize free sulphhydryl groups on proteins by the action of this transferase, resulting in the formation of mixed disulphides and decreased conformational stability. This may lead to the exposure of hydrophobic regions within the protein molecules, enhance the

aggregation and absorption of the proteins into intracellular membranes, and eventually lead to their degradation by fusion of the autophagosome with lysosomes (Khairallah et al. 1985). Likewise, the binding of the marked protein directly to the lysosomal membrane (microautophagy) may also provide a measure of selectivity to the proteolytic process (Beynon and Bond 1986).

Exercise likely influences the oxidation state of muscle cells in different ways. Strenuous exercise serves to increase the $NADH/NAD^+$ ratio, resulting in a more reduced state (Millward 1985). From this perspective, it would appear unlikely that proteins would be oxidized and marked for degradation during strenuous exercise by this type of reaction. This mechanism may help to explain the findings by some that muscle protein degradation occurs predominantly post-exercise when the cell returns to resting redox levels. However, it fails to explain myofibrillar degradation during exercise (Belcastro et al. 1988), which may be regulated through a different pathway.

In addition, exercise may influence redox state by an alternate mechanism. Through hormonal influences concomitant with exercise (Galbo 1986, Richter 1986, Kettelhut et al. 1988), adenylate cyclase is activated and cAMP levels increase. Increased cAMP levels have been shown to decrease thiol disulphide ratios in rat liver, resulting in a more oxidized state and thus increased protein breakdown (Isaacs and Blinkley 1977, Hopgood et al. 1980). This may account for the increased lysosomal activity found in liver with prolonged exercise (Booth and Watson 1985).

The phosphorylation of cell proteins may also be regulated through hormone-mediated cAMP production. Increases in cAMP levels due to glucagon and catecholamine release concomitant with exercise may serve to phosphorylate cell proteins by a cAMP-dependent protein kinase intermediate (Parkhouse 1988). This may provide

an additional means of regulation of protein turnover with exercise, and along with increased oxidation levels may account for increased lysosomal activity in liver with exercise (Beynon and Bond 1986).

ATP dependence is a feature of certain cytosolic degradative pathways, and may provide additional regulatory control under varying physiologic and metabolic conditions (Kettelhut et al. 1988). Proteolytic systems possessing an absolute requirement for ATP may be functionally important in explaining the variability in protein breakdown observed in exercise and fatigue states. The ATP-dependent ubiquitin conjugate proteolytic system has been described as a ligase system consisting of several proteins (Hershko 1988). The covalent conjugation of ubiquitin, a small molecular weight protein, to the substrate acts to brand the complex for degradation. The conjugation is an energy-requiring process, and is an obligatory intermediary step in the breakdown of the protein. Specific proteases exist which recognize and degrade only ubiquitin-conjugated substrate (Etlinger et al. 1985, Hershko 1988). If the ATP-dependent ubiquitin system does function to regulate proteolysis in muscle cells, then it would provide an explanation for the delay in myofibrillar breakdown post-exercise which has been reported by some researchers.

An additional proteolytic system which has been found to exist in the cytosol and likely plays a role in myofibrillar protein degradation are the calcium-dependent proteases, or calpains (Murachi 1985). These highly specific heterodimeric proteases have been found to exist in two forms in skeletal muscle, a millimolar calcium-requiring form and a micromolar calcium-requiring form. Though the millimolar-requiring form is not thought to be functional at physiological concentrations of calcium, it has been postulated that an autoproteolytic event occurs, resulting in an enzyme with increased

calcium sensitivity (Suzuki et al. 1987). Calpains are not thought to be involved in bulk hydrolysis of cellular proteins, but have been implicated in the initiation of degradation of myofibrillar proteins by disassembly of the proteins from their organized structure within the myofibril (Kulesza-Lipka and Jakubiec-Puka 1985, Perides et al. 1987). Loss of intact Z-lines which have been found following exhaustive exercise may be a result of calpain action through activation by high intracellular calcium concentrations (Belcastro et al. 1988). High intracellular levels of calcium may result from repetitive contractions which may serve to effect impairment of the calcium transport system concomitant with fatigue (Belcastro et al. 1985, 1988).

F. Protein Turnover and Hormonal Influences

Thyroid Hormones

Thyroid hormones such as thyroxine (T_4) or triiodothyronine (T_3) also provide a means by which muscle proteolysis is regulated (Demartino and Goldberg 1978, Millward 1985). Thyroid hormones regulate both synthesis and degradation of muscle proteins, causing increased rates of both processes (Kettelhut et al. 1988). These hormones have been found to possess different dose-response relationships on protein synthesis and breakdown, and precise levels are required for the normal growth of muscle and other tissues. High levels of thyroid hormones result in a net catabolic state, since degradation rates appear to increase more than rates of synthesis. Literature suggests that thyroid hormones enhance muscle protein breakdown by activation of both lysosomal and non-lysosomal pathways, including ATP-dependent ubiquitin (Demartino and Goldberg 1978, Millward 1985) and calpain-mediated (Zeman et al. 1986) proteolytic processes. It has

been suggested that a common chronic adaptation of muscle to disease is a fall in protein turnover as regulated by decreased levels of thyroid hormones (Morrison et al. 1988).

In spite of much research, the influence of physical exercise on thyroid function has not been thoroughly defined. Literature suggests that plasma concentrations of T_4 and T_3 are essentially unchanged during a single bout of exercise, however, prolonged exercise at a high intensity may effect an increase in T_4 levels (Galbo 1986). Detection of changes in thyroid hormone resulting from acute exercise bouts are difficult to assess, and evidence suggests that several days of increased physical activity may be required for detection. Physical training changes do not appear to affect secretion of thyroid hormones (Galbo et al. 1977). During exercise, hyperthyroid individuals exhibit higher core temperatures and hypothyroid individuals lower core temperatures, than euthyroid subjects. This indicates that thyroid status plays a thermoregulatory role during exercise, which may in itself have ramifications for protein turnover rates, as elevations in temperature have been shown to promote degradation (Baracos et al. 1984).

Glucocorticoids

Glucocorticoids have been shown to markedly influence muscle protein turnover (McGrath and Goldspink 1982, Simmons et al. 1984). Glucocorticoid administration tends to effect a decrease in the synthesis of myofibrillar proteins (Rouleau 1987, Kettelhut 1988) by reducing amino acid uptake by muscle, and by decreasing DNA and protein synthesis. Though the majority of literature substantiates a role for glucocorticoids in the stimulation of protein degradation, results vary depending on the muscle fibre type and nutritional status studied. Glycolytic fibres tend to be more

sensitive to the catabolic effects of adrenal steroids than oxidative fibres (Seene and Viru 1982, Dahlmann et al. 1986). Different results have also been reported with different nutritional states. In the fed state, large doses are required to effect increases in degradation. However, in a starved state, glucocorticoids have been shown to play a critical role in the promotion of muscle protein breakdown. This process is physiologically crucial in the gluconeogenic process. Simmons et al. (1984) have found that administration to human subjects of physiological levels of cortisol causes an increase in proteolysis, and that with prolonged chronic administration an adaptive response occurs where the proteolytic increase is found to drop. An explanation for this decrease in proteolysis, as well as for the variable results obtained within different nutritional states, points to a possible regulatory role of insulin in muscle response to steroids. If insulin levels are high, proteolysis will decline, indicating a possible requirement for low insulin levels concomitant with adequate glucocorticoid levels in order to effect a proteolytic increase (Smith 1988). It has been shown that with prolonged fasting, glucocorticoid levels tend not to show a continuous increase, but rather demonstrate a plateau effect over time. As plasma insulin levels decline in the fasted state glucocorticoids may impart their regulatory influence in relation to these levels (Smith 1988).

Literature supports the role of a non-lysosomal pathway in glucocorticoid-induced myofibrillar proteolysis (Lowell et al. 1986, Kayali et al. 1987). It has been suggested that its catabolic action may be exerted through either an ATP-dependent (Kettelhut et al. 1988) or a calcium-activated pathway (Kayali et al. 1987).

Insulin

Insulin is a major mediator of muscle protein turnover, and is intimately involved in the physiological regulation of protein balance in skeletal muscle (Davis and Karl 1986). High insulin levels have been found to promote net accumulation of cell protein within muscle, whereas low levels of insulin have been associated with a net breakdown of muscle protein (Goodman 1987). Insulin appears to have several anabolic actions on muscle, making it essential for normal muscle growth. It is widely reported to enhance protein synthesis by promoting the net uptake of amino acids and their incorporation into protein in vitro (Jefferson et al. 1974, Goldberg et al. 1980, Kettelhut et al. 1988). Interestingly, recent studies demonstrate that insulin stimulates the synthesis of myofibrillar proteins preferentially to cytosolic or sarcoplasmic proteins in vitro (Kettelhut 1988).

Insulin has also been found to inhibit protein degradation. In eviscerated rats, it has been demonstrated that physiological levels of insulin effect a decrease in protein degradation as measured by tyrosine release (Simmons et al. 1984). Such studies attribute the anabolic effects solely to insulin, and not to hyperglycemic effects. It appears that insulin regulates protein degradation through the lysosomal pathway. Some studies suggest that insulin prevents alterations in lysosomal enzyme distribution, preventing fusion of autophagic vacuoles with primary lysosomes (Demartino and Goldberg 1978). In perfused heart, withdrawal of insulin has been found to cause appearance of autophagic vacuoles as shown by electron microscopy (Jefferson et al. 1974). Findings suggest that actin is degraded solely by a non-lysosomal system (Lowell et al. 1986), and if this were true in an absolute sense it would follow that insulin would have no direct affect on myofibrillar protein degradation. Goodman et al. (1987) have

demonstrated this by refeeding starved rats a diet of carbohydrates and finding that this treatment does not spare the degradation of the myofibrillar pro- i...

G. The In Vitro Model

Both in vivo (Barrett et al. 1989) and in vitro techniques have been utilized in investigations of protein turnover (Hasselgren et al. 1988). In vitro methods offer several advantages over in vivo models in the study of muscle protein synthesis and degradation, including simplicity of technique, maintenance of strict control of substrate and hormone concentrations, and elimination of complicating interactions with other tissues (Kettelhut et al. 1988). Two experimental approaches have been widely used in the in vitro study of protein degradation, including incubated rat muscles (soleus, extensor digitorum longus, diaphragm, or epitrochlearis) and perfused sections of rat (hindlimbs, hindquarters, or hearts). Generally, isolated muscle preparations tend to be in a highly catabolic state, with the rate of protein synthesis exceeded by the overall rate of protein degradation. Research has shown that rates of protein synthesis may be 50-78% less in an in vitro model than in vivo (Baracos et al. 1989). By varying the incubation conditions, it is possible to lower overall rates of degradation and increase the rates of synthesis. Such conditions include incubating muscles in the presence of glucose, insulin, and amino acids (Fulks et al. 1975), maintaining in situ resting length (Baracos and Goldberg 1986, Hasselgren et al. 1990), controlling incubation temperature (Essig et al. 1985, Segal and Faulkner 1985) and continuous oxygenation of the medium (Baracos et al. 1989). Muscles with a large surface area relative to their weight are preferable for in vitro study, as incubated muscles must rely on diffusion for a supply of oxygen. Segal and Faulkner (1985) found that muscles which exceeded the critical radius demonstrated

development of anoxic cores, concomitant with large increases in extracellular space, abnormal reductions in ATP concentrations, and significant impairment of contractile function.

The rat epitrochlearis muscle has been utilized for metabolic studies by several researchers (Stirewalt and Low 1983, Wallberg-Henriksson 1986, Nesher 1980 a,b, Nie 1989 a,b,c, Zetan 1989). It has been demonstrated to be a suitable intact mammalian muscle preparation for in vitro incubations, and possesses several positive characteristics in this regard. The muscle can be readily dissected out intact, without reduction of the muscle's high-energy phosphate compounds. In addition, the epitrochlearis is a thin, flat muscle, possessing a large surface area relative to its weight. In rats weighing between 112-132 gms., the thickness of the muscle at its widest point is only 0.7-0.8 mm., and it weighs approximately 21-27 mg. (Wallberg-Henriksson 1987). The epitrochlearis muscle has a heterogeneous fibre composition, comprised of 70-80% type II B, 10-20% type II A, and 6-12% type I fibres. The muscle possesses a high glycolytic capacity, and despite its composition, it also possesses a relatively high oxidative capacity (Zetan 1989). The epitrochlearis muscle arises from the tendon of insertion of the latissimus dorsi muscle and inserts into the medial epicondyle of the humerus (Wallberg-Henriksson 1987).

Electrical stimulation of skeletal muscle has been used by a number of researchers in a variety of experimental models. Such studies have utilized surface stimulation in humans (Bergstrom and Hultman 1988), exposed in situ muscle (Petrofsky et al. 1980, Brooks and Faulkner 1990), perfused rat hindlimb (Bylund-Fellenius et al. 1984) as well as implanted electrodes (Schachat et al. 1988). In addition, electrical stimulation has been used in isolated muscle preparations (Nesher et al. 1980 a,b, 1985, Constable et al. 1988, Nie et al. 1989 a,b, Nie and Henriksson 1989, Hopp and Palmer

1990). Nesher et al. (1980a) investigated the mechanical performance of the rat epitrochlearis muscle under a variety of conditions and found that performance of the muscle was related to the rate of stimulation. These authors found that by using 2 msec. square wave pulses at between 10-20 volts, they were able to produce sustained maximum twitch tension without immediate signs of fatigue. The twitch tension developed by the contracting muscle was found to drop after 30 minutes to between 55-70% of initial values, depending on the stimulation frequency utilized. In addition, it was reported that at high stimulation rates (over 48 twitches/min) tissue levels of ATP and PCr fell to up to 45% of initial values. The viability of the preparation used in this study was indicated by lactate to pyruvate ratios of between 11-15.

Nie et al. (1989a) utilized a similar model to investigate the effects of contractile activity on myofibrillar protein degradation and amino acid metabolism in rat epitrochlearis muscle. These investigators studied both single twitch and repeated tetanic contractions using 2 msec. square wave pulses at 10 volts with varied frequencies for one hour. The authors found that voltages higher than 10 volts did not significantly increase contraction force, however, caused earlier muscle fatigue at the same frequency. The maximal twitch tension developed by the stimulated muscle was found to decrease rapidly with most stimulation patterns, with a loss of half the initial force observed in under 5 minutes, and final forces averaging 7-20% of initial values. High stimulation rates were also found to cause a drop in high-energy phosphate content, with reported values showing decreases of up to 45% of initial values, in agreement with Nesher et al. (1980a).

CHAPTER III - MATERIALS AND METHODS

A. Animals

Fourteen, male Sprague-Dawley rats were utilized for this study (mean weight 122. ± 0.86 gms., range 115-127 gms.). Rats were obtained approximately 2 days before the experiment from Charles River Laboratories Inc. and housed in wire cages. All rats were maintained on a diet of Purina rat chow and water ad libitum and were subjected to standard 12 hour light-dark cycles.

B. Muscle Preparation

Rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (0.189 c.c./gm., mean dose 23.1 ± 1.44 c.c.). The epitrochlearis muscle was exposed by blunt dissection and excised intact free of the surrounding fascia and musculature using tissue forceps and surgical scissors. The average time between disruption of the vascular supply and the freezing or mounting of the muscles in the incubation medium was under 3 minutes (mean = 140.8 ± 14.9 sec.).

Muscles were divided into 3 groups, following the experimental protocol presented in Figures 1A, B, and C. Group A muscles used for SDS-PAGE experiments were immediately frozen following dissection in liquid nitrogen and stored at -70°C. Group A muscles used for electron microscopy investigation were fixed in situ after exposure and prior to dissection.

