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Synthesis and assembly of myofibrillar proteins in differentiating muscle cells

by

Ronald Eugene Allen

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LIST OF ABBREVIATIONS

ADP = adenosine 5'-diphosphate

ATP = adenosine 5'-triphosphate

ATPase = adenosine triphosphatase

^{o}C = degrees centigrade

cm = centimeter

DNA = deoxyribonucleic acid

dpm = disintegrations per minute

EDTA = ethylenediaminetetra acetic acid

EGTA = 1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane

g = acceleration due to gravity = 960 cm/sec

hr = hour

IF = initiation factor

M = molar

mCi = millicurie

min = minute

ml = milliliter

mM = millimolar

mRNA = messenger ribonucleic acid

nm = nanometer

OD = optical density

Pi = inorganic phosphate

polyA = polyadenylic acid

RNA = ribonucleic acid

S = sedimentation coefficient

SDS = sodium dodecyl sulfate

sec = second

tcRNA = translational control ribonucleic acid

Tris = tris-(hydroxymethyl)-aminomethane

μCi = microcurie

μg = microgram

μM = micromolar

μm = micrometer

I. INTRODUCTION

Through the complex process of cell differentiation, cells of higher organisms become specialized to serve specific physiological functions in the total organism. Perhaps one of the most striking examples of this specialization is skeletal muscle cell differentiation. Mature mammalian skeletal muscle cells have several distinguishing characteristics. They are elongated, multinucleated and contain, as approximately one half of their total protein, a contractile organelle, the myofibril. This highly specialized organelle is responsible for rapid contraction and relaxation, the primary function of skeletal muscle.

The myofibril is a highly organized biological structure resembling a long thread of repeating contractile units. Each unit, or sarcomere, is composed of interdigitating thick (15 nm diameter) and thin (6-8 nm diameter) filaments. Sarcomeres are connected end to end by a structure called a Z line into which the thin filaments of adjacent sarcomeres insert. The myofibril is composed of only about eight different proteins which interact in a very specific manner to form the structural, the force generating, and the regulatory components of the system. Thick filaments are composed primarily of myosin which actually forms the shaft of the filament but also contain C-protein which binds around the lateral portions of the filament at regular intervals, and M-protein which forms part of the M line structures that interconnect thick filaments at their centers. The backbone of the thin filament is actin which binds the regulatory proteins tropomyosin and troponin, and possibly

β-actinin. The Z line is composed of α-actinin and perhaps other proteins, but the exact composition and structure of the Z line are not clearly understood.

During the terminal phase of muscle cell differentiation, many noticeable muscle characteristics appear. Mononucleated myoblasts align end to end, and their cell membranes fuse to form a single elongated multinucleated cell, the myotube. Concurrent with the fusion process, many muscle specific proteins are synthesized including acetylcholinesterase and myosin. Through undefined mechanisms, the contractile apparatus is then assembled enabling contraction to occur.

Elucidation of the mechanisms responsible for the proper de novo assembly of myofibrils during the terminal phase of differentiation is the broad objective of this research. Starting with the assumption that myofibrils self-assemble from newly synthesized proteins, the relative time sequence in which individual proteins accumulate and the relative amounts in which they accumulate should play a major role in regulating the assembly process. The experiments described in this thesis were designed to answer two questions:

1. When does the massive accumulation of key myofibrillar proteins begin during muscle cell differentiation?

2. What are the relative amounts in which key myofibrillar proteins accumulate?

Chick embryo muscle cell cultures were used as the source of differentiating muscle because the differentiation process can be controlled and synchronized to a greater extent than muscle cell differentiation in an embryo.

Four major myofibrillar proteins were chosen for these experiments because of their structural and functional significance. Actin and myosin were chosen because they are the two proteins responsible for force generation and are also the primary structural components of the thin and thick filaments, respectively. Tropomyosin was selected as a representative of the myofibrillar proteins which have a regulatory function. α-Actinin was selected because of its location in the Z line, a structure which forms the boundary of sarcomeres and therefore may play a vital role in the addition of new sarcomeres during myofibrillar growth. This thesis describes the development of techniques and methodology used to study these proteins in differentiating muscle cell cultures, the temporal and stoichiometric patterns of myofibrillar protein accumulation, and the resultant nascent myofibrillar structures that are assembled in myotubes.

II. LITERATURE REVIEW

A discussion of myofibrillar protein synthesis and myofibril assembly in differentiating muscle first requires a brief overview of two fundamental areas, myofibril structure and composition and muscle differentiation. These sections are intended to provide information that is relevant to subsequent discussions of myofibrillar protein synthesis and assembly and not as a comprehensive review of each respective topic. Therefore, this review has been organized into four sections: (1) myofibril structure and composition, (2) muscle cell differentiation, (3) ultrastructural observations on de novo myofibril assembly, and (4) biochemical observations on myofibrillar protein accretion in differentiating skeletal muscle cells. The goal of this review is to provide an adequate foundation for reporting and discussing the results of this research. Unless specified otherwise, all comments will refer to mammalian or avian skeletal muscle and its components.

A. Myofibril Structure and Composition

The myofibril, first described in detail by Huxley (1953 and 1957) and Huxley and Hanson (1954), is a threadlike structure of repeating sarcomeres. Each sarcomere is composed of thick filaments (15 nm) and thin filaments (8 nm). The thin filaments are attached to the Z line at one end and extend into the space between parallel thick filaments which form the A band at the center of the sarcomere. Cross sections through the terminal portions of thick filaments reveal a hexagonal array of six thin filaments surrounding each thick filament. In general,

thin filaments are a very consistent length of 1.0 μm and thick filaments are 1.5 μm in length. The Z line structure into which thin filaments of two adjacent sarcomeres insert serves to link sarcomeres end to end to make the long thread-like myofibril. The structure of the Z line remains a controversial topic but perhaps the early model of Knappeis and Carlsen (1962) provides the best working model. In this model, thin filaments approach the Z line as a four point square lattice. The end of each thin filament gives rise to four Z filaments which traverse the Z line to join with Z filaments from four thin filaments coming from the other side of the Z line. In addition to Z filaments, an amorphous or matrix component with an ill-defined structure is generally present and often serves to partially obscure the Z filament lattice.

The thick filament is composed primarily of myosin which is a large (467,000 daltons) protein (Godfrey and Harrington, 1970). Myosin is a 150 to 160 nm long molecule with a 95% α-helical coiled-coil tail and a globular head. Two heavy polypeptide chains (200,000 daltons) comprise the tail and part of the head, and four light polypeptide chains ranging from about 16,000 to 25,000 daltons comprise the rest of the head region.

Myosin is very susceptible to proteolytic cleavage by a number of proteases; this property actually enabled investigators to dissect the molecule (Lowey and Cohen, 1962; Lowey et al., 1969). Tryptic digestion acts on the mid-region of the molecule to cleave myosin into two parts. One part consisting of a portion of the tail is termed light meromyosin and is essentially 100% α-helical, does not bind actin, or have ATPase activity. The globular head plus the remaining portion of

the tail is termed heavy meromyosin and contains both the actin binding and ATPase activity of the myosin molecule (Lowey and Cohen, 1962; Lowey et al., 1969).

Myosin is soluble at high ionic strengths but insoluble at ionic strengths approximating those found in the living cell. Under these conditions, myosin molecules spontaneously form filaments resembling native thick filaments in muscle (Huxley, 1963). The tail portion of the molecule forms the backbone of the filament and the head region protrudes out from the filament and is referred to as a cross-bridge. The cross-bridge is the locus of the ATPase activity and actin binding site of the molecule (Gergely, 1950; 1953) and is the portion of the molecule that moves during contraction (Huxley, 1972).

As mentioned, myosin has the ability to hydrolyze ATP to ADP and inorganic phosphate. The physiologically significant ATPase activity of myosin is Mg^{++} dependent. Kinetics of this reaction have been investigated in great detail (see reviews by Mannherz and Goody, 1976 and Taylor, 1972) and the rate limiting step is the release of products, ADP and inorganic phosphate (Pi), from myosin. In muscle, this slow step is greatly accelerated by the association of actin with the myosin·ADP·Pi complex; this is the basis of the actin stimulation of Mg^{++}-dependent myosin ATPase. Lymn and Taylor (1971) have proposed an elegant cyclic scheme for the actomyosin ATPase reaction responsible for muscle contraction. The cyclic nature of the scheme readily lends itself to adaptation to the cyclic movement of myosin cross-bridges during contraction. Evidence for cross-bridges movement was obtained by Reedy et al. (1965) through low angle X-ray diffraction and electron microscope

studies of glycerinated insect flight muscle. In going from rigor to the relaxed state (no actin-myosin interaction), the angle of the cross-bridge changed from 45° to 90° with respect to the myosin filament. This movement has been proposed to be correlated with ATP cleavage on myosin (Lymn and Taylor, 1971). Furthermore, the change from the relaxed to the rigor state resulted in a change in cross-bridge angle from 90° back to 45° (Reedy et al., 1965). Lymn and Taylor (1971) suggested that this change corresponded to actin activation of product release. Various investigations have been conducted using ATP analogues and various other experimental approaches; however, the movement of cross-bridges has yet to be correlated experimentally with the kinetics of actomyosin ATPase.

The second major protein of the thick filament is C-protein with a molecular weight of 135,000 to 140,000 daltons (Offer, 1972). This protein binds in a series of stripes transversely along the middle one third of each end of the thick filament (Moos et al., 1975; Offer, 1972; Pepe and Drucker, 1975). The spacing between stripes is 43 nm which is also a characteristic spacing of light meromyosin paracrystals. Moos et al. (1975) concluded from their observations that the periodicity of binding reflects the periodicity of the underlying light meromyosin portion of the myosin molecule. The protein's function is unknown since Moos et al. (1975) demonstrated that C-protein is not necessary for thick filament assembly from purified myosin.

A third protein associated with the thick filament is M-protein which has a molecular weight of about 160,000 (Masaki and Takaiti, 1974; M. Stromer, Iowa State University, personal communication, 1974). M-protein presumably composes

part of the M line structure in the center of the A band and helps to maintain thick filaments in register with one another.

Thin filaments are composed of an actin backbone with associated regulatory proteins. Actin, the primary protein, is a globular protein of about 42,000 daltons. The most striking property of this protein is its ability to aggregate from monomeric G-actin into a long, double-stranded filamentous form of globular units referred to as F-actin. Aggregation is accomplished with the hydrolysis of bound ATP-Mg^{++} or Ca^{++} at elevated salt concentrations (Laki et al., 1950; Straub and Feur, 1950). The right-handed F-actin helix (Depue and Rice, 1965) has a repeating periodicity along the filament of 36 nm with each unit containing 13 monomers in the two chains (Moore et al., 1970; O'Brien et al., 1971). Although actin, like myosin, spontaneously forms filaments, the filaments are a random assortment of lengths instead of the precise 1 μm found in a myofibril. Ultrastructurally, however, the synthetic F-actin filaments appear similar to the native actin filaments in muscle if consideration is given to the masking effects that tropomyosin and troponin have on the beaded structure of the F-actin backbone in native filaments.

In recent years, actin has been found in motile systems in virtually every organism or cell type studied (Pollard and Weihing, 1974). Actin also appears to be one of the most highly conserved molecules known. This is not surprising since even in skeletal muscle this one small protein must exhibit many specific properties. In addition to the ability to interact with itself to form unidirectional filaments, actin must also have specific sites for interacting with myosin during

contraction, for binding tropomyosin and troponin-I to regulate contraction and relaxation, and for binding α-actinin at the Z line end and possibly β-actinin at the other end of the filament. It would seem very difficult to modify the structure of actin by substituting amino acids without having a detrimental effect on some of its properties.

β-Actinin is a protein present in small amounts which is thought to be bound to the free end of F-actin filaments. β-Actinin has been postulated to be the protein responsible for regulating thin filament length (Kawamura and Maruyama, 1970, 1972; Maruyama, 1966). The protein is not well characterized, its function has not been rigorously confirmed, and even its existence has been questioned.

As mentioned previously, other proteins are bound to actin in the thin filament. Tropomyosin, a dimeric protein of about 70,000 daltons, is one of the regulatory proteins on the thin filament. Two very similar polypeptides, α and β (Cummins and Perry, 1973), with molecular weights of 33,000-36,000, respectively, form a rod-like molecule 40 nm long by 2 nm in diameter. The molecule is essentially a 100% α-helical coiled coil (Cohen and Holmes, 1963). One strand composed of tropomyosin molecules aligned in a head-to-tail arrangement lies in the groove on each side of the F-actin filament. Tropomyosin, under the control of the troponin complex, is thought to roll into the groove permitting interaction of actin and myosin during contraction, and then rolls out of the groove preventing interaction of actin and myosin during relaxation (Haselgrove, 1972; Huxley, 1972).

The second regulatory protein, troponin, is bound to tropomyosin and, in response to changes in Ca^{++} concentration, regulates contraction.

In the absence of Ca^{++}, troponin serves to inhibit the interaction of actin and myosin, thereby permitting relaxation. When free intracellular Ca^{++} concentration increases above $10^{-7}M$, troponin binds Ca^{++} and undergoes a conformational change resulting in the movement of tropomyosin into the actin groove. As previously mentioned, this movement of tropomyosin permits actin-myosin interaction and contraction.

Troponin is a 72,000 dalton protein composed of one molecule of each of three nonidentical subunits (Greaser and Gergely, 1971). The largest of the three subunits is troponin-T (33,000 daltons) which binds the complex to tropomyosin (Jackson et al., 1975). Troponin-C (18,000 daltons) is the Ca^{++} binding subunit and is bound to troponin-T and troponin-I (Collins et al., 1973). Troponin-I (21,000 daltons) is bound to troponin-C and, in the absence of bound Ca^{++} on troponin-C, is also bound to actin (Wilkinson and Grand, 1975). By binding to actin, troponin-I alone can inhibit the Mg^{++} activated actomyosin ATPase, and when associated with the rest of the troponin-tropomyosin complex, troponin-I and tropomyosin both function in inhibiting the Mg^{++}-activated ATPase and, consequently, contraction. When troponin-C binds Ca^{++}, troponin-I is dissociated from actin permitting the movement of tropomyosin into the groove which ultimately results in contraction.

Just as the Z line structure, the structure to which thin filaments are attached, is not fully understood, its composition has also been elusive. α-Actinin has been identified as a Z line component by selective proteolytic digestion of Z lines (Goll et al., 1969), extraction of Z lines (Robson et al., 1970; Stromer et al., 1967, 1969) and by antibody localization (Masaki et al., 1967; Schollmeyer, 1976).

This 200,000 dalton protein is 40-50 nm by 3.5-4.5 nm (Suzuki et al., 1976) and binds very strongly to F-actin. Actin binding is strongest at low temperatures but α-actinin also binds weakly to F-actin at 37°; however, at 37° it can be displaced by tropomyosin (Goll et al., 1972; Stromer and Goll, 1972). The available antibody binding evidence (Schollmeyer, 1976) suggests that α-actinin makes up the amorphous or matrix component of the Z line, however, this point has not been unequivocally demonstrated. Anti-tropomyosin has also been reported to bind to Z lines in tadpole muscle which have little of the amorphous component (Schollmeyer, 1976) and therefore suggests that tropomyosin is a component of the Z filaments. The probability that new, unknown proteins exist in the Z line seems small. It seems more likely however that proteins such as actin and tropomyosin that are found in thin filaments could also be components of the Z line.

B. Muscle Cell Differentiation

Cell differentiation in general is accomplished through differential gene action. Consequently, only genes coding for enzymes and structural proteins characteristic of a particular cell at a given point in differentiation are expressed. The regulation of differential gene action in differentiating muscle cells or any eukaryotic cell is poorly understood; therefore, only the morphological and biochemical results of this process can be presented.

Prior to the terminal stages of differentiation, myogenic precursor cells are biochemically and morphologically indistinguishable from many

other cells. They are mononucleated cells that have the capacity to proliferate and possess only the contractile proteins common to most cell types, the "cytoplasmic contractile system" (Pollard and Weihing, 1974). They do not possess any "muscle specific" characteristics. At some late stage in the differentiation process, cell proliferation ceases; individual mononucleated myogenic cells then align end to end and fuse into a long multinucleated myotube (Stockdale and Holtzer, 1961). These events account for the multinucleated, nonproliferating state of differentiated muscle cells. Fusion has been demonstrated to be a very specific process. Only myogenic cells spontaneously fuse with each other; mixed cultures of chick skeletal muscle cells and liver, kidney, or heart cells produce myotubes which are derived only from the skeletal muscle cells (Yaffe and Feldman, 1965). Even though cultured muscle cells will not fuse with different cell types from the same species, hybrid myotubes can be formed with myogenic cells from different species such as calf, rat, rabbit or chick muscle cells (Yaffe and Feldman, 1965). The fusion process and the basis of muscle cell recognition have not been elucidated on a molecular level.

Concomitant with cessation of proliferation and fusion, several enzymes characteristic of muscle cells are synthesized. Mature muscle has tremendous energy demands primarily because ATP hydrolysis provides the energy for contraction. Therefore, the accumulation of enzymes active in the energy metabolism of skeletal muscle has been studied. Several of these enzymes have been found to increase dramatically in differentiating muscle cells when proliferation ceases and fusion begins. These enzymes include those involved in the metabolism of

glycogen, such as glycogen phosphorylase and synthetase (Shainberg et al., 1971; Wahrmann et al., 1973), as well as the glycolytic enzyme aldolase (Turner et al., 1974). Creatine kinase which is responsible for the short term storage of energy in the form of creatine phosphate also increases rapidly during this stage of muscle differentiation (Shainberg et al., 1971; Turner et al., 1974).

In addition to the enzyme pathways responsible for supplying the energy needs of muscle cells, the excitation-contraction coupling system is also developing. Fambrough and Rash (1971) first described the development of acetylcholine sensitivity in differentiating muscle cell cultures. They measured resting membrane potentials with microelectrodes and the sensitivity of these membranes to acetylcholine. Resting potentials increased as cells progressed from short bipolar mononucleated myogenic cells through long bipolar cells to fused myotubes. Furthermore, only the long bipolar cells and the myotubes were sensitive to acetylcholine. These results have been verified by histochemical studies of acetylcholinesterase activity in differentiating muscle cell cultures (Fluck and Strohman, 1973; Paterson and Prives, 1973).

The sarcoplasmic reticulum is the other critical part of this excitation-contraction system and is responsible for getting the signal from the nerve to the myofibril. The sarcoplasmic reticulum is a membrane system surrounding the myofibril that releases Ca^{++} in response to depolarization of the sarcolemma. The wave of depolarization is conducted from the sarcolemma into the cell by the T tubule. Troponin-C on the thin filament binds the Ca^{++} and initiates contraction

as described in the preceding section. Following the wave of depolarization, the sarcoplasmic reticulum actively sequesters Ca^{++} with the energy provided by a sarcoplasmic reticulum ATPase. Lowering intracellular free Ca^{++} to 10^{-8} or less causes the release of Ca^{++} from troponin and results in relaxation of the myofibril. As with the other muscle specific systems, the sarcoplasmic reticulum's ability to bind Ca^{++} and the sarcoplasmic reticulum ATPase activity start to increase prior to and during fusion of myoblasts in differentiating muscle cell cultures (Lough et al., 1972). Ultrastructural studies of differentiating muscle cells confirm the biochemical observations; membrane systems resembling the sarcoplasmic reticulum may be found developing near nascent myofibrils (Walker et al., 1975).

There is also evidence that myosin (Coleman and Coleman, 1968) and actin (Paterson et al., 1974) are synthesized for myofibril assembly in concert with these other changes that have been discussed. A more detailed discussion of myosin and actin synthesis will follow in later sections of this review.

In summary, myogenic precursor cells, about which very little is known, undergo a major change in their protein synthetic program and start synthesizing proteins that result in formation of structures and ability to carry out functions characteristic of terminally differentiated muscle cells. They cease proliferation, fuse to form multinucleated myotubes, synthesize the enzymes necessary for supplying the energy needs of a contracting muscle cell, develop the capability to transfer a signal from a motor neuron to myofibrils, and synthesize and assemble myofibrillar proteins. These changes seem to be initiated virtually

simultaneously and collectively represent the last major step in muscle cell differentiation.

C. Ultrastructural Events during Myofibril Assembly

The origin of myofibrils in developing muscle has been a controversial topic for many years. Early scientists, equipped with light microscopes and fertile imaginations, provided the first insights into myofibril assembly. Godlewski (1902) presented one of the first popular theories, the precursor granule theory; cytoplasmic granules were believed to align in rows parallel to the long axis of the cell and fuse to form myofibrils. With the discovery of new organelles, new theories attributing myofibril formation to those organelles arose. Mitochondria, which normally become elongated and oriented with the long axis of a muscle cell, were assigned a primary role in myofibril formation (Duesberg, 1910; Meves, 1909; Naville, 1922). Nuclear material (Eycleshymer, 1904; McGill, 1910) and centrioles (Wolbach, 1927) also were suggested as myofibril precursors.

Weed (1936) finally brought the argument back to a cytoplasmic granule theory. She noted changes in mitochondrial staining during myofibril formation but found no evidence for the direct transformation of mitochondria into myofibrils. Myofibrils were formed at the expense of certain cytoplasmic granules, and were produced in a manner which Weed claimed was similar to the elaboration of materials in secretory cells.

Until electron microscopy became a useful tool, many of the early hypotheses could not be confirmed or refuted. In addition to electron microscopy, new knowledge of myofibril structure, the sliding filament theory of muscle contraction, and new insights into the process of protein synthesis stimulated renewed interest and prompted many new investigations into the assembly of myofibrils. As many of the old theories of myofibril development were easily discarded with the advent of electron microscopy, many new problems surrounding the details of filament assembly developed.

The most important electron microscope observation regarding myofibril formation has been that thick and thin filaments are formed first and then are assembled into myofibrils. The details of the process of filament formation, however, have been a controversial subject described differently by several investigators. With respect to filament formation, three theories have emerged: (1) thin filaments are formed first, (2) thick filaments are formed first, and (3) thick and thin filaments are formed simultaneously.

In support of the theory that thin filaments form before thick filaments, Allen and Pepe (1965) reported free thin filaments less than 1 μm in length in negatively stained embryonic chick muscle homogenates prior to the presence of any thick, myosin-containing filaments. Allen (1973) and Hilfer et al. (1973) also observed, in additional electron microscope studies on fixed, embedded, and sectioned material, that free thin filaments appear first in developing chick embryo. From these observations, it has been concluded that actin is synthesized first during muscle development. These authors have not, however,

investigated the question of whether or not these thin filaments are actually components of the cytoplasmic contractile system common to many cells (Pollard and Weihing, 1974).

On the other end of the spectrum, Firket (1967) reported that thick (myosin) filaments, 9-12 nm diameter, appeared first as wavy, scattered filaments in the cytoplasm of cultured chick muscle explants. These filaments had not yet attained their full size. Heuson-Stiennon (1965) had previously suggested that free myosin filaments in the sarcoplasm, increased in diameter and complexity prior to incorporation into myofibrils. These arguments are not very convincing in view of the fact that seemingly normal, full-sized synthetic myosin filaments can be formed without any detectable intermediate structural form from solutions of myosin (Huxley, 1963). Furthermore, a separate class of 10 nm filaments not composed of actin or myosin have been described by Ishikawa et al. (1969). These 10 nm "intermediate" filaments appear as free, wavy filaments in the cytoplasm of many cell types including mononucleated fibroblasts and myoblasts and multinucleated myotubes. It is conceivable that the developing thick filaments described by Firket (1967) and Heuson-Stiennon (1965) may have been intermediate filaments and not true myosin thick filaments.

The third theory proposes that thick and thin filaments appear simultaneously in developing muscle cells. Hay (1963), studying differentiating muscle in the salamander tail, and Przybylski and Blumberg (1966), studying chick embryo muscle differentiation, reported that two filament populations are present as scattered filaments, 6-7 nm thin filaments and 10-12 nm thick filaments. The same important questions

can be applied to these results. Were these filaments actually actin and myosin filaments destined for future assembly into myofibrils or were they actin filaments from the cytoplasmic system and 10 nm intermediate filaments found in several cell types? Studies by Fischman (1967) and by Shimada et al. (1967) partially answer this question. These workers have reported free thin filaments 6-7 nm in diameter and less than or equal to 1.1 μm in length and free thick filaments less than or equal to 1.6 μm in length and 16-17 nm in diameter. Thick filaments described in these studies more closely resemble thick filaments in mature muscle (15 nm in diameter and 1.5 μm long). Fischman stated that there may be seven to ten times more actin filaments than myosin filaments but he found no cell with only one of the two types of filament. He concluded that there is no sequence of myofibrillar protein synthesis as suggested by Allen and Pepe (1965).

After the formation of myofilaments, assembly into nascent myofibrillar structures takes place. Just as there is little agreement regarding the filament formation sequence, there are several different descriptions of the sequence of the assembly process. Hay (1963) reported the presence of Z bodies and clumps of thin filaments without any associated thick filaments in differentiating muscle of the salamander tail. She proposed that these represented important sites of myofibril organization. She did, however, comment that thick and thin filament aggregates were often present without any associated Z line material. Heuson-Stiennon (1965) also observed Z bodies early in development, and these Z lines appeared to be pinching off from the cell membrane. She also proposed that these Z lines were important sites of early

myofilament aggregation. Before accepting these observations at face value, it should be noted that both of these studies were conducted with material fixed only in osmium tetroxide and not in gluteraldehyde. Therefore optimum preservation had not been attained. Also OsO_4 alone would have destroyed actin not previously fixed with gluteraldehyde or actin not protected by tropomyosin (Pollard[1]). Furthermore, the concept of a common cytoplasmic contractile system had not evolved. The cytoplasmic contractile system is frequently found closely associated with the cell membrane and binds antibodies to α-actinin, a Z line component (Schollmeyer, 1976) at locations resembling Z bodies. Consequently, these investigators may have been observing the poorly preserved cytoplasmic actin and dense bodies of the cytoplasmic contractile system.

Hay (1963) and Heuson-Stiennon (1965) also noted that bundles of thick and thin filaments were present; this observation was subsequently reported by others (Allen and Pepe, 1965; Firket, 1967; Fischman, 1967; Przybylski and Blumberg, 1966; Shimada et al., 1967). In cross section, these bundles of thick and thin filaments are packed in the hexagonal array characteristic of mature myofibrils. Thick and thin filament aggregates range in size from a few filaments to arrays greater than 0.4-0.5 μm in diameter. These bundles are often found around the periphery of the cell, but they are not associated with the cell membrane. Fischman (1967), Shimada et al. (1967), Firket (1967), Przybylski and Blumberg (1966) and Allen and Pepe (1965) have all proposed that the hexagonal packing of thick and thin filaments represents the initial

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[1]Pollard, T. D., Harvard Medical School, personal communication, 1975.

form of aggregation during myofibril assembly. Z material subsequently associates with the ends of the thin filament and links adjacent bundles end to end. The Z bodies become more condensed and finally resemble mature Z lines. M line and I band formation occurs shortly after or concurrent with initial Z line formation but has not been documented in detail.

At least two other laboratories have proposed that there is simultaneous aggregation of thick and thin filaments and Z material, instead of sequential states of aggregation (Dessouky and Hibbs, 1965; Hilfer et al., 1973). Dessouky and Hibbs (1965), however, reported that the M line is not seen until after A and I bands and Z lines are formed.

In summary, Z lines have been proposed as the initial aggregates, as have thick and thin filament aggregates and also the combination of the two. These three proposals have been presented to explain the initial aggregation of myofibrillar structures. There are three points in the myofibril where filaments are linked transversely, the M line, Z line, and myosin cross-bridges. The initial bonding forces in myofibril assembly, according to the majority of the evidence, appear to be due to myosin cross-bridges of the thick filament interacting with actin in the thin filament. Z line formation is of secondary importance in establishing and maintaining the proper three dimensional aggregation of thick and thin filaments; the M line does not appear to have a crucial role in this process. Further support for the view that the M line may not be essential is that functional skeletal muscle exists in the cockroach (Hagopian and Spiro, 1968), in Rhodnius prolixus (a South American blood-sucking insect) (Toselli and Pepe, 1968) and in

the dragon fly (Smith, 1966) which have no M lines. In addition, no M lines have been observed in a wide sampling of invertebrate hearts (Edwards and Challice, 1960; North, 1963; Smith, 1963).

D. Myofibrillar Protein Synthesis during Muscle Cell Differentiation

Ultrastructural observations provided evidence for the existence of myofibrillar proteins in differentiating muscle by describing structures that are presumed to contain these proteins; fluorescent antibodies to specific myofibrillar proteins were also used to detect their presence. Myosin was first identified in embryonic muscle by the binding of fluorescein-labeled anti-myosin (Holtzer et al., 1957). Ogawa (1962) also identified both myosin and actin by antibody localization in chick embryos. Actin was first observed after 72 hours of incubation, and myosin appeared after 96 hours. Consequently, the concept of sequential synthesis of myofibrillar proteins was again proposed. The results of these two studies should be viewed with caution, because the techniques available to purify and monitor the purity of the proteins used to elicit the antibody response were not sufficient to insure that a pure antigen was used. Therefore, the antibodies employed in these experiments could easily have been made in response to probable contaminants in the antigen preparation.

Biochemically oriented studies, necessary to substantiate the ultrastructural evidence, have been slower in developing. The first detailed biochemical experiments (Baril and Herrmann, 1967) demonstrated that

embryonic myosin from 11 to 14 day embryos gave the same elution profile as myosin from 8 day post-hatched chicks when chromatographed on DEAE cellulose. Embryonic myosin was also immunologically indistinguishable and had similar ATPase activities when compared with 8 day post-hatch chick myosin. The rate of accumulation was slow from the 9th to the 14th day of embryonic age but increased more rapidly from day 18 in the embryo to 8 days after hatching. During the slow period of myosin accumulation, myogenic cells were making the transition from proliferating mononucleated cells to multinucleated, nonproliferating fibers. By 18 days in embryonic development, fusion was nearly complete (Herrmann et al., 1970).