Group B muscles were immediately positioned into a resting-length clip following dissection, and placed in a pre-incubation medium for a period of 30 minutes. A change of medium was performed, following which a further incubation period was imposed,

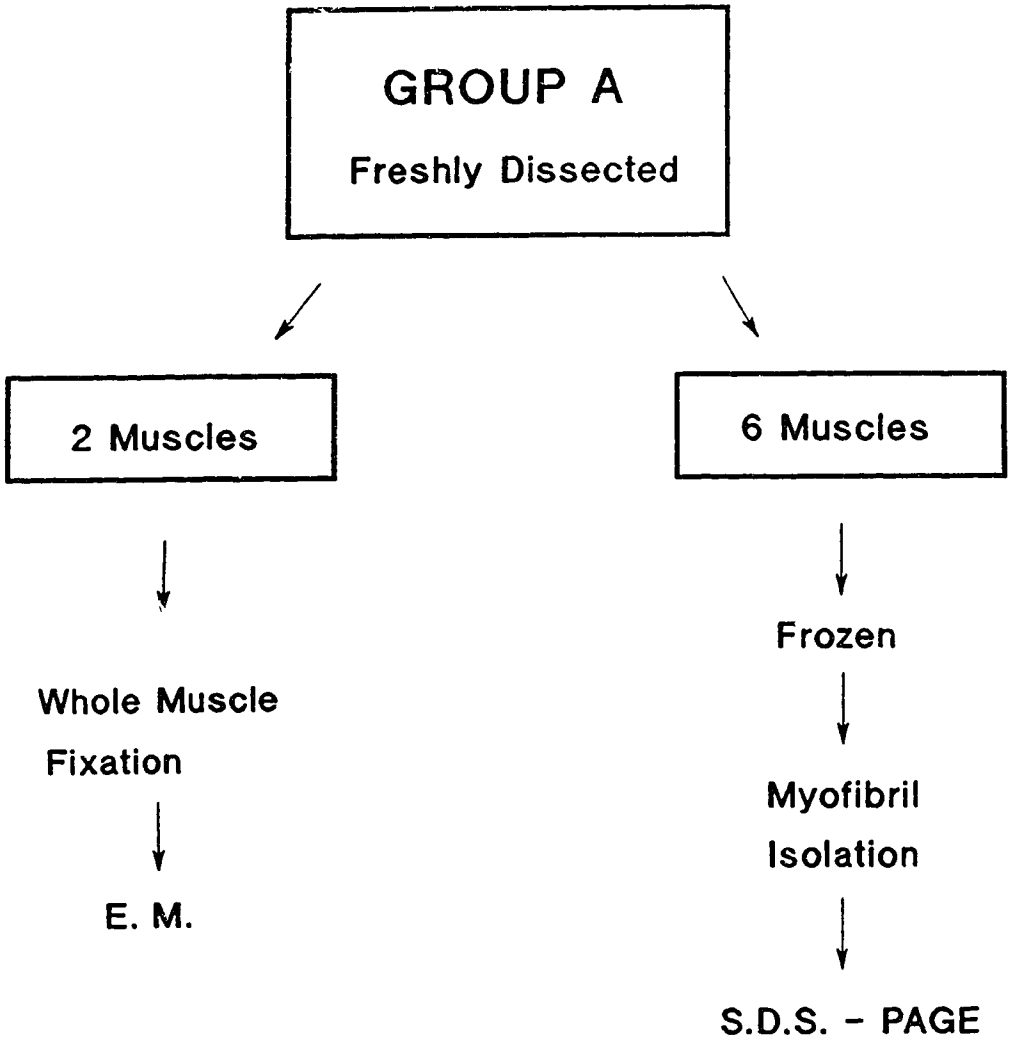


Figure 1A Experimental Protocol for Group A

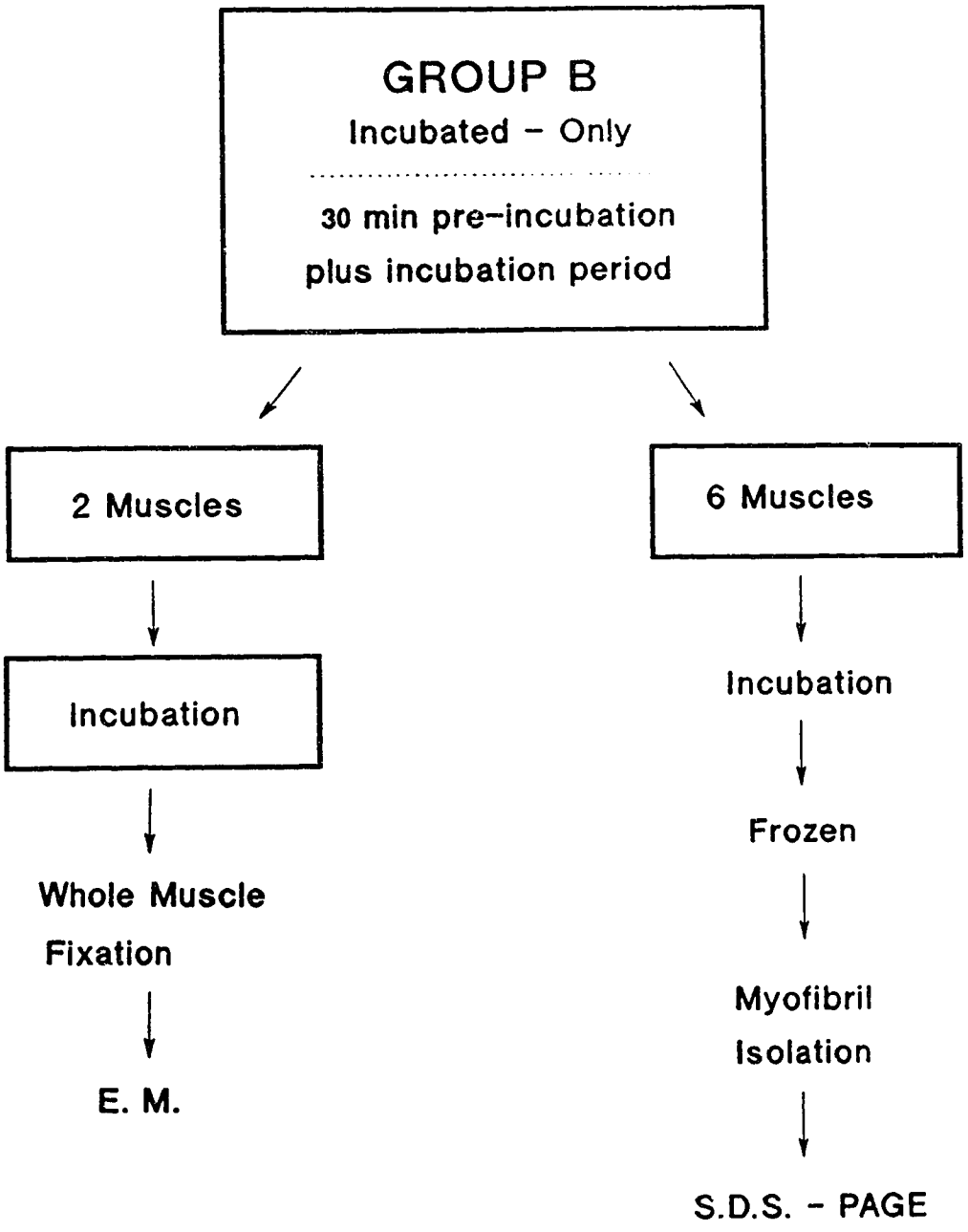


Figure 1B Experimental Protocol for Group B

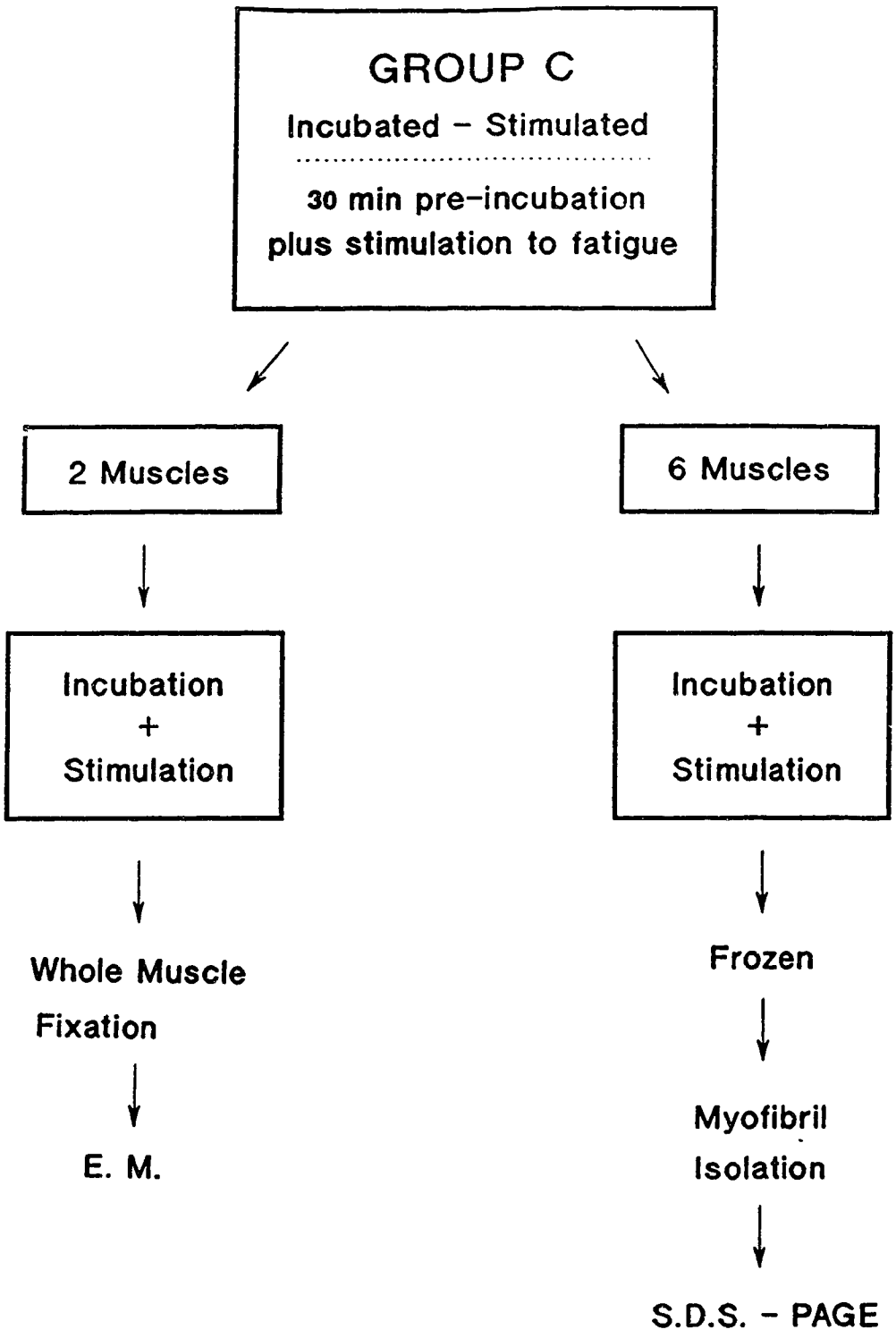


Figure 1C Experimental Protocol for Group C

equal in duration to the period to fatigue of the stimulated contralateral muscle of group C. Following the incubation, the muscles were either immediately fixed (E.M.) or frozen (SDS-PAGE) as discussed previously.

Group C muscles were mounted on clips and placed onto a stimulation apparatus where a resting tension of 500 mg. was imposed. The apparatus was placed into a cuvette containing incubation medium, and a pre-incubation period of 30 minutes was administered. A change of medium was performed, as in group B, and after the equilibration period electrical stimulation was started. Stimulation of the mounted muscle continued until the point of exhaustion, which was defined as that point where the peak twitch tension decreased to 40 mg., or approximately 15% of the initial peak twitch tension. This represented a 85% reduction in force output from initial tension values. Muscles which reached exhaustion in less than 35 minutes were discarded. The muscles were subsequently treated for E.M. or SDS-PAGE procedures, as described previously.

C. Muscle Incubations

The muscles were incubated in spectrophotometric cuvettes (Canlab) containing 1.2 mls. each of a modified Krebs Ringer bicarbonate buffer at a pH of 7.4. Constituents of the standard buffer included NaCl (118 mM), KCl (4.7 mM), Ca $Cl_2 \cdot 2H_20$ (2.5 mM), $KH_2\ PO_4$ (1.2 mM), $MgSO_4 \cdot 7H_20$ (1.2 mM) and $NaHCO_3$ (25 mM). Modifications to the standard buffer included the following: 5 mM glucose, all 20 amino acids at physiological concentrations (Mallette et al. 1969), 5 mM curare, and 5 mM HEPES. The medium was gassed continuously with a 5% $C0_2$ -95% 0_2 mixture via polyethylene tubing and microhematocrit capillary tubes (Fisher Scientific), and maintained at a

constant temperature of 37° C.

Incubated muscles comprising group B were maintained at an approximate in situ resting length by mounting of the muscle on a support made of 22-gauge orthodontic wire.

D. Stimulation

Microclamps made of orthodontic wire (22-gauge) were fastened to each end of the excised muscle. The distal microclamp was subsequently attached to the base of a stimulation rig made from plexiglass and dental acrylic (Figure 2). The rig was then lowered into the incubation cuvette, which was vertically mounted within a temperature-controlled cuvette holder (Pye Unicam Ltd.) fastened on a light microscope stage (Carl Zeiss). The proximal microclamp was attached by 4-0 silk suture (Davis and Geck) to a force-displacement transducer (Model #FT03C, Grass Instrument Co.). Electrical stimulation was by means of platinum foil plate electrodes (Johnson Matthey Ltd.) contained within the stimulation rig. Lead wires attached to the plate electrodes were connected to an electronic stimulator (Model SD-9B, Grass Instrument Co.), which provided square wave impulses of 2 msec. duration at a frequency of 2.0 Hz. A potential difference of 10 volts was maintained across the electrodes. A filtered DC power supply was utilized for the stimulation procedure (Model NFBR, Electro Products Laboratories, Inc.), and stimulation parameters were monitored by a digital storage oscilloscope (Model 468, Tektronix). Muscle performance was monitored and recorded by a chart recorder (Model 1200-Linear) which was powered by a triple output power supply (Model 6236-B, Hewlett-Packard).

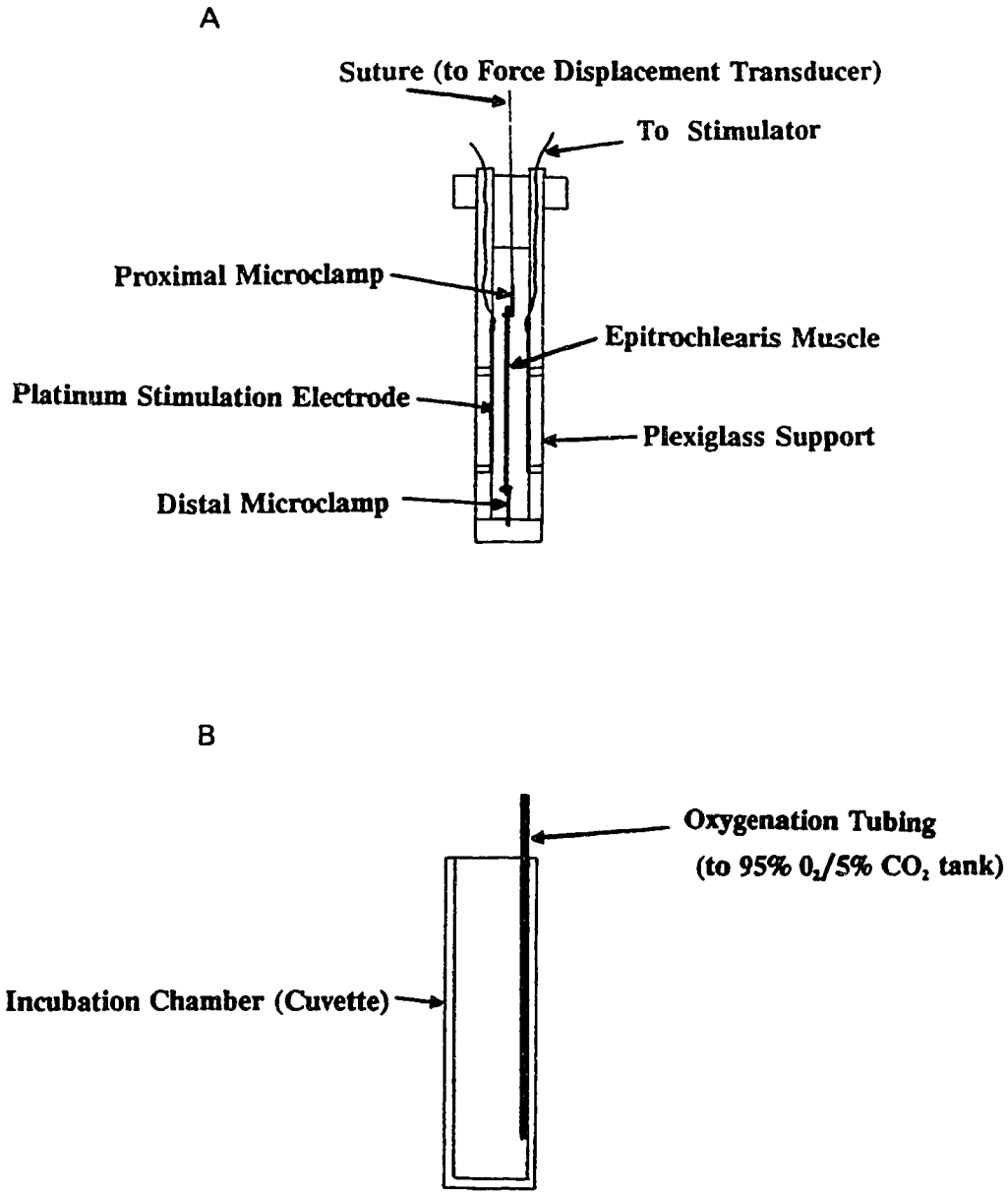


Figure 2 Stimulation Apparatus

A. Stimulation Rig

B. Incubation Cuvette

E. Biochemical Analysis

Myofibril fractions were isolated for both SDS-PAGE and electron microscopy procedures. Muscle tissue was homogenized using a mortar and pestle apparatus in a borate-KCl buffer at pH 7.1 (39 mM Na borate, 50 mM Tris, 5 mM Ethylenediamine Tetraacetic Acid (EDTA), 1 mM phenyl methyl sulphonyl fluoride (PMSF) and 1 mM iodoacetate). Briefly, the homogenate was centrifuged at 3000 rpm for 12 minutes (Damon 1 EC-centra-7R centrifuge) at 4°C, then resuspended in the same buffer and centrifuged again. The pellet was then resuspended in 39 mM Na borate and 5 mM Tris (pH 7.1) with 1 mM PMSF and 1 mM iodoacetate, and centrifuged twice as above. In addition to the removal of EDTA, the myofibril fraction was washed twice in a wash solution containing 50 mM Tris, 5 mM NaN_3, 100 mM KCl, 0.5% Triton-x-100, 1 mM PMSF and 1 mM iodoacetate at a pH of 7.4. This procedure allows for the isolation of myofibril proteins with minimal contamination (Perry and Corsi 1958, Belcastro et al. 1980). In addition, the PMSF and iodoacetate provide the means to minimize endogenous proteolytic activity (Uchida et al. 1977). The final two steps consisted of resuspending the pellet twice in a suspension medium consisting of 150 mM KCl and 50 mM Tris at pH 7.4. The pellets used for electron microscopy study were immediately taken through the fixation procedure, whereas for SDS-PAGE experiments, small aliquots of the final suspension were taken for protein determination by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as a standard. The myofibril fractions were combined by group and adjusted to a concentration of 2 mg/ml using the final suspension medium.

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed on discontinuous slab gels by the method of Laemmli (1970). Gels were

prepared from acrylamide and methylene bis-acrylamide (BIS) and polymerized using a combination of ammonium persulphate (APS) and tetramethylene diamine (TEMED) as catalysts. 12% or 7.5% separating gels, and 4% stacking gels were utilized for all runs. Gel conditions and reagents used are presented in Table 2.

Prepared samples were mixed 1:1 with sample buffer and placed in a 95° C water bath for 4 minutes, in order to dissolve the samples prior to electrophoresis. Known amounts of sample were loaded onto the gels, and run at a constant current of 35 mA for approximately 8 1/4 hours (Protein II slab gel, Biorad Laboratories; Power supply #2002, Fisher Scientific). Gels were stained for 6-8 hours in 0.1% Coomassie Blue R-250 in fixative (40% MeOH, 10% HOAc). Destaining was accomplished in approximately 6 hours using repeated changes of a 40% MeOH/10% HOAc solution. Each gel was scanned wet using a laser densitometer (LKB Ultroscan XL) (see Appendix C) and subsequently photographed.

F. Electron Microscopy

Electron micrographs were prepared from both intact muscle tissue and isolated myofibril pellets. Samples were fixed in situ or immediately post-treatment in 2.5% glutaraldehyde fixative in 0.1 M cacodylate buffer at pH 7.2. After 2 hours fixation at room temperature, tissues were rinsed and post-fixed in 1% osmium tetroxide, rinsed again in cacodylate buffer, and dehydrated through a graded series of ethanol (10 minutes in each of 50%, 70%, 85%, 95% and twice in absolute ethanol). Samples were then immersed in propylene oxide for 20 minutes, followed by infiltration in a 1:1 mixture of propylene oxide and Araldite embedding resin. The specimens remained in this mixture overnight, after which embedding took place in pure Araldite with

Table 2 SDS-Page Reagents and Gel Conditions

Stacking Gel	Separating Gel	Running Buffer	Sample Buffer
0.125 M Tris-HCl, pH 6.8	0.375 M Tris-HCl, pH 8.8	0.025 M Tris-HCl, pH 8.3	0.063 M Tris-HCl, pH 6.8
0.1% ($^{W}/_{V}$) SDS	0.1% ($^{W}/_{V}$) SDS	0.192 M.glycine	10% glycerol
0.05% APS	0.05% APS	0.1% SDS	2% SDS
0.1% ($^{V}/_{V}$) TEMED	0.05% ($^{V}/_{V}$) TEMED		5% 2-MCE
4% acrylamide, Acrylamide :BIS (37:1)	7.5%, 12% acrylamide, Acrylamide :BIS (37:1)		0.01% bromophenol blue

polymerization carried out at 60°C for 48 hours. Following embedding, the blocks were trimmed with a razor blade, and thin sections were cut with a glass knife on the Reichert Om U2 ultramicrotome. Sections were picked up on formvar-coated 200-mesh copper grids and stained in 5% uranyl acetate in absolute methanol for 20 minutes. Grids were examined with a Phillips EM 300 transmission electron microscope at 60 KV.

G. Data Analysis

Electron microscopy and SDS-PAGE investigations were assessed qualitatively and are descriptively reported. Gels and corresponding scans were studied for alterations in protein content, and the approximate molecular weight regions relating to these alterations were identified. Original densitometric scans (see Appendix C) were superimposed on each other to facilitate direct comparisons between incubated-only and incubated-stimulated, and incubated-only and freshly dissected samples. Electron micrographs were studied for evidence of morphological alterations resulting from incubation and/or stimulation conditions. Such alterations looked for included evidence of Z-line dissolution, loss of myofilaments, mitochondrial abnormalities, S.R. or T-tubule disruptions, and vacuole formation.

Mechanical performance data, and other data where appropriate, are represented as means ± standard errors of the mean (S.E.).

CHAPTER IV - RESULTS

A. Mechanical Performance

The average time taken for the stimulated muscles to reach exhaustion (the state at which the force output constituted only 15% of the initial peak twitch tension) was 47 ± 2.6 minutes. The contractile response to electrical stimulation over the duration of the stimulation period is illustrated in Figure 3.

The decline in force output observed throughout the period of stimulation can be seen to constitute several phases, as demonstrated by the changing slopes observed in Figure 3, and in the data presented in Table 3. An initial phase of rapid decline in twitch tension (from an initial average peak tension of 262 ± 10.7 mg.) was observed over the first eight minutes. A slight moderation was observed in the rate of force decrement from minutes 8-35. A third and final phase was noted after the 35th minute, when a slow rate of diminution of tension was observed until fatigue.

B. SDS-PAGE

Gel electrophoresis demonstrated a selective protein loss in samples which were incubated only, and a more generalized loss of protein in samples which were incubated and stimulated. The gels are presented with the unknown protein bands labelled for identification. Densitometric scans are also presented to provide a more precise measure of differences in protein quantity. The scans are superimposed to facilitate the comparisons between different conditions.

Plate 1 shows the 12% gel which was run to separate the lower molecular weight proteins of the samples (gel #1). Lanes A, B, and C contained 70 µg each from all three

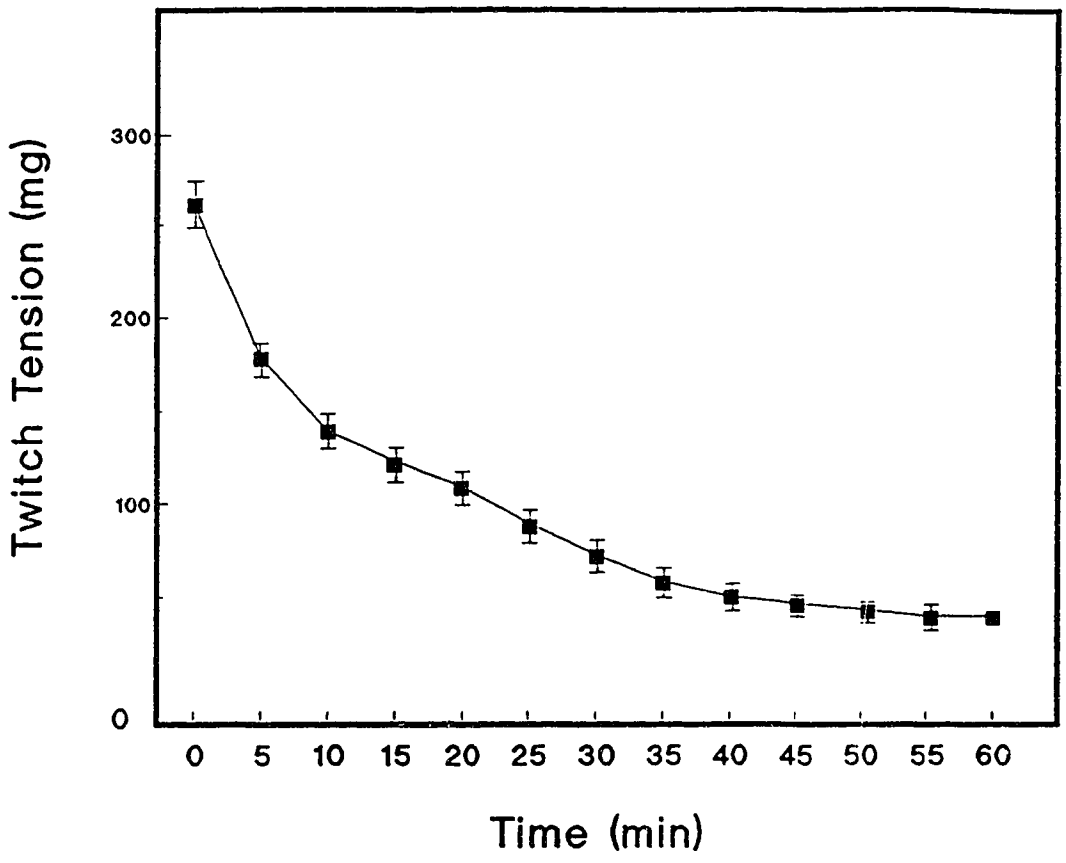


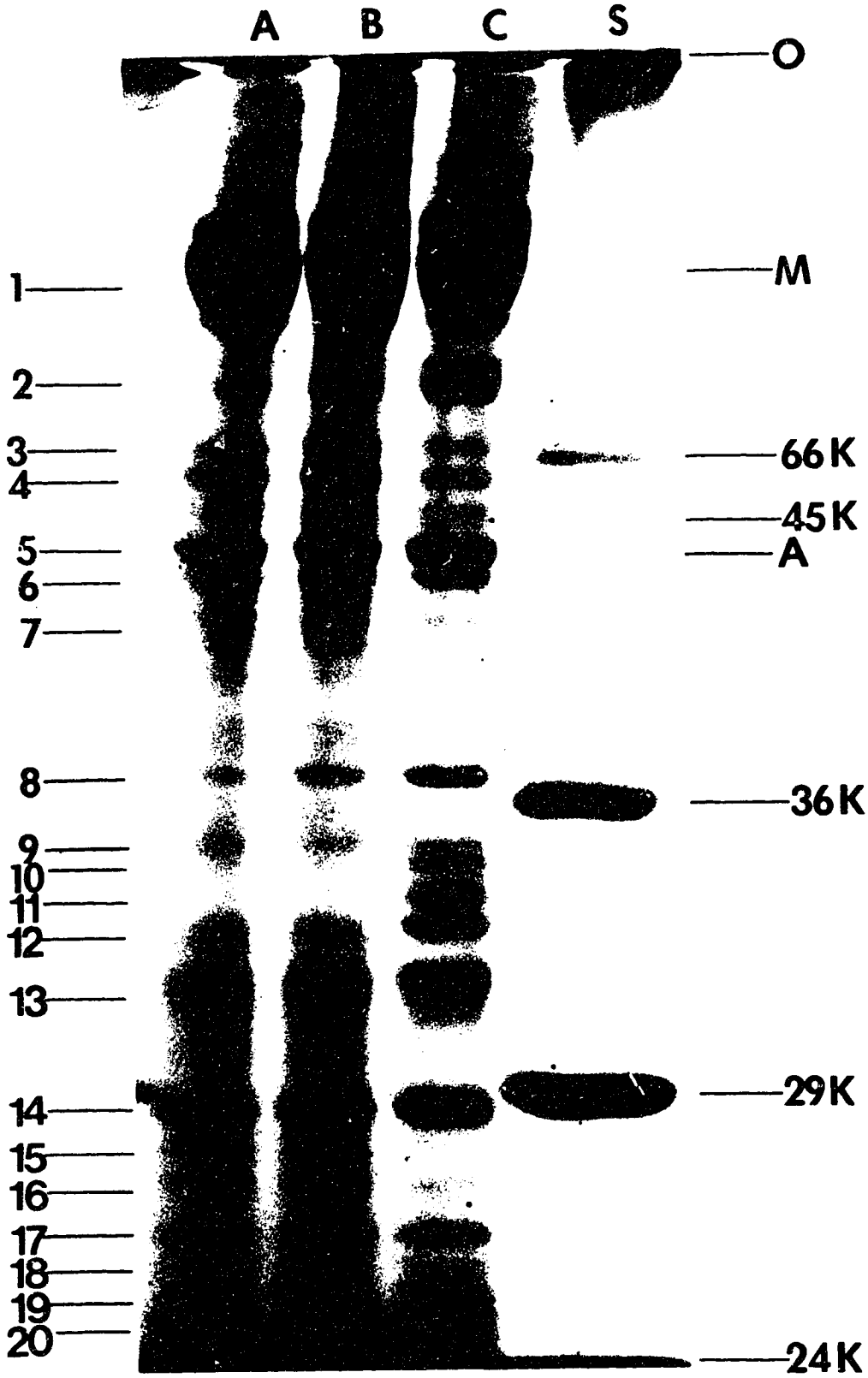
Figure 3 Average Peak Twitch Tension to Fatigue
Values are means ± SE.

Table 3 Twitch Performance Throughout Stimulation Procedure

Minutes	0	1	2	3	4	5	6	7	8	9	10	15	20	25	30	35	40	45	50	55	60
Average Peak Twitch Tension (mg) (mean ± S.E.)	261.9 ±10.75	241.9 ±9.52	226.3 ±7.89	210.6 ±5.34	192.5 ±5.79	180.3 ±7.70	168.8 ±6.23	158.8 ±6.23	150.0 ±6.12	145.6 ±5.26	137.4 ±5.03	123.1 ±4.92	109.4 ±3.36	90.0 ±3.95	73.1 ±4.41	58.8 ±3.39	50.6 ±3.12	47.0 ±1.79	43.8 ±2.08	40.0 ±7.07	40.0
Average % of Initial P.T.T.	100	92	86	80	74	69	64	61	57	56	53	47	42	34	28	22	19	18	17	15	15
Average Rate * of Decline (%/min.)		8.0	6.0	6.0	6.0	5.0	5.0	3.0	4.0	1.0	3.0	1.2	1.0	1.6	1.2	1.2	0.4	0.2	0.2	0.4	

* calculated over period from previous force recording

Plate 1 Gel #1 - 12% SDS-PAGE of Rat Myofibrils. Lanes A, B, and C contained 70 μg each from all three conditions. Lane S contained low molecular weight standards. Numbers on left indicate unknown protein bands. Approximate molecular weights are indicated.
A = Incubated-Stimulated; B = Incubated-only;
C = Freshly Dissected; S = Molecular Weight Standards;
O = Origin; M = Myosin Heavy Chain; A = Actin;
K = Kilodaltons.


A
B
C
S
O
M
66K
45K
A
36K
29K
24K
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

treatments, and lane S contained low molecular weight standards (Sigma Chemical Co.). Initial inspection of the gel demonstrated banding patterns similar to those found by others who have studied rat myofibrils (Sender 1971, Snyder et al. 1984, Belcastro et al. 1988). Some differences were noted within the gel between the different lanes. Specifically, heavier staining of unknown band (8) in lane C (freshly dissected sample) was observed, as well as the presence of 4 heavily stained bands (bands (9), (10), (11), and (12)) in the same lane. Also, additional low molecular weight fragments, indicated by bands (18), (19), and (20), were seen. It appeared that lanes A and B (incubated-stimulated, and incubated-only samples) contained a greater number of diffuse bands, demonstrated by the more pronounced background staining found within these lanes as compared to lane C (freshly dissected sample). This may be indicative of a more pronounced degradatory process within the samples of these lanes. Lane C demonstrated a more distinct banding pattern. No obvious differences were noted between lanes A and B upon visual inspection of the gel.

Figures 4A and 4B show the plots of log molecular weight versus relative mobility of the low molecular weight standards of the gel shown in Plate 1 (gel #1). Linear regression analysis of the five visible standards (Figure 4A) revealed a relatively poor fit ($r = 0.9478$), resulting from an apparent deviation of the highest molecular weight standard from the rest which migrated linearly. Figure 4B presents the regression analysis performed using the lower four visible standards, which demonstrates a strong linear relationship in the range of 24-45 Kda ($r = 0.9998$).

The relative mobilities of the sample myofibrillar proteins, and their approximate molecular weights as determined by fitting to the standard curve of Figure 4B are presented in Table 4. Bands (1)-(4) are out of the range of linearity of the standard

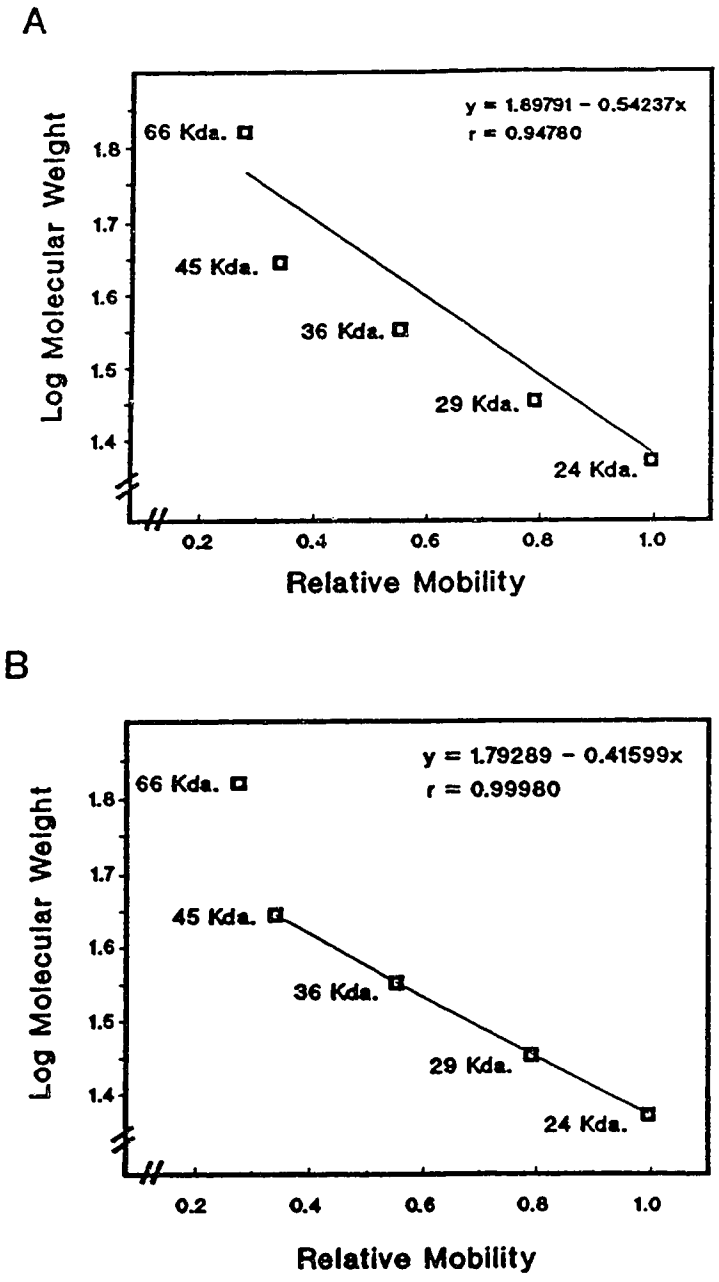


Figure 4 Log Molecular Weight (of Standards) vs. Relative Mobility (12% Gel).
A = Regression Calculated Using 5 M.W. Standards;
B = Regression Calculated Using 4 M.W. Standards.

Table 4 Relative mobilities (Rm)* of unknown protein bands and approximate molecular weights - 12% gel

Unknown Band	Rm.	Approx. M.W.(Kda)**	Possible Identity
1	0.159	O.O.R.	
2	0.235	O.O.R.	
3	0.288	O.O.R.	
4	0.309	O.O.R.	
5	0.376	43.3	Actin
6	0.394	42.6	M-Creatine Kinase?
7	0.429	41.2	Eu-Actinin?
8	0.544	36.9	Troponin T?
9	0.593	35.2	Troponin T?
10	0.606	34.7	Troponin T?
11	0.633	[illegible]	Troponin T?
12	0.659	32.0	Tropomyosin?
13	0.704	31.6	Tropomyosin?
14	0.801	28.8	
15	0.823	28.2	
16	0.854	27.4	Myosin Light Chain?
17	0.898	26.3	Myosin Light Chain?
18	0.925	25.6	Myosin Light Chain?
19	0.947	25.1	Myosin Light Chain?
20	0.973	24.4	Myosin Light Chain?