Although the relationship of myosin synthesis to muscle cell differentiation has received the most attention, several investigators have sought to discern the relationship of synthesis of other myofibrillar proteins to myosin synthesis during embryonic development of muscle. Potter and Herrmann (1970) investigated the accumulation of myosin and tropomyosin. Tropomyosin increased more slowly than myosin so that the proportion of tropomyosin to myosin was much lower in early embryonic development than in hatched chicks. Consistent with these findings were those of Hitchcock (1970) in which Ca^{++} sensitivity of natural actomyosin ATPase was studied in developing embryonic chick muscle. Actomyosin prepared from embryonic muscle 9-19 days after incubation showed steadily increasing $Mg^{++}Ca^{++}$ATPase/Mg^{++} EGTA ATPase ratios during this time. This indicates that the functioning of the regulatory protein system is delayed with respect to actin and myosin accumulation during development.

Contrary to the results of Potter and Herrmann (1970) and Hitchcock (1970), Roy et al. (1976) isolated the Ca^{++} regulatory complex from embryonic chicken skeletal muscle and found it to be very similar to adult muscle. Tropomyosin contained both α and β subunits but the β form was present in larger quantities. In the adult muscle, the β form decreased and the α form increased. They concluded that the Ca^{++} regulatory proteins are not deficient in embryonic muscle, although there seemed to be differential expression of the two tropomyosin subunits.

Additional evidence for sequential accumulation of myofibrillar proteins was provided by Heywood and Rich (1968). Their experiments identified polyribosomal fractions from sucrose gradients that synthesized myosin, actin, and tropomyosin along with other proteins. The evidence for sequential accumulation of proteins consisted of changes in polyribosomal profiles at three stages of embryonic development. At 10 days, the actin containing peak was largest. At 14 days the myosin peak had increased in size with respect to the actin peak, and by 18 days the tropomyosin-containing peak had increased relative to the actin and myosin peaks. From these results they claimed that actin synthesis preceded myosin and both actin and myosin were synthesized before tropomyosin. It is important to note that these peaks were not exclusively actin, myosin, or tropomyosin polysomes; actin, myosin, and tropomyosin polysomes comprised 12%, 50%, and 40% of their respective peaks.

More recently, John (1976) compared the myofibril composition of 19-day fetal and adult mice by quantitative disc gel electrophoresis

using fast green as a stain. He found that the molar ratio of actin to myosin was constant, but the molar ratio of actin to tropomyosin decreased from 4.6 in the fetus to about 2.9 in the adult. The amount of myosin light chain three also increased. This research is in agreement with Potter and Herrmann (1970), Hitchcock (1970), and Heywood and Rich (1968) who suggested the sequential accumulation of actin and myosin and of tropomyosin, one member of the relaxing protein system.

Evidence refuting the sequential synthesis of myofibrillar proteins in chick embryo muscle has been reported by Masaki and Yoshizaki (1972) in studies using fluorescent antibody localization techniques. They prepared antibodies to myosin, tropomyosin, troponin, α-actinin, β-actinin, and M-protein. At about 50 hours of incubation, positive staining was first seen and it was seen for all of the proteins studied. Therefore, they concluded that there is no sequence of myofibrillar protein synthetic events.

Experiments with embryonic muscle have not produced definitive information concerning the time of synthesis and relative amounts of myofibrillar proteins synthesized during muscle differentiation. One major difficulty with these experiments is the fact that muscle cells in an embryo do not differentiate in synchrony. The final step in differentiation may occur any time during the last half of the incubation period. Consequently, at any point in time, myogenic cells in various stages of differentiation may be found. This point becomes critical when one recalls that nonmyofibrillar actin and myosin are present in virtually all embryonic cells long before they differentiate into myofibril-producing myoblasts. The biochemical experiments

discussed previously have not considered this problem; therefore it is not surprising that actin and myosin appeared to accumulate before tropomyosin.

Recognition of this problem has led to the use of muscle cell cultures to study the events of muscle cell differentiation. The myogenic cell population is synchronized, and the large majority of these cells undergo the terminal step in differentiation during a 24 hour period, as compared to a period of 10 to 12 days in an embryo. Coleman and Coleman (1968) provided the first detailed description of myosin synthesis in cultured muscle cells. They measured myosin by first precipitating it with antibodies to purified myosin and then measured the ATPase of the precipitate. As a percent of total culture protein, myosin comprised 0.5% of 3-day cultures and 5% of 7-day cultures of pure myotubes. Pulse labeling studies demonstrated that the rate of myosin synthesis increased steadily up to 7 days in culture. Fluorescent anti-myosin staining demonstrated the presence of myosin in myotubes and also in long bipolar mononucleated myoblasts.

Paterson and Strohman (1972) provided the next description of myosin synthesis in differentiating muscle cell culture. They also found that the rate of myosin synthesis increased dramatically in 2-day cultures, about the time that cell fusion occurs.

In an effort to determine the relationship between myoblast fusion and myosin synthesis, Paterson and Strohman (1972) attempted to follow myosin synthesis in cultures that were prevented from fusing by the addition of EGTA, a Ca^{++} chelating agent, to the culture medium.

Shainberg et al. (1969) first described the effect of Ca^{++} concentration on cell fusion. By supplementing Ca^{++}-free medium with various concentrations of $CaCl_2$, they found that myoblast fusion could be effectively inhibited by maintaining Ca^{++} concentrations below 270 μM. Upon addition of Ca^{++} to a concentration of 1400 μM, fusion was initiated and myotubes formed within 3-4 hours. Growing cultures in low Ca^{++} concentration did not decrease the ^{3}H thymidine or ^{14}C uridine incorporation and only slightly decreased ^{3}H leucine incorporation. Therefore, the removal of Ca^{++} did not grossly alter major cell functions.

Paterson and Strohman altered Shainberg's procedure, and instead of adding Ca^{++} to Ca^{++}-free medium, they added EGTA to complete medium. The required EGTA concentration was determined beforehand in a pilot experiment by using various EGTA concentrations. When they blocked cell fusion until 72 hours in culture by chelating Ca^{++} with EGTA, the myosin synthesis rate did not increase until after 72 hours. They concluded from their results that cell fusion was required to initiate myosin synthesis in cultured muscle cells. Yaffe and Dym (1972) also found that increased myosin synthesis followed fusion of myoblasts into myotubes. They found that myosin mRNA is synthesized before fusion and suggested that it might be stored and translated after fusion. Przybyla and Strohman (1974) found no myosin heavy chain mRNA in early unfused cultures or in EGTA-blocked cultures but only in fusing cultures or myotubes. They suggested that the mRNA for myosin heavy chain was not associated with ribosomes prior to fusion. In agreement with these experiments, Buckingham et al. (1974) presented evidence that myosin synthesis starts 5-8 hours after fusion and that prior to fusion, myosin

heavy chain mRNA is associated with a ribonucleoprotein particle but not polyribosomes or single ribosomes. After fusion, myosin mRNA was detected as polysomal mRNA.

Paterson et al. (1974) demonstrated the synthesis of an actin-like protein from a poly A-containing RNA in a wheat germ cell-free system. The protein co-migrates with actin and gives a tryptic peptide map that resembles that of actin. In 72-hour cultures, actin accounts for 8% of the protein synthesized by the wheat germ system whereas in fibroblasts, prefusion myoblasts and EGTA fusion-blocked myoblasts, actin accounts for only 1%. They concluded that both actin synthesis and myosin synthesis were controlled by myoblast fusion.

Emerson and Beckner (1975) investigated myosin synthesis in differentiating muscle cell cultures and also found an increase in myosin synthesis during the fusion period and very little myosin synthesis prior to fusion. They also studied myosin synthesis in EGTA fusion-blocked cultures. They used medium, however, that had been previously "conditioned" (Konigsberg, 1971) to promote withdrawal from the cell cycle and used EGTA to block their cells from 24 to 96 hours in culture. Their experiments demonstrated an increase in myosin synthesis in the fusion-arrested cultures. The rate of synthesis was essentially the same as in control fusing cultures, a maximum of 30,000 myosin heavy chain molecules/nucleus/minute. They demonstrated that myosin synthesis does not require fusion but seems to be coordinated with withdrawal of myogenic cells from the cell cycle. Young et al. (1975) also demonstrated myosin heavy chain synthesis and the presence of myosin heavy chain-synthesizing polyribosomes in 24-hour prefusion cultures.

In addition, Holtzer et al. (1975) presented electron microscope evidence of thick and thin filaments in mononucleated myogenic cells. Several other investigators have recently presented biochemical and ultrastructural evidence for myosin synthesis in fusion-blocked cells (Merlie and Gros, 1976; Moss and Strohman, 1976; Trotter and Nameroff, 1976; Vertel and Fischman, 1976). The recent evidence indicates that there is no obligatory relationship between the process of cell fusion and myosin synthesis, thereby virtually eliminating the idea that fusion controls myosin synthesis.

The relationship of the synthesis of other myofibrillar proteins to that of myosin in muscle cell cultures has not been investigated very extensively. Duprat et al. (1975) used fluorescent antibodies to actin and myosin to demonstrate their simultaneous appearance in differentiating muscle cells in culture. Reports from the laboratory of H. Holtzer (Chi et al., 1975; Holtzer et al., 1974; Rubinstein et al., 1974) claim that myosin and actin are synthesized and degraded in a coordinated manner. The myosin to actin ratios ranged from near 2.8 for pure myotube cultures to 0.9 for mononucleated nonmyogenic cells and presumptive myoblasts. The half-lives for the degradation of myosin and actin were essentially equal in all cultures examined. In pure cultures of myotubes, the half-life for the turnover of actin and myosin was about 6 days while in presumptive myoblasts and fibroblasts, the half-life was 2.5-3 days.

The previous studies have dealt primarily with the accumulation of myofibrillar proteins with respect to muscle differentiation; at this point a brief review of the investigations into the control

of myofibrillar protein synthesis in embryonic muscle will be presented.

Most of this work has dealt with the isolation and characterization of myosin mRNA and its translation in cell free systems. Heywood et al. (1967) was first to develop a procedure for isolating polyribosomes from embryonic chick muscle. They were able to demonstrate that the ^{14}C labeled amino acids were incorporated into protein by the polyribosomes. With the addition of carrier myosin, ^{14}C labeled protein could be recovered, and some of the labeled protein was demonstrated to co-migrate with myosin on 12M urea polyacrylamide gels. When polyribosomes were separated on linear 15-40% sucrose gradients, the myosin-like protein was demonstrated to be synthesized primarily from the polyribosomes in the lower one third of the gradient. Polyribosomes from this portion of the gradient, when negatively stained, were shown to have 50 to 60 ribosomes per polyribosome.

A 26S mRNA from the lower part of the polyribosome gradients was isolated (Heywood and Nwagwu, 1969) and subsequently purified (Morris et al., 1972; Sarkar et al., 1974). In a cell-free system, this 26S RNA directed the synthesis of a polypeptide chain that had the properties of myosin heavy chain and upon proteolytic digestion with trypsin and α-chymotrypsin, produced the same peptide map as myosin heavy chain (Rourke and Heywood, 1972). They also found that the synthesis of myosin in a reticulocyte system required initiation factors from embryonic skeletal muscle. The specific initiation factor required was IF3 which forms the initiation complex with the 40s ribosomal subunit and the mRNA.

Subsequent studies on the specificity of IF3 (Heywood et al., 1974), revealed that two IF3 fractions from red muscle could be separated by phosphocellulose chromatography. In a system containing both myoglobin and myosin mRNA's, one IF3 fraction specifically stimulated myoglobin synthesis while the other stimulated myosin synthesis. This study also reported a new RNA isolated from the IF3 fraction that inhibited the translation of heterologous mRNA's.

A report of this new RNA, termed translational control RNA (tcRNA), indicated that tcRNA from muscle inhibits the binding of globin mRNA to 40S ribosomal subunits in rabbit reticulocyte cell free systems (Kennedy et al., 1974). Furthermore, two classes of tcRNA have now been isolated from embryonic muscle (Bester et al., 1975); one class was isolated from messenger ribonucleoprotein particles (mRNP-mRNA) and inhibited the translation of muscle mRNP-mRNA while having very little effect on polysomal mRNA. About 50% of this tcRNA was uridylate residues that formed stable hybrids with poly A. The other tcRNA had no effect on mRNP-mRNA but stimulated the translation of muscle polysomal mRNA. Both muscle tcRNA's inhibit globin synthesis. A model relating the tcRNAs to heterogeneous nuclear RNA and transcription has been proposed. Most of this research has yet to be confirmed by other laboratories; and the relationship of the proposed specific initiation factor and tcRNA controls of myosin synthesis to the problem of muscle cell differentiation and myofibril assembly has yet to be determined.

Many structural and biochemical properties of the myofibril are known, and many of the changes that accompany muscle cell differentiation have also been described. There is, however, a gap in the knowledge

concerning the manner in which differentiating muscle cells accumulate and assemble myofibrillar proteins into functional myofibrils. Ultrastructural descriptions of myofibril assembly have been provided, and biochemical investigations have presented a detailed description of myosin synthesis in differentiating muscle. The total pattern of myofibrillar protein accumulation, however, and its relationship to myofibril assembly has not been elucidated. The goal of the research described in this thesis is to describe the patterns of accumulation of myosin, actin, tropomyosin, and α-actinin in differentiating muscle cell cultures and to relate these patterns to light and electron microscopic observations of myofibril assembly in these cultures.

III. MATERIALS AND METHODS

A. Preparation and Radioisotope Labeling of Muscle Cell Cultures

Fertile eggs were purchased from Hy-Line Hatchery (Dallas Center, Iowa) and incubated at 37°C and 95% humidity. All culture manipulations were done under sterile conditions using sterile equipment and solutions. The leg muscles of 12-day chick embryos were placed in complete culture medium which consisted of 85% Eagles Minimum Essential Medium (Eagle, 1959), 10% horse serum, 5% chick embryo extract, 50 units/ml of penicillin, and 2.5 μg/ml of fungizone. The embryonic muscle was dissociated into a single cell suspension with a vortex Super Mixer (Curtin-Matheson Scientific, Elk Grove Village, Illinois) at maximum speed for 30 sec. Single cells were filtered through 200 x 200 mesh nylon cloth in Swinney filter holders (Millipore Corporation, Bedford, Massachusetts) followed by filtration through lens paper. The filtered cell suspension was sedimented by centrifugation at 700 x g_{max} for 5 min at room temperature. Cells were resuspended in complete medium by vortexing for 5-10 sec and plated at a density of 1.4×10^5 cells/cm^2 in 6, 10, or 15 cm diameter collagen coated tissue culture dishes. Dishes were collagen-coated by rinsing with a solution of 0.2 mg/ml soluble bovine achilles tendon collagen in 1% acetic acid. Cells growing in 6, 10, and 15 cm dishes were fed 2, 5, and 15 ml of complete medium, respectively, every 24 hr. They were incubated at 37°C in an atmosphere of 95% air and 5% carbon dioxide.

In some experiments, myoblast fusion was blocked by the addition of 1.6-1.8 mM 1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane (EGTA)

to complete medium. The concentration of EGTA required to block fusion was determined empirically for each new batch of complete medium in a test experiment using several EGTA concentrations.

Radioactive labeling of culture proteins was accomplished by adding 4, 5 ^{3}H,L-leucine (5 mCi/mole) (New England Nuclear, Boston, Massachusetts) to complete medium. In cultures grown in the presence of radioisotope for 2 to 4 days, a concentration of 5 μCi/ml was used.

B. Fusion Index

Percentage fusion was determined in cultures that had been Giemsa stained. For the Giemsa procedure, cultures first were rinsed in buffered saline solution (0.137 M NaCl, 0.0027 M KCl, 0.001 M $MgCl_2$, 0.001 M $CaCl_2$, 0.00015 M NaH_2PO_4, 0.00136 M Na_2HPO_4, 0.006 M $NaHCO_3$, 0.0055 M glucose, pH 7.4) at 37°C, then were fixed in absolute methanol for 5 min, and finally were stained with Giemsa stain for 15 min at room temperature. Nuclei within 10 random microscope fields were counted and scored as being either within mononucleated cells or within multinucleated cells. The percentage fusion was calculated as the total number of nuclei within multinucleated cells divided by the total number of nuclei within all cells.

C. Myofibrillar Protein Preparation from Cultures

Unless specified otherwise, all solutions, extractions, centrifugations, and related procedures described in this section were done at 0-2°C.

1. Myosin

Cells were scraped from the dishes with a plastic spatula and homogenized in 0.1 M sodium phosphate, 1 mM 2-mercaptoethanol, pH 7.0. Cell homogenates were extracted for two 4-hr periods in 0.1 M sodium phosphate, 1 mM 2-mercaptoethanol, pH 7.0, to remove sarcoplasmic proteins. After centrifugation at 14,000X g_{max} for 30 min, the supernatant fractions were discarded. The pellets were resuspended and extracted for 1 hr in 0.1 M sodium pyrophosphate pH 7.5. After centrifugation at 14,000 X g_{max} for 30 min, the supernatant fraction was salted out from 0 to 50% ammonium sulfate saturation with solid ammonium sulfate, and the myosin precipitate was sedimented at 22,000 X g_{max} for 45 min. The myosin-containing pellet was washed with cold distilled water, resuspended in 50 µl of 1% SDS, 10 mM sodium phosphate, 1 mM 2-mercaptoethanol, pH 7.0. Prior to heating at 100°C for 10 min, 25 µl of 4.6 M 2-mercaptoethanol, 0.03% bromphenol blue, 5.2% SDS, 60 mM sodium phosphate, pH 7.0, and 20% glycerol was added to each sample.

2. Actin and α-actinin

Culture medium was decanted and cultures were rinsed with 0.25 M sucrose. Three ml of a glycerination solution containing 50% glycerol, 0.1 M KCl, 10 mM Tris-HCl, 5 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.8 were added to each 10 cm culture dish, and the cells were scraped from the dish with a plastic spatula. The cell suspensions were stored in glycerination solution at 2°C for 48-96 hr. The purpose of the glycerination procedure was to remove sarcoplasmic proteins. The glycerinated cell suspension was centrifuged at 3,000 X g_{max} for 30 min, and the

suspension fraction was discarded. Pellets were resuspended in 3 ml of 50 mM KCl, 10 mM Tris-HCl, 0.5 mM 2-mercaptoethanol pH 7.6; they were then centrifuged again for 15 min at 3,000 X g_{max}. To remove the glycerol and soluble proteins, the sample was washed three times in 50 mM KCl and sedimented at 3,000 X g_{max} for 15 min. The final pellet was resuspended in 3 ml of cold acetone and sedimented at 3,000 X g_{max} for 15 min. Each sample was washed with acetone and sedimented a total of three times and allowed to dry overnight at room temperature.

G-Actin was extracted from these acetone powders in 0.2 ml of 0.5 mM ATP, 0.5 mM 2-mercaptoethnaol, 0.2 mM $CaCl_2$, pH 7.8 for 30 min at 2°C. The supernatant fraction was removed with a disposable pipette, centrifuged at 200,000 X g_{max} for 1 hr and the resulting pellet was discarded. G-Actin from the supernatant was polymerized to F-actin by adding 0.1 volume of 1.1 M NaCl, 0.1 M $MgCl_2$ and letting the solution stand at room temperature for 1.5 hr. After polymerization, the samples were placed in an ice bath for 30 min to enhance the binding of α-actinin to F-actin (Goll et al., 1972; Stromer and Goll, 1972). The F-actin-α-actinin samples were pelleted by centrifugation at 200,000 X g_{max} for 1 hr and were prepared for SDS-polyacrylamide gel electrophoresis as described for myosin.

3. Tropomyosin

Tropomyosin was prepared by the same procedure used for actin and α-actinin through the 50 mM KCl wash steps. Instead of precipitating the cell residue with acetone, the residue was resuspended in 3 ml of cold 95% ethanol. The precipitate was sedimented at 3,000 X g_{max}

for 15 min and then resuspended again in 3 ml of cold 95% ethanol. This suspension was centrifuged at 3,000 X g_{max}, the pellet was resuspended in cold diethyl ether and sedimented again at 3,000 X g_{max}. This last step was repeated a second time, and the final pellet was dried for 4-5 hr. The dried pellet was resuspended in 1 ml of 1.0 M NaCl, 25 mM Tris-HCl, 0.1 mM $CaCl_2$, 5 mM 2-mercaptoethanol, pH 8.0 and allowed to extract at room temperature overnight. The suspension was sedimented at 3,000 X g_{max} for 15 min, the supernatant fraction was removed and was salted out between 40 and 70% ammonium sulfate saturation. The P_{40-70} precipitate was resuspended in 1 ml of 1 M NaCl, 20 mM sodium acetate, pH 4.6 and sedimented at 16,650 X g_{max} for 30 min. The tropomyosin pellet was prepared for electrophoresis as previously described for myosin.

4. Crude myofibrils

Myotube cultures were scraped from the dish in 0.1 M KCl, 20 mM potassium phosphate, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3, pH 6.8 (standard salt solution), and myotubes were fragmented by vortexing for 2 min at top speed. Fragmented myotubes were sedimented at 3,000 X g_{max} for 30 min. The supernatant fraction was discarded, and the pellet was resuspended in standard salt solution. The suspension was again mixed with a vortex mixer at top speed for 1 min and resedimented as before. The pellet was then resuspended in 3 ml of 0.1 M NaCl, 5% Triton X-100, 9 mM Tris-HCl, pH 7.6, and vortexed at top speed for 1 min. Following sedimentation at 3,000 X g_{max} for 15 min, the pellet was resuspended in the same solution with 3 strokes of a Dounce homogenizer type B pestle

and sedimented at 3,000 x g_{max} for 15 min. The combined myofibril pellets were homogenized in 10 ml of 0.1 M NaCl, 5 mM sodium phosphate, pH 7.0 with 10 strokes of a Dounce homogenizer, type B pestle.

D. Electrophoresis and Quantitation of Myofibrillar Proteins in Sodium Dodecyl Sulfate Polyacrylamide Gels

Quantitation of muscle culture myofibrillar proteins was essential to the experiments proposed; therefore, a quantitative procedure for analyzing proteins on SDS-polyacrylamide gels had to be developed. An extensive study of the parameters involved in establishing standard curves for myofibrillar protein quantitation was conducted.

The general experimental design employed in our experiments utilized two separate preparations of each purified myofibrillar protein with twenty protein concentrations per preparation and triplicate gels per concentration. To determine the influence of destaining time on gel quantitation, each gel was scanned twice at one week and twice at three weeks after electrophoresis.

To determine the effect of distance of migration, experiments were performed with two concentrations of actin, approximately 1 μg and 30 μg. Triplicate sets of gels were electrophoresed to tracking dye migration distances of 2, 3, 4, 5, and 6 cm. The gels were scanned and integrated three weeks after electrophoresis.

1. Purified porcine skeletal muscle myofibrillar proteins

To obtain accurate standard curves for the quantitation of myofibrillar proteins, highly purified myofibrillar proteins were required.

Myosin was prepared from freshly exised hind limb muscles of the pig according to the method of Seraydarian et al. (1967). The myosin was then further purified by DEAE cellulose (Whatman, DE-52) column chromatography. The protein was applied to the column in 40 mM potassium pyrophosphate, 1 mM EDTA, pH 7.5. Myosin was eluted by using a linear (0 to 0.5 M) KCl gradient in the presence of 40 mM potassium pyrophosphate, 1 mM EDTA, pH 7.5. The myosin peak was collected by salting out from 30 to 45% ammonium sulfate saturation.

Actin was also prepared essentially according to the method of Seraydarian et al. (1967). Conversion of G- to F-actin, however, was accomplished in the presence of 0.5 M KCl, instead of 0.1 M KCl, to reduce the amount of tropomyosin and troponin contamination (Spudich and Watt, 1971). Actin prepared in this way still contained approximately 1% of its protein as α-actinin. α-Actinin was removed from G-actin by using gel permeation chromatography (LKB Ultrogel AcA 34) in 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, pH 7.8. The contaminating α-actinin was eluted well ahead of the main G-actin peak. The G-actin was collected and used for gel quantitation. Purified α-actinin was prepared by using the procedure of Suzuki et al. (1976).

Tropomyosin and troponin were purified according to the procedures of Arakawa et al. (1970). This procedure consists briefly of: (1) preparation of a tropomyosin-troponin complex from a low ionic strength extract of washed myofibrils, (2) separation of troponin and tropomyosin by iso-electric precipitation, and (3) purification of tropomyosin and troponin by ammonium sulfate fractionation. The tropomyosin did not require further chromatographic purification.

Troponin, however, was further purified by ion exchange chromatography on DEAE cellulose (Whatman DE-52). Troponin was applied to the column in a solution of 50 mM KCl, 20 mM Tris-acetate, pH 7.5, and eluted with a 50 mM to 300 mM linear KCl gradient in the presence of 20 mM Tris-acetate, pH 7.5. The troponin fraction was collected by ammonium sulfate fractionation.

Purified pig muscle myofibrils were prepared from the red and white portions of the semitendinosus muscle by the procedure of Goll et al. (1974).

2. Electrophoresis

Three concentrations of each protein solution, approximately 1.5 mg/ml, 0.15 mg/ml, and 0.015 mg/ml, were prepared, and the exact concentration was determined by the Folin-Lowry procedure as modified by Goll et al. (1964). All Folin determinations were subsequently corrected back to a biuret protein concentration basis (Gornall et al., 1949), which is not as dependent upon amino acid composition. The correction factors were determined carefully by simultaneous comparison of biuret and Folin protein analyses of common solutions of each purified protein. After determination of protein concentrations, one volume of a solution containing 1.64 ml of 13.1% SDS, 1.33 ml of 2-mercaptoethanol, 0.3 ml of 0.4% bromphenol blue, and 0.83 ml of glycerol was added to two volumes of each protein solution. The three protein solutions were heated to 80°C for 10 min in tightly capped 5 ml plastic tubes. A Hamilton syringe was used to load 10-80 µl volumes of each protein

solution; this procedure provided a range of protein loads from approximately 0.1 to 50 μg.

Proteins were electrophoresed on 8 cm 7½% polyacrylamide gels containing 74 parts monomer to 1 part crosslinker. A current of 8 milliamperes/gel was applied until the tracking dye had traveled 6 cm into the gel. The cathode and anode buffer were identical to those employed by Weber and Osborn (1969). Gels were stained for 16-20 hr (generally overnight) in 0.1% Coomassie Brilliant Blue R 250 (Sigma Chemical Company, St. Louis, Missouri), 50% methanol, and 7% acetic acid and were destained electrophoretically in a Canalco Quick Gel Destainer (Canalco, Inc., Rockville, Maryland) in 7% acetic acid for 15 min. The gels were then stored in a destaining solution containing 50% methanol and 7.5% acetic. The destaining solution was changed daily for the first week and weekly thereafter.

3. Densitometry

A Zeiss PMQ II Spectrophotometer (Carl Zeiss, Oakbrook, Illinois) was equipped with a thin layer chromatogram scanning attachment which was modified to hold cuvettes horizontally. Peak integration was performed by a System I Computing Integrator (Spectra-Physics, Santa Clara, California) that received input directly from the spectrophotometer. A Honeywell Electronik 194 recorder (Honeywell, Inc., Fort Washington, Pennsylvania) was added in series with the spectrophotometer and computing integrator in order to visually monitor the scan.

Gels were scanned at a wavelength of 550 nm, a slit width of 0.2 nm, and a scan speed of 20 mm per min. The computing integrator was programmed

to correct for baseline drift by re-establishing baseline before and after each peak. In the case of fused peaks, as with the troponin subunits, baseline was established before and after the fused peak group and horizontal or trapazoidal corrections were applied to each peak.

4. Data analysis

The mass of protein on each gel was corrected for the purity of the protein preparation used in each experiment. Percentage purity was estimated as the percentage of total peak area contributed by the peak of interest. The protein quantity loaded on each gel was corrected for the purity of the respective protein standard. The corrected protein concentration of each gel and the peak areas were subjected to analysis of variance and various regression analyses.

The data were first graphically evaluated to arrive at some general models which could be used to fit curves to the data by using regression analysis techniques. The most consistent and the best fit for the proteins in this study was provided by evaluating the data as two curves joining at a "break" point between low and high concentration ranges. The data in each portion of the curve were fitted independently with the general model: $y = b_0 + b_1x$; the dependent and independent variables are peak area (y) and protein concentration (x), respectively. Variance estimates for each portion of the curve were determined from these analyses and subsequently used to weight the data for the final regression analysis. The error structure for the second portion of the curve seemed to be a function of protein concentration; therefore

the deviations from the predicted line were regressed on concentration to determine this function. This equation, of the form $\sigma = b_0 + b$ (concentration-break point) was subsequently used as an estimate of the standard deviation for the high concentration portion of the curve. In the final regression analysis, the data from each portion of the curve were weighted by dividing by their respective standard deviation estimates, and the two portions of the curve were fitted as a single broken curve. The break point in each curve was determined by substituting various break point concentration values in the regression analysis to determine the break point which resulted in the smallest mean square error.

Analysis of variance was performed on the combined data obtained from the one and three week scanning times, and the sources of variation that were partitioned out were protein concentration, gels within concentration, time, concentration by time interaction, gel by time within concentration, and scans within gels.

E. Gel Slicing and Liquid Scintillation Counting

The radioactivity in a protein band was determined by freezing the gel and cutting 0.8 mm slices through the band using a Mickle gel slicer (Brinkmann Instruments, Westbury, New York). Gel slices were placed in polyethylene minivials and were dissolved in 0.2 ml of 30% H_2O_2 at 50°C for 3 hr before adding 4 ml of Aquasol. Samples were counted in a Model 3320 Packard Liquid Scintillation Spectrometer,

and raw counts per minute were converted to disintegrations per minute by using an automatic external standardization method.

F. Light and Electron Microscopy

1. Light microscopy

For bright field and phase microscopy, a Zeiss Photomicroscope I equipped with a 16x neofluor bright field objective, a 40x neofluor phase objective, and a 100x planapochromat phase objective was used. Cultures for bright field microscopy were stained with Giemsa stain as previously described.

2. Electron microscopy

Samples were prepared for electron microscopy by washing cell cultures twice in buffered saline solution prior to fixation with 2.5% glutaraldehyde which was obtained as a 25% solution from Aldrich Chemical Company (Milwaukee, Wisconsin) and was neutralized with barium carbonate before use in the fixative solution. Following fixation, cultures were stored in buffered saline solution at 2°C until all cultures in the experiment were ready to be dehydrated and embedded. Prior to dehydration, cultures were post fixed in 1% osmium tetroxide, 20 mM sodium phosphate buffer for 30 min at room temperature and rinsed three times with buffered saline solution. The cultures were dehydrated with a graded ethanol series of 20%, 50%, and 70% ethanol, 5 min in each step. Two 15 min transition steps in 80 and 90% hydroxypropyl-methacrylate in ethanol were followed by two 15 min steps in 100% hydroxypropylmethacrylate. Infiltration was accomplished with 1:1

(v/v) mixture of hydroxypropylmethacrylate:Epon-Araldite for 1 hr. This mixture was replaced with 100% Epon-Araldite for 15 min. After decanting the 100% Epon-Araldite, half-filled gelatin capsules containing 100% Epon-Araldite were inverted and placed on the culture. The cultures were allowed to stand at room temperature overnight and were then polymerized for 36-48 hr in 60°C oven.

Thin sections were cut on an LKB Ultrotome III with a glass knife. Sections were collected on copper grids and stained for 13 min in 2% methanolic uranyl acetate. The grids were dipped rapidly in 100% methanol, 50% methanol, and finally in distilled water. After the grids were allowed to dry for 1 hr, they were stained for 11 min in lead citrate (Reynolds, 1963).

G. Determination of Time of Initiation and Rate of Myofibrillar Protein Synthesis

The experiments were originally designed to answer the following question: what is the temporal sequence in which myosin, actin, tropomyosin, and α-actinin are synthesized and available for myofibril assembly in differentiating muscle cells? Because these experiments evolved over a three year period, the fundamental idea was continually modified and refined. Consequently, three basic forms of the experiment were employed to obtain the data presented in this thesis.

Due to the unique size and solubility properties of myosin, investigators had been able to quantitatively separate myosin from crude culture homogenates on SDS-polyacrylamide gels and directly measure the

amount of myosin in a culture. However, the other myofibrillar proteins of interest comigrate with a host of additional proteins on SDS-polyacrylamide gels; therefore these myofibrillar proteins could not be measured in crude culture homogenates. Furthermore, attempted purification from such small amounts of tissue would not be expected to result in quantitative recovery of the protein or even in consistent yields. With these limitations in mind, an isotope dilution approach to the problem was chosen.

In isotope dilution experiments, the measurement made on the unknown sample is a specific radioactivity determination. The specific radioactivity, or specific activity, is the radioactivity of the sample divided by its mass, i.e. dpm/μg. The advantage this procedure offers over the conventional direct approach is that only an aliquot of the total sample is required to measure the specific activity. Therefore the requirement of quantitative recovery of purified protein is circumvented. Consequently, this procedure permits the study of proteins which are difficult to quantitatively isolate and purify. In these experiments, the culture proteins were partially purified, as previously described, and isolated in a pure form as a band on SDS-polyacrylamide gels. From this gel band of pure protein, the mass of protein in each band was determined by densitometry, and the radioactivity was determined by slicing the band from the gel and counting in a liquid scintillation counter, as described previously.

1. Modified isotope dilution in pulse labeled muscle cell culture (Form I)

The first form of the isotope dilution experiment was used to follow the accumulation of myosin and actin in both regular muscle cultures and in fusion-blocked cultures. This experiment was designed to determine the temporal relationship between the initiation of rapid synthesis of actin and myosin in these cultures.

In this series of experiments, cultures were pulse labeled with ^{3}H,L-leucine for 4 hr at the beginning of the experiment. At regular intervals, starting with the end of the pulse labeling period, triplicate cultures were harvested and subjected to specific activity determinations on the protein of interest, as previously discussed. As the cells synthesized additional unlabeled protein after the pulse, the specific activity of the protein in the cells declined. Therefore the reciprocal of specific activity reflected the increase in protein accumulation with time.

An assumption inherent in this experiment is that the originally labeled protein is not significantly degraded during the experiment, or if it is, labeled myosin and actin are degraded in a coordinated fashion so that the resulting accumulation curves will contain the same relative error. An attempt to verify these assumptions led to the second modification of the basic isotope dilution experiment.

2. Modified isotope dilution in unlabeled cultures with added radioactive culture homogenate (Form II)

These experiments were designed to eliminate the assumptions about turnover of labeled myofibrillar proteins and were used to study the

temporal relationship among actin, myosin, tropomyosin, and α-actinin accumulation. In these experiments, cultures were not labeled with radioactive leucine, but after they were harvested and prior to subsequent protein purification steps, a constant aliquot of radioactive muscle culture homogenate was added to each sample. The radioactive homogenate was obtained from muscle cultures grown in the presence of ^{3}H,L-leucine for 2 to 4 days. The labeled cultures were scraped from the dish in 0.1 M sodium phosphate, 1 mM 2-mercaptoethanol, pH 7.4 and vigorously homogenized with a Dounce homogenizer, type B pestle. A constant volume of culture homogenate, equivalent to approximately 0.2 labeled muscle culture, was added to each unlabeled culture sample.

It should also be noted that the actin and myosin accumulation profiles obtained from these experiments revealed the same relationship to each other and to muscle cell differentiation as the actin and myosin accumulation profiles in the preceding pulse labeling experiments. Therefore, the turnover of labeled myofibrillar protein did not appreciably affect the outcome of the earlier experiments.

These first two forms of the experiment provided data on the relative increases in protein accumulation; therefore, they only permitted comparisons of the time at which muscle cells increased their accumulation of various myofibrillar proteins. The relative rates of accumulation of the individual proteins could not be determined by this approach. This shortcoming prompted the refinement of the experiment to a true isotope dilution procedure.

3. Quantitative isotope dilution of myofibrillar proteins in muscle cell cultures (Form III)

The third form of the experiment was designed to determine the absolute rate of accumulation of each myofibrillar protein; this had not been possible with the previous two forms of the experiment.

True isotope dilution experiments require, in addition to the specific activity of the unknown sample, the specific activity of the radioactive protein added to the sample and the precise amount of radioactive protein added. With this information, the amount of unlabeled protein in the original sample can be determined as follows:

$$C_2 = \frac{C_1(SA_1 - SA_2)}{SA_2}$$

C_1 = amount of labeled protein added

SA_1 = specific activity of the labeled protein

C_2 = amount of unlabeled protein in the sample

SA_2 = specific activity of unknown sample with added labeled protein.

In the two previous modified forms of the isotope dilution experiment, C_1 and SA_1 were not available; therefore the results showed only relative increases in protein accumulated.

In this third form of the experiment, a similar format was followed as in Form II. Instead of adding labeled culture homogenate, however, labeled culture myofibrils were prepared as previously described, and added to the unknown culture samples. An aliquot of labeled culture myofibrils was prepared for SDS-polyacrylamide gel electrophoresis, and triplicate gels of four different loads (generally 10, 20, 40,

and 80 μl) were electrophoresed and quantitated by densitometry. The densitometry provided the mass of each protein in the gel and allowed the calculation of the original concentration of that protein in the myofibril sample. This provided C_1 for each of the four proteins. The SA_1 was determined by slicing the band and counting the radioactivity and dividing the radioactivity by the previously determined mass of protein in the band. From these three measurements, SA_1 and C_1 from labeled myofibrils and SA_2 from the culture sample, the amount of protein, C_2, in the original culture was determined.

The rates of accumulation were determined by linear regression of C_2 per fused nucleus on time in culture for the rapid myofibrillar protein synthesis phase. The slope of the regression line provided the amount of protein accumulated per fused nucleus per hour. Since cultures can vary from experiment to experiment, these rates of accumulation were expressed as ratios of rates for individual proteins, i.e. actin rate/myosin rate, actin rate/tropomyosin rate, etc. Furthermore, the cultures used for these experiments were grown in the presence of fluorodeoxyuridine from 48 hr through the termination of the experiment. Fluorodeoxyuridine was not added to younger cultures because myogenic cell proliferation would have been inhibited. By 48 hr, myogenic cell proliferation had generally ceased; therefore only fibroblast proliferation was inhibited. By inhibiting the proliferation of fibroblasts, the contribution of cytoplasmic contractile system synthesis to the observed synthetic patterns was reduced.

IV. RESULTS

The purposes of these experiments were to describe the myofibrillar protein accumulation patterns in differentiating muscle cells grown in culture and to relate the accumulation patterns to assembly of myofibrils. Modified isotope dilution experiments were developed to monitor the onset of rapid accumulation of myosin, actin, tropomyosin, and α-actinin in muscle cell cultures. The rate of accumulation of each of these proteins was also measured to complete the description of myofibrillar protein accumulation.

In addition to the protein accumulation experiments, several preparative and analytical procedures had to be developed or adapted in order to perform the myofibrillar protein accumulation experiments. Consequently, the results of the preparative and analytical procedures will be presented before the results of accumulation experiments. The results have been organized as follows: (A) preparation of myofibrillar proteins from cultured muscle cells, (B) SDS polyacrylamide gel electrophoresis and quantitation of myofibrillar proteins, (C) light and electron microscopy of cultured muscle cells, and (D) myofibrillar protein accumulation patterns.

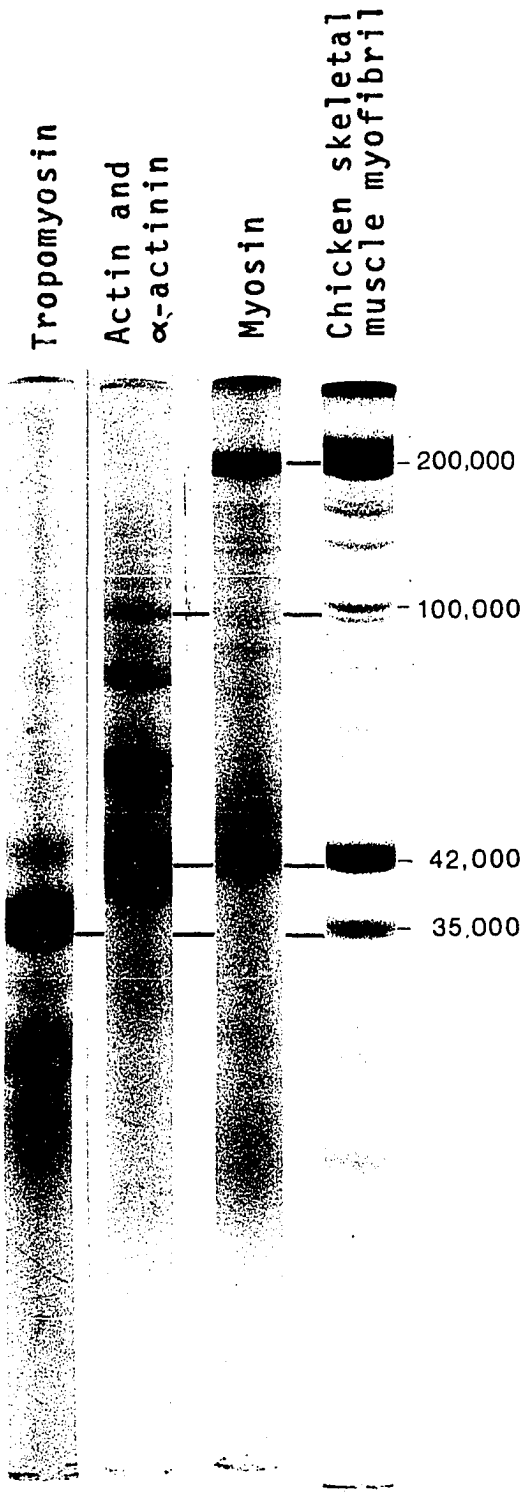
A. Preparation of Myofibrillar Proteins from Cultured Muscle Cells

There were two reasons for preparing myofibrillar proteins from cultured muscle cells as described in the Materials and Methods section. The first was to provide an aliquot of each individual, purified myofibrillar protein in order to permit the measurement of protein mass

and radioactivity. As pointed out in the Review of Literature, other investigators have attempted to monitor myofibrillar protein synthesis by analyzing crude culture homogenates (Chi et al., 1975; Holtzer et al., 1974; Paterson and Strohman, 1972; Rubinstein et al., 1974). The protein they measured was of questionable purity; consequently the results they obtained are difficult to interpret. The second function of the protein preparation procedures was to provide evidence for the identity of the protein being isolated. Because the mass of tissue in each sample at the beginning of the procedure was generally less than 50 mg, conventional assays for the myofibrillar proteins could not be utilized. Therefore, only key purification steps commonly used to purify each myofibrillar protein were employed. Purification characteristics and mobility on SDS polyacrylamide gels provided evidence for the identity of each protein.

A typical preparation of myosin from muscle culture is shown in Figure 1. Evidence for the identity of myosin was provided by its solubility properties and its mobility on SDS-gels. Both culture myosin and skeletal muscle myosin are soluble in high ionic strength (100 mM sodium pyrophosphate in this procedure) and can be precipitated from solution at 50% ammonium sulfate saturation. The primary criterion used in the identification of myosin was its mobility on SDS polyacrylamide gels. Very few proteins have been reported to have polypeptide chains in the molecular weight range of myosin heavy chain (200,000 daltons). If other proteins co-purified with myosin heavy chain and also had a subunit molecular weight of 200,000 daltons, they would not be expected to be present in muscle cultures in large enough

Figure 1. Seven and one-half percent SDS polyacrylamide gels of muscle culture myofibrillar proteins. The protein preparative procedure used to obtain the fraction loaded onto the respective gels is denoted at the top of each gel. The gel of chicken myofibrils is included to serve as a reference for the mobility of myofibrillar proteins in the other gels. The 35,000 dalton band in the tropomyosin preparation co-migrates with chicken myofibril tropomyosin. The 42,000 dalton band and the 100,000 dalton band in the actin and α-actinin preparation co-migrate with chicken myofibril actin and α-actinin respectively. Myosin heavy chain in the chicken myofibril gel co-migrates with the 200,000 dalton band in the gel representing the myosin preparation. Protein loads for each of the culture protein gels were the total yield of the respective protein isolation procedure from one 100 cm muscle culture dish.


Tropomyosin
Actin and α-actinin
Myosin
Chicken skeletal muscle myofibril
200,000
100,000
42,000
35,000

quantities, with respect to myosin, to alter the experimental results significantly.

Muscle culture actin and α-actinin (Figure 1) were isolated with the same preparative procedure. The first crucial steps in this procedure were the acetone precipitations of the glycerinated cells. The primary purpose of this step was to precipitate the myosin in the cell suspension. An actin-like protein was then extracted from the dried acetone residue in a low ionic strength solution containing ATP, Ca^{++}, and 2-mercaptoethanol. The actin-like protein could not be sedimented by centrifugation at 200,000 x g_{max} for 1 hr, but after adding NaCl and $MgCl_2$ to a final concentration of 100 mM and 10 mM respectively, the actin-like protein could be sedimented by centrifugation at 200,000 x g_{max} for 1 hr. These same properties are encountered with the extraction of skeletal muscle G-actin from acetone powder and its polymerization into F-actin when the ionic strength and Mg^{++} concentration are increased. The primary protein resulting from this procedure had the same mobility on SDS polyacrylamide gels as purified skeletal muscle actin.

α-Actinin is present in very small amounts in muscle; therefore, it would be exceedingly difficult to use the conventional α-actinin extraction procedure (Suzuki et al., 1976) to isolate it. Since, α-actinin is the most persistent contaminant of purified actin, α-actin was isolated as the 95,000 to 100,000 daltons contaminant in muscle culture actin preparations. The primary evidence for its identity was its mobility on SDS-gels and its ability to bind to F-actin and co-purify with it.

One possible contaminant of the actin preparation should be acknowledged. The tropomyosin-binding subunit of troponin (troponin T) from chicken muscle has a mobility on SDS polyacrylamide gels very close to that of actin. Therefore, when tropomyosin contaminates muscle culture actin preparations, there is a real possibility that small amounts of troponin-T may be bound to the tropomyosin. This troponin T could be indistinguishable from the actin band on SDS-gels. The actin gel in Figure 1 does not show appreciable amounts of tropomyosin; however, small amounts of tropomyosin were occasionally observed in some culture actin preparations. For this reason, trace amounts of troponin-T could have been present.

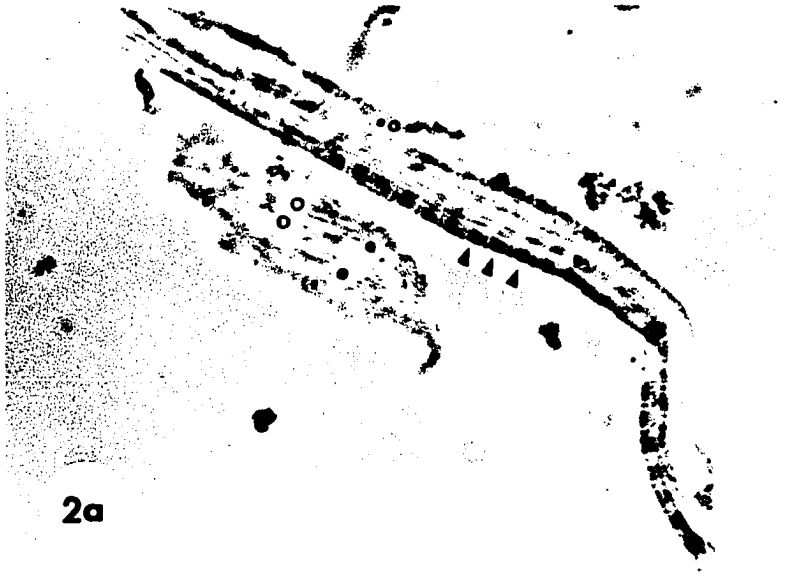
Tropomyosin (Figure 1) was isolated from muscle cell cultures by using a modification of Bailey's procedure (1948). The culture tropomyosin was extracted from ethanol-ether dried residue in 1 M NaCl and then was salted out between 40 and 70% ammonium sulfate saturation. This procedure would be expected to result in the isolation of tropomyosin from skeletal muscle. Because skeletal muscle tropomyosin is insoluble at pH 4.6, the P_{40-70} pellet of culture protein was resuspended in 1 M NaCl, 20 mM sodium acetate, pH 4.6. This step removed the ammonium sulfate which interferes with electrophoresis and insured that the tropomyosin remained insoluble.

Several experiments required muscle culture myofibrils; these myofibrils were prepared with a procedure that resembles the skeletal muscle myofibril procedure routinely used in our laboratory (Goll et al., 1974). Individual sarcomeres can be identified (Figure 2a) as well as contaminating nuclei (Figure 2b). Nuclear contamination in

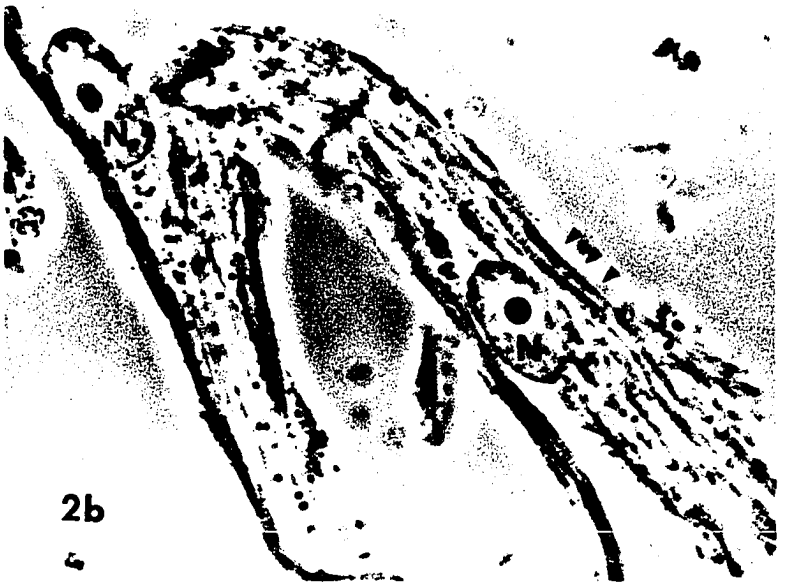
Figure 2. Myofibrils prepared from five day muscle cell culture.

a. and b. Myofibrils prepared from 5 day muscle cell cultures. Phase micrographs of muscle culture myofibrils reveal banding pattern (arrow heads) characteristic of myofibrils. Nuclei (N) also contaminate these preparations (2b), X1640.

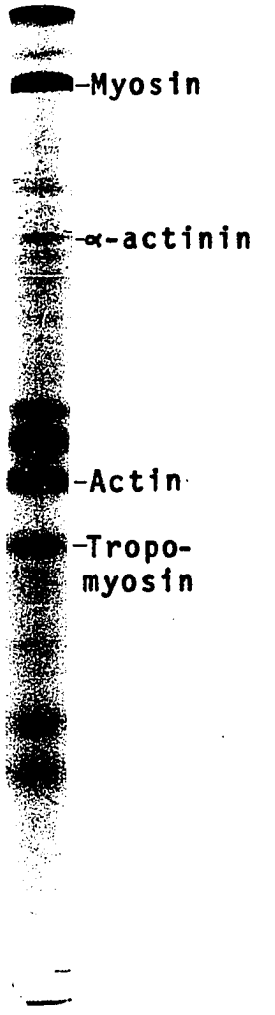
c. Seven and one-half percent SDS polyacrylamide gel of muscle culture myofibrils. Myosin, α-actinin, actin, and tropomyosin bands have been identified in this gel.



2a



2b



2c

these preparations was often extensive and probably accounts for the additional proteins visible in gels of cultured myofibrils (Figure 2c). Nuclei contaminated these preparations primarily because nuclei were not separated from myofibrils by the centrifugation steps in this procedure and because they represented a larger proportion of cultured muscle cells, as compared to mature skeletal muscle; furthermore, due to the small amount of tissue present and the presumed delicate nature of forming myofibrils, the culture myofibril procedure was not as rigorous as the procedure used for preparing myofibrils from mature skeletal muscle. Protein bands with the mobility of myosin, actin, α-actinin, and tropomyosin were present on myofibril gels (Figure 2c). These bands could have contained small amounts of contaminating protein with identical mobilities.

B. SDS Polyacrylamide Gel Electrophoresis and Quantitation of Myofibrillar Proteins

To measure the specific activity of cultured proteins, the mass of protein in a particular band of a SDS polyacrylamide gel had to be accurately measured. The development of SDS polyacrylamide gel densitometry as an analytical tool entailed not only the establishment of standard curves for each protein of interest, but also the analysis of the relative errors associated with various aspects of the procedure and estimates of the amount of variation to be expected with each protein determination.

Gels of the purified proteins used to prepare the standard curves are shown in Figure 3. The respective proteins loaded on these gels were greater than 95% pure. Standard curves of peak areas, determined at one week, versus protein mass are shown in Figures 4-10. The center lines in these curves were determined by regression analysis of the scan area and protein mass data, and the outer lines encompass a 95% confidence interval based on the data variation associated with each portion of the curve. Table 1 presents the regression equations for area as a function of protein concentration (mass/gel). The curve was not forced through the origin but was allowed to seek its own intercept; therefore, the intercepts are included in the equations and designated as either not differing (superscript a) or differing (superscript b) significantly from 0. The R^2 terms for each regression analysis are also presented in Table 1 to show the proportion of the variation in the data for each curve that is explained by the regression equation. With only one exception, greater than 98% of the variation in peak area can be accounted for by the determined relationship to protein concentration.

The most striking feature of these curves is the biphasic nature of the area response with respect to increasing protein concentration. Other investigators have reported deviations from linearity at high protein concentrations (Fischbein, 1972; Fenner et al., 1975; Kruski and Narayan, 1974), however, never as low as the concentration range observed in these experiments. The low concentration portion of the curve obeys Beer's Law; however, as the protein concentration increases beyond the low concentration range, the response no longer obeys Beer's

Figure 3. SDS-polyacrylamide gels of purified myofibrillar proteins used to develop standard curves for quantitative electrophoresis and densitometry. The identity of the purified protein on each gel is indicated at the top of each gel. The three subunits of troponin are designated as T, I, and C, and correspond to the troponin-T, troponin-I, and troponin-C subunits, respectively.

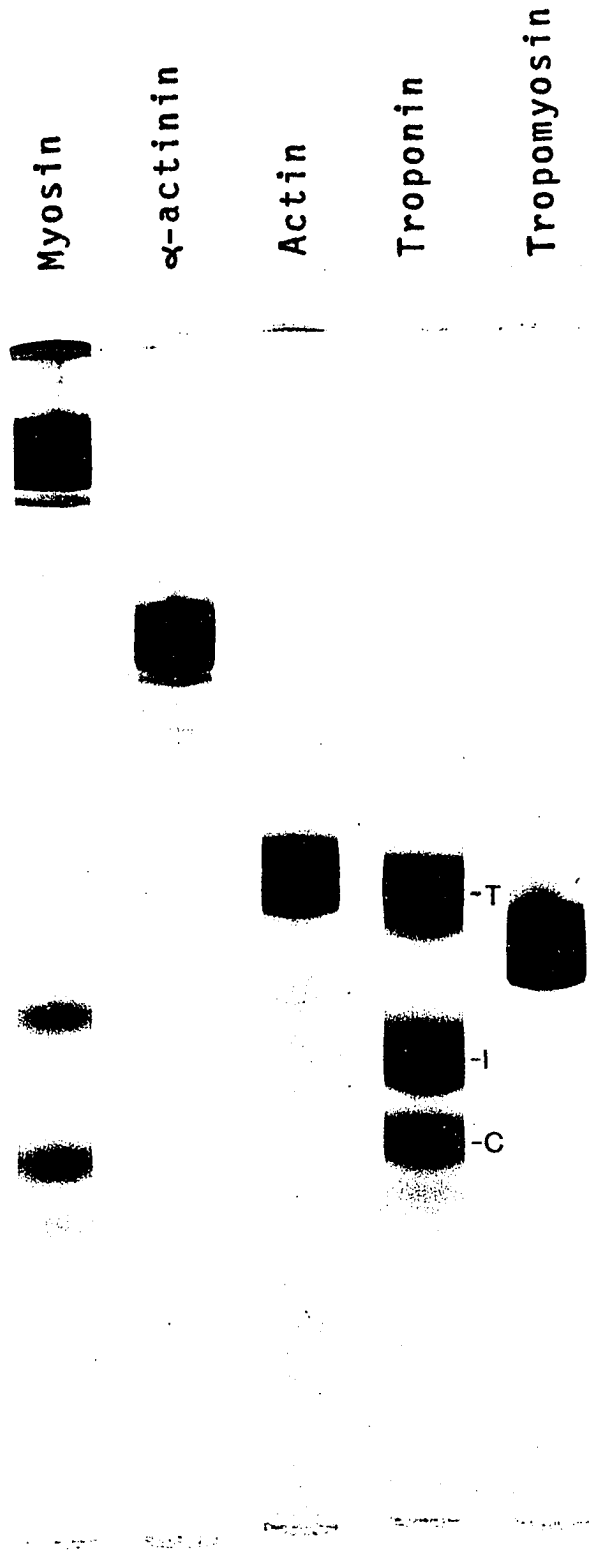

Myosin
α-actinin
Actin
Troponin
Tropomyosin
-T
-I
-C

Figure 4. Quantitative electrophoresis and densitometry: standard curve for myosin heavy chain scanned one week after electrophoresis. The center line is the regression line representing the change in peak area with increasing protein loads. The two outer lines encompass a 95% confidence interval. Points (+) represent the average of 6 scans, triplicate gels and two scans per gel. The Y axis has been extended to accommodate the lower confidence limit at low protein concentrations. The intercept of the regression line was not significantly different from zero.