* Rm. = distance of protein migration/distance of tracking dye migration

** calculated from least squares regression line (Fig. 4B) fitted to lower four molecular weight standards (y = 1.79289 - 0.41599x; r = 0.99980)

O.O.R. = out of range of linearity of standard curve

curve, and thus their approximate molecular weights cannot be estimated. However, it seems likely that myosin heavy chain comprises the heavy band in the sample lanes at Rm = 0.159, since it is the principal myofibrillar protein. Suggested identities of the proteins are based on published molecular weights for those proteins (Murakami and Uchida 1985, Schmalbruch 1985, Craig 1986, Alberts et al. 1989).

A comparison of the densitometric scans of the two incubated samples is presented in Figure 5A, in which lanes A (incubated-stimulated) and B (incubated-only) are superimposed on each other. Differences in absorption were noted to varying extents in certain regions of the scan. There appeared to be a marked reduction in protein content in the region above myosin (200 Kda range and heavier) in the incubated-stimulated sample. The open scan of lane B at about 200 Kda was indicative of a high absorbance which had exceeded the scale of the plotter. Interestingly, a more generalized reduction in protein was demonstrated at the majority of the remaining peaks. Most notable of the regions below 200 Kda which demonstrate differences appeared to be a series of peaks between approximately 200 and 43 Kda, and single peaks at approximately 43 Kda, 37 Kda, 32 Kda, and 29 Kda.

A similar comparison is made between incubated and non-incubated samples in Figure 5B, in which a superimposition of lanes B (incubated-only) and C (freshly dissected) is presented. Losses in protein were noted in the incubated sample in the regions representing approximately 37 Kda, and a triplet at about 34-35 Kda. The scan representing the incubated-only sample shows greater background absorption throughout the full range of the gel, as well as greater absorption in the peaks representing approximately 200 Kda, and a doublet which migrated about 35-40 mm. from the origin.

Plate 2 presents gel #2 (7.5% gel) which provided the opportunity to separate

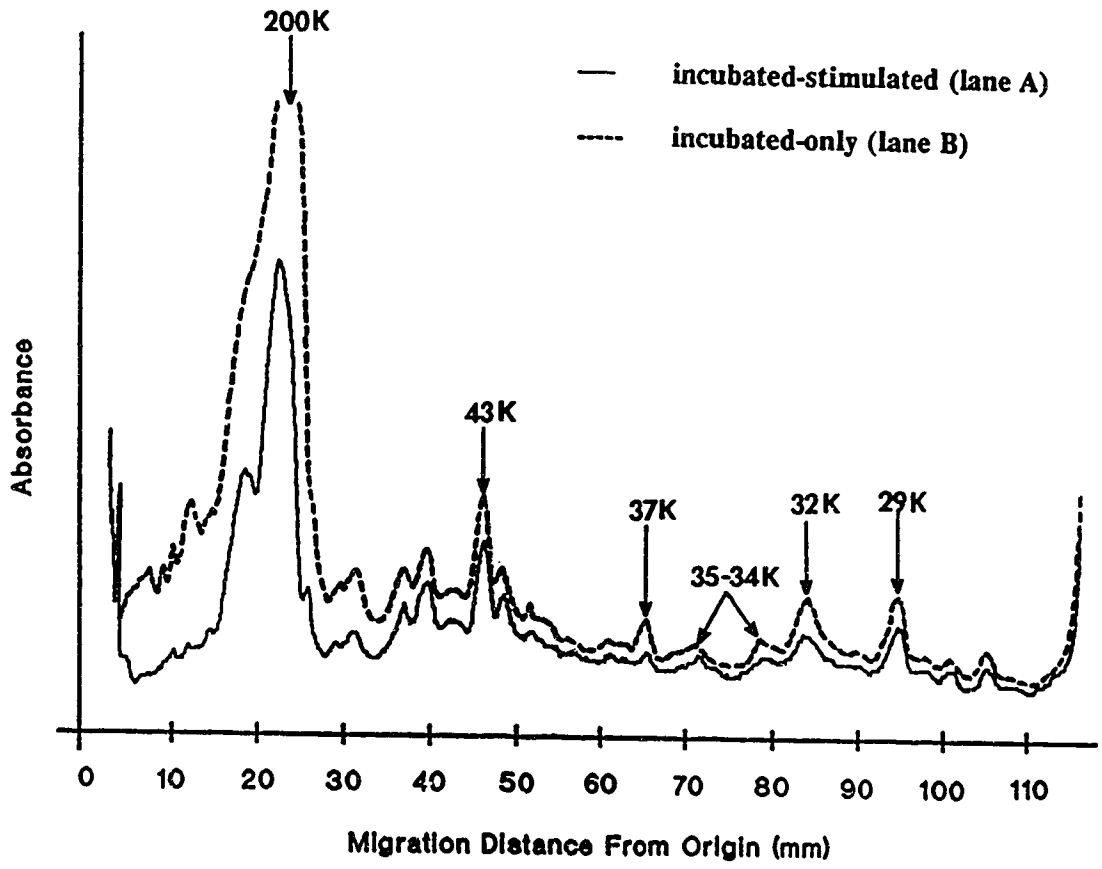


Figure 5A Superimposition of densitometric scans of lanes A (incubated-stimulated) and B (incubated-only) of Gel #1 (12% gel). Approximate molecular weights are indicated.
K=Kilodaltons; Solid line = incubated-stimulated (lane A); Broken line = incubated-only (lane B).

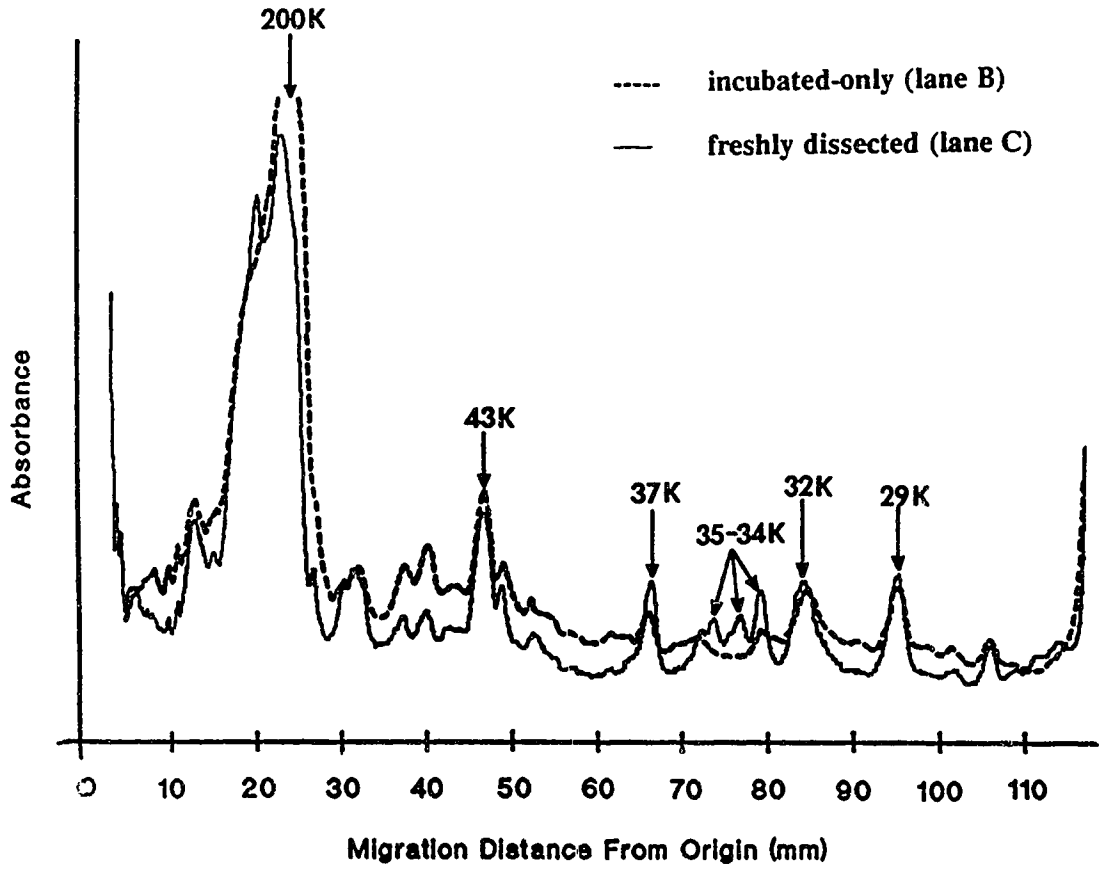
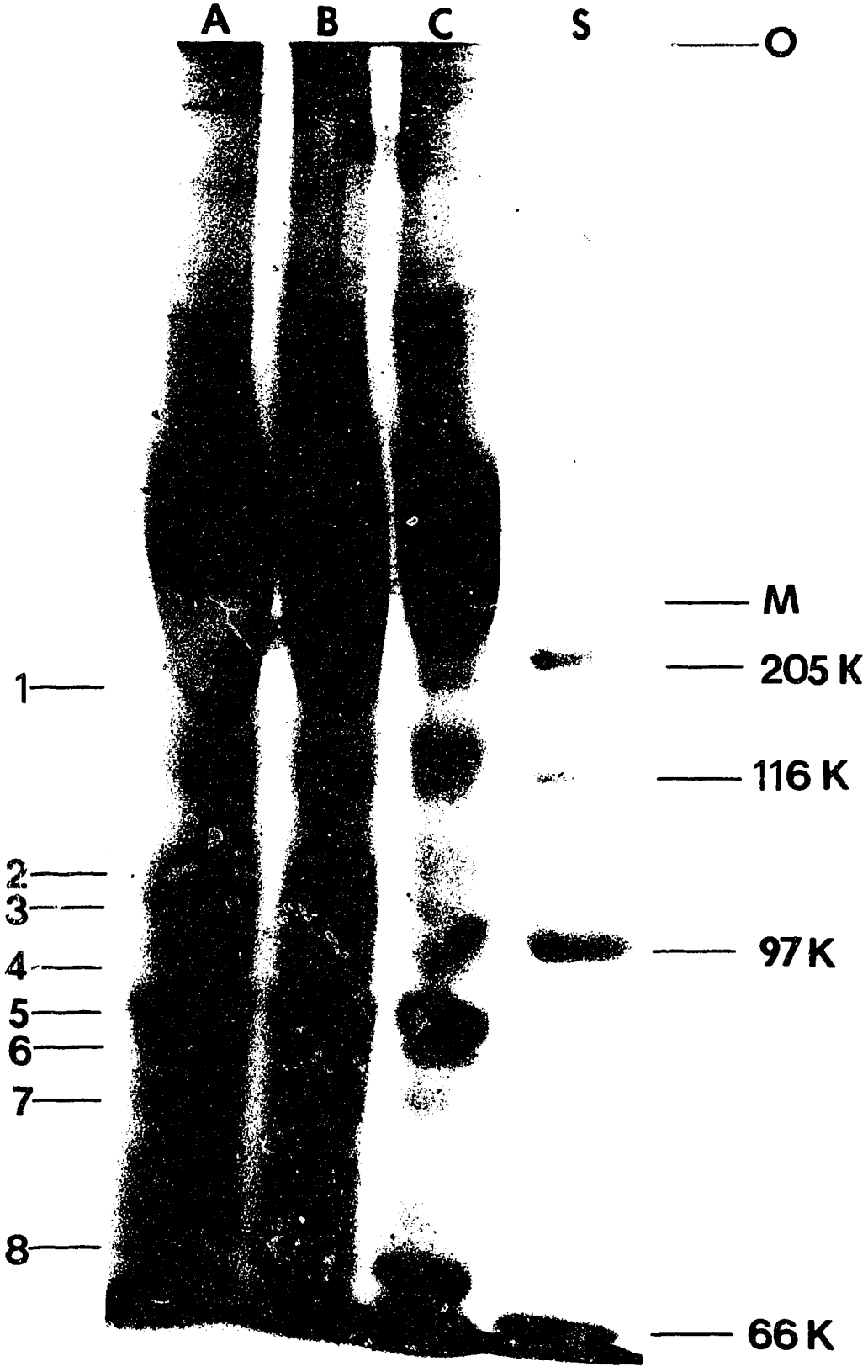


Figure 5B Superimposition of densitometric scans of lanes B (incubated-only) and C (freshly dissected) of Gel #1 (12% gel). Approximate molecular weights are indicated.
K= Kilodaltons; Solid line = freshly dissected (lane C); Broken line = incubated-only (lane B).

Plate 2 Gel #2 - 7.5% SDS-PAGE of Rat Myofibrils.
Lanes A, B, and C contained 70 μg each from all three conditions. Lane S contained high molecular weight standards. Numbers on left indicate unknown protein bands. Approximate molecular weights are indicated.
A = Incubated-Stimulated; B = Incubated-only;
C = Freshly Dissected; S = Molecular Weight Standards;
O = Origin; M = Myosin Heavy Chain; K = Kilodaltons.


A
B
C
S
O
M
205 K
116 K
97 K
66 K
1
2
3
4
5
6
7
8

the proteins of higher molecular weights. 70 μg of myofibrillar protein from all three treatments were run separately in lanes A, B, and C, and high molecular weight standards (Sigma Chemical Co.) were run in lane S. A general increase in background in this gel resulted in some loss in clarity of some of the bands. Some differences among the lanes, however, were noted. Bands (2) and (3), which are evident in lanes A and B, appeared to be missing in lane C. In addition, band 8 in lane C showed much heavier staining than the corresponding band in lanes A and B. As observed in gel #1, a more pronounced background staining in the incubated lanes (A and B) was found relative to lane C. This may be indicative of a more pronounced degradatory response under these conditions.

The log of the high molecular weight standards in gel #2 is plotted against relative mobility in Figures 6A, B. Linear regression analysis of the four visible standards (Figure 6A) revealed a poor fit ($r=0.93$) resulting from an apparent deviation of the highest molecular weight standard from the rest which migrated in a linear fashion. A regression analysis of the lower three visible standards is shown in Figure 6B, which demonstrates a strong linear relationship in the range of 66-116 Kda ($r=0.9999$).

Table 5 presents the relative mobilities and estimated molecular weights of the sample myofibrillar proteins in gel #2. The relative mobilities of bands (2)-(8) were fitted to the standard curve in the range of linearity (Figure 6B), and their approximate molecular weights were calculated by using the appropriate regression equation.

Figure 7A presents a comparison of the densitometric scans of the two incubated samples. The scans of lanes A (incubated-stimulated) and B (incubated-only) are presented, superimposed on each other. An apparent generalized loss of protein was observed in the scan of the incubated-stimulated group (lane A) at all peaks which are

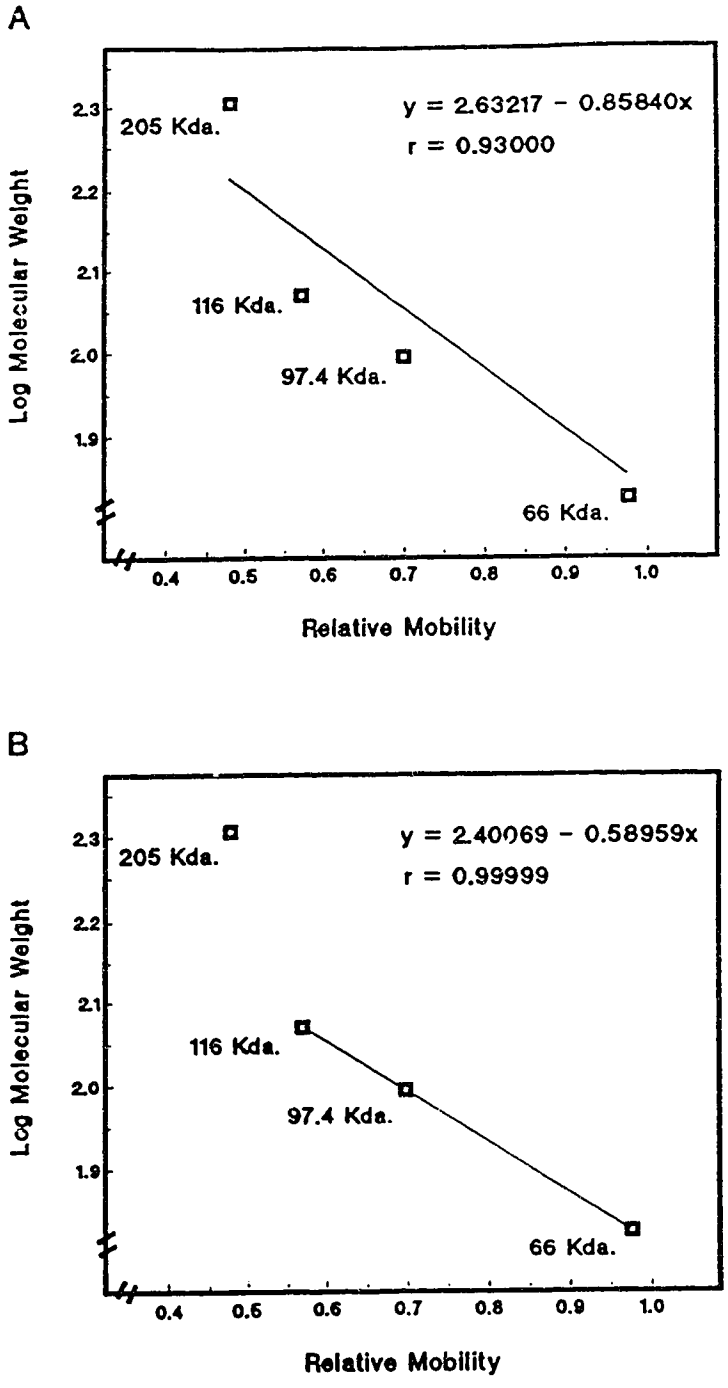


Figure 6 Log Molecular Weight (of Standards) vs. Relative Mobility (7.5% Gel).
A = Regression Calculated Using 4 M.W. Standards;
B = Regression Calculated Using 3 M.W. Standards.

Table 5 Relative mobilities (Rm)* of unknown protein bands and approximate molecular weights - 7.5% gel

Unknown Band	Rm.	Approx M.W.(Kda)**	Possible Identity
1	0.542	O.O.R.	
2	0.637	105.9	
3	0.669	101.5	
4	0.708	96.2	α-actinin?
5	0.755	90.3	α-actinin?
6	0.783	86.9	Amorphin?
7	0.821	82.5	Amorphin?
8	0.952	69.1	

* Rm. = distance of protein migration/distance of tracking dye migration

** calculated from least squares regression line (Fig. 6B) fitted to lower three molecular weight standards (y=2.40069 - 0.58959x; r=0.99999)

O.O.R. = out of range of linearity of standard curve

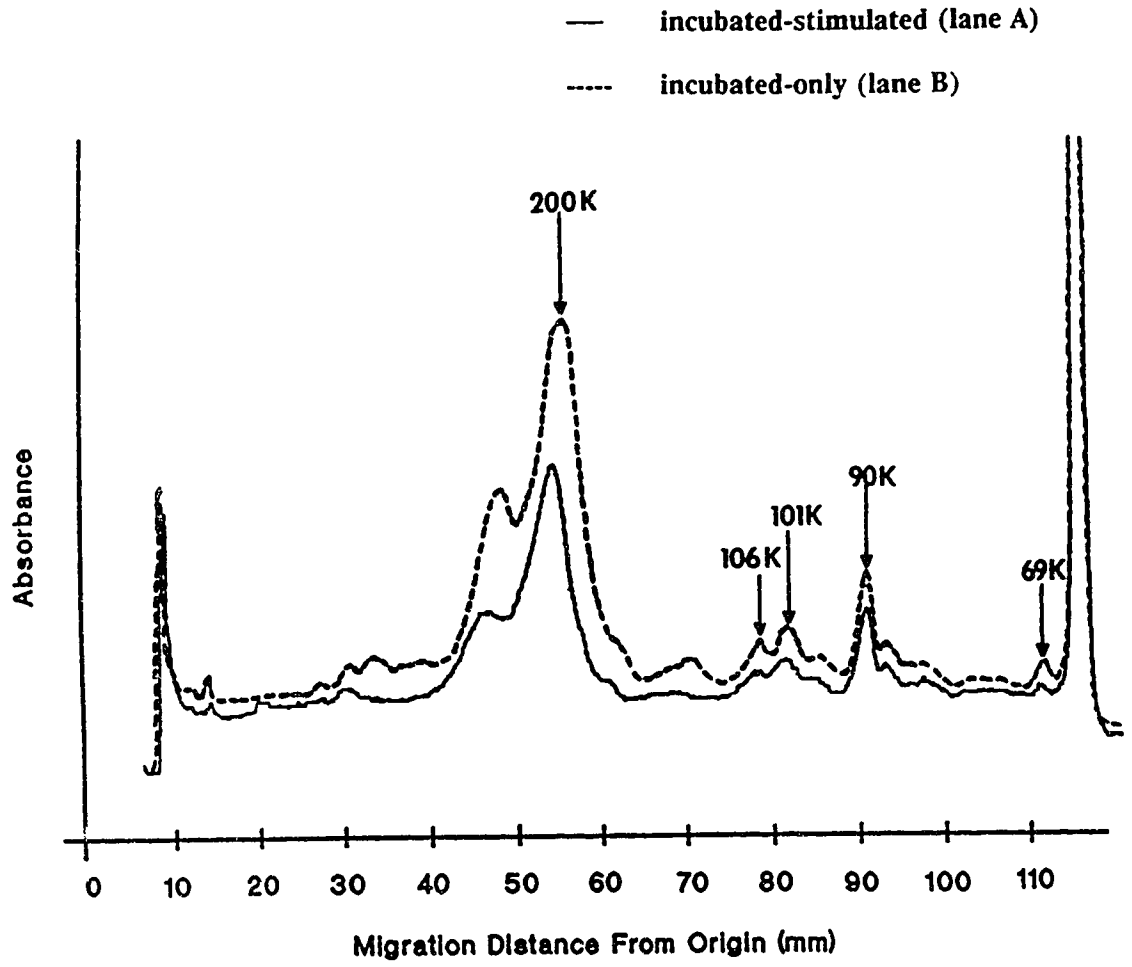


Figure 7A Superimposition of densitometric scans of lanes A (incubated-stimulated) and B (incubated-only) of Gel #2 (7.5% gel). Approximate molecular weights are indicated.
K = Kilodaltons; Solid line = incubated-stimulated (lane A); Broken line = incubated-only (lane B).

represented.

Figure 7B presents a similar comparison between incubated and non-incubated samples. The densitometric scans of lanes B (incubated only) and C (freshly dissected) are shown superimposed on one another. Lane C demonstrates less baseline absorbance in the lower molecular weight regions (75-110 mm. from the origin), as well as an absence of certain peaks, notably in the regions of 200 Kda, 106 Kda, and 101 Kda. The freshly dissected sample (lane C) also demonstrates a couple areas of increased absorption - one peak in the region between 200 and 106 Kda (at about 66 mm. from the origin), and another peak at about 69 Kda.

Plate 3 presents the 12% gel (gel #3) which was run to determine the consistency of the protein migrations across the different lanes and between different gels. Lanes A-E each consisted of 25 μg of myofibrillar protein from freshly dissected muscles, and lane S contained low molecular weight standards as in gel #1. Similar banding patterns were noted among the lanes, and these patterns appeared consistent with those seen in lane C of gel #1 (Plate 1), which contained an identical sample.

C. Electron Microscopy

Electron microscopy of intact tissue sections revealed no obvious indications of myofibrillar protein loss. Nevertheless, several interesting observations were made. Muscles which were fixed in situ showed essentially normal morphology in the majority of sections which were studied. The one notable exception to this general rule was the occasional finding of swollen or disrupted mitochondria, which were randomly distributed, demonstrating no apparent localization. This finding, however, was observed in relatively few of the in situ sections which were studied.

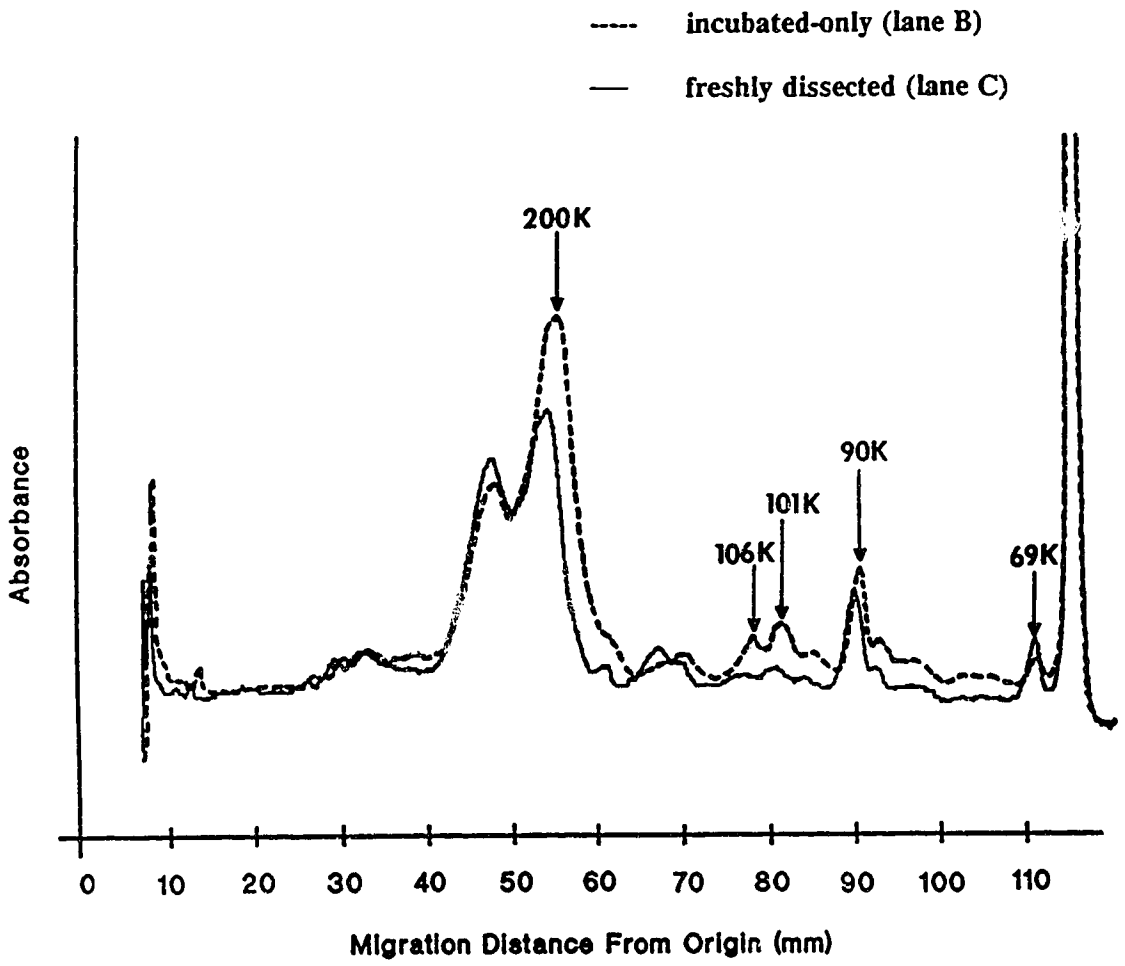
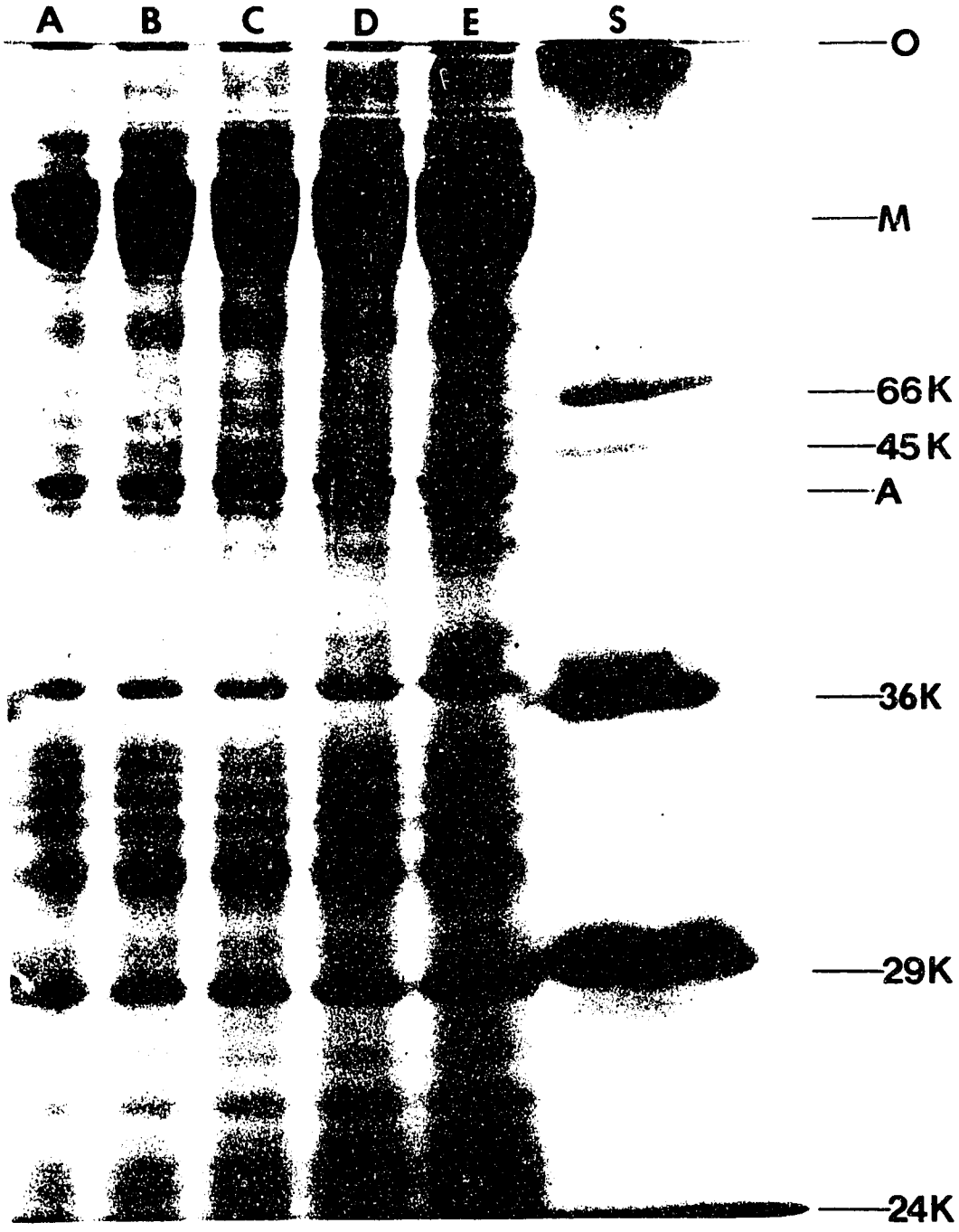


Figure 7B Superimposition of densitometric scans of lanes B (incubated-only) and C (freshly dissected) of Gel #2 (7.5% gel). Approximate molecular weights are indicated.
K = Kilodaltons; Solid line = freshly dissected (lane C);
Broken line = incubated-only (lane B).

Plate 3 Gel #3 - 12% SDS-PAGE of Rat Myofibrils. Lanes A, B, C, D, and E contained 25 μg each of isolated myofibrillar protein from freshly dissected rat epitrochlearis muscle. Lane S contained low molecular weight standards. Approximate molecular weights are indicated.O = Origin; M = Myosin Heavy Chain; A = Actin;
K = Kilodaltons.


A
B
C
D
E
S
O
M
66K
45K
A
36K
29K
24K

Mitochondrial abnormalities, including swelling and vocuolation, were prominent features of the sections viewed from incubated-only and incubated-stimulated conditions. The presence of these abnormalities were widespread and generalized, being observed in the majority of fibres which were viewed. They did not appear localized to a specific region.

Samples from the incubated-stimulated group demonstrated two notable morphological alterations. A misalignment of sarcomeric structure was a generalized finding which was observed in the majority of sections which were viewed. In addition, prominent vacuolation was a common feature of most sections. These clear vacuoles, some of which contained membranous structures within, appeared to be randomly distributed throughout different regions of the muscle fibres.

Plates 4 and 5 show representative sections of freshly dissected epitrochlearis muscles which were fixed in situ. Plate 4 shows seven adjacent myofibrils lined up in register. The Z-bands are intact, with no evidence of disruption. Mitochondria are easily recognizable near the Z-lines, as are T-tubules and terminal cisternae (comprising the "triad") near the A-I junction. The A, I, and H bands are readily observed, as is the M-line. Glycogen granules and sections of membrane, probably comprising the S.R., can be seen between adjacent myofibrils.

Plate 5 is a greater magnified view of two adjacent myofibrils in the region of the Z-line. Myofilaments comprising both the I and A bands can be readily seen, as can the large mitochondria adjacent to the Z-band. T-tubules can be found in this section, as can glycogen granules, which are evident between the myofibrils.

Plates 6-9 represent longitudinal sections of epitrochlearis muscle which has been incubated for 68 minutes (30 min. pre-incubation + 30 min.). Plate 6 shows well-aligned

Plate 4 Transmission electron micrograph of rat epitrochlearis muscle fixed in situ. Z-bands (Z) appear intact, with no evidence of disruption. Mitochondria (Mi) are easily recognizable adjacent to the I-bands (I). T-tubules (T) and terminal cisternae of the S.R. (C) are also visible, showing no evidence of alterations. The A-band (A), H-band (H), and M-line (M) are also shown (x27,825).

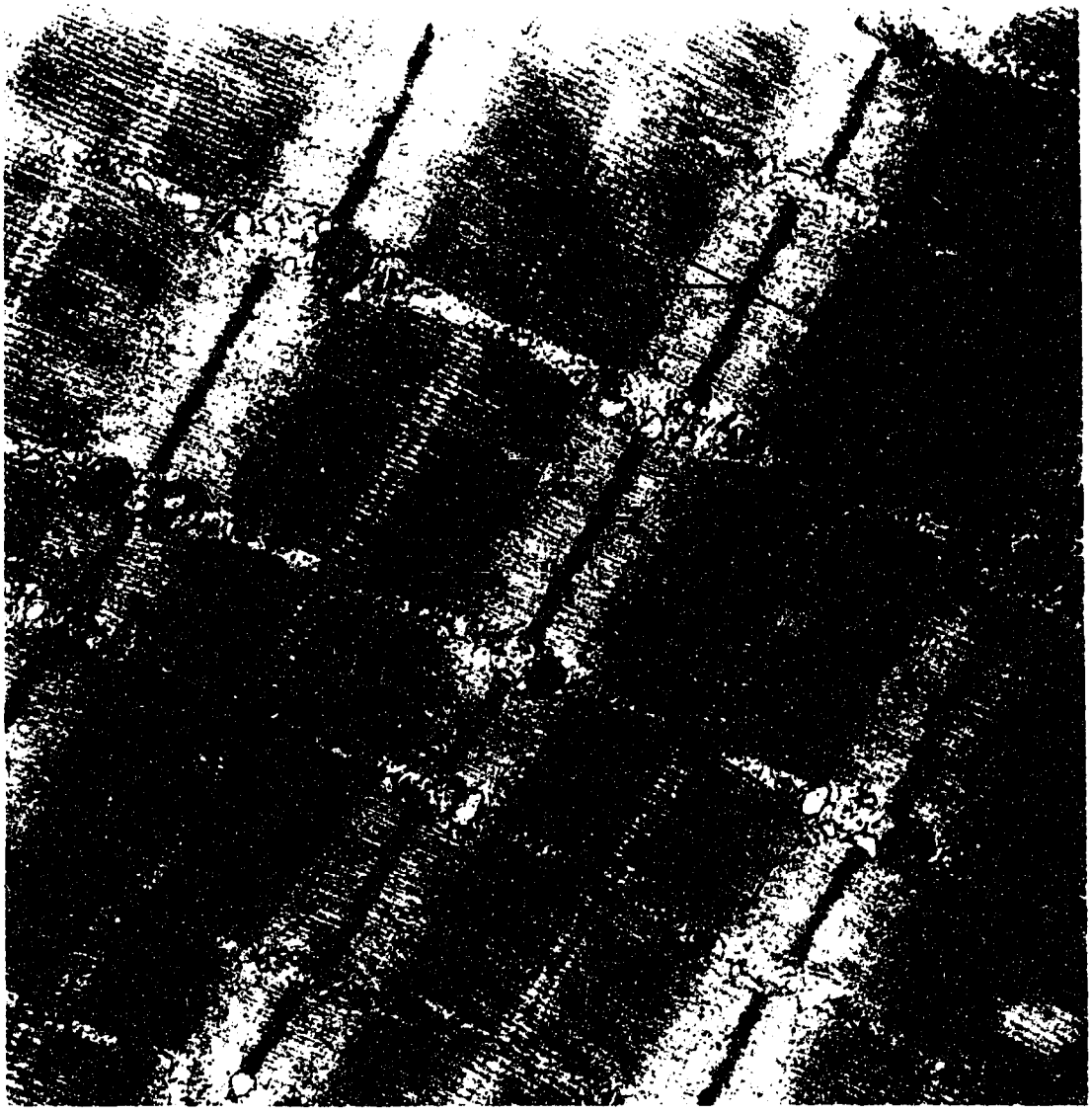


Plate 5 Transmission electron micrograph of rat epitrochlearis muscle fixed in situ. Two adjacent myofibrils (Mf) can be seen. Mitochondria (Mi) and T-tubules (T) are also present. Glycogen granules (g) can be seen between adjacent myofibrils (x76,810).