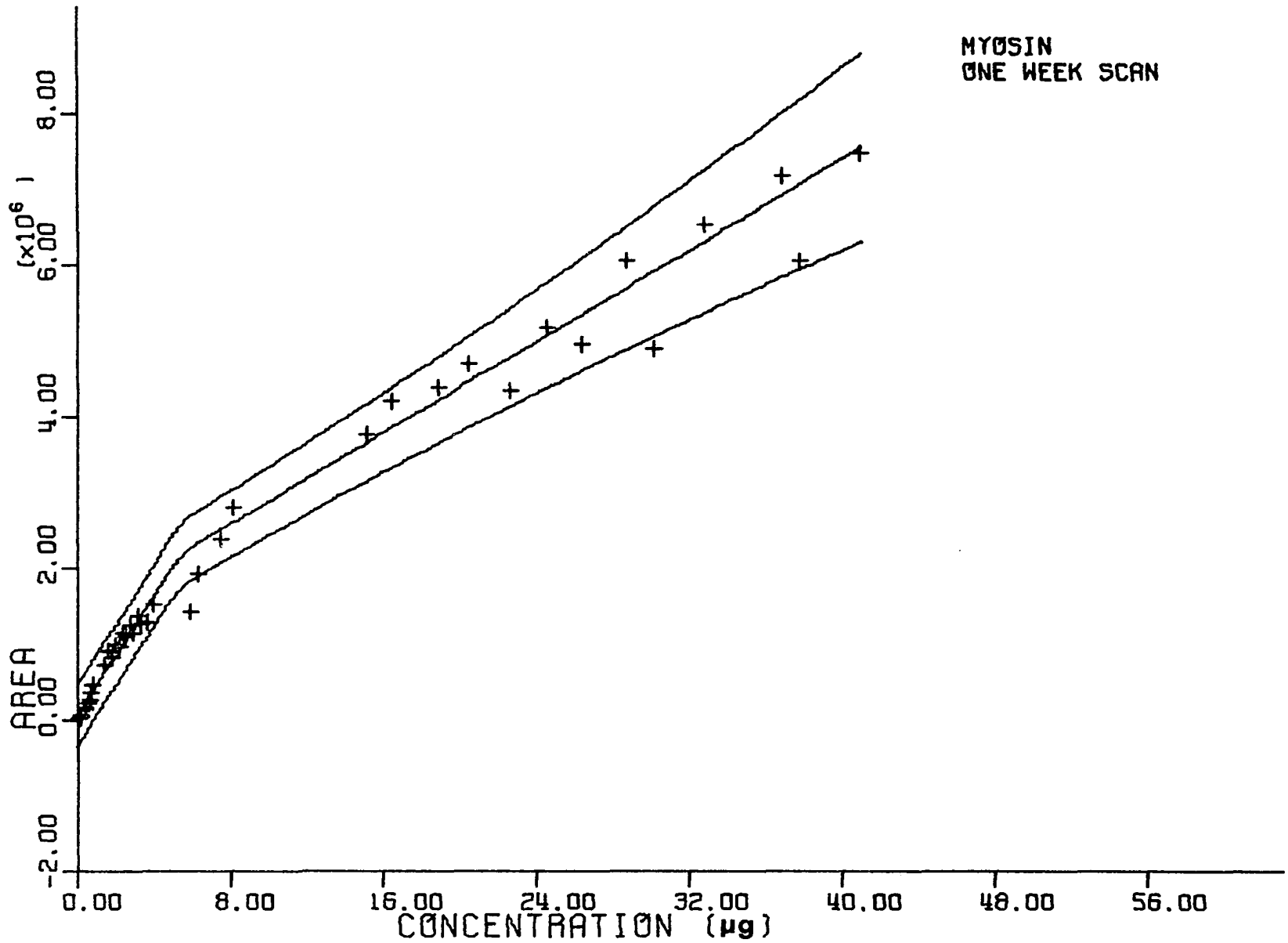

MYOSIN
ONE WEEK SCAN
AREA (x10^6)
8.00
6.00
4.00
2.00
0.00
-2.00
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
CONCENTRATION (µg)

Figure 5. Quantitative electrophoresis and densitometry: standard curve for the 100,000 dalton α-actinin subunit scanned one week after electrophoresis. The center line is the regression line representing the change in peak area with increasing protein loads. The two outer lines encompass a 95% confidence interval. Points (+) represent the average of 6 scans, triplicate gels and two scans per gel. The Y axis has been extended to accommodate the lower confidence limit at low protein concentrations. The intercept of the regression line was not significantly different from zero.

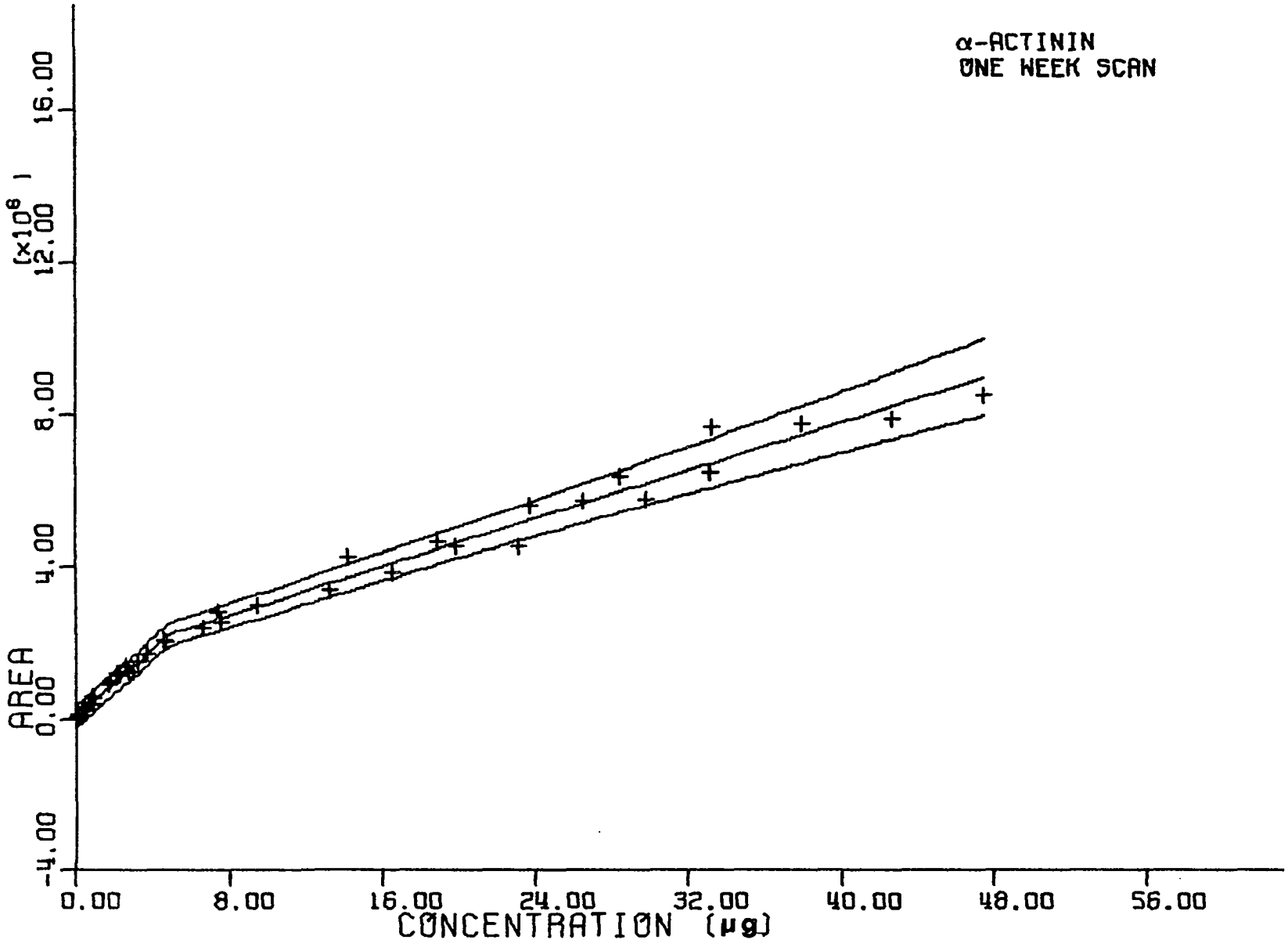

α-ACTININ
ONE WEEK SCAN
AREA (×10⁶)
16.00
12.00
8.00
4.00
0.00
-4.00
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
CONCENTRATION (µg)

Figure 6. Quantitative electrophoresis and densitometry: standard curve for actin scanned one week after electrophoresis. The center line is the regression line representing the change in peak area with increasing protein loads. The two outer lines encompass a 95% confidence interval. Points (+) represent the average of 6 scans, triplicate gels and two scans per gel. The Y axis has been extended to accommodate the lower confidence limit at low protein concentrations. The intercept of the regression line was not significantly different from zero.

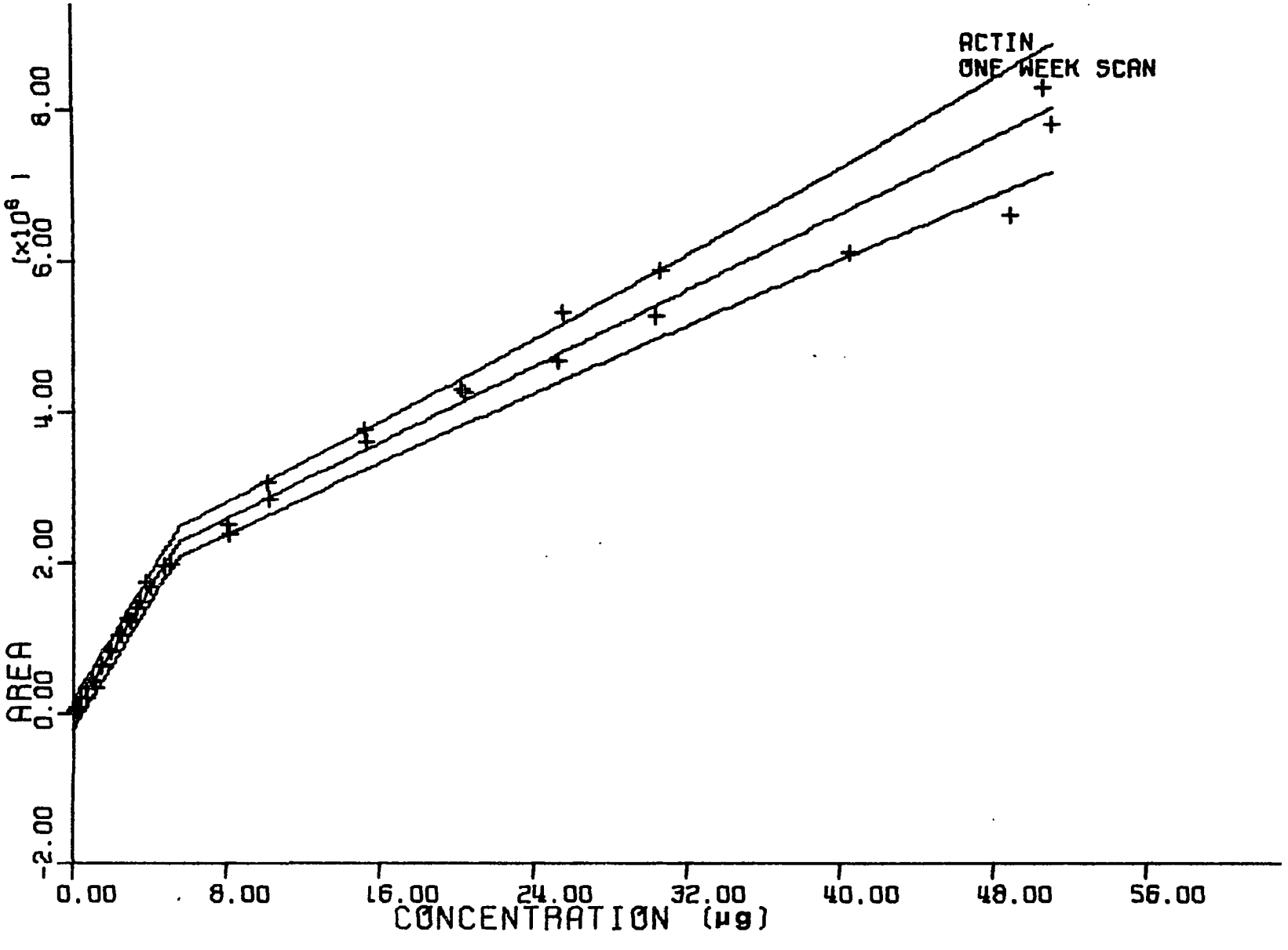

ACTIN
ONE WEEK SCAN
AREA (x10^6)
8.00
6.00
4.00
2.00
0.00
-2.00
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
CONCENTRATION (µg)

Figure 7. Quantitative electrophoresis and densitometry: standard curve for tropomyosin scanned one week after electrophoresis. The center line is the regression line representing the change in peak area with increasing protein loads. The two outer lines encompass a 95% confidence interval. Points (+) represent the average of 6 scans, triplicate gels and two scans per gel. The Y axis has been extended to accommodate the lower confidence limit at low protein concentrations. The intercept of the regression line was not significantly different from zero.

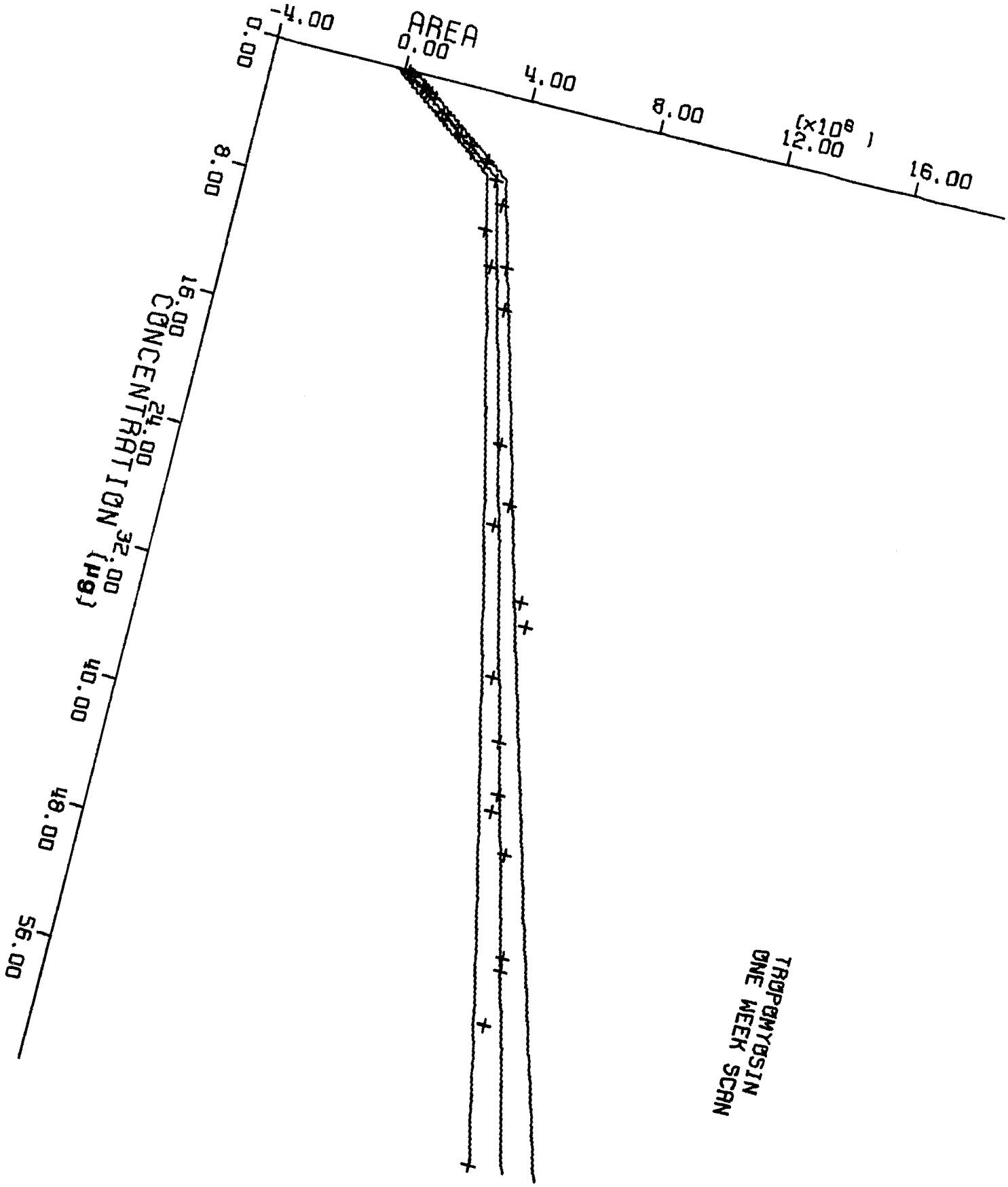

AREA
-4.00
0.00
4.00
8.00
12.00
16.00
(x10^8)
0.00
8.00
16.00
24.00
32.00
40.00
48.00
56.00
CONCENTRATION (nM)
TROPOMYOSIN
ONE WEEK SCAN

Figure 8. Quantitative electrophoresis and densitometry: one week standard curve for troponin-T. The center line is the regression line representing the change in peak area with increasing protein loads. The two outer lines encompass a 95% confidence interval. Points (+) represent the average of 6 scans, triplicate gels and two scans per gel. The Y axis has been extended to accommodate the lower confidence limit at low protein concentrations. The intercept of the regression line was not significantly different from zero.

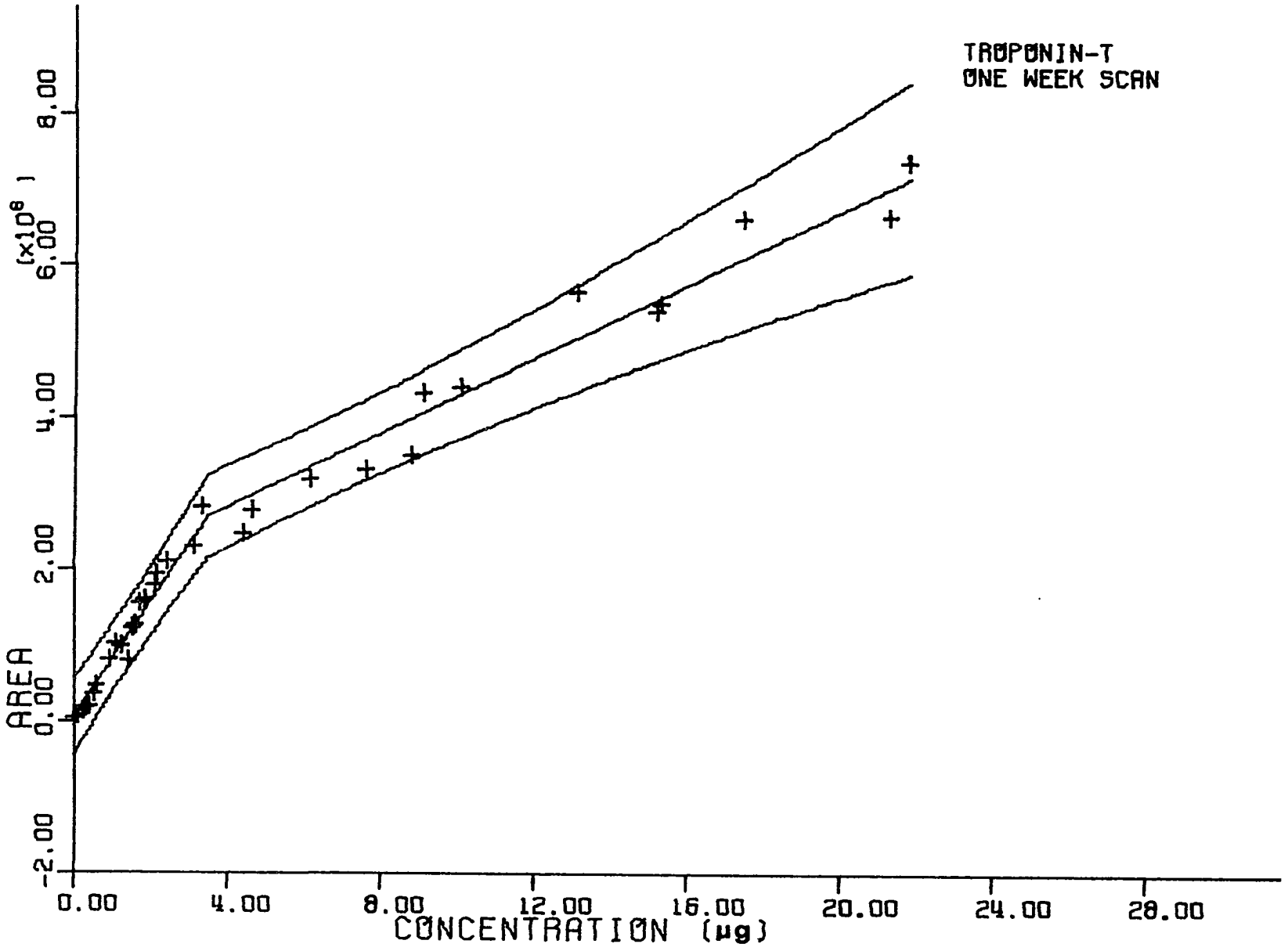

TROPONIN-T
ONE WEEK SCAN
AREA (x10^6)
8.00
6.00
4.00
2.00
0.00
-2.00
0.00
4.00
8.00
12.00
16.00
20.00
24.00
28.00
CONCENTRATION (µg)

Figure 9. Quantitative electrophoresis and densitometry: one week standard curve for troponin-I. The center line is the regression line representing the change in peak area with increasing protein loads. The two outer lines encompass a 95% confidence interval. Points (+) represent the average of 6 scans, triplicate gels and two scans per gel. The Y axis has been extended to accommodate the lower confidence limit at low protein concentrations. The intercept of the regression line was not significantly different from zero.

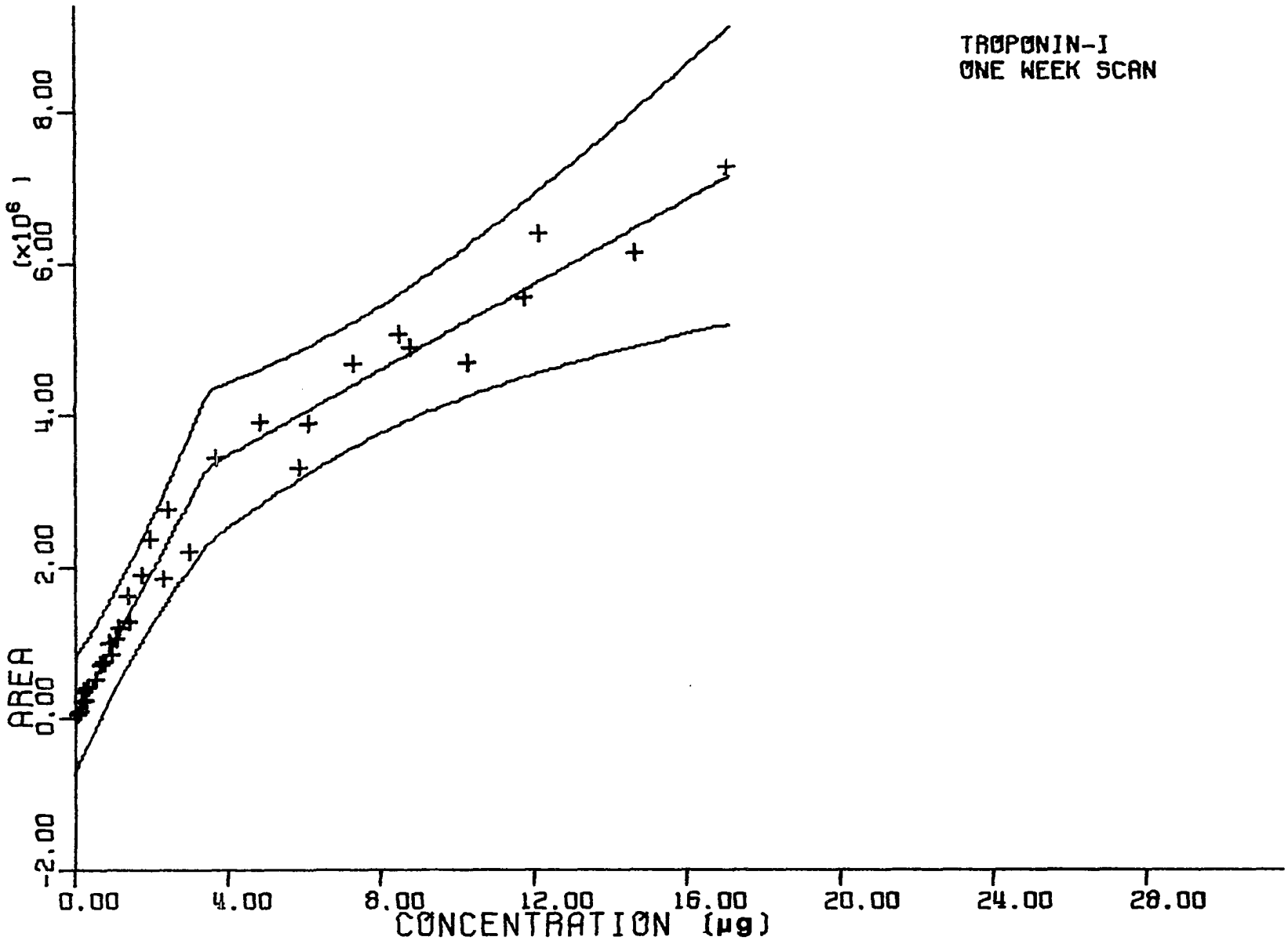

TROPONIN-I
ONE WEEK SCAN
AREA (x10^6)
8.00
6.00
4.00
2.00
0.00
-2.00
0.00
4.00
8.00
12.00
16.00
20.00
24.00
28.00
CONCENTRATION (µg)

Figure 10. Quantitative electrophoresis and densitometry: one week standard curve for troponin-C. The center line is the regression line representing the change in peak area with increasing protein loads. The two outer lines encompass a 95% confidence interval. Points (+) represent the average of 6 scans, triplicate gels and two scans per gel. The Y axis has been extended to accommodate the lower confidence limit at low protein concentrations. The intercept of the regression line was significantly different from zero.

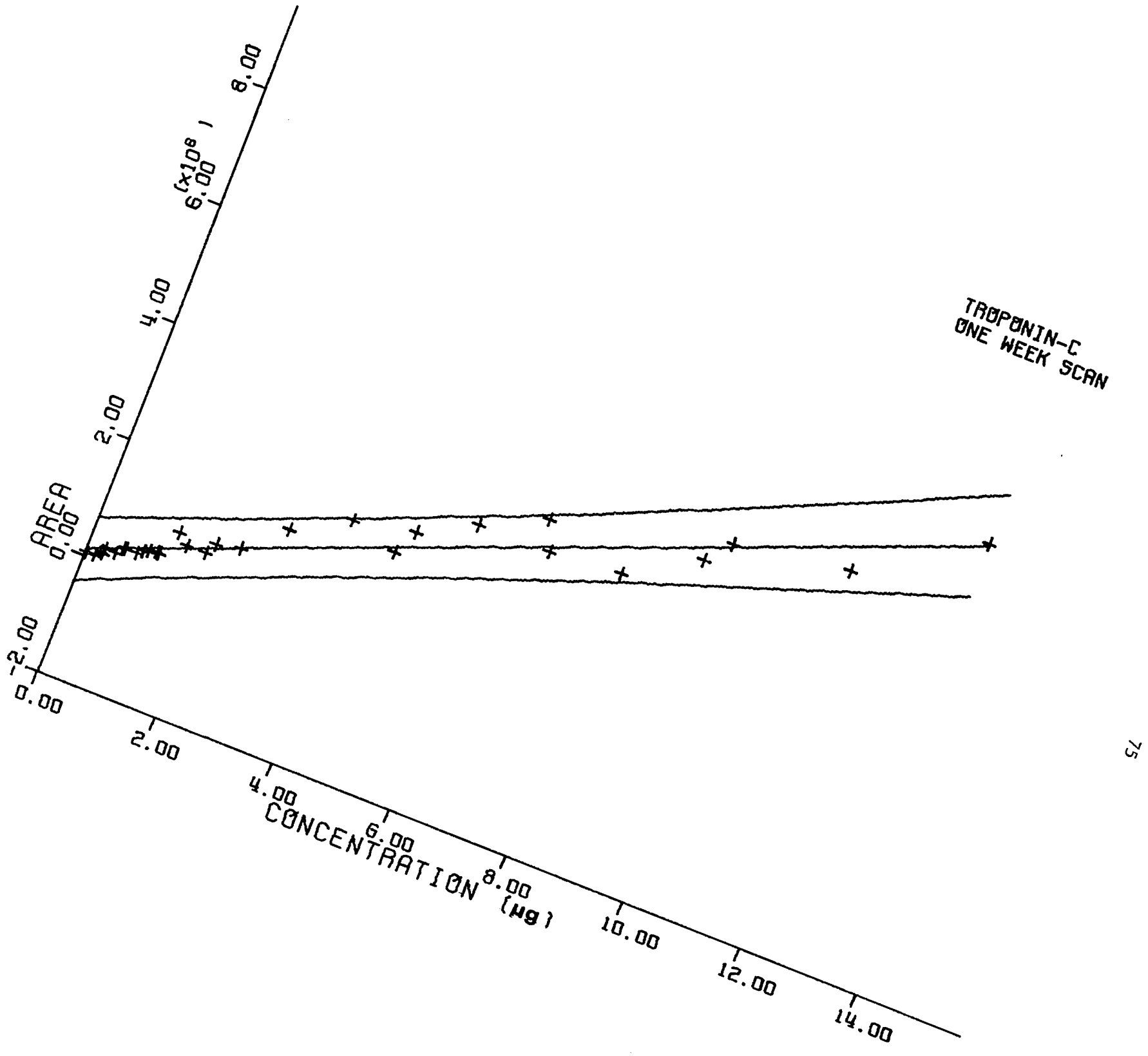

TROPONIN-C
ONE WEEK SCAN
AREA
8.00
6.00
(x10⁸)
4.00
2.00
0.00
-2.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
CONCENTRATION (µg)

Table 1. Quantitative electrophoresis and densitometry: regression equations for one- and three-week standard curves for myofibrillar proteins

Protein	One week	Three week
Myosin	$R^2 = 0.98$	$R^2 = 0.99$
	$\leq 5.5\mu g$ Area = 63,154.38[a] + 390,758.98 (conc)[c]	$\leq 5.5\mu g$ Area = 1,004[a] + 371,596 (conc)
	$\geq 5.5\mu g$ Area = 1,303,047.36[b] + 150,778.44 (conc)	$\geq 5.5\mu g$ Area = 1,148,623[b] + 162,929 (conc)
α-actinin	$R^2 = 0.99$	$R^2 = 0.99$
	$\leq 5.0\mu g$ Area = 80,908.15[a] + 437,474.71 (conc)	$\leq 5.1\mu g$ Area = 60,499[b] + 480,338 (conc)
	$\geq 5.0\mu g$ Area = 1,477,258.26[b] + 158,204.69 (conc)	$\geq 5.1\mu g$ Area = 1,717,598[b] + 155,417 (conc)
Actin	$R^2 = 0.99$	$R^2 = 0.99$
	$\leq 5.0\mu g$ Area = -3,332.66[a] + 414,437.99 (conc)	$\leq 5.3\mu g$ Area = -37,683[b] + 429,740 (conc)
	$\geq 5.5\mu g$ Area = 1,580,346.32[b] + 126,496.36 (conc)	$\geq 5.3\mu g$ Area = 1,584,654[b] + 123,639 (conc)
Tropomyosin	$R^2 = 0.99$	$R^2 = 0.99$
	$\leq 5.0\mu g$ Area = 66,212.57[a] + 692,840.15 (conc)	$\leq 4.8\mu g$ Area = 22,751[a] + 729,502 (conc)
	$\geq 5.0\mu g$ Area = 2,877,954.56[b] + 130,491.75 (conc)	$\geq 4.8\mu g$ Area = 2,814,214[b] + 147,947 (conc)
Troponin-T	$R^2 = 0.99$	$R^2 = 0.99$
	$\leq 3.5\mu g$ Area = 62,652.03[a] + 759,834.05 (conc)	$\leq 2.7\mu g$ Area = -64,085[a] + 980,612 (conc)
	$\geq 3.5\mu g$ Area = 1,876,203.95 + 244,247.79 (conc)	$\geq 2.7\mu g$ Area = 1,859,718[b] + 268,093 (conc)
Troponin-I	$R^2 = 0.99$	$R^2 = 0.99$
	$\leq 3.5\mu g$ Area = 53,422.54[a] + 939,397.91 (conc)	$\leq 2.5\mu g$ Area = -4,892[a] + 1,118,453 (conc)
	$\geq 3.5\mu g$ Area = 2,355,906.15[b] + 281,545.45 (conc)	$\geq 2.5\mu g$ Area = 1,894,400[b] + 360,693 (conc)
Troponin-C	$R^2 = 0.99$	$R^2 = 0.99$
	no break Area = 128,957[b] + 399,375.64 (conc)	no break Area = 193,241[a] + 426,776 (conc)

[a] Does not differ significantly from zero ($p < 0.05$).