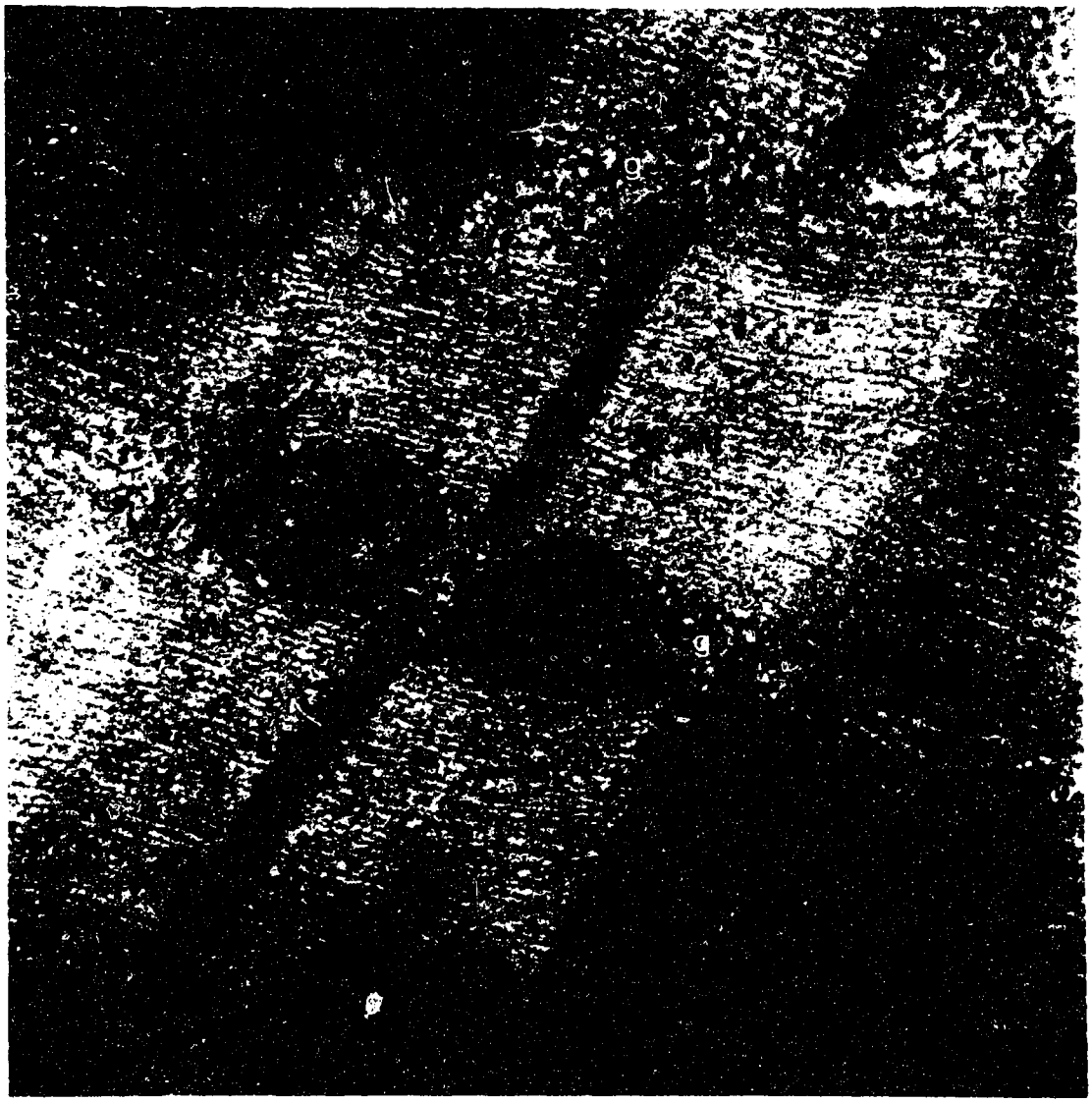


Plate 6 Transmission electron micrograph of rat epitrochlearis muscle which has been incubated at resting length for 68 minutes. Well-aligned sarcomeric structure is evident. Disruption of centrally-located mitochondria (Mi) can be seen, as can membranous bodies (mb) within the mitochondria. Glycogen granules (g) are also present (x27,825).


mb
mb

Plate 7 Transmission electron micrograph of rat epitrochlearis muscle which has been incubated at resting length for 68 minutes. Disrupted mitochondria (Mi) are seen. Membranous bodies (mb) are present, some containing electron dense particles (p). Fragmented cristae (cr) can also be seen (x23,645).


cr
cr
p
Mi
Mi
cr
mb

Plate 8 Transmission electron micrograph of rat epitrochlearis muscle which has been incubated at resting length for 68 minutes. Disrupted mitochondrion containing membranous bodies (mb) is present, as are fragmented cristae (cr). T-tubules (T) are readily seen, as are numerous glycogen granules (g) (x76,810).


mb
cr
g
mb
mb
g

Plate 9 Transmission electron micrograph of rat epitrochlearis muscle which has been incubated at resting length for 68 minutes. Disruption of mitochondria is not seen in this section, however, an elongated mitochondrion (eMi) can be seen. Myofibrils appear hypercontracted. T-tubules (T) and numerous glycogen granules (g) are evident (x27,825).


g
T
eMi
g
T

sarcomeric structure. The myofibrils generally appear intact, and glycogen granules are abundant. Disruption of the centrally-located mitochondria is notable, with multiple membranous vacuoles comprising the majority of these structures. Remnants of cristae can be seen around the periphery of these organelles. The darkly stained region evident in the upper left of this section is most likely artifactual in origin.

Plate 7 shows more extensive vacuolation between adjacent myofibrils. Swelling of mitochondria and corresponding pallor of matrix space is evident. Remnants of mitochondrial cristae can be found. The mitochondria near the bottom of this section appear to be normal, as does the general ultrastructure of the myofibrils.

Plate 8 shows a more highly magnified view of a vacuolated mitochondrion. Cristae can be seen in peripheral areas of the structure. Numerous glycogen granules are evident. An abnormally elongated mitochondrion can be seen in Plate 9, in which section the general ultrastructure otherwise appears normal. Darkly stained spots seen on this section are likely preparation artifact.

Plates 10-13 represent longitudinal sections of epitrochlearis muscle which has been electrically stimulated for 38 minutes following a 30 minute pre-incubation period. The section shown in Plate 10 demonstrates normal-looking myofibrillar structure, with an absence of enlarged, vesicular mitochondria and vacuolated sarcotubular system. However, misalignment of adjacent sarcomeres is more noticeable than control sections. Glycogen granules are evident.

Plate 11 demonstrates a section of muscle containing numerous membranous-bound interfilamentous vesicles which are prevalent in one of the myofibrils. Some of the myofibrils seen in this section appear intact, with no mitochondrial or sarcotubular alterations evident.

Plate 10 Transmission electron micrograph of fatigued rat epitrochlearis muscle which has been pre-incubated for 30 minutes followed by 38 minutes of electrical stimulation. Mitochondria (Mi) appear intact. T-tubules also appear normal. A misalignment of adjacent sarcomeres is evident (x27,825).


Mi
Mi

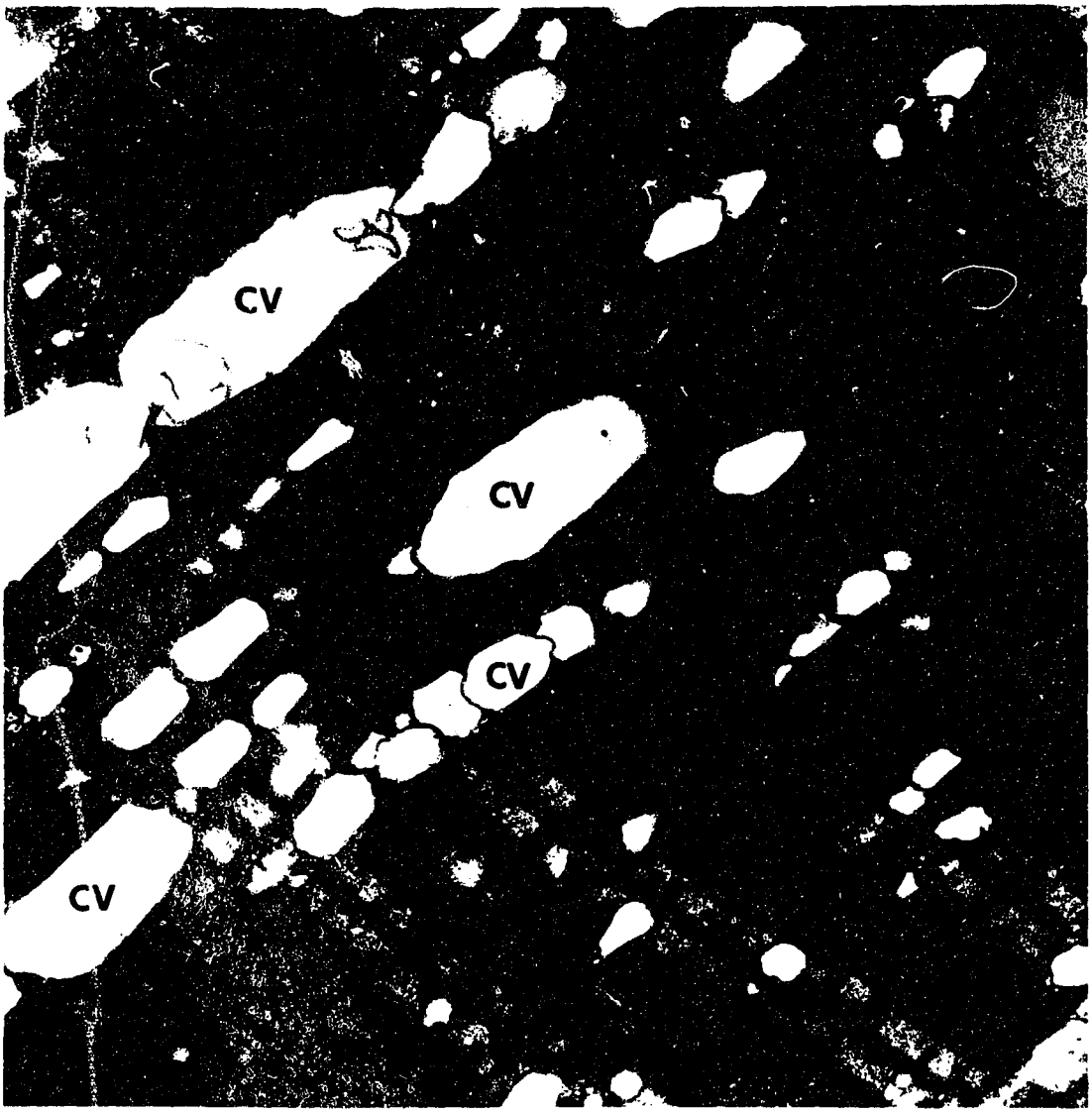
Plate 11 Transmission electron micrograph of fatigued rat epitrochlearis muscle which has been pre-incubated for 30 minutes followed by 38 minutes of electrical stimulation. Numerous membrane-bound interfilamentous vesicles (v) are seen (x76,810).


V

Plate 12 Transmission electron micrograph of fatigued rat epitrochlearis muscle which has been pre-incubated for 30 minutes followed by 38 minutes of electrical stimulation. Large clear vacuoles (CV) are seen, some of which contain membranous material (m). Misalignment of sarcomeres is seen (x6,600).


CV
m
CV

Plate 13 Transmission electron micrograph of fatigued rat epitrochlearis muscle which has been pre-incubated for 30 minutes followed by 38 minutes of electrical stimulation. Large clear vacuoles (CV) are seen throughout the muscle fibre (x11,715).


CV
CV
CV
CV

Plates 12 and 13 demonstrate large clear vacuoles, some containing membranous material, randomly distributed throughout the muscle fibres in view. Plate 12 shows portions of two fibres, with vacuolation being prevalent in one of the fibres. It is notable that both of these sections demonstrate misalignment of adjacent sarcomeres.

CHAPTER V - DISCUSSION

This study was designed to identify compositional and morphological changes in skeletal myofibrils resulting from an exhausting bout of induced isometric contractions. Such changes have previously been demonstrated in rats run to exhaustion (Belcastro et al. 1988). In an effort to isolate the specific effects of contractile activity on the degradation of myofibrillar protein from hormonal, neurogenic and nutritional systemic effects, Nie et al. (1989) used an electrically-stimulated isolated muscle preparation and also showed evidence of myofibrillar degradation. The present study utilized a similar in vitro model in an effort to descriptively present observed alterations induced by exhausting isometric contractions.

A. Mechanical Performance

The initial isometric tensions recorded in the present study are in poor agreement with those reported by Nesher et al. (1980) and Nie et al. (1989) with this muscle preparation and similar stimulation parameters. The average initial twitch tension recorded in the present study was less than 0.3 gms., varying considerably from values between 3.0 and 4.0 gms. reported in the literature. One explanation for this discrepancy is the longer dissection time required to dissect and mount the muscle (1 1/2 minutes longer on average) in the present study. However, this seems to be an improbable cause. Results indicate that when dissection times fell into the range of those employed in previous studies, no increases in initial tension were recorded. A more likely explanation for this observed force discrepancy may be attributed to the design of the stimulation apparatus which was employed in the present study. Foil plate

electrodes, located approximately 1.5 mm on either side of the mounted muscle, were utilized in this study (Figure 2). It is possible that a portion of the electrical current failed to pass through the mounted muscle, but rather passed around the muscle through the incubation medium, which represented a pathway of lower resistance. As a consequence, a lower number of fibres were stimulated to contract, resulting in a decreased twitch tension. It is conceivable that the utilization of point electrodes in direct contact with the muscle may have resulted in a greater current reaching the muscle, and thus effect a greater force output comparable to those reported in similar studies (Nesher et al. 1980, Nie et al. 1989).

The decline in twitch performance over time is in reasonable agreement with that reported by others (Nesher et al. 1980, Nie et al. 1989). The present study found an initial rapid decline in tension development within the first eight minutes, which levelled off gradually until exhaustion. An explanation for the observation of several distinct phases in the rate of force diminution will require further research, however, a possible changeover in utilizable substrate within the stimulation period, and/or failure of the T-tubule system to propagate a signal may be hypothesized. It is unlikely that fatigue was due to limited energy stores, as previous reports demonstrate adequate levels of high energy phosphates ATP and PCr after one hour of stimulation (Nesher et al. 1980, Nie et al. 1989).

B. SDS-PAGE

Both 7.5% and 12% gels were run, in an effort to separate both the higher and lower molecular weight proteins of the samples. In both gels, a slight deviation in the expected migration of the highest molecular weight standard was encountered. The

linear migration patterns of the lower molecular weight standards permitted a relative degree of confidence in the assignment of approximate molecular weights to the unknown bands lying within these regions. However, the assignment of molecular weights falling outside the linear range required greater caution.

There exist several possible explanations for the observed anomalies in standard migrations. One possibility is that the standards were contaminated in some way. This appears unlikely, however, since a similar phenomenon was found in both the high and low molecular weight standards. Another explanation for this observed deviation may be that the gel runs were started before polymerization was complete. This could potentially have resulted in a greater band migration near the top of the gel. The precise source of the deviation can only be speculated.

Since the use of SDS-PAGE as an analytical tool is not without problems (see Chapter II), and in light of the potential risks involved with presentation of the gel data in too specific a fashion, an effort will be made in the following discussion to present the more general features of the compositional protein alterations observed.

MUSCLE RESPONSE TO INCUBATION

It is interesting to note the differences between the samples which were incubated-only and the samples which were freshly dissected. Both scan comparisons (Figures 5B, 7B) revealed greater amounts of protein in the incubated samples, with a few notable exceptions. This was an unexpected finding, as incubated muscles, especially in the absence of insulin, would be expected to be in a net catabolic state (Baracos et al. 1986, Kettelhut et al. 1988). Nevertheless, there are a couple of possible explanations which may account for these observations. Firstly, it is conceivable that the lanes which

contained the freshly dissected samples were loaded with a greater amount of protein than the lanes containing the incubated samples. Despite the effort made to load the lanes with as close to equal amounts of protein as possible, a component of error may have been encountered in attempting to achieve this end. A possible source of this error may have been a discrepancy in the final concentrations of sample added to each lane. It must be noted that we experienced difficulty in achieving uniform protein concentrations of our different samples. During sample preparation, we found that the isolated myofibrils had a tendency to adhere to the glass tubes being used for isolation and transfer. Potentially, this may have resulted in alterations in the final concentrations of sample which were loaded. A second possibility is that the greater absorbance noted with the freshly dissected samples, at least in the lower molecular weight regions, may have resulted from selective proteolysis of higher molecular weight proteins which could have resulted in the accumulation of lower molecular weight fragments.