[b] Differs significantly from zero ($p < 0.05$).

[c] Conc = protein concentration (μg/gel).

Law and becomes a function of Beer's Law plus an additional phenomenon. During the course of these experiments, it was noted that the point at which the curve breaks often corresponds to a concentration range where the peak height begins to exceed 3.0 OD units. Beyond 3.0 OD, stray light could be expected to significantly depress the measured absorbance of the protein band thus resulting in a reduced area per unit of protein compared with lower concentration regions.

This explanation can also be used to interpret the distance of migration effect observed by Fischbein (1972) and also in these experiments with large protein loads (Figure 11). The effect of distance of migration on actin quantitation at 1.3 μg/gel and 32 μg/gel, which would normally fall within the low and high concentration portions of the standard curve respectively, is presented in Figure 11. The gels corresponding to these distances of migration and protein concentrations are shown in Figure 12. A large increase in peak area with increasing distance of migration was observed for the 32 μg gels while no change in peak area occurred with increasing distance of migration for the 1.3 μg gels. At low concentrations, the peak never approached 2 OD; therefore, as the band migrated further and became more diffuse there was no change in absorbance. At higher concentrations, however as the band became more diffuse due to greater distance of migration, the peak height diminished, more of the peak area remained under 3 OD and therefore absorbed according to Beer's Law. This resulted in greater peak areas as the protein migrated further into the gel. These experiments cannot disprove the hypothesis that the distance of migration effect is due to the inability of narrow, compact protein bands to bind as much dye

Figure 11. Effect of distance of protein migration on peak area. Triplicate gels of purified actin at two protein loads, 1.3 and 32 μg, were migrated to tracking dye distances of 2, 3, 4, 5, and 6 cm. The horizontal axis, however, indicates the migration distance of the protein band and not the migration distance of the tracking dye. Vertical bars over points represent plus or minus one standard deviation unit.

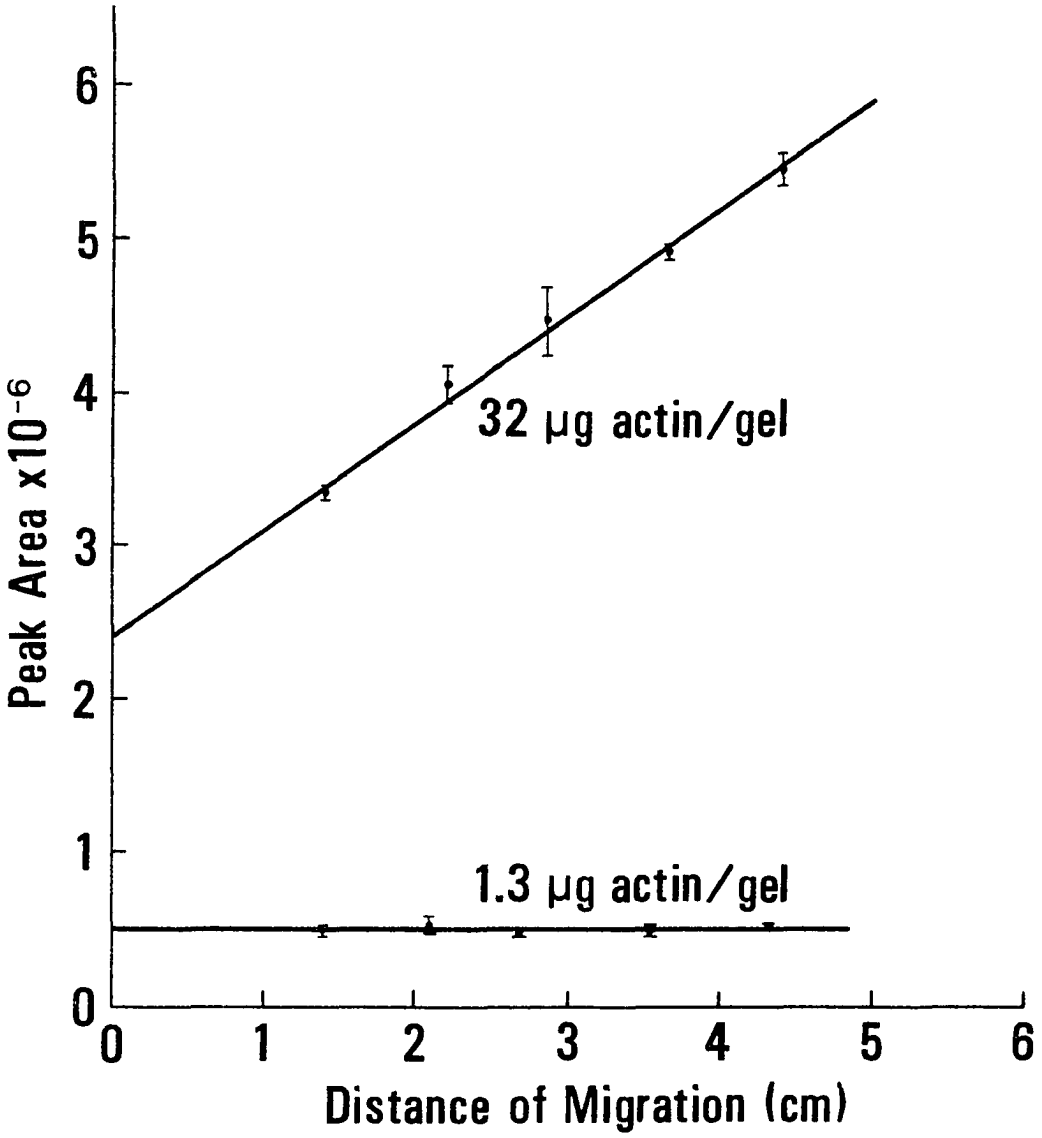
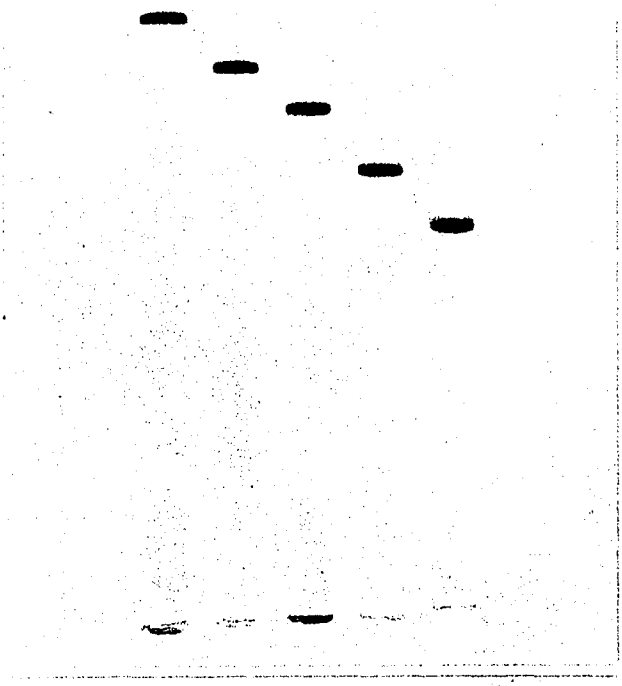
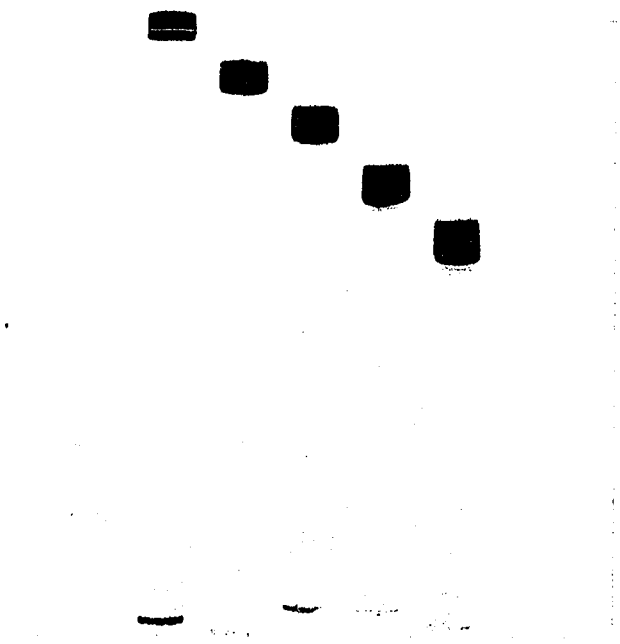

Peak Area x10⁻⁶
0
1
2
3
4
5
6
32 µg actin/gel
1.3 µg actin/gel
0
1
2
3
4
5
6
Distance of Migration (cm)

Figure 12. Seven and one-half percent polyacrylamide gels of purified actin migrated to five distances. These gels are representatives of each set of triplicate gels of the two protein loads (1.3 and 32 μg) and the five distances of migration.

1.3 μg Actin



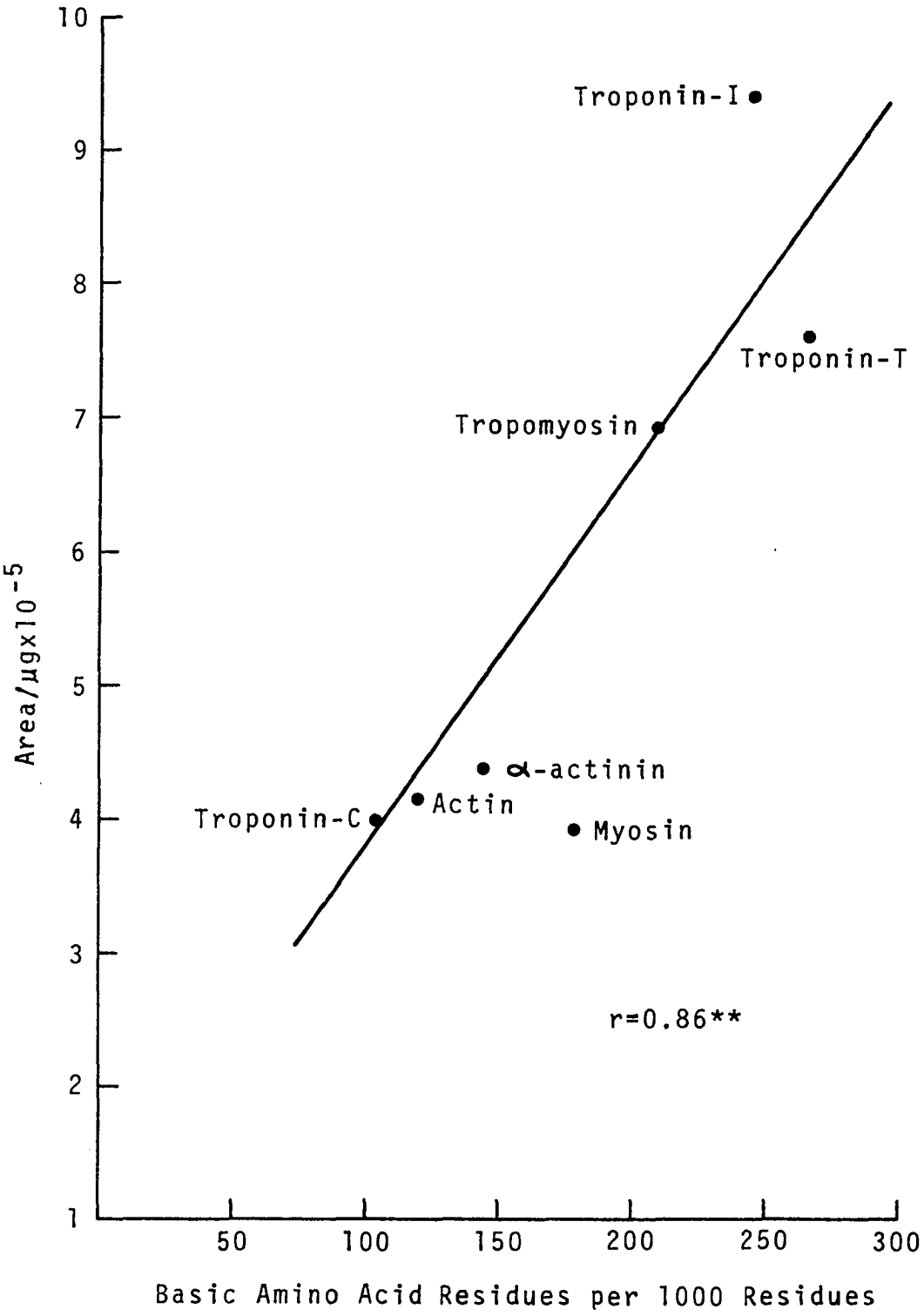
32 μg Actin



as diffuse bands (Fischbein, 1972). Fenner et al. (1975), however, reported that the relationship between protein load and eluted Coomassie Blue dye remains linear over a large range indicating that compaction of protein bands does not depress dye binding. Furthermore, Kruski and Narayan (1974) provided additional evidence that nonlinearity at higher concentration ranges is probably due to instrumental limitations in measuring large absorbance. It is important at this point to acknowledge the potential inaccuracy of OD measurements above 2-3 OD. From a purely empirical point of view, however, the results obtained from these measurements, under carefully controlled conditions, are reasonably reliable and repeatable, although certainly not as accurate as measurements from low concentration ranges.

The equations also provide information regarding dye binding properties of these proteins. The slopes of the curves at low concentrations, before the break point, represent the peak area/μg, and are found to vary from 3.71×10^5 to 11.18×10^5 (Table 1). Because Coomassie blue is a negatively charged molecule and has been postulated to bind electrostatically to proteins, the relationship between dye binding and basic amino acid composition of these myofibrillar proteins has been evaluated (Figure 13). A positive relationship ($r = 0.86$) between bound dye and basic amino acid composition exists for the proteins used in our experiments. A more complex relationship may also exist, however, which involves the effect of the proximity of basic residues to acidic residues which could tend to negate the electrostatic attraction between the dye molecule and the basic amino acid. Although the dye binding cannot be fully explained,

Figure 13. The relationship between Coomassie Brilliant Blue R binding and basic amino acid composition of myofibrillar proteins. The peak area per microgram of protein was determined for each protein from the slopes of the regression equations in the pre-breakpoint, low concentration region of the curve (Table 1). The basic amino acid composition (sum of lysine, arginine, and histidine) per one thousand residues were determined from the following sources: myosin (Lowey and Holt, 1972; Lowey et al., 1969), α-actinin (Suzuki et al., 1973), actin (Elzinga et al., 1973), tropomyosin (Phillips, 1976), troponin-T (Jackson et al., 1975), troponin-I (Wilkinson and Grand, 1975), and troponin-C (Collins et al., 1973). Linear regression analysis of peak area per microgram and basic amino acid composition yields a highly significant ($p < 0.01$) correlation ($r = 0.86$).


10
9
8
7
6
5
4
3
2
1
Area/μgx10^{-5}
Troponin-I
Troponin-T
Tropomyosin
α-actinin
Actin
Troponin-C
Myosin
r=0.86**
50
100
150
200
250
300
Basic Amino Acid Residues per 1000 Residues

an important point emerges from this data. Different proteins can vary widely in their ability to bind Coomassie Blue dye. Research which attempts to draw quantitative conclusions from gel densitometry without considering the problem of differential dye binding of proteins should be viewed with a great deal of caution.

The relative error associated with densitometry and standard curve preparation was evaluated by using analysis of variance (Table 2). The mean sums of squares (MSS) are presented, and their relative magnitudes within a given protein provide an indication of the relative error associated with each source of variation in the densitometry process. Several trends emerged when all seven proteins were studied; the most striking observation was the consistently small source of variation contributed by the duplicate scans of each gel (scans/gel). To some degree, the small MSS for scans/gel was probably responsible for the fact that most of the other sources of variation were significant. Furthermore, significant differences between one and three week scans were observed in five of the seven proteins studied along with significant concentration by time interactions and gel by time interactions within concentration. Although the relative magnitude of these sources of variation differed from one protein to the next, they were all consistently large when compared with duplicate scans of the same gel.

The analysis of variance results suggest that instrumental errors were negligible when compared to the between gel variation attributable to sample preparation and loading. Furthermore, the destaining process was also an important potential source of error, as indicated by the significant differences between one and three week scanning times and

Table 2. Analysis of variance of SDS-polyacrylamide gel quantitation

Source of variation	Myosin heavy chain df[a]	Myosin heavy chain MSS[b]	α-Actinin df	α-Actinin MSS	Actin df	Actin MSS	Tropomyosin df	Tropomyosin MSS
Concentration	59	6.0132×10^{13}	38	8.3025×10^{13}	39	6.7033×10^{13}	39	1.3705×10^{14}
Gel within concentration	120	6.8998×10^{10}**	78	2.4658×10^{11}**	80	3.5330×10^{10}**	80	2.8172×10^{11}**
Time	1	1.9242×10^{10}	1	1.4087×10^{12}**	1	2.8957×10^{11}*	1	7.7990×10^{12}**
Concentration × time	59	2.9467×10^{11}**	38	2.3932×10^{11}**	39	1.2320×10^{11}**	39	7.5505×10^{11}**
Gel × time within concentration	120	3.5076×10^{10}**	78	1.1985×10^{11}**	80	3.6799×10^{10}**	80	1.6660×10^{11}**
Scans/gel (residual)	360	3.4597×10^{9}	246	2.6395×10^{9}	238	2.1809×10^{9}	240	5.2150×10^{9}
Total	719	4.9776×10^{12}	479	6.6695×10^{12}	477	5.5046×10^{12}	479	1.1313×10^{13}

[a]df = degrees of freedom.

[b]MSS = mean sums of squares.

*$p < 0.01$.

**$p < 0.001$.

Table 2. Continued.

Source of variation	Troponin-T df	Troponin-T MSS	Troponin-I df	Troponin-I MSS	Tropinin-C df	Tropinin-C MSS
Concentration	35	5.8657×10^{13}	35	5.9475×10^{13}	32	2.9161×10^{13}
Gel within concentration	72	3.1383×10^{11}**	72	3.2082×10^{11}**	66	6.1803×10^{11}**
Time	1	2.6468×10^{12}**	1	3.4454×10^{12}**	1	2.2629×10^{12}
Concentration x time	35	7.2556×10^{10}*	35	1.2336×10^{11}**	32	6.2578×10^{11}
Gel x time within concentration	72	3.3924×10^{10}**	72	3.9667×10^{10}**	66	5.0441×10^{11}**
Scans/gel (residual)	216	3.5790×10^{9}	216	3.3165×10^{9}	198	8.6904×10^{8}
Total	431	4.8353×10^{12}	431	4.9096×10^{12}	395	2.6068×10^{12}

the significant concentration by scan time and gel by scan time interactions (Table 2). In view of these sources of variation, the most reliable results can be obtained by standardizing the destaining procedure and by scanning the gels at a fixed time after electrophoresis to eliminate the confusing gel variations and changes with time. Protein determinations from gels reported in this thesis were obtained by using the procedure developed from these quantitation experiments. Furthermore, it should be noted that virtually all culture protein gels were quantitated in low concentration ranges where quantitation was most accurate; this was primarily due to the small amounts of protein isolated from cultured cells.

The gel quantitation technique was also applied to the quantitation of myosin, actin, α-actinin, and tropomyosin in porcine skeletal muscle myofibrils. Myofibrils were evaluated from two portions of the semitendinosus muscle, the red portion composed of a high proportion of slow-twitch, oxidative fibers and the "white" portion which is composed predominately of fast-twitch, glycolytic fibers (Beecher et al., 1965). Ratios of various myofibrillar proteins (Table 3) from skeletal muscle are important because they provide a standard with which to compare the composition of culture muscle myofibrils and the relative molar rates of accumulation of myofibrillar proteins during differentiation and assembly. Difference between red and white myofibrils will not be discussed; the purpose of providing both sets of figures is to give a range of ratios, since the fiber type of the myotubes in these cultures has not been determined.

Table 3. Quantitative electrophoresis and densitometry of myofibrils. Molar ratios of myofibrillar proteins[a]

Source of myofibrils	Actin: myosin heavy chain	Actin: tropomyosin subunit	Actin: α-actinin subunit	Myosin heavy chain: tropomyosin subunit	Myosin heavy chain: α-actinin subunit	Tropomyosin subunit: α-actinin subunit
Pig semitendinosus red portion	2.79 ± 0.53[b]	2.75 ± 0.12	20.08 ± 1.79	1.05 ± 0.10	8.20 ± 0.67	7.84 ± 0.34
Pig semitendinosus white portion	2.60 ± 0.24	2.74 ± 0.07	20.59 ± 2.75	1.12 ± 0.08	8.64 ± 1.32	8.69 ± 0.64
5-day chick embryo muscle cell culture	4.86 ± 0.14	4.59 ± 0.27	48.00 ± 10.70	0.94 ± 0.04	7.77 ± 4.3	8.93 ± 4.3

[a]Purified myofibrils were prepared according to Goll et al. (1974) and electrophoresed on seven and one-half percent polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue and scanned one week after electrophoresis. Only peak areas falling within the low concentration regions of the respective standard curves were used.

[b]The ± represents plus or minus one standard deviation.

Of the myofibrillar proteins of importance in this research, only myosin, actin, α-actinin, and tropomyosin have been quantitatively evaluated. The values of 2.6 to 2.8 molecules of actin per molecule of myosin heavy chain are somewhat lower than the value of 3.17 reported by Potter (1974) but are in the same range as the value of 2.8 calculated from the mass ratios of myosin:actin reported by Tregear and Squire (1973). Potter (1974) also reported molar ratios of 3.65 moles of actin per mole of tropomyosin subunit; this again is higher than the ratio of 2.75 obtained in our research. A ratio of myosin heavy chain:tropomyosin subunit of about 1.1 did agree very well with Potter's values of 1.15. The major discrepancy resides in the actin quantitation. This discrepancy has several possible explanations; it could reflect a fundamental difference between rabbit and pig myofibril composition; this seems unlikely but cannot be ruled out. A more plausible explanation is that either Potter's procedure could be selectively losing tropomyosin and myosin or that our myofibril preparations selectively lose actin relative to tropomyosin and myosin.

Our values of 20 actin molecules per α-actinin subunit are somewhat higher than expected values. Calculating on the basis of a 1.06 μm thin filament with 13 actins per repeat, and a repeat distance of either 35 nm (Huxley, 1963) or 37.8 nm (Hanson et al., 1972), each thin filament would contain 393 or 365 actin molecules, respectively. These calculations, when combined with the molar ratios of actin to α-actinin (Table 3), produce a range of figures of 9.12 to 9.85 α-actinin molecules (2 subunits/molecule) per thin filament. If one α-actinin molecule comprises each Z filament of the Z line, there should only be 2

α-actinins per thin filament; however, if α-actinin is the amorphous component (Schollmeyer, 1976), there is no structural basis on which to estimate how many α-actinins would be expected.

Myofibrils from cultured muscle cells have also been quantitated (Table 3). The results suggest that myosin heavy chain, α-actinin, and tropomyosin are present in the same relative proportions as in skeletal muscle myofibrils. Ratios of these three proteins to actin, however, show that there is a large excess of actin, when compared with mature skeletal muscle myofibrils. Although the source of this excess actin is not readily apparent, actin has been reported to be present in the nucleus (McIntosh et al., 1975) and in the cytoplasmic system of contaminating fibroblasts (Pollard and Weihing, 1974) and could account for a portion of this excess actin. Troponin-T might be co-migrating with actin and could also be responsible for the inflated actin ratios. It is also conceivable that these large ratios could actually reflect the actual composition of forming and newly formed myofibrils.

C. Light and Electron Microscopy of Cultured Muscle Cells

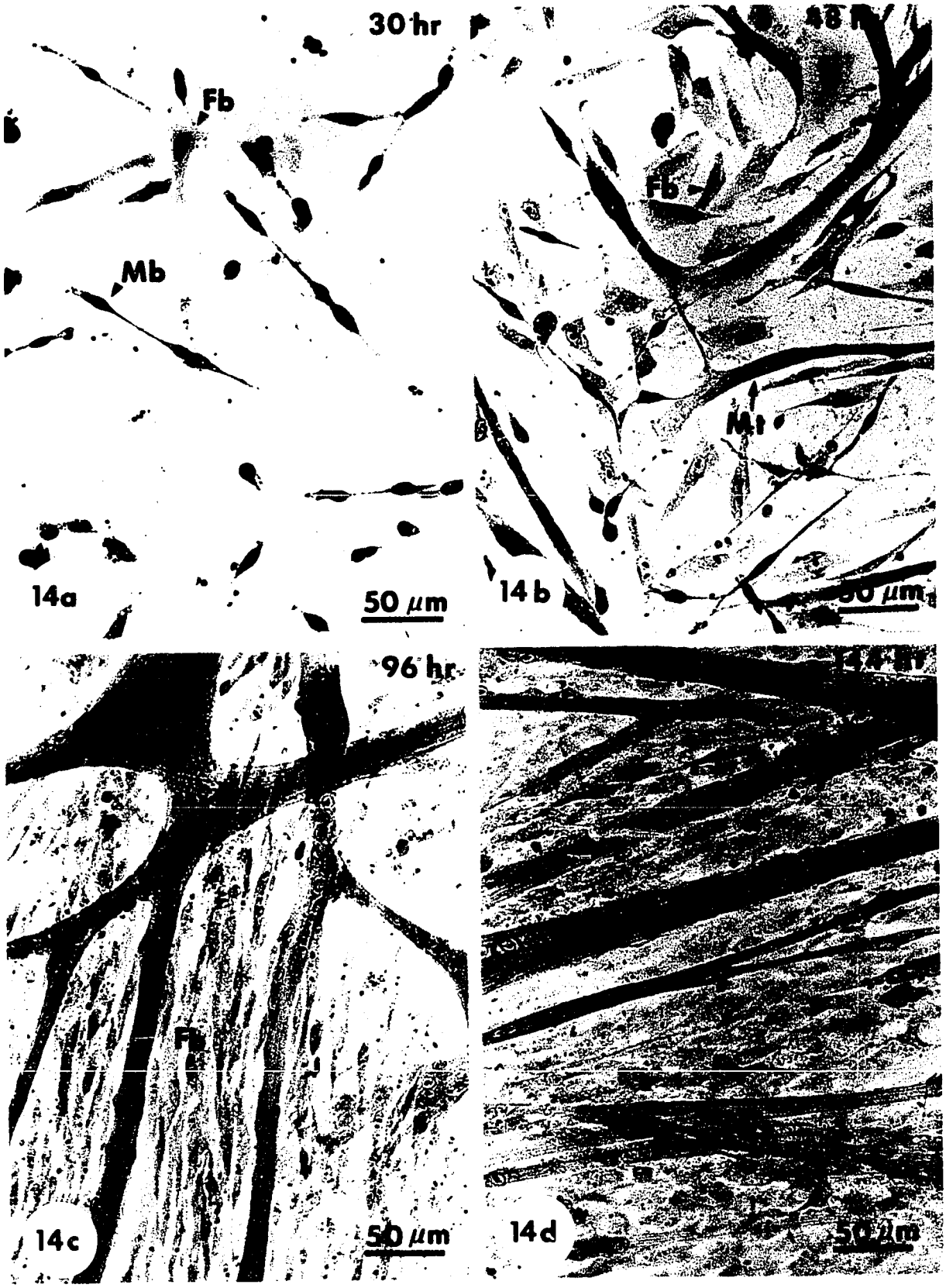
An important aspect of this research was the structural investigation. The light microscope observations were intended to provide an assessment of muscle cell differentiation for correlating with and interpreting the biochemical data. Furthermore, the electron microscope observations were designed to permit the comparison of biochemical events with the progress of sarcomere assembly. The electron microscope study was not designed to be a comprehensive structural investigation

in and of itself but it was intended to permit comparisons with structural events observed by many other investigators (refer to Section C of Literature Review) and to place these events in the proper frame with respect to the biochemical changes being monitored.

Under our culture conditions, muscle cells grown in complete medium, with no modifications, attached to the collagen-coated dish within the first twenty-four hours. By 30 hr in culture, mononucleated bipolar cells, presumably myoblasts, and irregularly shaped mononucleated fibroblasts were in the process of proliferating (Figure 14a). At 48 hr in culture, many myoblasts had ceased proliferating and had fused into myotubes (Figure 14b). Many fibroblasts may be seen between the myotubes. The fibroblasts continued to proliferate throughout the culture period and by 96 hr (Figure 14c) were rapidly covering the dish between myotubes. Fusion was essentially complete, and a large network of branching myotubes was present. Evidence of myofibril formation may be seen in the cytoplasm of these myotubes (Figure 14c). Myofibrils were easily identified in myotubes at 144 hr (Figure 14d) by the characteristic striated appearance caused by individual sarcomeres. By this time in culture, fibroblasts had completely packed the spaces between myotubes. As a result of the extensive fibroblast proliferation, the percentage of fused nuclei had dropped from the peak value attained between 48 and 72 hr.