However plausible the foregoing explanations of the unexpected absorbances may be, certain peak discrepancies are evident and must be noted. Specifically, peaks which represent molecular weights of approximately 69 Kda, 34-35 Kda, and 37 Kda show a significant loss in the incubated samples. It is difficult to suggest what proteins are represented by these peaks, however, troponin T and tropomyosin have been reported to have molecular weights between 30 Kda and 35 Kda (see Table 1). It is conceivable that proteolytic action may have resulted from the incubation procedure, and that a calcium-activated neutral protease (calpain) may have been responsible, since troponin T and tropomyosin have been shown to be degraded by this protease in vitro (Azanza et al. 1979, Toyo-oka and Masaki 1979).

It is well documented that incubated muscles tend to be in negative protein

balance (Kettelhut et al. 1988, Baracos et al. 1989, Hasselgren et al. 1990). As previously discussed, certain incubation conditions have been shown to influence the catabolic process, including the presence of glucose, insulin, and amino acids (Fulks et al. 1974). In the present study, an effort was made to create an environment as physiologic as possible, so as to minimize experimental artifact. A notable exception to this general principle was the exclusion of insulin and other hormones from the medium, a requisite in accordance with our experimental objectives. Literature suggests, however, that it is unlikely that the absence of insulin from our medium could directly account for the myofibrillar losses we observed. Hasselgren et al. (1990), using a preparation similar to the present work, found that myofibrillar protein degradation, as indicated by 3-methyl histidine, was not altered by the presence of insulin. These researchers attribute the protein loss seen in insulin-deprived preparations solely to the non-myofibrillar protein component. This supports the general belief that the two protein components are regulated independently and by different pathways (Kadowaki et al. 1989). Reports of insulin-influenced decreases in proteolysis in similar preparations have failed to separate out differential affects on myofibrillar and non-myofibrillar components (Faulks et al. 1975, Stirewalt and Low 1983).

Certain caution must be exercised in the interpretation of results in which 3-methyl histidine is utilized as an indicator of myofibrillar degradation. Three-methyl histidine is the post-translational product of histidine residues found only in actin and in the myosin heavy chain of type II muscle fibres. It is therefore obvious that, as such, it is not necessarily representative of the myofibrillar proteins as a whole. Research suggests that, in addition to the variability in turnover rates between myofibrillar and non-myofibrillar proteins, heterogeneity also exists in the turnover rates of the individual

proteins constituting the myofibrils (Bates et al. 1983, Bates and Millward 1983). The breakdown of actin alone may account for 70-100% of the 3-methyl histidine measured in isolated muscle preparations (Bates et al. 1983). It thus seems plausible that the selective protein loss observed with incubation in the present study may not have occurred concomitant with actin loss, and would not have been demonstrated in 3-methyl histidine data.

A major consideration with isolated muscle preparations is the adequacy of nutrient exchange by diffusion. In this regard, temperature, tissue oxygenation and tissue dimensions are important variables which must be considered (Segal and Faulkner 1985, Maltin and Harris 1985, 1986, Fagan and Tischler 1989). The effect of increased temperature on muscle protein metabolism and preparation viability is well documented (Baracos et al. 1984, Essig et al. 1985, Segal and Faulkner 1985). It has been demonstrated that as temperature decreases, the distance into isolated muscle where O_2 tension falls to zero increases exponentially. The reduction in rates of protein synthesis and degradation concomitant with lower temperatures coincide with decreases in metabolic rate, both showing similar Q_{10} values (Essig et al. 1985). Therefore, it is possible that a lower incubation temperature than was utilized in the present study may have resulted in a decreased proteolytic response.

The level of tissue oxygenation is also crucial in the maintenance of a viable preparation. Oxygen deprivation has been found to decrease protein synthesis and increase protein degradation (Maltin and Harris 1985, Fagan and Tischler 1989), and contribute to the development of anoxic cores (Segal and Faulkner 1985). The present study utilized a continuous aeration technique which has been shown to be effective in the promotion of tissue viability (Baracos et al. 1989). In addition, evidence exists which

suggests that the epitrochlearis muscle is thin enough to insure adequate diffusion of oxygen and substrates to all regions of the tissue (Wallberg-Henriksson 1987). Since biochemical assays determining ATP, PCr, glycogen and lactate were not performed in the present study, it is difficult to ascertain the viability of the incubated samples with certainty, and the affect this may have had on the degradative response. However, the consideration of results from investigations which have utilized similar models and conditions suggests that it is not unreasonable to assume that the present preparation remained viable throughout the duration of the experiment.

MUSCLE RESPONSE TO STIMULATION

The loss in protein content is observed across all peaks, indicating generalized net myofibrillar protein breakdown in response to fatiguing isometric contractions. This is in agreement with Nie et al. (1989) who found increased 3-methyl histidine release using a similar stimulation protocol. This evidence suggests that contractile activity per se affects a net breakdown in myofibrillar protein in the absence of hormonal influence. The presence of amino acids in the absence of insulin did not appear to prevent this proteolytic response, which was also observed by Nie et al. (1989) in the absence of amino acids. The precise response, if any, to the presence of amino acids in our medium cannot be determined from the present results since different analytical methods have been used.

Belcastro et al. (1988), using an in vivo exercise model, reported a selective protein loss from the contractile apparatus, having found losses in bands representing approximately 58 Kda and 95 Kda. This contrasts with the present study in which a generalized degradation pattern has been indicated. A possible explanation for these

disparate results is that the present model did not allow for hormonal, nutritional, or neurogenic influences, which have all been implicated in playing a role in muscle protein turnover. It is quite possible that certain hormones which rise concomitant with an acute bout of exercise may have a regulatory influence on certain proteolytic pathways within muscle. Such influences have been investigated, and the possible mechanisms by which this control may be mediated have been hypothesized (Seene and Viru 1982, Zeman et al. 1986, Balon et al. 1990). It seems plausible that the influence of the contractile activity itself may somehow be modulated by hormonal interactions. We cannot rule out the possibility that certain endocrine factors may somehow activate a more selective degradative system, perhaps targeting important Z-line components such as desmin and α-actinin. Such an observation has been made by Gutierrez et al. (1990), who observed a 2-stage degradative process in myofibrils of mice injected with snake venom. An initial stage of selective proteolysis was seen, characterized by the loss of desmin and α-actinin. This was thought to be due to a calcium influx through a damaged plasma membrane, and was followed later by a more widespread degradation of myofibrillar protein. A similar 2-stage process has been described by Ishiura et al. (1984), and it has been hypothesized that the selective degradation of desmin may cause the release of α-actinin from the Z-band, thus compromising the integrity of this region. It has been postulated that such a process may be due to the action of calcium-activated neutral proteases (calpains), which have been found localized to the Z-band region (Ishiura 1982, Baker et al. 1987, Riley et al. 1987). Previous research has also suggested that calpains may act as mediators in myofibrillar protein turnover, and that calpain-induced release of α-actinin from the Z-disk may be a switching on factor for a more generalized and complete breakdown (Kulesza-Lipka 1985, Baker et al. 1987). The resultant dissolution of the Z-

band region has been implicated in the failure of myofibrillar force transmission and has been linked to the process of muscular fatigue (Belcastro et al. 1988).

Of the hormones which have been shown to respond to exercise, those which have been demonstrated to have a direct influence on the regulation of protein turnover include insulin, thyroid hormones, and glucocorticoids. However, it is unlikely that insulin has a direct regulatory affect on myofibrillar protein turnover, since research shows that insulin influences the lysosomal pathway (Jefferson et al. 1974, Goodman 1987), and lysosomes are reportedly not involved in the degradation of myofibrillar protein (Lowell 1986). Both thyroid hormones and glucocorticoids have been reported to play a role in the regulation of myofibrillar proteolysis. Increased plasma thyroxine levels, however, do not appear until up to several hours after exercise, so it is unlikely that the catabolic influence of this hormone affects the proteolytic response observed during an acute exercise bout. On the other hand, it is quite likely that glucocorticoids may exert regulatory influence in this regard, since cortisol levels have been found to increase at workloads as low as 25% VO_2 max. during acute exercise (Galbo 1986).

In light of the foregoing, it is tempting to postulate a role for cortisol in the selective myofibrillar degradation observed in in vivo exercise studies. Increased cortisol levels, which would be anticipated from a stress-inducing exhaustive exercise bout, may somehow activate a non-lysosomal proteolytic response. Cortisol, being a steroid hormone, exerts its influence by diffusion across the sarcolemma and binding to receptors within the cytoplasm. Certain receptors may hypothetically be responsible for the regulation of either calpains or ATP-dependent proteases within the cytosol. Danlman et al. (1986), lend support to this notion, by reporting an increase in "easily releasable myofilaments" upon glucocorticoid administration, and further suggesting that

calpains may be responsible.

Recent research supports the concept that increased protein degradation seen with exercise occurs in the non-lysosomal compartment. Kasperek and Snider (1989), reported exercise-induced increases in total protein breakdown, and found that chloroquine, a weak base which inhibits lysosome function, did not significantly alter breakdown rates. It is important to note that these researchers failed to demonstrate a myofibrillar component to the proteolytic process, however, this is not a surprising finding since 3-methyl histidine was utilized as an index for myofibrillar breakdown. As mentioned previously, changes in specific myofibrillar proteins like desmin and α-actinin, as seen in the Belcastro (1988) study, would not be represented in 3-methyl histidine release, but rather would contribute to the release of tyrosine. Tyrosine is often used as an index for the quantification of total protein breakdown (Kasperek and Snider 1989).

If hormones do indeed play a central role in the regulation of the selective proteolytic response observed in in vivo exercise models (as postulated previously), then the possible mediators of the generalized response seen with contractile activity alone must also be discussed. From a purely hypothetical standpoint, it would not be unreasonable to suggest that the effect of contractile activity alone might be to influence non-lysosomal pathways which demonstrate less specificity than the calpain system. Such pathways may include the ATP-ubiquitin-dependent pathway, or the protease complex ("proteasome") (Kettelhut et al. 1988, Furuno et al. 1990). The specificities of these particular pathways, however, remain to be elucidated.

C. **Electron Microscopy**

Electron microscopic investigation was performed in conjunction with the biochemical procedures in order to identify possible morphological alterations resulting from the incubation and stimulation conditions. Electron microscopy did not demonstrate evidence of myofibrillar protein loss in any of the whole muscle sections which were examined. In contrast, with SDS-PAGE, a general myofibrillar protein loss was observed with stimulation. In light of this apparent discrepancy, it may be possible that the generalized proteolytic response which was observed with electrophoresis was not extensive enough to be demonstrated morphologically with the electron microscope. Another possibility for these apparently disparate findings relates to one of the limitations of this study, in that the intact muscles used from each condition may not have been representative of that group as a whole.

Despite the inability to find morphological evidence of myofibrillar proteolysis, certain alterations in morphology were observed among the different conditions and are worthy of mention. Muscles which were fixed in situ demonstrated essentially normal ultrastructure in the majority of sections studied. The only specific alteration observed in this group was the occasional isolated finding of swollen mitochondria, however, this was a relatively rare finding in sections belonging to this group. Mitochondrial swelling was a prominent feature of samples from incubated-only and incubated-stimulated conditions. This often included observations of matrix space pallor and concentric membrane formations within these structures. These were generalized findings, and were randomly distributed throughout the viewed sections, demonstrating no apparent specific localization. The etiology of these features is unknown, however, literature suggests they may be an artifact of the fixation procedure (Mair and Tomé 1972,

Carpenter and Karpati 1984, Engel and Banker 1986). It is difficult to explain why these findings were more prevalent under the incubated-only and incubated-stimulated conditions.

The sarcomeric misalignment observed in incubated-stimulated samples was a generalized feature specific to this group, and was seen in the majority of the sections viewed. It would appear unlikely that such an apparent anomaly could be accounted for by a mechanical disruption, since the present study utilized isometric contractions of low force. The significance of this finding requires further investigation.

The most notable morphological alteration observed in the incubated-stimulated samples was the appearance of large clear vacuoles localized to different areas of certain muscle fibres. This phenomenon was not observed in either the freshly dissected or incubated-only samples, and was found in the majority of viewed sections of the stimulated muscle. It is possible that this vacuolation represents a dilatation of the sarcotubular system (Gonzalez-Serratos et al. 1978, Casademont et al. 1988). Similar observations have been made in certain pathological conditions such as dermatomyositis, polymyositis, and McArdle's disease (Casademont et al. 1988), as well as in muscles subject to acute trauma (Fisher et al. 1990). A plausible explanation for this observed phenomenon is that prolonged muscular contractions may result in an increased Na^+ concentration in the T-tubule system, resulting from either an enhanced T-tubule Na^+/K^+ - ATPase activity or as a result of pH-induced Na^+ pump inhibition. A concomitant influx of Cl^- to maintain the electrical gradient is followed by the entry of water, resulting in dilatation of the T-tubules (Casademont et al. 1988). Such a mechanism has been implicated as a possible factor in the fatigue process, since a dilated T-tubule system could possibly compromise the propagation of an action potential.

CHAPTER VI - SUMMARY, CONCLUSION AND RECOMMENDATIONS

A. Summary

The purpose of this study was to investigate any morphological and compositional changes in skeletal myofibrils resulting from an exhausting bout of isometric contractions. Epitrochlearis muscles from fourteen male Sprague-Dawley rats were dissected and assigned to one of three experimental conditions - freshly dissected, incubated-only, and incubated-stimulated groups. The muscles were then analyzed using SDS-PAGE and electron microscopy and the results descriptively reported.

Densitometric scanning indicated greater amounts of protein in samples which were incubated than in samples which were freshly dissected, except for a few notable areas in which the incubated samples displayed significant protein losses. This finding of greater protein quantities in the incubated samples was unexpected, and could most likely be attributed to extra protein loading in the incubated lane, or to selective degradation of the very high molecular weight proteins within those samples. The incubated samples demonstrated specific protein losses in the regions of 69 Kda, 37 Kda, and 34-35 Kda, and it is possible that these losses included the myofibrillar proteins troponin T and tropomyosin.

Muscles which were stimulated demonstrated a widespread, generalized loss of protein throughout the full molecular weight range examined. This suggests a non-specific net proteolytic response to contractile activity in the absence of hormonal influence. However, whether or not this represents an increased rate of degradatory activity, or a decreased rate of synthesis under these conditions cannot be ascertained. A hypothesis was put forth, in which a role for glucocorticoids was postulated in the

regulation of a selective degradatory response in response to exercise in vivo.

Electron microscopy study failed to demonstrate evidence of myofibrillar breakdown in incubated and stimulated muscles. Nevertheless, several interesting observations were noted. Swollen mitochondria, some of which appeared to contain membrane formations, were observed in all three experimental groups, however, were more prevalent in the samples which were incubated. It was suggested that these were artifactual in nature, resulting from the fixation procedure, however, the precise origin and significance of this finding is unknown. Widespread vacuolation of the sarcotubular system was observed in the majority of the stimulated sections which were viewed. This was a significant finding, as it was not observed in either of the other two groups, and may have influenced the onset of fatigue in the stimulated muscles. The finding of sarcomeric misalignment was also a feature unique to the stimulated muscles, however, the specific etiology and significance of this finding will require further research.

B. Conclusion

1. Muscles which were incubated and not stimulated demonstrated a loss of myofibrillar protein with molecular weights approximately 69 Kda, 37 Kda, and 34-35 Kda.
2. Muscles which were incubated and stimulated to exhaustion demonstrated a generalized, widespread loss of myofibrillar protein throughout the full molecular weight range investigated.
3. Muscles which were incubated and stimulated to exhaustion demonstrated widespread vacuolation, probably of the sarcotubular system, which may have contributed to the onset of fatigue.

4. Muscles which were incubated and stimulated to exhaustion demonstrated a misalignment of sarcomeres, the significance of which is unknown.

C. Recommendations

1. In future studies of this nature, analysis of ATP, PCr, lactate and glycogen should be undertaken as an index of preparation viability and a useful information source with which to correlate mechanical performance.
2. If possible, individual myofibril samples from each particular muscle should be run in separate gel lanes in order to discern inter-sample differences, and to possibly correlate this information with each individual muscle's mechanical performance. To this end, it will be necessary to devise improved isolation and sample transfer procedures which utilize plastic vessels having a lower affinity for the myofibril particles. Positive consequences of this may be less loss of sample than found using conventional glass, and a greater likelihood of achieving uniform concentrations of the myofibril samples.
3. A greater number of gels should be run in future studies of this type. Different percentage gels would increase the likelihood of discerning specific differences in proteins which lie in different molecular weight regions.
4. Molecular weight standards should be run several times on practice gels prior to running experimental samples, in order to establish linearity throughout the full range of molecular weights represented.
5. Future research should attempt to evaluate 3-methyl histidine and tyrosine release in conjunction with SDS-PAGE and electron microscopic evaluations

within the same study. By utilizing a multi-analytical approach such as this, it would be possible to directly relate the different indices. This may enable researchers to answer some important questions concerning some of the current methodologies in widespread use.

6. An interesting follow-up study would be to selectively alter the stimulation medium with different endocrine factors in order to evaluate the affect these have on protein breakdown and mechanical performance. A comparison could then be made to in vivo exercise models. Resultant findings may help to elucidate the precise role played by contractile activity and specific systemic influences in the degradative response seen with exercise.