The large mass of fibroblasts also pose a problem in studying myofibrillar protein synthesis because they possess several of the same contractile proteins found in myotubes. These contractile proteins in fibroblasts and in myogenic precursor cells comprise the cytoplasmic

Figure 14. Differentiating muscle cell cultures observed in the bright field microscope. Cultures were fed complete medium for the duration of the experiment. Mononucleated fibroblasts (Fb) are shown in series a, b, c, and d. Mononucleated myoblasts (Mb) in 30 hr cultures align and fuse into myotubes (Mt) as indicated in 48 hr, 96 hr, and 144 hr cultures. The banding pattern of myofibrils is evident in the myotubes of 144 hr cultures. Cultures were stained with Giemsa stain, X262.


30 hr
Fb
Mb
14a
50 μm
Fb
Mb
14b
96 hr
Fb
14c
50 μm
14d
50 μm

contractile system (Pollard and Weihing, 1974). The organization of this contractile system differs drastically from highly ordered myofibrils found in skeletal muscle. Contractile proteins, predominately actin, are organized in long bundles of parallel thin filaments (Figure 15). Thick filaments, analogous to myosin thick filaments in myofibrils, have not been visualized with electron microscopy although a form of myosin is present in these cells. Antibodies to α-actinin have been localized in these filament bundles at the electron microscope level (Schollmeyer, 1976) in areas of increased density, presumably due to α-actinin cross links between actin filaments. Tropomyosin has not been localized at the electron microscope level but the existence of a tropomyosin-like molecule in these structures has been demonstrated by immunofluorescence (Lazarides, 1975, 1976). The cytoplasmic contractile system seems to be dismantled to a large extent in myotubes, since it is not frequently observed.

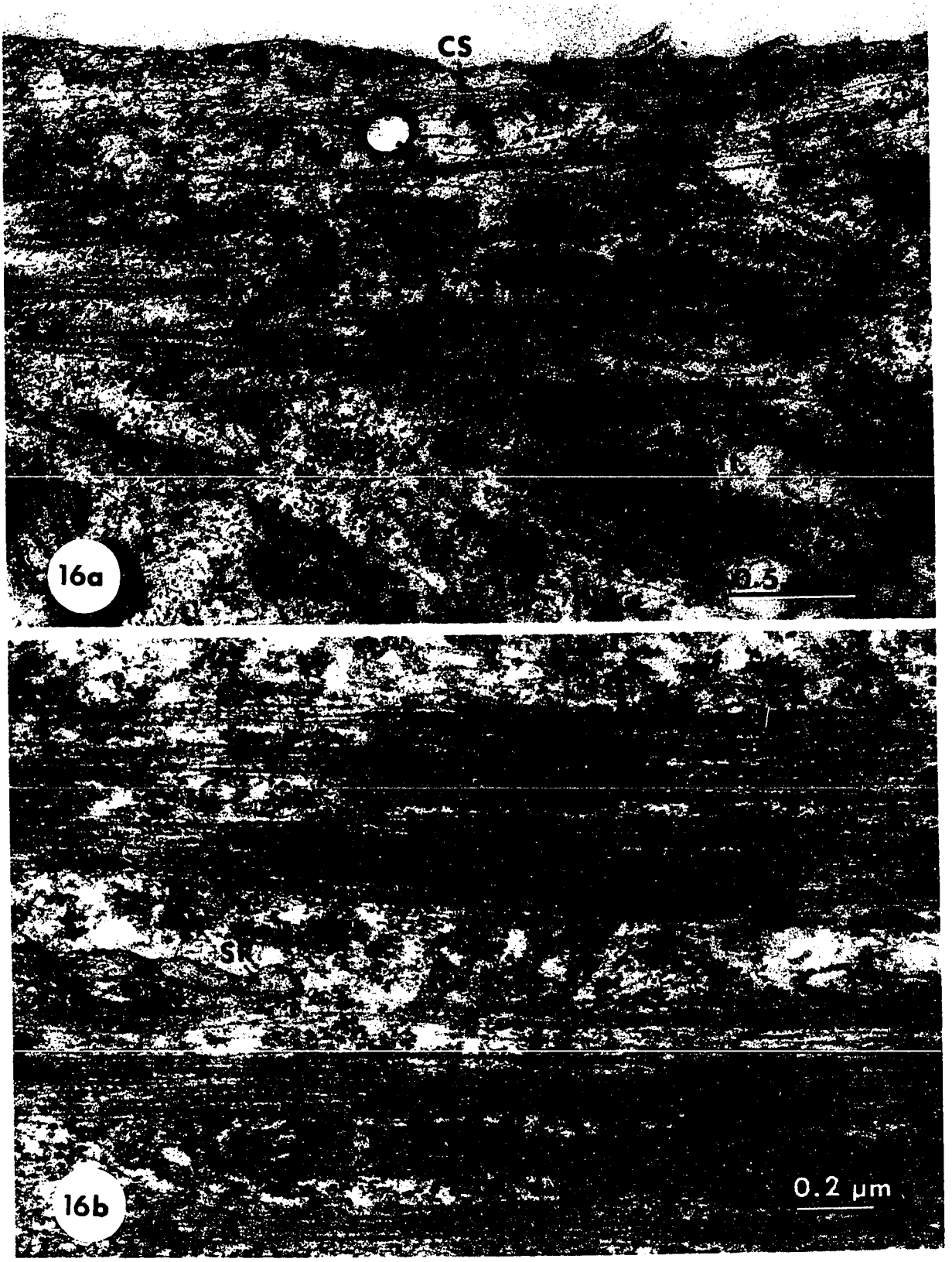
In young myotubes, 48-72 hr in culture, the earliest observable myofibrillar precursors were aggregates of thick and thin filaments (Figures 16a and b). Remnants of the cytoplasmic contractile system may also be seen close to the cell membrane (Figure 16a). The aggregates were somewhat randomly oriented, but closer examination of these thick and thin filament bundles showed that the filaments were organized in an alternating fashion (Figure 16b). There was no evidence of a precursor Z line structure in these aggregates; however, this does not rule out the existence of a few molecules associated with the ends of thin filaments.

Figure 15. Survey electron micrograph of muscle cell culture fibroblast. The cytoplasmic contractile system (CS) of the fibroblast is found near the cell membrane. Other organelles, rough endoplasmic reticulum (RER) and microtubules (Tu), are also found scattered throughout the cytoplasm, X27,830.


15

Figure 16a. Early myofibrillar protein structures in 60 hr cultured myotubes. Remnants of the cytoplasmic contractile system (CS) can be found beneath the cell membrane. Aggregates of thick and thin filaments (Tt) are somewhat randomly oriented, X43,180.

Figure 16b. A higher magnification view of myofibrillar protein aggregates. Individual thick (T) and thin (t) filaments are associated in the characteristic interdigitating arrays. Evidence of sarcoplasmic reticulum (SR) development is also present, X66,040.


CS
16a
0.2 µm
16b
0.2 µm

In a slightly more advanced state of assembly, 72-96 hr in culture (Figure 17), thick and thin filament aggregates had started to associate end to end. Dense bodies of Z line precursor material were seen between the ends of adjacent filament bundles. There was no evidence of an I band region possibly indicating that the thick and thin filaments were positioned in a contracted state. M line regions were evident in these sarcomeres, although some myofibrils in a more advanced state (Figure 19a) showed no evidence of M line formation. The sarcoplasmic reticulum membrane system was evidently developing simultaneously with the assembly of myofibrils (Figure 17).

Examination of the filament aggregates at higher magnifications (Figure 18) clearly showed the interdigitation of thick and thin filaments and some evidence of cross-bridge attachment of thick to thin filaments. M line structures were present in some sarcomeres (Figure 18a) although it was difficult to discern much structural detail. A Z line, present in Figure 18b, showed a filamentous structure, in addition to the amorphous material, which was not apparent in Z-line dense material from more primitive myofibrils. Regardless of whether α-actinin forms the Z filaments of the amorphous material, it was probably present in this structure at this stage of assembly. A distinct I band region was also observed and may indicate that the proteins and structural organization required for relaxation were present.

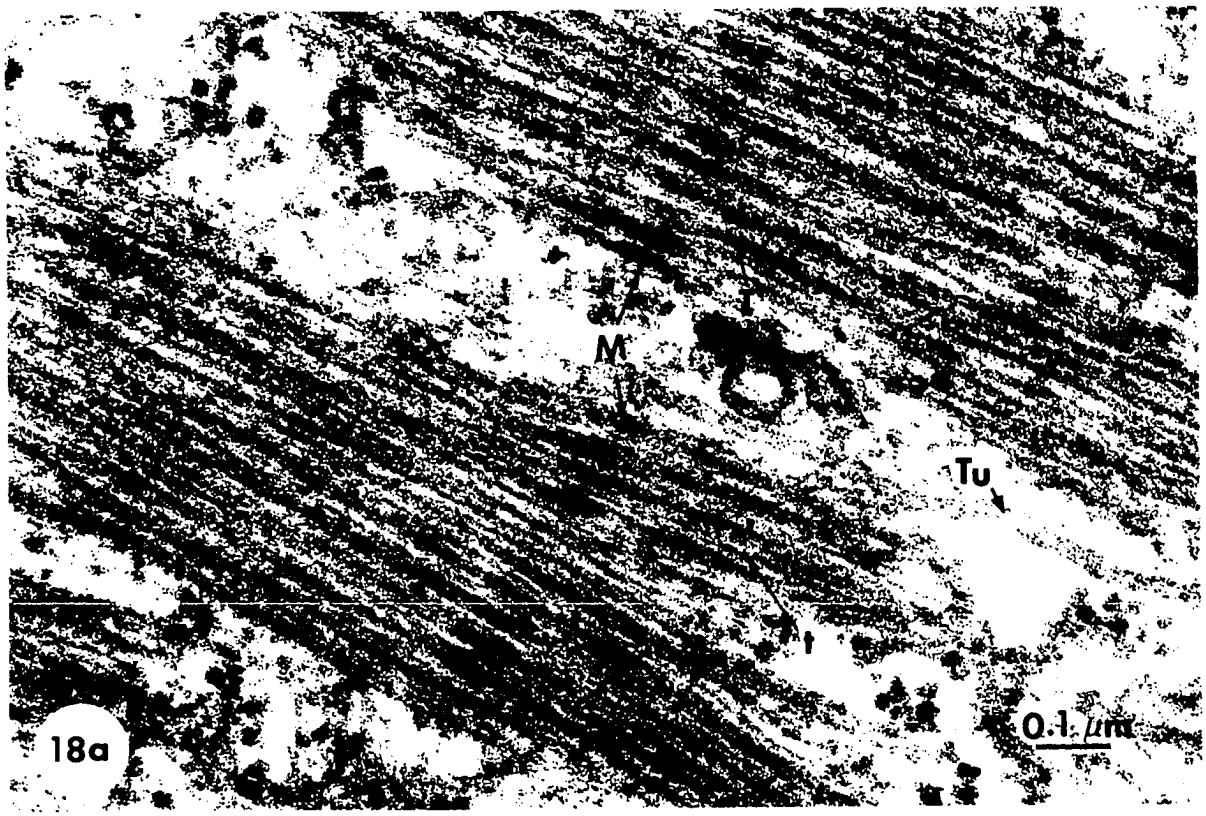
As previously noted, Z lines appeared to start as dense regions of Z line material, seemingly without a filamentous structure (Figure 19a). In addition to having a somewhat amorphous structure, the discontinuous Z material frequently was not in register within the myofibril, and the

Figure 17. Myofibril formation in a 72 hr cultured myotube. Thick and thin aggregates (Tta) are aligning end to end and Z line precursors (Zp) are developing between myofibrils. Developing M lines are also evident and the sarcoplasmic reticulum (SR) is seen near the nascent myofibril. Microtubules (Tu) and a large polyribosome (P) can be seen in the cytoplasm near the developing myofibril, X32,760.

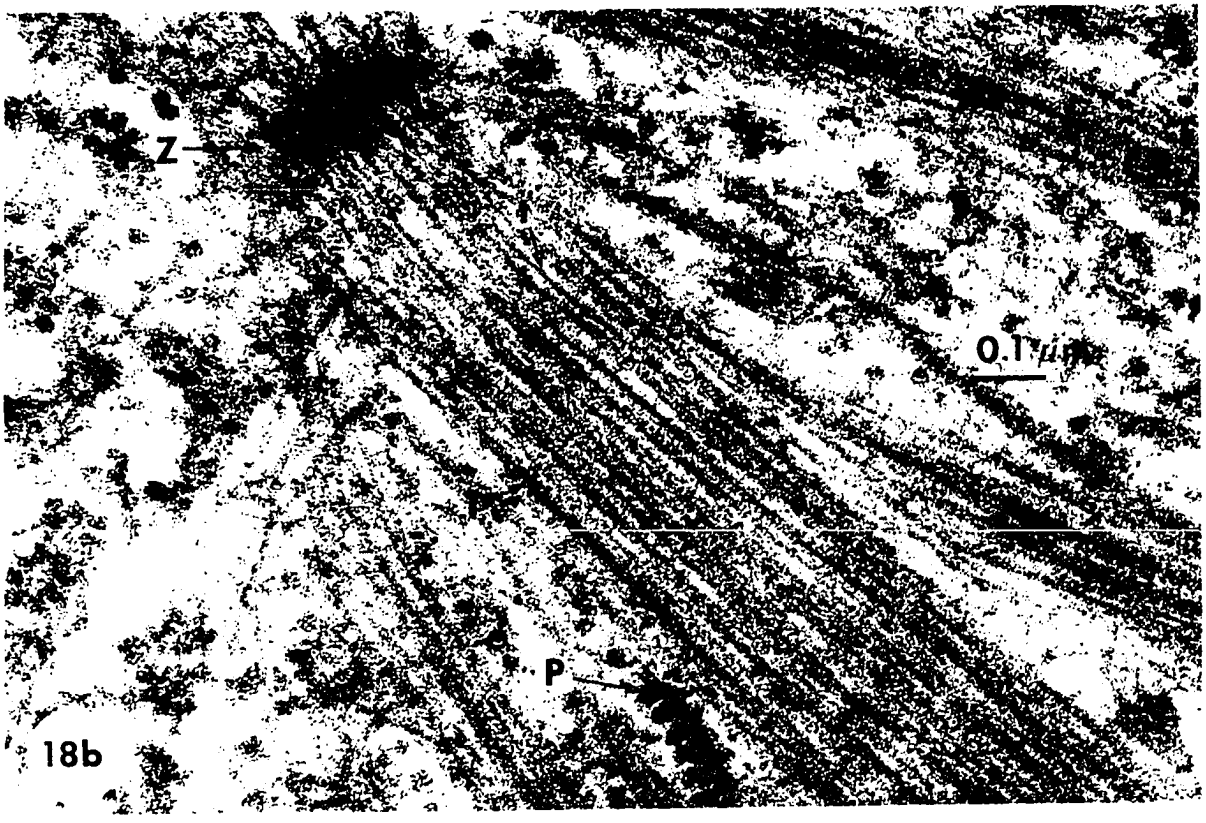

P
M
17

Figure 18a. Interaction of thick and thin filaments in nascent myofibrils. The alternating pattern of thick (T) and thin (t) filaments is evident. The M line (M) is readily seen in the myofibril as well as a microtubule (Tu) running parallel to the forming myofibrils, X96,900.

Figure 18b. Evidence of cross-bridge interaction between thick and thin filaments (arrow heads) and a well formed Z line (Z), with amorphous and filamentous structure, are characteristic of 96 hr cultured muscle cells, X96,900.



18a



18b

Figure 19a. Z line formulation in developing myofibrils from 96 hr cultured myotubes. Figure 19a shows developing Z lines; regions of Z line precursor bodies (Zp) are found associated with ends of thin filaments. The Z line precursors have not yet formed a continuous straight Z line (Z) within myofibrils as shown in 19b, X32,760.

Figure 19b. Mature myofibrils in a myotube from a 96 hr muscle cell culture. Sarcomeres with mature Z lines (Z), well formed I bands (I) and A bands (A), and evidence of M line structure have been assembled in this myotube, X32,760.

19a

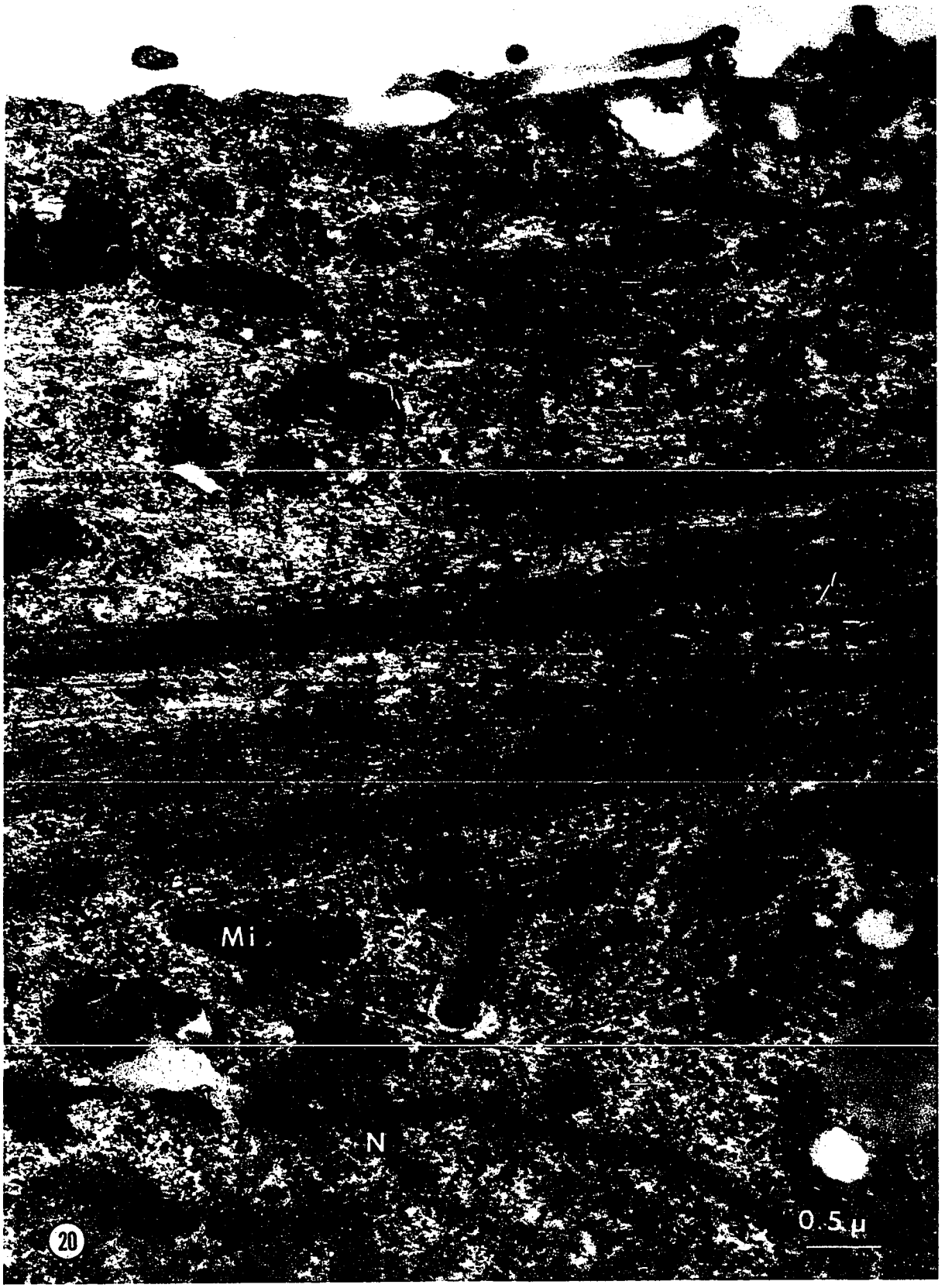
19b

Z lines of adjacent myofibrils also were not in register. These sarcomeres also lacked I band regions. Myofibrils in the most advanced state examined, 96 hr in culture, (Figure 19b) displayed well organized Z lines and thick and thin filaments. Myofibrils were in register side to side, and the sarcoplasmic reticulum was developing adjacent to the myofibril. I bands and M lines were also evident.

A lower magnification micrograph of a portion of a developing myotube (Figure 20) revealed several important myofibrillar structures. Well formed myofibrils with distinct A bands, I bands, and Z lines were seen in the center of the micrograph. In the same region, however, thick and thin filament aggregates with amorphous Z line structures and even small aggregates of thick and thin filaments without any apparent Z line material were observed. The myotube shown (Figure 20) illustrates a very important point. Myofibril assembly is not a process of sequential events but is a continuing process, as evidenced by the range of states of myofibril assembly found in the same region of the cell at the same time.

The observations presented in this section are in general agreement with those presented in other electron microscope investigations (Fischman, 1967, 1970; Przybylski and Blumberg, 1966; Shimada et al., 1967). Small aggregates of thick and thin filaments were the first identifiable myofibrillar structures, followed by the association of amorphous Z line material at the ends of thin filaments. Z lines became more organized and eventually showed evidence of Z filament structure. During this time, sarcomeres aligned end to end to form myofibrils and finally aligned side to side with Z lines, I bands, and A bands in

Figure 20. Low magnitude overview of a 96 hr cultured myotube. Several organelles are present including the nucleus (N), numerous polyribusomes (P), mitochondria (Mi), and microtubules (Tu). The entire range of developing myofibrillar structures can be seen, from primitive thick and thin filament aggregates (Tta) to mature myofibrils with distinct A bands (A), I bands (I) and Z lines (Z). In addition, intermediate stages of assembly are found in which the Z line precursors (Zp) are still associating with the ends of thin filaments, X23,400.


Mi
N
0.5 μ
20

register. A distinct M line could not always be observed, even in sarcomeres which had all the other bands characteristic of a mature myofibril.

D. Accumulation of Myofibrillar Proteins in Cultured Muscle Cells

This section is concerned with the primary goal of this research which was to describe the myofibrillar protein accumulation patterns in differentiating muscle. Two parameters of these accumulation patterns will be described in separate subsections:

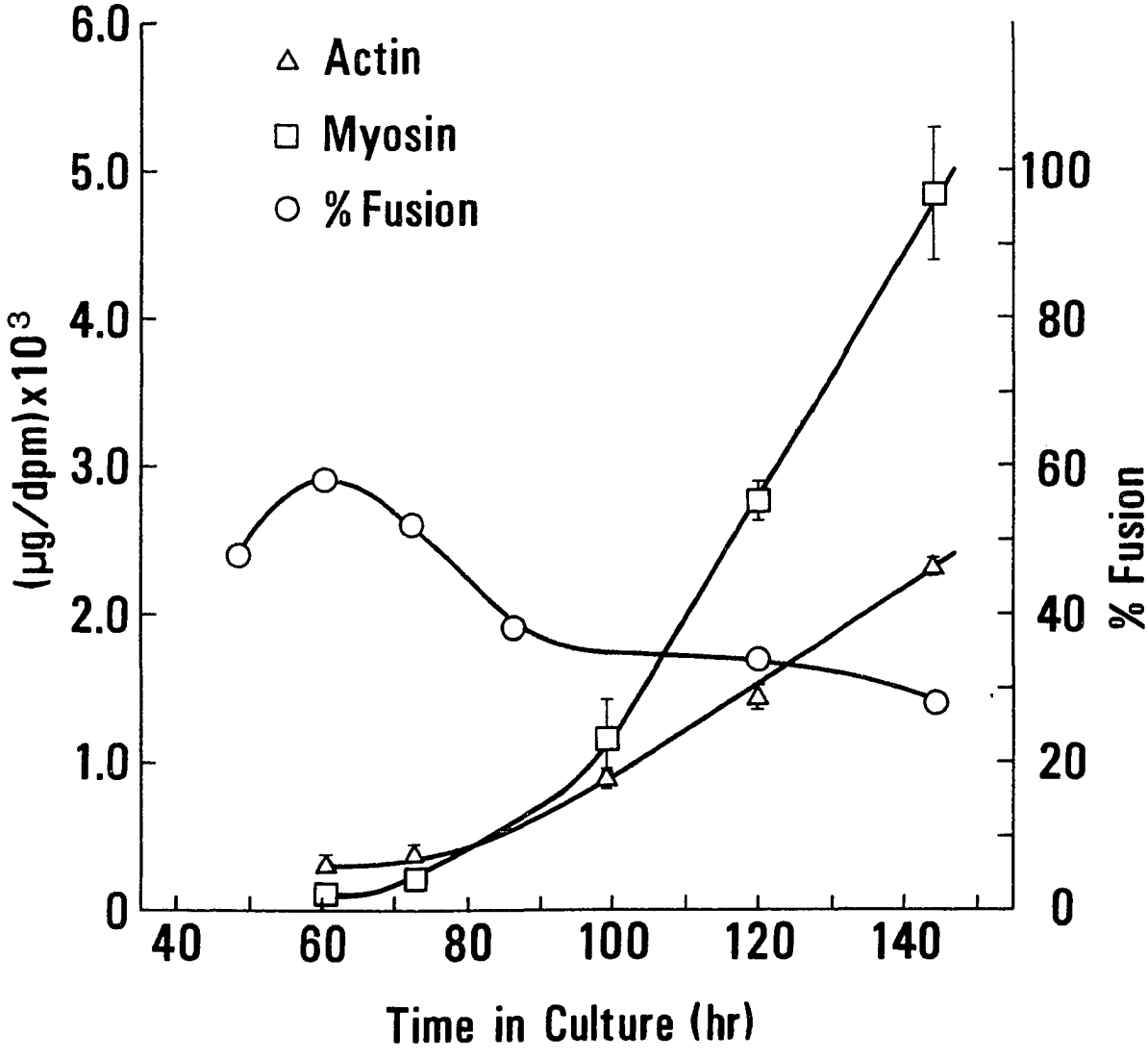
(1) the temporal sequence in which the increased accumulation of each of the four myofibrillar proteins is initiated,

(2) the relative rates at which these four myofibrillar proteins accumulate after increased accumulation is initiated.

1. Initiation of increased accumulation of myofibrillar proteins

As pointed out in the Materials and Methods section, these experiments employed the first two modified forms of isotope dilution analysis. The first experiment was designed to monitor the time at which myosin and actin accumulation began to increase during differentiation. For this analysis, form I of the isotope dilution experiment was employed. Cells grown in complete medium were pulse labeled for four hours prior to taking the first culture samples. As the cells accumulated unlabeled protein, beyond the pulse label period, 1/specific activity of a purified aliquot of actin or myosin increased. The profiles of this measure of accumulation (Figure 21) demonstrated an increase in both actin and myosin accumulation between 72 and 96 hr. This increase occurred soon after the

Figure 21. Actin and myosin heavy chain accumulation in cultured muscle cells as a function of time in culture. Form I of the isotope dilution experiment in which cultures were pulse-labeled was used to obtain the actin and myosin profiles. The percentage fusion has been superimposed over these accumulation profiles to provide a frame of reference with respect to differentiation. Vertical bars associated with points on the curve represent plus or minus one standard deviation unit.


Actin
Myosin
% Fusion
(μg/dpm)x10³
% Fusion
Time in Culture (hr)
6.0
5.0
4.0
3.0
2.0
1.0
0
100
80
60
40
20
0
40
60
80
100
120
140

peak of fusion in these cultures. The kinetics of myosin accumulation is consistent with numerous other investigations that have been reported (Coleman and Coleman, 1968; Paterson and Strohman, 1972; Young et al., 1975). The point of significance in this experiment, however, was that myosin and actin displayed the same increase in accumulation at the same time; this provides evidence that they are synthesized in a co-ordinated manner in differentiating muscle cells as suggested, but not adequately demonstrated, by Holtzer's group (Chi et al., 1975; Holtzer et al., 1974; Rubinstein et al., 1974, 1976).

At the time these experiments were conducted, the prevailing assumption was that myofibrillar protein synthesis was somehow dependent upon muscle cell fusion and was not initiated unless fusion had occurred (Paterson and Strohman, 1972; Paterson et al., 1974). Therefore, the next experiment was designed to test this hypothesis. The same experimental format was followed with one exception; cell fusion was prevented from 24 hr to 96 hr by the addition of a predetermined concentration of EGTA to the culture medium. Normally fusion is complete in regular cultures by 72 hr. As indicated in Figure 22, 24 hr cultures appeared very similar to the 24 hr control cultures (Figure 14); however at 96 hr, less than 1% fusion had occurred and the bipolar cells had become very elongated (Figure 22b). When fusion was permitted by feeding complete medium without EGTA, the elongated myotubes fused within the following 24 hours, and the percent fusion exceeded 50%. The kinetics of myosin and actin accumulation were also monitored in these cultures (Figure 23). Contrary to the results of Paterson and Strohman (1972) and Paterson et al. (1974), actin and myosin accumulation increased in a coordinated

Figure 22. Morphology of cultured muscle cells in EGTA fusion arrest experiments. Individual fibroblasts (Fb) and myoblasts (Mb) are seen at 24 hr in panel a. At 24 hr, EGTA was introduced into the culture medium and fusion was inhibited until 96 hr as indicated by the elongated myoblasts (Mb) in panel b. At 96 hr, EGTA was removed from the medium and fusion was allowed to proceed, panel c. Bright field light micrograph of Giemsa stained cells, X262.

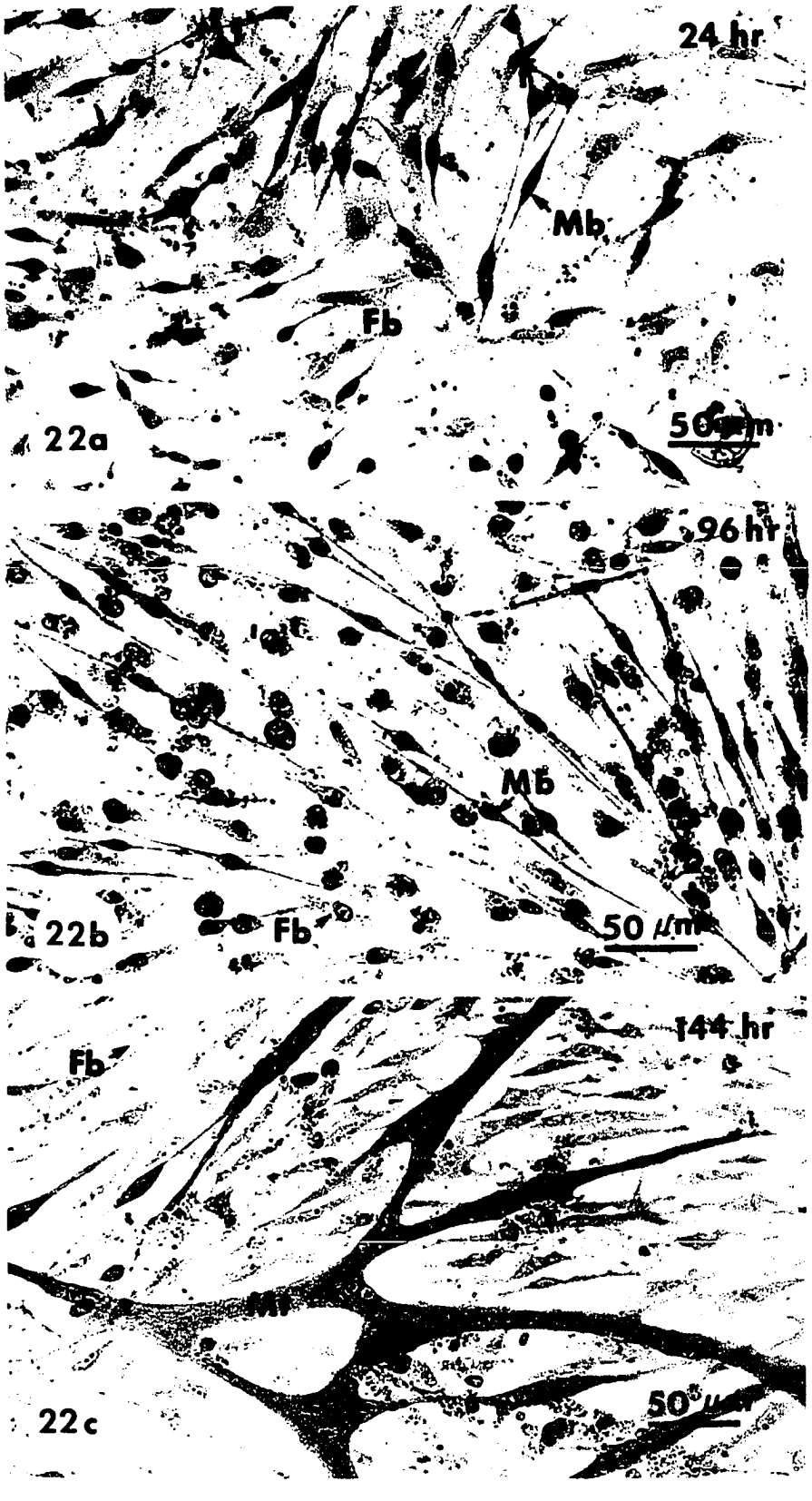
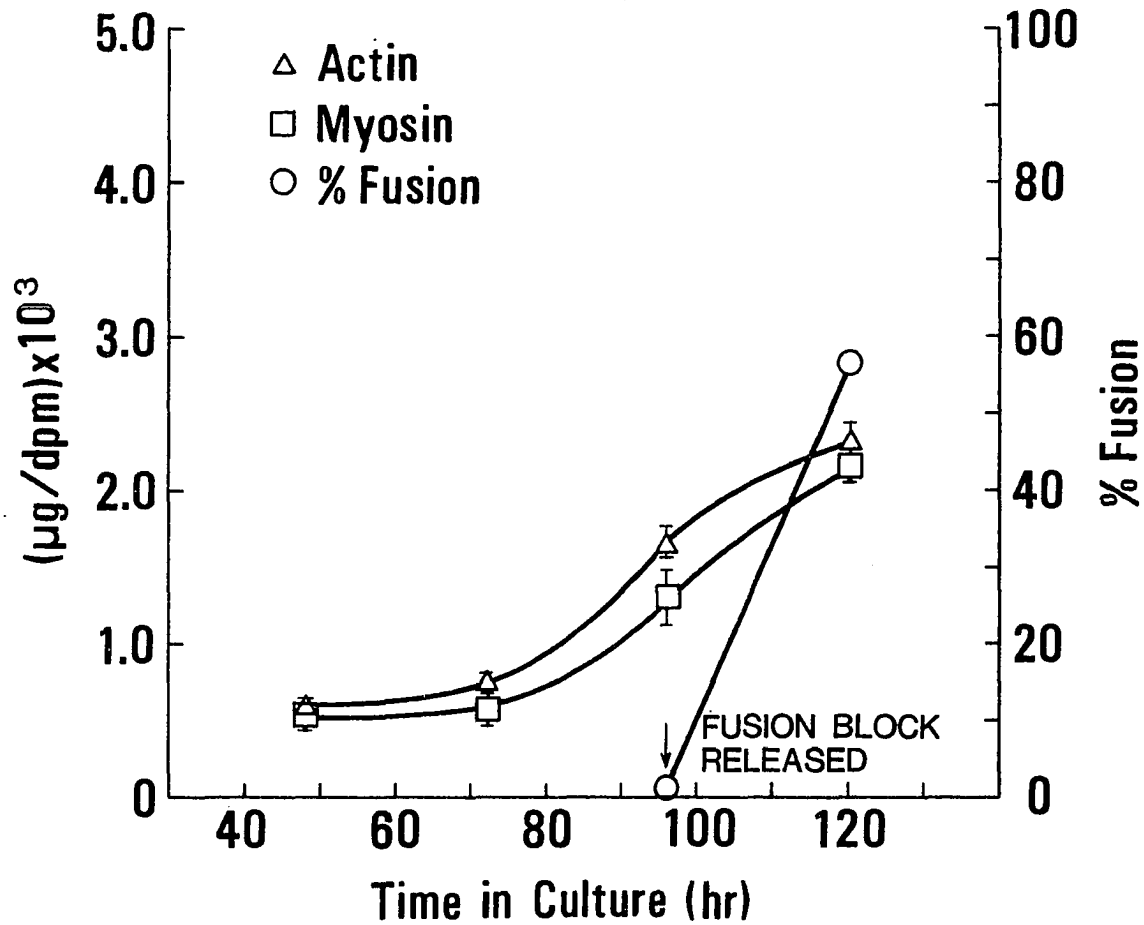

24 hr
Mb
Fb
22a
96 hr
Mb
Fb
50 µm
22b
144 hr
Fb
22c

Figure 23. Actin and myosin heavy chain accumulation in EGTA fusion arrested muscle cell cultures. Points represent mean values and vertical bars represent plus or minus one standard deviation unit. The percentage fusion is designated at 96 and 120 hr.


5.0
4.0
3.0
2.0
1.0
0
100
80
60
40
20
0
△ Actin
□ Myosin
○ % Fusion
(μg/dpm)x10³
% Fusion
FUSION BLOCK
RELEASED
40
60
80
100
120
Time in Culture (hr)

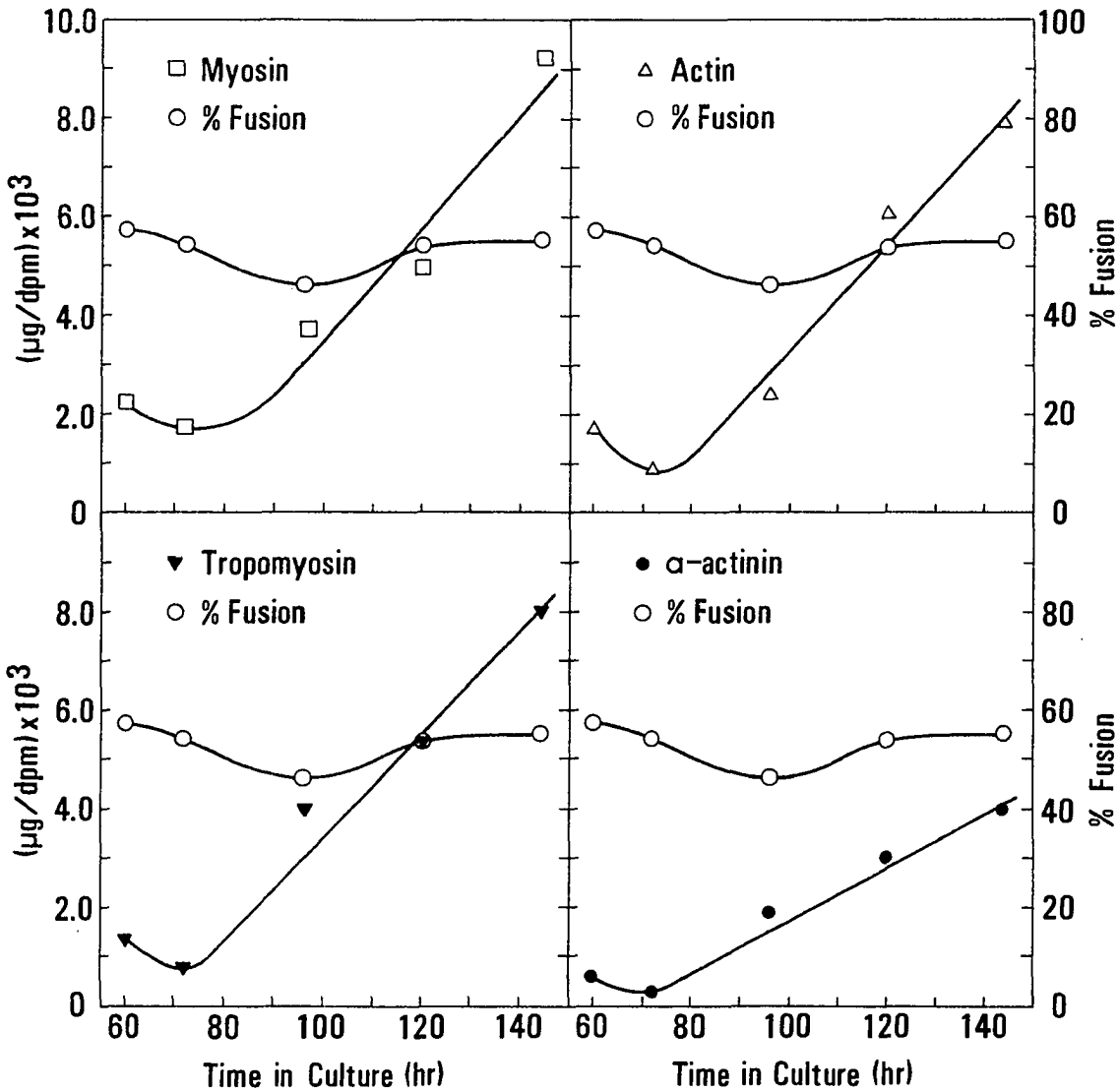
manner at least 24 hours prior to the release of the fusion block. During the course of the experiments reported in this thesis, Emerson and Beckner (1975) reported that myosin synthesis could occur in EGTA fusion-blocked muscle cultures. Subsequent to that report, other investigators have demonstrated the synthesis of myosin in fusion-blocked cultures (Merlie and Gros, 1976; Moss and Strohman, 1976; Trotter and Nameroff, 1976; Vertel and Fischman, 1976).

The accumulation kinetics of myosin, actin, tropomyosin and α-actinin were investigated by using the second modification of the isotope dilution experiment, form II. As discussed in the Materials and Methods section, one of the assumptions associated with form I of the isotope dilution experiment was that degradation of the original pulse-labeled pool of protein was insignificant and did not alter the results of the experiment. Form II of the isotope dilution experiment was developed to circumvent the protein degradation problem by eliminating the pulse labeling step. Instead, a constant amount of ^{3}H-leucine-labeled muscle culture homogenate was added to each experimental culture after harvest and prior to protein purification.

Accumulation profiles of the four myofibrillar proteins are presented in Figures 24a-d. Figures 24a and b demonstrate the same pattern of actin and myosin accumulation observed with form I of the isotope dilution experiment. Therefore, it was concluded from these experiments that the assumptions concerning protein degradation in form I of the isotope dilution experiment were valid.

Accumulation of actin and myosin in these experiments increased at the same time in culture; furthermore, tropomyosin and α-actinin

Figure 24. Accumulation profiles of myosin, actin, tropomyosin, and α-actinin in differentiating muscle cell cultures. Form II of the isotope dilution experiment was used to obtain these results; this experiment involved adding radioactive muscle culture homogenate to each sample prior to protein purification.


Myosin
% Fusion
Actin
% Fusion
Tropomyosin
% Fusion
α-actinin
% Fusion
(μg/dpm)x10³
% Fusion
Time in Culture (hr)
Time in Culture (hr)
10.0
8.0
6.0
4.0
2.0
0
100
80
60
40
20
0
60
80
100
120
140

accumulation also increased at about the same time (Figures 24c and d). These results suggested that the accumulation of all myofibrillar proteins is coordinated in differentiating muscle cells. At the same time, these results provide evidence against the sequential synthesis of myofibrillar proteins in differentiating muscle as proposed by Allen and Pepe (1965), Heywood and Rich (1968), and Hitchcock (1970).

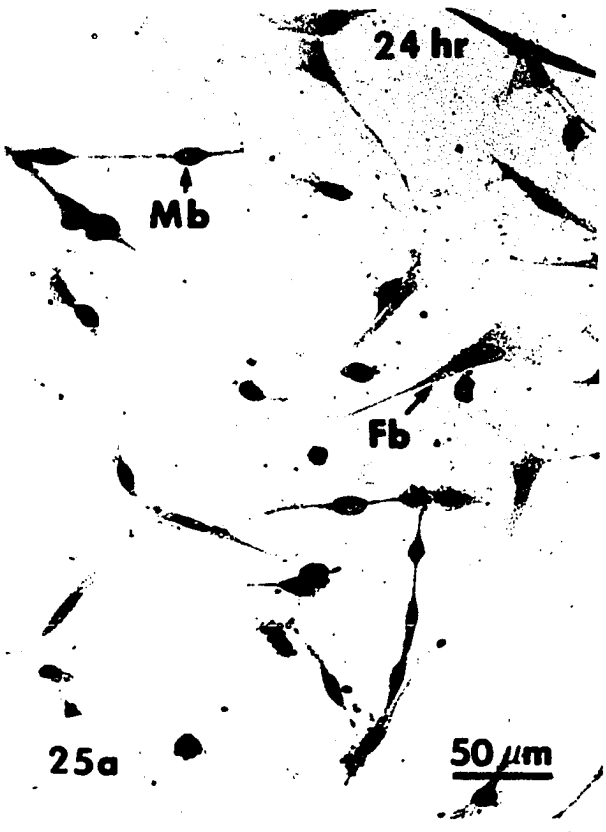
2. Rates of accumulation of myofibrillar proteins

The previous section has provided a somewhat qualitative picture of myofibrillar protein accumulation and suggests that the process is coordinated, at least with respect to the time at which each of these proteins begins to accumulate. If myofibrillar protein accumulation is a highly regulated process, it might be expected that both the relative times and relative rates of accumulation of these myofibrillar proteins would be coordinated.

A third and final modification of the original isotope dilution experiment (form III) was developed to monitor the rate of protein accumulation. This experiment was a true quantitative isotope dilution experiment. A constant volume of radioactive culture myofibril preparation was added to each culture sample. The radioactive culture myofibril preparation was subsequently assayed for mass and specific activity of each protein of interest. Knowing the mass of each protein added to the sample, its specific activity, and the final specific activity of the culture protein sample, the mass of protein in the culture at the time of harvest was calculated.

For these experiments, the muscle culture procedure was also modified. As demonstrated in Figure 14, conventional muscle cultures contained an increasing number of fibroblasts as the time in culture progressed. Furthermore, fibroblasts contain several contractile proteins that would have been indistinguishable from some of the myofibrillar proteins with the preparative and analytical techniques employed in these experiments. By 48 hr in culture, most of the myogenic cells had stopped dividing and had fused or were preparing to fuse, but the fibroblasts were continuing to proliferate. In an effort to minimize the influence of fibroblast contamination on the experiments, cultures were grown in the presence of 2.4×10^{-7} M fluorodeoxyuridine from 48 hr through the termination of the experiment. The addition of fluorodeoxyuridine inhibits thymidylate synthetase and consequently inhibits DNA synthesis. When examined in the bright field light microscope (Figure 25), fibroblast contamination in 24 hr cultures (Figure 25a) and in the 48 hr (Figure 25b) cultures appeared to be comparable to the conventional cultures at 30 and 48 hr (Figures 14a and b). Fibroblast contamination between myotubes, however, did not appear to increase substantially in these same cultures following the addition of fluorodeoxyuridine (Figures 25c and d). The inhibition of fibroblast proliferation was especially noticeable when contrasted with conventional cultures (Figure 14) in which fibroblast numbers increased steadily beyond 48 hr to finally fill the inter-myotube spaces. In conventional cultures (Figure 21), the percentage fusion peaked and then steadily decreased due to the increasing number of fibroblasts; however, in fluorodeoxyuridine cultures (Figure 26), percentage fusion remained relatively constant beyond

Figure 25. Muscle cell cultures with stationary fibroblast populations. Myoblasts (Mb), fibroblast (Fb) and myotubes (Mt) in these cultures show the same morphology as in conventional cultures (Figure 14). At 48 hr, however, fluorodeoxyuridine was introduced into the medium to inhibit DNA synthesis in proliferating fibroblasts. In 96 and 120 hr cultures, only a few fibroblasts are seen between myotubes. The fibroblasts have not become confluent as in untreated cultures (Figure 14d), X262.

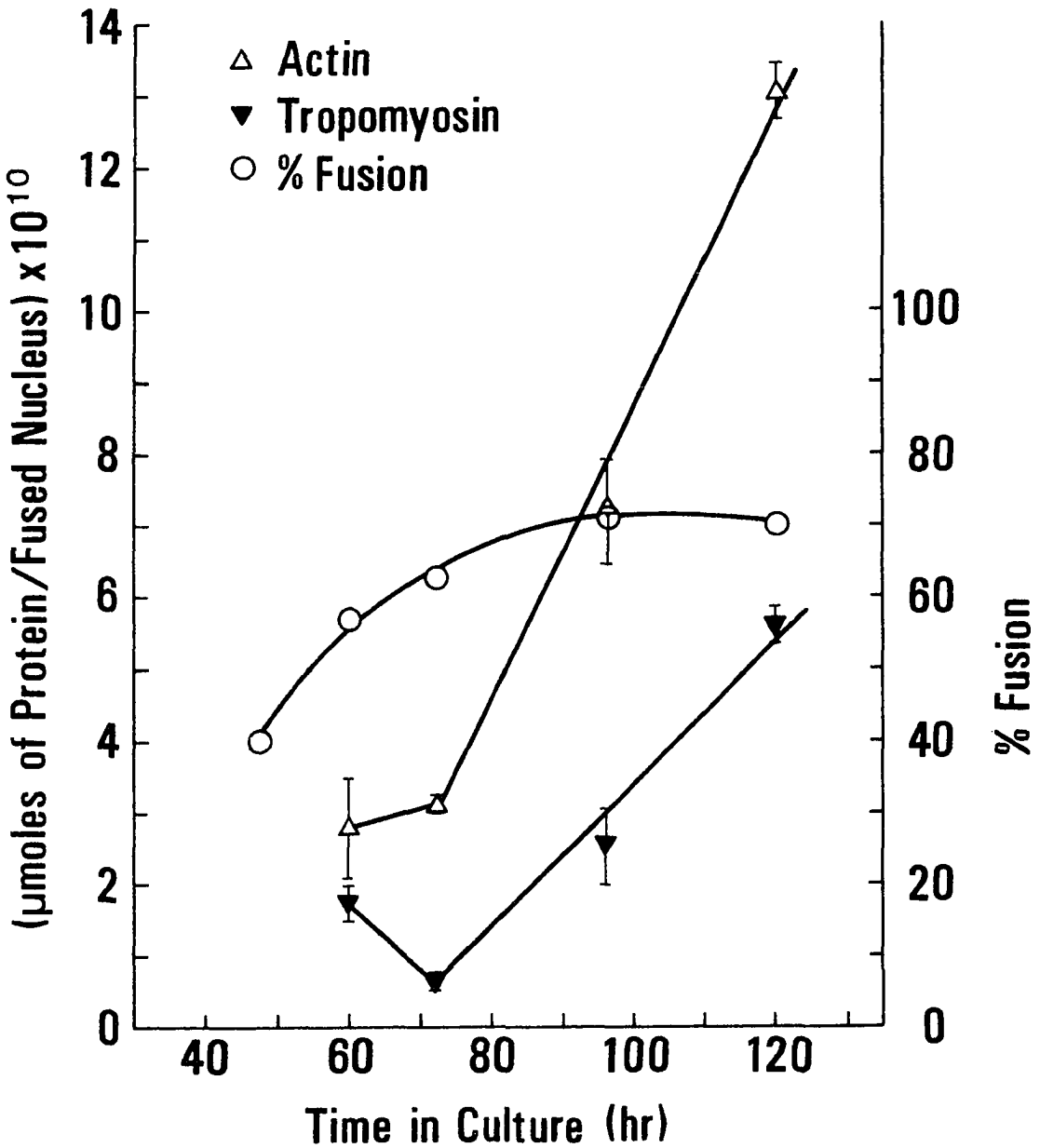

24 hr
Mb
Fb
25a
50 µm


25b


96 hr
Fb
25c


144 hr
Fb
Mb
25d

Figure 26. Actin and tropomyosin accumulation in muscle cell cultures. Form III of the isotope dilution experiment, which entailed the addition of radioactive muscle cell culture myofibrils, was used. The percentage fusion is not decreasing significantly with increasing culture age due to fluorodeoxyuridine inhibition of fibroblast proliferation. The slopes of the accumulation curves reflect the relative rates of actin and tropomyosin subunit accumulation.


Actin
Tropomyosin
% Fusion
(μmoles of Protein/Fused Nucleus) x10^10
% Fusion
Time in Culture (hr)
0
2
4
6
8
10
12
14
0
20
40
60
80
100
40
60
80
100
120

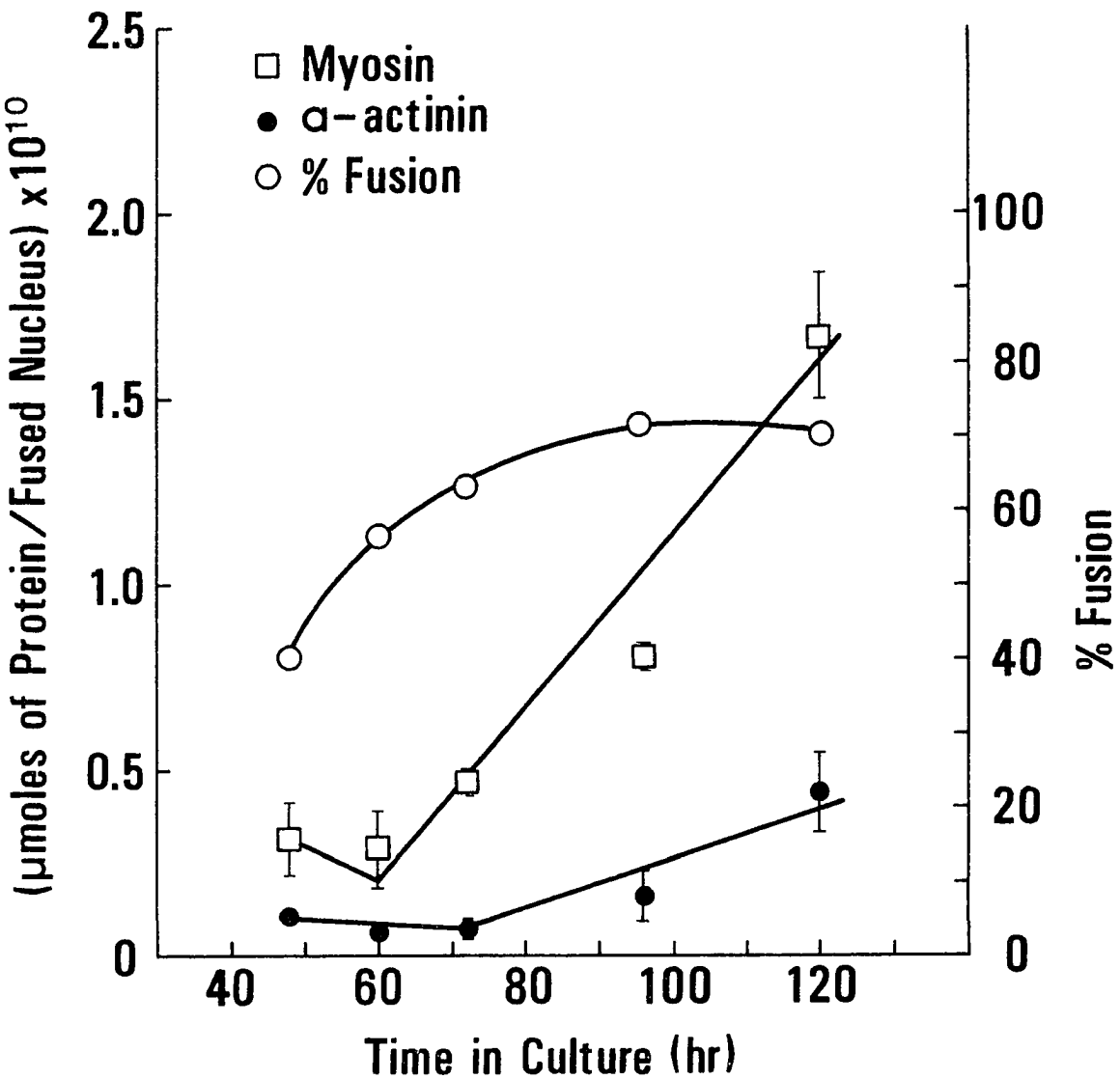
48 hr, reflecting the cessation of fibroblast proliferation. Myoblasts in the fluorodeoxyuridine-treated cultures (Figure 25) fused and formed myotubes which ultimately elaborated myofibrils (Figure 25d). There was no readily observable deviation from normal muscle cell growth due to the addition of fluorodeoxyuridine.

Since fibroblasts did not increase in number, and often decreased slightly due to cell death and subsequent detachment from the dish, it is reasonable to assume that there was no substantial accumulation of fibroblast contractile protein beyond the level present when cell division was blocked. If this assumption is valid, the subsequent accumulation of contractile proteins may be attributed almost exclusively to myotubes. Therefore, the rates of accumulation in these cultures reflected myofibrillar protein accumulation in myotubes.

The same general patterns of accumulation were observed in these experiments as had been seen in the earlier studies. Actin and myosin (Figure 26 and 27) again increased during the cell fusion period as before, but on the basis of the moles of each protein accumulated per fused nucleus, actin molecules were accumulated at a much faster rate than myosin. Tropomyosin and α-actinin also were accumulated during this time, but the molar rates of accumulation were clearly different from each other and from actin or myosin.

The manner in which the results were expressed frequently caused the initial portions of the curves to show a negative slope. Because the mass of protein measured in cultures was standardized back to a fused nucleus basis and because the initial determinations were made before fusion was complete, the values calculated for μ moles/fused

Figure 27. Accumulation of myosin heavy chain and α-actinin subunit in fluorodeoxyuridine treated muscle cell cultures. These results were obtained from Form III isotope dilution experiments. As in Figure 26, the slopes of the accumulation curves represent the rates of accumulation and the percentage fusion is not decreasing in older cultures.


Myosin
α-actinin
% Fusion
(μmoles of Protein/Fused Nucleus) x10^10
2.5
2.0
1.5
1.0
0.5
0
100
80
60
40
20
0
% Fusion
40
60
80
100
120
Time in Culture (hr)

nucleus decreased as fusion increased thus increasing the denominator of this expression. If results were expressed on a per culture or a per total nucleus basis, the slopes would virtually be zero indicating very little accumulation during the early period.

In an effort to determine the pattern of accumulation rates, three separate experiments were conducted to evaluate the accumulation rates of actin, myosin, tropomyosin and α-actinin. For each protein, the rate of accumulation during the rapid accumulation phase was calculated as the slope of the line determined by linear regression of μ moles of protein/fused nucleus on time in culture. To standardize the rates of accumulation between experiments, the rates of accumulation were expressed as ratios of accumulation rates for every combination of two proteins (Table 4). This procedure reduced the experimental variation which was often large as evidenced by the large standard deviations obtained when the individual rates of accumulation were averaged over the three experiments (Table 5). A consistent pattern of synthesis rates was observed throughout the series of experiments. Actin was always accumulated at the fastest molar rate, followed by tropomyosin, myosin, and finally α-actinin. The ratios of accumulation rates also remained reasonably constant from one experiment to the next. A summary of rates of myofibrillar protein accumulation, total muscle cell culture myofibrillar protein composition, muscle culture myofibril composition, and mature pig skeletal muscle myofibril composition has been presented in Table 6.

The individual rates of accumulations, averaged across all three experiments, may be used to calculate the number of molecules of each

Table 4. Ratios of molar accumulation rates of myofibrillar proteins in myotubes[a]

	Actin: myosin heavy chain	Actin: tropomyosin subunit	Actin: α-actinin subunit	Myosin heavy chain: tropomyosin subunit	Myosin heavy chain: α-actinin subunit	Tropomyosin subunit: α-actinin subunit
Number of experiments[b]	3	3	2	3	1	1
Mean	4.56	1.90	16.35	0.42	3.31	7.44
Standard deviation	0.18	0.27	0.93	0.04	–	–

[a]Ratios of accumulation rates were determined from isotope dilution experiments with cultured muscle cells.

[b]Number of experiments indicates the number of experiments in which the two proteins in the ratios were measured simultaneously.