CHAPTER VII - BIBLIOGRAPHY

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Appendix A. Procedure Data Sheet

RAT SPECIFICS:

Date:

#
Wgt(g)
Anesthetic Dose(cc)

Epi Dissection Times:
S /
C /

Remarks:

PRE-INCUBATION SPECIFICS:

KRB Medium ( ) Glucose
( ) Insulin
( ) AAs
Temperature

	S	C
Start		
Change Medium		
Begin Stimulation		

STIMULATION SPECIFICS:

Chart Scale(mg/div):
Frequency:
Duration:
Voltage:

Running Time (minutes)	Avg Peak Twitch Tension (divisions)	Avg Baseline Tension (mg)

Appendix B. Raw Twitch Performance Data

PEAK TENSION RECORDINGS- TEN MINUTES

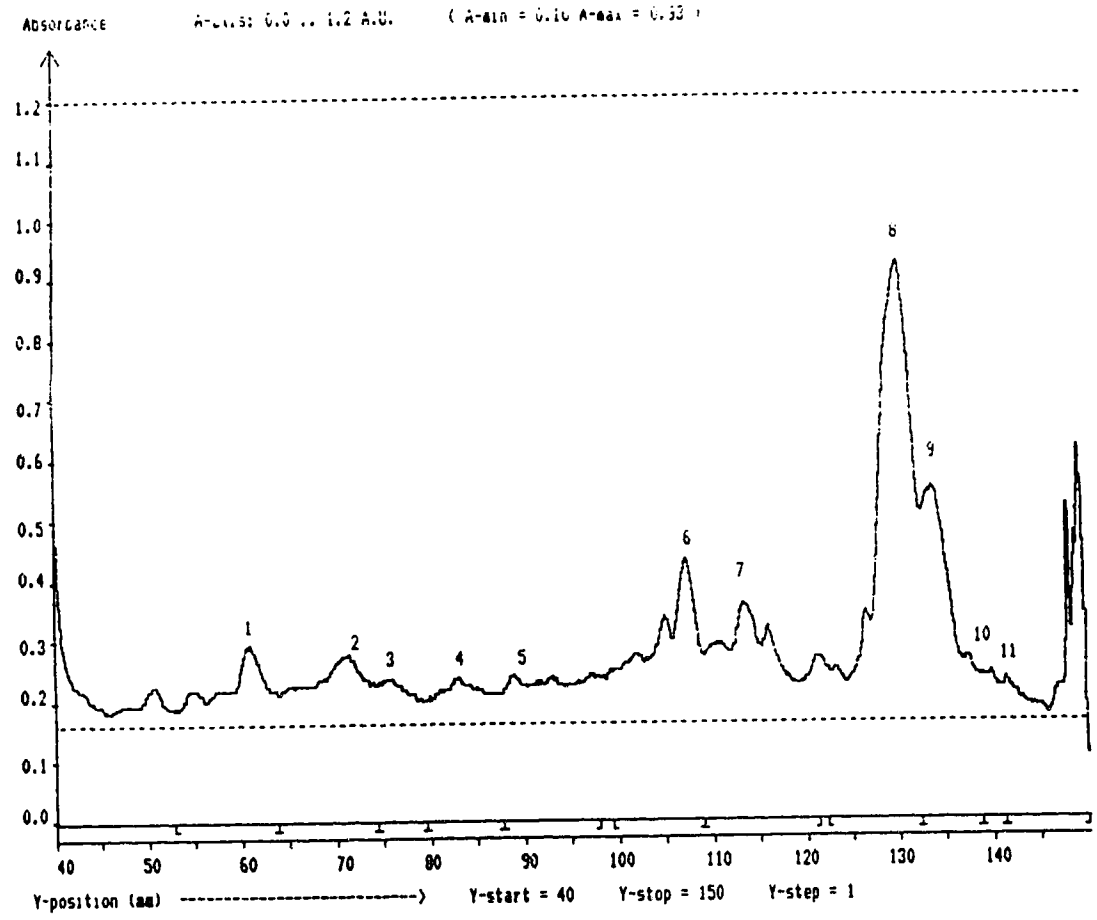
AVGPTT1-(mg) Minutes	0	1	2	3	4	5	6	7	8	9	10
E-56(S)	270	270	260	240	230	230	210	200	190	180	170
E-58(S)	260	240	240	220	200	190	180	170	160	150	140
E-60(S)	220	210	200	200	180	170	160	150	140	140	135
E-66(S)	220	200	190	190	180	160	160	160	150	150	150
E-70(S)	290	260	230	210	190	170	160	150	140	140	130
E-74(S)	300	260	240	220	200	190	170	150	150	140	140
E-90(S)	240	220	210	195	180	170	160	150	140	140	130
E-182(S)	295	275	240	210	180	160	150	140	130	125	120
AVGPTT.	261.9	241.9	226.3	210.6	192.5	180	168.8	158.8	150	145.6	139.4
%PTT.	100	92	86	80	74	69	64	61	57	56	53

PEAK TENSION RECORDINGS-TO FATIGUE

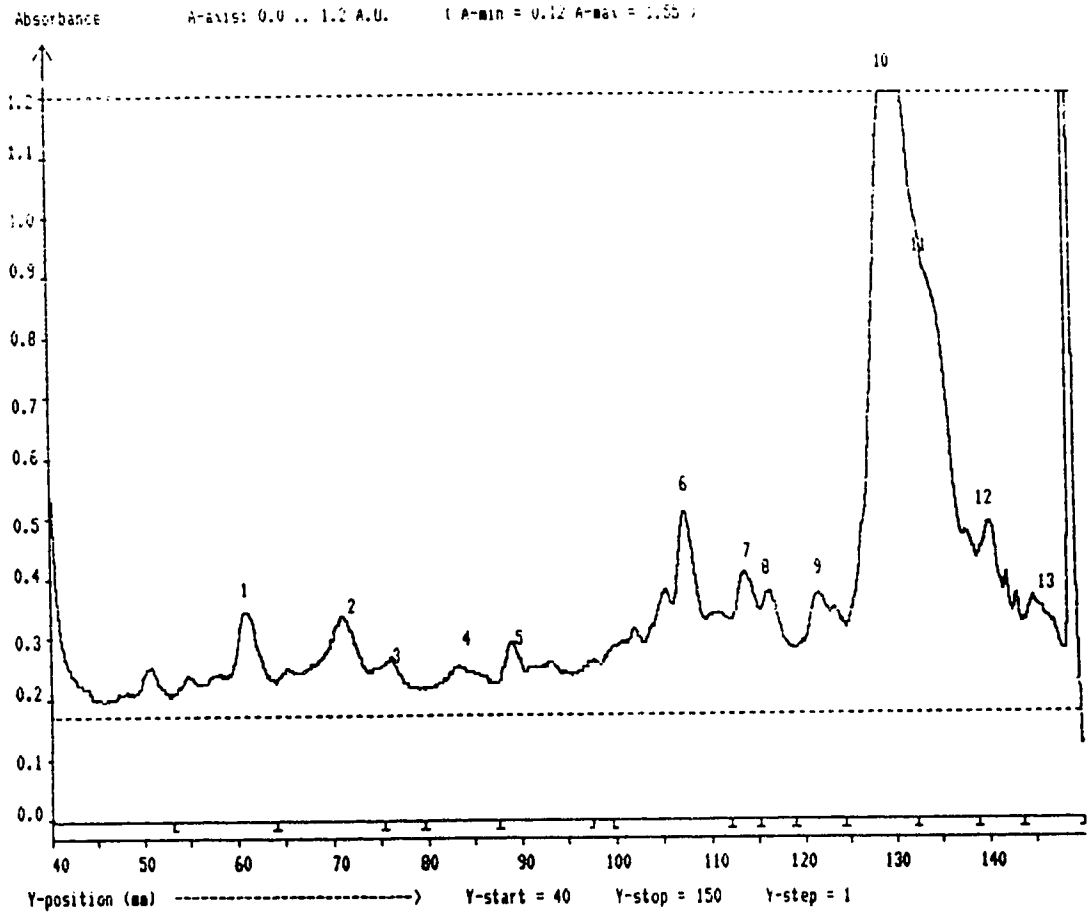
AVGPTT2-(mg) Minutes	0	5	10	15	20	25	30	35	40	45	50	55	60
E-56(S)	270	220	170	150	130	110	90	75	60	50	50	50	40
E-58(S)	260	190	140	130	110	90	70	50	40				
E-60(S)	220	170	135	120	110	90	80	70	60	50	40		
E-66(S)	220	160	150	130	100	90	70	60	60	50	45	30	
E-70(S)	290	170	130	100	100	80	65	50	40		0		
E-74(S)	300	190	140	120	110	90	80	60	55	45	40		
E-90(S)	240	170	130	125	115	100	85	60	50	40			
E-182(S)	295	160	120	110	100	70	50	45	40				
AVGPTT	261.9	178.8	139.4	123.1	109.4	90	73.1	58.8	50.6	47	43.8	40	40
%PTT	100	69	53	47	42	34	28	22	19	18	17	15	

Appendix C. Original Densitometric Scans

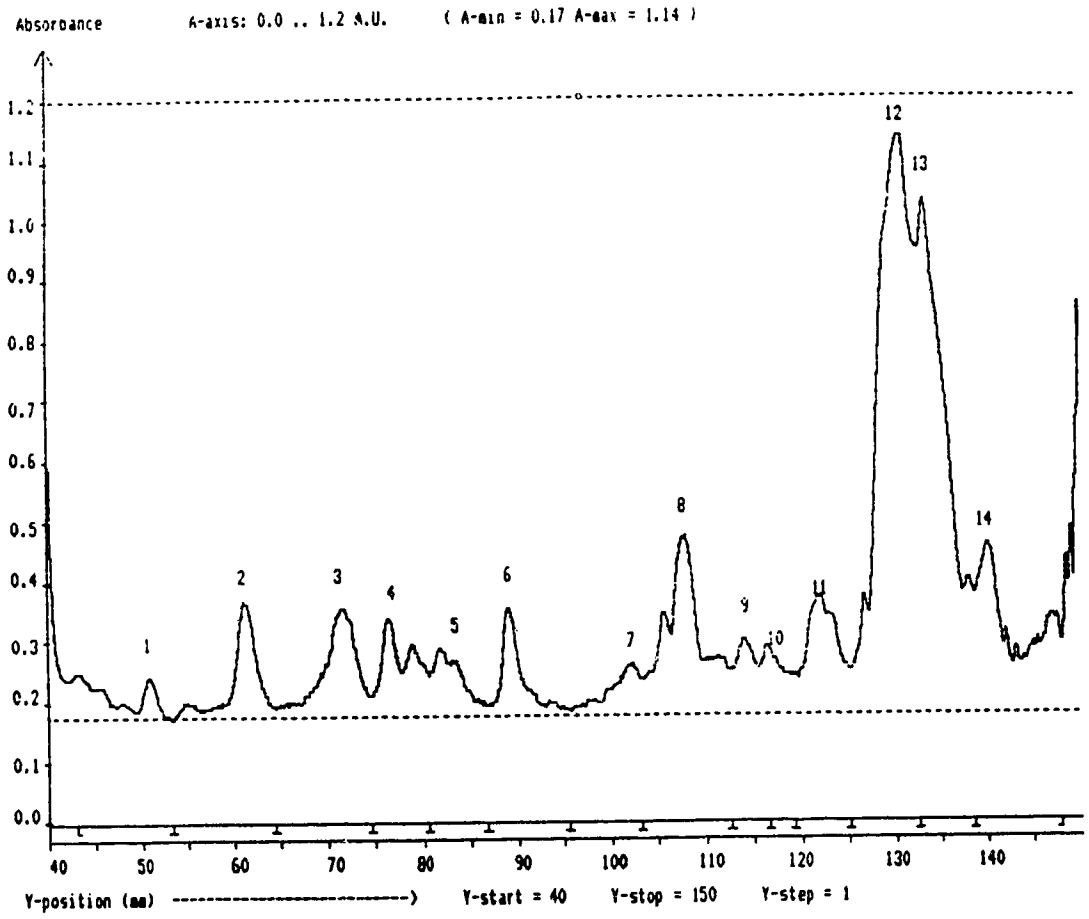
Scan #1 - Incubated-stimulated sample - Lane A (12% gel)



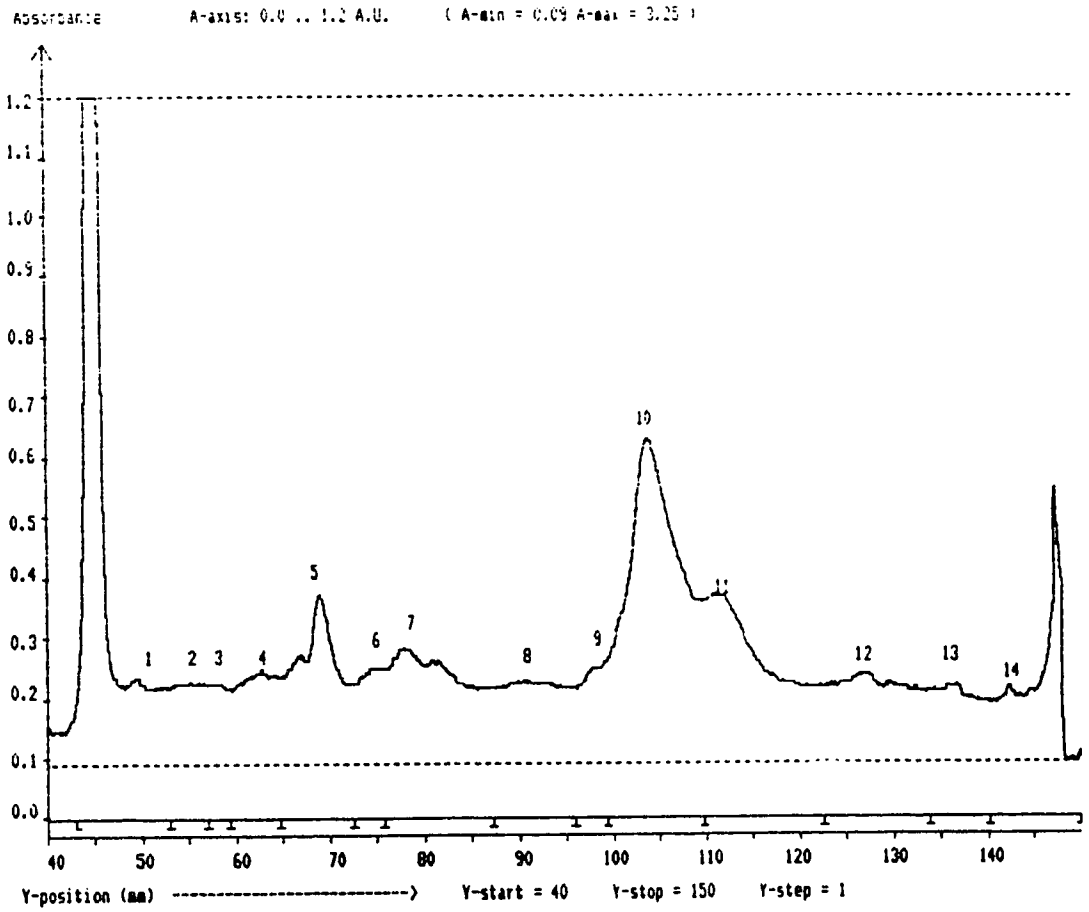
Scan #2 - Incubated-only sample - Lane B (12% gel)



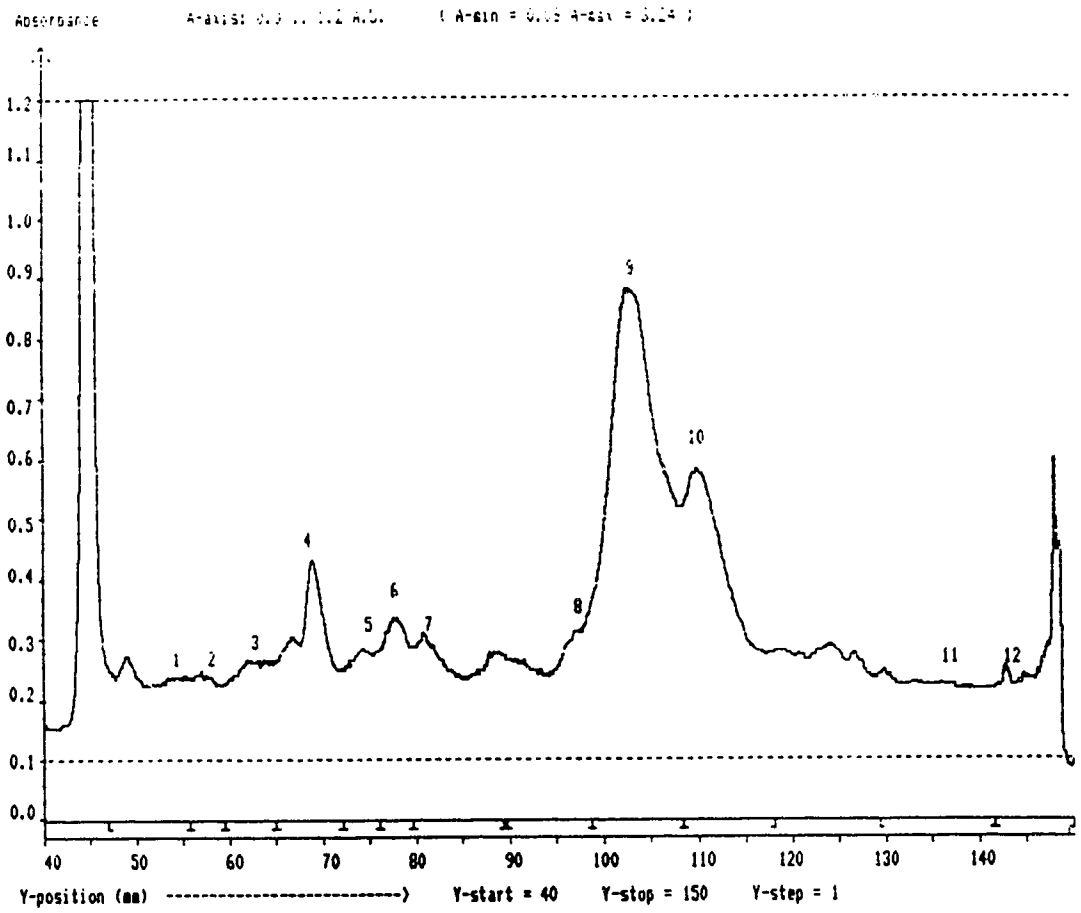
Scan #3 - Freshly dissected sample - Lane C (12% gel)



Scan #4 - Incubated-stimulated sample - Lane A (7.5% gel)



Scan #5 - Incubated-only sample - Lane B (7.5% gel)



Scan #6 - Freshly dissected sample - Lane C (7.5% gel)