Table 5. Rates of myofibrillar protein accumulation (molecules/fused nucleus/minute)[a]

	Actin	Myosin heavy chain	Tropomyosin subunit	α-Actinin subunit
Number of experiments	3	3	3	2
Mean	131,090	31,920	74,320	6,924
Standard deviation	46,230	12,630	26,980	147

[a]Accumulation rates were determined from isotope dilution experiments with cultured muscle cells.

Table 6. Summary of myofibrillar protein accumulation rate and myofibrillar protein composition ratios[a]

	Actin: myosin	Actin: tropomyosin	Actin: α-actinin	Myosin: tropomyosin	Myosin: α-actinin	Tropomyosin: α-actinin
Accumulation rates in myotubes[b]	9.12 ± 0.36	3.80 ± 0.54	32.70 ± 1.86	0.42 ± 0.04	3.31	7.44
Composition of total muscle culture[c]	12.18 ± 1.22	4.26 ± 0.56	44.60 ± 15.8	0.41 ± 0.11	3.79	7.04
Composition of muscle culture myofibrils[d]	9.72 ± 0.28	9.18 ± 0.54	96.00 ± 21.4	0.94 ± 0.04	7.77 ± 4.3	8.93 ± 4.3
Composition of pig muscle myofibrils[e]	5.38 ± 0.26	5.50 ± 0.02	40.68 ± 0.72	1.09 ± 0.05	8.42 ± 0.31	8.26 ± 0.60

[a]Ratios are expressed as ratios of whole molecules instead of polypeptide subunits as in Tables 3, 4 and 5.

[b]Ratios of accumulation rates determined from isotope dilution experiments with cultured muscle cells.

[c]Ratios of total amounts of protein present in myogenic and fibrogenic cells in 5-day muscle cultures determined from isotope dilution experiments.

[d]SDS polyacrylamide gel quantitation of myofibrils prepared from 5-day muscle cell cultures.

[e]SDS polyacrylamide gel quantitation of pig skeletal muscle myofibrils. Values represent means and standard deviations of red and white myofibril ratios from Table 3.

protein accumulating per fused nucleus per minute (Table 5). The number of molecules accumulating per fused nucleus per minute was very large and quite variable due to differences among the three experiments; however, these figures are probably good estimates. Emerson and Beckner (1975) reported a synthesis rate for myosin in cultured quail muscle of 30,000 molecules per fused nucleus per minute. The myosin accumulation rate determined from these experiments, about 32,000 molecules per fused/nucleus per minute, was similar, considering the expected variation.

In summary, the time of initiation of rapid accumulation and the relative rates of accumulation for the four myofibrillar proteins studied formed a very consistent pattern. This consistent pattern indicates that the metabolism of these myofibrillar proteins in differentiating muscle cells is a closely regulated process.

V. DISCUSSION

The purposes of this research were to describe the accumulation patterns of actin, myosin, tropomyosin, and α-actinin in differentiating skeletal muscle cells in culture and to relate the accumulation patterns to the assembly of myofibrils. Consequently, the onset of the accelerated accumulation of these four myofibrillar proteins and their rates of accumulation were determined. In order to interpret the significance of these accumulation patterns, in terms of the final assembled myofibril, the composition of myofibrils from mature porcine skeletal muscle and from muscle cell cultures was analyzed and compared. The assembly of myofibrils in differentiating muscle cell cultures was monitored with electron microscopy to relate biochemical changes to structural events in myofibril assembly. To accomplish these goals, both preparative and analytical techniques had to be developed.

A. Preparation and Analysis of Myofibrillar Proteins from Cultures

The primary problem in studying myofibrillar proteins in muscle cell cultures is that they cannot be isolated in a pure form without substantial preparative losses. Many investigators have claimed to have done this for myosin (Chi et al., 1975; Holtzer et al., 1974; Paterson and Strohman, 1972; Rubinstein et al., 1974, 1976); however, recent experiments by Young[1] have demonstrated that conventional procedures for isolating myosin are very inconsistent. Furthermore the yields vary according to culture

[1]Young, R. B., Michigan State University, personal communication, 1976.

age. In view of the limitations in quantitating myosin from cultured cells, presumably the least difficult of the myofibrillar proteins because of its relative abundance, quantitation of actin in muscle cultures must be viewed with a great deal of caution. Holtzer's laboratory (Chi et al., 1975; Holtzer et al., 1974; Rubinstein et al., 1974, 1976) claims to have quantitated actin from SDS-polyacrylamide gel scans of crude actomyosin preparations of muscle cultures. By quantitating actin and myosin in pure myotube cultures, they arrived at an actin:myosin heavy chain ratio of about 1.9:1; their ratio is substantially lower than any actin:myosin ratio obtained in the experiments reported here. The discrepancy may be due to large losses of actin during their actomyosin preparation. Their preparative procedure would not have retained the thin filaments which were not incorporated into sarcomeres. It is unlikely that the actin measured in those experiments was either pure or representative of the total amount present in the cultures.

Paterson et al. (1974) also studied actin synthesis in differentiating muscle cell cultures, and many of the same arguments apply to their experiments. Their actin preparation method, however, was similar to the initial steps in the procedure used in this thesis. Cultures were glycerinated and, after removal of the glycerol and soluble proteins, the preparation was applied to SDS-polyacrylamide gels. They did not however utilize acetone powder preparations and the subsequent extraction of G-actin from the acetone powder, as reported in this thesis. Therefore, any insoluble 40,000-45,000 dalton polypeptide would have co-migrated with actin and consequently contaminated their preparations.

In order to circumvent the problems of quantitative yield and protein purity, an isotope dilution approach to studying protein accumulation was used. This approach circumvented the need for quantitative yields of protein, and allowed more extensive purification steps to be used for the isolation of myofibrillar proteins. This gave greater assurance that the isolated culture myofibrillar proteins were less likely to also contain contaminants of similar molecular weight and mobility on SDS gels. The procedures for the isolation of myosin and actin from muscle cell cultures are more extensive than any procedures reported previously. In addition, procedures for the isolation of α-actinin and tropomyosin from cultured skeletal muscle cells have not been reported by others.

B. SDS-Polyacrylamide Gel Electrophoresis and Quantitation

Although the isotope dilution technique was an important key to this research project, to get specific activity measurements from the isotope dilution technique, procedures for accurately measuring the mass of protein in SDS-polyacrylamide gel bands had to be developed. Emphasis was placed on both the mechanics of the electrophoresis and scanning techniques as well as the relative magnitude of error introduced at each step in the entire process.

One of the initial observations from the gel quantitation experiments was that all of the curves, with the exception of troponin-C, were biphasic. At low protein loads, they obeyed Beer's Law, but when the maximum absorbance reached about 3.0 OD, the peak area response

was suppressed. Thus, the peak area/μg of protein was lower as protein loads were increased above those which gave an absorbance of 3.0 OD.

Deviations from Beer's Law with Coomassie Blue stain have been reported by other investigators (Fenner, et al., 1975; Fischbein, 1972; Kruski and Narayan, 1974). The deviation from Beer's Law and the increase in peak area/μg that occurred as distance of migration increased can be explained by the same phenomenon. When bands are absorbing near or above 3.0 OD, the actual amount of transmitted light is very small (0.1% of the incident light), and more importantly, the small amount of stray light present in any spectrophotometric analysis system is no longer insignificantly small compared to the actual transmitted light. The stray light adds to the transmitted light and results in a diminished absorbance reading and consequently, less peak area/μg. As protein bands are migrated further into the gel they become more diffuse and a smaller portion of the total peak area is contributed by absorbance in the critical region above 3.0 OD. Therefore, area/μg would be expected to increase with increasing distance of migration.

With heavy protein loads that would be measured on the second portion of the curve, accuracy is diminished. This probably results from two factors. First, distance of migration is an important variable because of the tendency for protein bands to become diffuse and to widen as distance of migration increases. Second, when absorbance is very high, it results in larger instrumental errors. It should be pointed out that all gel scans were done at a constant slit width.

The analysis of variance points out the primary sources of error in the quantitation procedure. The results demonstrate that rigorous

discipline is required in the electrophoresis, staining, destaining, and the scanning procedure. It is also very important to have several replications of gels of a given protein sample at the same protein load. There is, however, little advantage in scanning an individual gel more than once.

Another critical variable in the densitometry process is dye binding. Coomassie Blue dye binding per unit of protein varied more than 2-fold within the seven polypeptide chains evaluated in the experiments. There was a significant positive relationship between the amount of dye bound and the basic amino acid composition of the protein. This finding supports the contention that binding is due, at least in part, to electrostatic attraction between the negatively charged dye molecule and the positively charged amino acid residues.

Quantitative densitometry has not been studied in comparable detail by other investigators; although others have pointed out some of the potential sources of error. The research reported in this thesis suggests that when densitometric analysis of polyacrylamide gels is performed without considering distances of migration, staining and destaining protocol, and differential dye binding, the results are subject to large errors. Such results would give only approximations of the amounts of protein present in the various bands.

C. Accumulation and Assembly of Myofibrillar Proteins in Cultured Muscle Cells

The myofibrillar protein accumulation patterns and the electron microscope observations on myofibril assembly have been described in the Results section. It is important, however, to compare the accumulation patterns and the myofibril assembly process and to examine the relationships between the two. The role of myofibrillar protein accumulation in the myofibril assembly process may be described with one of three simple models.

1. The assembly of myofibrils is accomplished by the sequential accumulation of specific myofibrillar proteins.

This hypothesis has, in effect, been proposed by several investigators. On the basis of electron microscope observations, Allen and Pepe (1965) claimed that actin must be synthesized before myosin. Heywood and Rich (1968) reported a sequential increase in density gradient polyribosome peaks from embryonic chick muscle. Polyribosomes from various peak regions synthesized actin, myosin, and tropomyosin, in that order with increasing embryonic age. Hitchcock (1970) also proposed that actin and myosin are accumulated prior to proteins of the Ca^{++} sensitizing system, tropomyosin and troponin.

2. The accumulation of myofibrillar proteins is a coordinated process in which accumulation of all proteins is initiated simultaneously and proceeds with relative accumulation rates that reflect the composition of assembled myofibrils.

Electron microscope studies by Fischman (1967, 1970) led him to propose the simultaneous appearance of actin and myosin in differentiating muscle. Holtzer's laboratory (Chi et al., 1975; Holtzer et al., 1974; Rubinstein et al., 1974, 1976) has also presented evidence for the simultaneous accumulation of actin and myosin. They also reported that actin and myosin are present in mature myotubes in the same proportion as in adult skeletal muscle. This hypothesis provides an example of the ultimate in self-assembly; if all parts are present at the same time and in proper amounts they will assemble into the complete structure.

3. Myofibrillar proteins begin to accumulate simultaneously, but the individual rates of accumulation do not reflect the composition of the final structure.

This hypothesis is a permutation of ideas presented in the two preceding hypotheses and has not been championed by any investigator or group of investigators. Fischman (1967, 1970) has acknowledged, however, that even though actin and myosin appear simultaneously there seems to be 7 to 10 times more actin than myosin in differentiating muscle cells. Allen and Pepe (1965) also noted the presence of many more thin filaments than thick filaments early in assembly.

The two essential features of these three models will be considered in order and the supporting or refuting evidence will be presented.

1. Sequence of initiation of myofibrillar protein accumulation

With respect to the sequence of myofibrillar protein accumulation, the results of this research have clearly demonstrated that at least four myofibrillar proteins, actin, myosin, tropomyosin, and α-actinin, are accumulated simultaneously in differentiating muscle cells. These results are consistent with proposals concerning actin and myosin from Holtzer's group (Chi et al., 1975; Holtzer et al., 1974; Rubinstein et al., 1974, 1976), Fischman (1967, 1970) and Masaki and Yoshizaki (1972). Furthermore, the experiments which demonstrated actin and myosin accumulation in EGTA fusion-blocked cells clearly showed that the synthesis of myofibrillar proteins is not dependent on myoblast fusion, in agreement with the recent studies of myosin synthesis in fusion-blocked cells (Emerson and Beckner, 1975; Merlie and Gros, 1976; Moss and Strohman, 1976; Vertel and Fischman, 1976). The earlier experiments of Paterson and Strohman (1972) and Paterson et al. (1974) led them to propose that blocking myoblast fusion also blocks myofibrillar protein synthesis; therefore, they suggested that fusion is the signal that initiates the terminal step in muscle cell differentiation. These apparently erroneous results have been explained (Emerson and Beckner, 1975; Moss and Strohman, 1976) by the observations that EGTA blocking may retard the differentiation of muscle cells. Therefore, in Paterson's experiments (Paterson and Strohman, 1972; Paterson et al., 1974) the cells may have been released from the fusion block before cells were ready to initiate the synthesis of myofibrillar proteins. When measures were taken to accelerate the differentiation of EGTA-blocked cells by the addition of conditioned medium (Emerson and Beckner,

1975; Moss and Strohman, 1976) or when cultures were blocked for longer periods of time (Emerson and Beckner, 1975 and in the experiments reported in this thesis), myosin synthesis increased before the cells were allowed to fuse.

From the results of this research it can be concluded that the synthesis of actin, myosin, tropomyosin, and α-actinin is a coordinated process, is independent of fusion, and is associated with the terminal phase of myogenic cell differentiation. This process probably reflects the same change in the program for gene expression that is responsible for the many other differentiated muscle characteristics. For example as a myogenic cell proceeds through the final differentiation step, the expression of genetic programs for sarcoplasmic reticulum development, changes in energy metabolism, acetylcholinesterase sensitivity, cell fusion, and myofibrillar protein synthesis is initiated. The evidence indicates that the first model for myofibril assembly must be rejected because myofibrillar proteins are clearly not synthesized in sequence.

2. Rates of accumulation of myofibrillar proteins

The two remaining models deal with the relative rates of accumulation of myofibrillar proteins in differentiating skeletal muscle cells, as compared to the relative amounts of each myofibrillar protein in the myofibril. To facilitate comparisons between experiments, rates of protein accumulation have been reported here as ratios of accumulation rates for pairs of proteins. Therefore, ratios of rates of

accumulation will be discussed in terms of how closely they agree with the molar ratios of proteins in mature porcine skeletal muscle myofibrils.

The myofibril composition ratios obtained in these experiments are in reasonable agreement with the values reported by Potter (1974) and Tregear and Squire (1975). Some differences do exist however; the actin:myosin ratio of 5.38 is very close to the ratio of 5.6 reported by Tregear and Squire (1975) but is somewhat lower than Potter's value of 6.34. Based on myosin and actin extraction experiments, if myosin is 55% of the myofibril and actin is 20% (Maruyama and Ebashi, 1970), a value of 4.1 actin molecules for every myosin molecule can be calculated. This figure is substantially lower than any of the values published from gel quantitation (Potter, 1974; Tregear and Squire, 1975) or reported in this thesis. If tropomyosin is 5% of the myofibril and α-actinin is 2% (Maruyama and Ebashi, 1970), the following molecular ratios can be calculated: actin:tropomyosin = 6.7, myosin:tropomyosin = 1.6, actin:α-actinin = 47.5, myosin:α-actinin = 11.8, and tropomyosin:α-actinin = 7.2. Although the tropomyosin:α-actinin and the actin:α-actinin ratios agree reasonably well with the pig myofibril values reported in this thesis of 8.26 and 40.68, respectively, the actin:tropomyosin, myosin:tropomyosin and myosin:α-actinin ratios are larger than the gel quantitation data reported in this thesis (Table 6). A large range of values for ratios of myofibrillar proteins can be calculated either from myofibrillar protein extraction experiments (Maruyama and Ebashi, 1970) or gel quantitation (Potter, 1974). The myofibrillar protein ratios reported in this thesis are either in agreement with ratios from previously published myofibril composition

data or well within the range of composition ratios from either extraction or gel quantitation experiments.

Ratios of accumulation rates of actin, myosin, α-actinin, and tropomyosin in cultured embryonic chick muscle myotubes are clearly different than the stoichiometry of the proteins in pig skeletal muscle myofibrils (Table 6). Myotubes accumulate excess actin, tropomyosin, and α-actinin with respect to myosin, as evidenced by the large actin: myosin ratio and the small myosin:tropomyosin and myosin:α-actinin ratios. The ratio of accumulation rates of actin:tropomyosin and of actin:α-actinin were less than the respective protein ratios in pig myofibrils. Therefore, when compared with actin, myotubes also appear to accumulate more tropomyosin and α-actinin than is present in the mature myofibril. Ratios of tropomyosin:α-actinin molar accumulation rates, however, were very near the molar ratios of tropomyosin:α-actinin in pig myofibrils.

The possibility exists that the accumulation of contractile proteins measured in myotubes could consist of both myofibrillar and cytoplasmic system contractile proteins. The cytoplasmic contractile system is seldom seen in electron micrographs of older myotubes although portions of this contractile system may be found in young myotubes (Figure 16a). Therefore, compared to the massive accumulation of myofibrillar contractile proteins in myotubes, the contribution of cytoplasmic contractile protein synthesis to net accumulation may be insignificant. If, however, the accumulation of cytoplasmic contractile proteins is significant in young myotubes which have accumulated only small quantities of myofibrillar contractile proteins, the measured

accumulation of myofibrillar actin, for example, would be artificially large at early ages. This artifact in young myotubes would actually cause the slope of the accumulation curve, the rate of accumulation, to be smaller than the true rate of myofibrillar actin accumulation. This would only accentuate the discrepancy between the actin:myosin accumulation rate ratio and the actin:myosin pig myofibril composition ratio. It is, obviously, impossible to assess the contribution of the cytoplasmic contractile system within myotubes to total contractile protein accumulation, but the assumption that the effect is negligible, or at least within the realm of experimental error in these experiments, seems justified.

The results of the accumulation rate experiments indicate that, with the exception of the tropomyosin:α-actinin ratio, myofibrillar proteins accumulate at relative rates that do not reflect the stoichiometry of the mature myofibril. The data argue for rejection of the second model of myofibril assembly and acceptance of the third model, which requires simultaneous accumulation of myofibrillar proteins but nonstoichiometric rates of accumulation.

Because the accumulation rate ratios did not consistently reflect the stoichiometry of myosin, actin, tropomyosin, and α-actinin in mature myofibrils, it would be interesting to know where the excess actin, tropomyosin, and α-actinin accumulated. Molar ratios of these myofibrillar proteins in the entire unfractionated culture (Table 6), which includes both myogenic and fibrogenic cells, reveal an even greater actin:myosin ratio than the myofibrillar protein accumulation rates would predict. This result is not totally unexpected in view of the

presence of the constant background level of fibroblasts, arrested by fluorodeoxyuridine treatment, with their actin-rich cytoplasmic contractile system. Ratios of the other myofibrillar proteins, myosin, tropomyosin, and α-actinin, from the entire culture were similar to the myofibrillar protein accumulation rate ratios. This indicates that these proteins may not be present in significant quantities in the background fibroblast cytoplasmic system. Therefore, the total culture appears to be composed of a myofibrillar compartment, in which all proteins are present, and a fibroblast cytoplasmic system compartment composed predominately of actin. When total cultures were fractionated by preparing crude myofibrils, the actin:myosin ratio in the culture myofibrils was lower than the ratio of amounts of actin and myosin present in total culture. This can be interpreted to indicate that some of the total culture actin existed in a nonsedimentable actin pool that was not isolated with the myofibrils. The key question is whether this nonsedimentable actin represents the excess actin accumulated by myotubes or the background actin from the cytoplasmic contractile system of fibroblasts. This question cannot be unequivocally answered.

In comparing the compositions of culture myofibrils and mature pig muscle myofibrils, myosin, α-actinin, and tropomyosin were present in the same relative proportions. In culture myofibrils, however, actin was present in excess as indicated by larger actin:myosin, actin:tropomyosin, and actin:α-actinin ratios when compared to muscle myofibrils. These results can be explained if, during the fractionation and isolation of myofibrils from cultures, bundles of actin rich thin filaments from fibroblasts were isolated with the myofibrils. If one

compares the size of an individual myofibril with that of the cytoplasmic contractile system thin filament bundles, it seems reasonable that they might sediment together in the fractionation procedure. This would increase the proportion of actin in a preparation of myofibrils isolated from muscle cell cultures which also contain fibroblasts.

Additional support for the contention that the excess actin in culture myofibril preparations came from the fibroblast cytoplasmic system is provided by comparing the amounts of actin, α-actinin and tropomyosin present in culture myofibrils with the ratios of accumulation rates for these three proteins in myotubes. The ratio of tropomyosin:α-actinin present in culture myofibrils was nearly equal to the tropomyosin:α-actinin accumulation rate ratio. The ratios of actin:tropomyosin and actin:α-actinin present in isolated culture myofibrils, however, were much larger than both the respective accumulation rate ratios in myotubes and the ratios of these proteins in mature myofibrils. Contamination of culture myofibrils with an actin-rich fibroblast cytoplasmic system could produce these results.

In the discussion of nonsedimentable pools of actin in total cultures, the proposal was made that this actin pool could be attributed either to the fibroblast cytoplasmic system or to excess actin accumulated by myotubes. If the proposal is accepted that the culture myofibril preparations contain both assembled myofibrils and fibroblast cytoplasmic system proteins, the nonsedimentable pool of actin, which could not be isolated with culture myofibrils, can be attributed to the excess of actin accumulating in myotubes. The nonsedimentable

pool may correspond to the "free" thin filaments in myotubes which have often been reported in electron microscope studies of myofibril assembly (Allen and Pepe, 1965; Fischman, 1967; Hay, 1963, Hilfer, 1973; Przybylski and Blumberg, 1966; Shimada et al., 1967). It is also possible that fibroblasts in the cultures may contain some of these "free" thin filaments.

The nonsedimentable actin pool could also have associated with it a large portion of the excess tropomyosin and α-actinin being accumulated in myotubes (excess with respect to myosin accumulation and myofibril composition). As previously mentioned, molar ratios of myosin:tropomyosin and myosin:α-actinin present in culture myofibrils revealed that these three proteins were present in the same proportion as in mature pig myofibrils, but the respective accumulation rate ratios were only about one half as large. This indicates that there must have been large amounts of tropomyosin and α-actinin present in nonsedimentable pools that could not be isolated with culture myofibrils. Much of the excess tropomyosin and α-actinin may, therefore, be associated with actin in the proposed soluble pool of free thin filaments that are accumulating; however, in myotubes tropomyosin and α-actinin are even accumulating in excess of actin, when compared with the composition of pig myofibrils. Consequently, there may also be a pool of "free" tropomyosin and α-actinin, both of which are soluble under intracellular ionic strength and pH conditions.

The following pattern of myofibrillar protein accumulation can be proposed from the preceding discussion. The accumulation of each of the myofibrillar proteins studied, actin, myosin, α-actinin, and

tropomyosin, is initiated simultaneously. The relative rates at which these proteins accumulate, when compared to mature myofibril composition, result in an excess amount of actin, tropomyosin and α-actinin with respect to myosin. These three proteins associate into thin filaments, some of which aggregate with newly formed myosin thick filaments, while the remaining thin filaments exist as the "free" thin filaments described by many electron microscopists (Allen and Pepe, 1965; Fischman, 1967, 1970; Hay, 1963, Hilfer et al., 1973; Przybylski and Blumberg, 1966; Shimada et al., 1967). Small pools of excess α-actinin and tropomyosin may also be present as soluble molecules in the cytoplasm.

D. Ultrastructural Observations of Myofibril Assembly

If the myofibrillar protein accumulation pattern plays a role in myofibril assembly, the pattern should be compatible with the ultrastructural observations of myofibril assembly. Electron microscopy of thin sections of embedded muscle cultures was the technique used to assess the state of myofibril assembly. As previously discussed, the accumulation of all four of the myofibrillar proteins studied was initiated simultaneously. The electron microscopy results could be interpreted to demonstrate the simultaneous appearance of actin and myosin, since thick and thin filaments are the first discernible myofibrillar structures. Fischman (1967, 1970) has also stated that free thick and thin filaments appear simultaneously in developing muscle cells. In the results reported in this thesis, however, Z line structures

do not appear simultaneously with thick and thin filament aggregates; therefore delayed accumulation of α-actinin, a Z line component, with respect to actin and myosin would have been predicted from the microscopy observations. Consequently, the accumulation pattern and the ultrastructural observations are in apparent disagreement. It is conceivable that the first few molecules of α-actinin or small Z line precursor aggregates might not have been detected. In contrast to the observations in this thesis, Dessouky and Hibbs (1965) and Hilfer et al. (1973) have reported the simultaneous appearance of Z lines and thick and thin filament aggregates. This would agree with the accumulation patterns reported here.

Ultrastructural observations would predict rates of actin:myosin accumulation comparable to the proportions present in myofibrils, because an abundance of free thin filaments was not observed. Other investigators, however, have claimed that free thin filaments are present in excess of myosin filaments during early stages of assembly (Allen and Pepe, 1965; Fischman, 1967, 1970; Hay, 1963; Hilfer et al., 1973; Przybylski and Blumberg, 1966; Shimada et al., 1967). Therefore the accumulation rates would be consistent with their observations. Furthermore, these biochemical studies have been interpreted to actually predict the presence of free thin filaments.

α-Actinin has been localized in Z lines (Goll et al., 1969; Stromer et al., 1967, 1969; Schollmeyer, 1976) and Z line analogues in many contractile systems (Schollmeyer, 1976). Due to the resemblance of Z line precursors in assembling myofibrils and Z line analogues in other systems, it is logical to conclude that the precursor Z line bodies in

developing myotubes contain α-actinin. Because Z lines are slower to develop than thick and thin filament aggregates, the rate of accumulation of α-actinin might be expected to be slower than the rates of actin and myosin accumulation. Therefore the accumulation pattern again disagrees with the ultrastructural observations.

The electron microscope observations also indicated that Z line formation started with the amorphous component of the Z line forming Z line precursor bodies. The filamentous nature of the Z line developed later. This process has been reported by many other investigators (Allen and Pepe, 1965; Fischman, 1967, 1970; Hay, 1963), but it is in apparent contrast with the observation that the Z line of tadpole, Rana pipens, myofibrils are primarily filamentous with very little amorphous component while in the adult frog both amorphous and filamentous components are present. In both the tadpole and adult frog, however, the myofibrils are completely assembled. Therefore, it is not clear whether the amorphous or the filamentous component is actually assembled first. The difference in the Z lines of tadpoles and adult frogs may, instead, reflect a difference in the physiological role of the fibers, as in the case of Z line differences between red and white muscle fibers of mammalian muscles. It is also possible that the filamentous and amorphous Z line components are being assembled at the same time, but the filamentous nature of the Z line is not detected due to the lack of organization and alignment of thin filaments inserting into these structures.

Because tropomyosin cannot be detected directly by electron microscopy of cultured muscle cells, it is difficult to relate the rate

of accumulation to myofibril assembly. There is, however, indirect evidence for the presence of tropomyosin; if tropomyosin were not present, α-actinin binding would not be restricted to the Z line region but could bind along the thin filament. If α-actinin bound along the thin filament, one would expect to see cross connections between adjacent thin filaments as described by Stromer and Goll (1972). Such structures were not seen. Furthermore, the development of distinct I bands may also reflect the presence of tropomyosin. I bands occur in relaxed myofibrils, and in relaxed myofibrils, at 10^{-7} M Ca^{++} concentration, the troponin-tropomyosin complex prevents the interaction of actin and myosin and causes relaxation. In vitro, tropomyosin alone causes the inhibition of actomyosin ATPase, an in vitro measure of contraction. Therefore, if tropomyosin was present on these F-actin filaments without troponin, a relaxed state with distinct I bands would be possible. Alternatively, the appearance of I bands may just reflect the presence of well formed Z lines that serve to hold the relaxed sarcomeres in a position with less thick and thin filament overlap. The elaboration of a functional sarcoplasmic reticulum, capable of maintaining low Ca^{++} concentrations, as well as the existence of troponin in these structures could also play a role in maintaining the relaxed state. The existence of troponin and a functional sarcoplasmic reticulum, however, have not been confirmed; therefore, the precise conditions responsible for I band formation in developing myofibrils are unknown.

The electron microscope study of myofibril assembly agrees with the structural observations of several other investigators, but the patterns of accumulation of myosin, actin, tropomyosin, and α-actinin differ from

those predicted from the observed nascent myofibrillar structures. The explanation for this discrepancy may exist in an important concept of metabolic control. In the control of metabolism, synthesis of an enzyme is a coarse form of metabolic control, when compared to other forms of control such as competitive and allosteric inhibition or enzyme modification. In the metabolic control of myofibril assembly, the synthesis or accumulation patterns of myofibrillar proteins may likewise represent a coarse form of control over the assembly process. This coarse control may serve to establish the proper conditions for other unknown processes, such as amino acid side chain modification or selective proteolysis. These other processes may function as finer controls on the myofibril assembly process.

Many questions regarding myofibril assembly have not been answered by this research. For example, the aggregation of thick and thin filaments into hexagonal arrays has been noted to be the initial form of myofibril aggregation; however, the maintenance of these actin-myosin bonds is difficult to understand. Without Z lines, what prevents actin and myosin filaments from contracting past one another, assuming conditions are favorable for contraction? If conditions do not favor contraction and relaxation is favored, what forces maintain the thick and thin filaments in position relative to one another? The only conditions under which free thick and thin filaments would remain in stable positions relative to one another is in the absence of ATP. This condition constitutes rigor and does not seem very likely. One possible explanation is that certain conditions exist in embryonic muscle which allow actin-myosin interaction without cross bridge movement

and subsequent contraction; however, there is no basis for postulating such a mechanism. An alternative explanation is that free thick and thin filaments are continually contracting past one another until Z line formation inhibits further movement. This continual contracting movement of free thick and thin filaments could serve to align these filaments into a continuous parallel stream which would subsequently become a myofibril. Unfortunately, the lack of knowledge concerning the presence and functionality of troponin and the sarcoplasmic reticulum make it impossible to understand the contractile state of actin and myosin filaments during myofibril assembly.

The formation of Z lines is also difficult to explain. The primary reason, however, is that the structure and protein interactions in Z lines of mature skeletal muscle myofibrils are not fully understood. If myofibrillar proteins do not accumulate in a sequential manner, why does Z line formation become apparent at a later stage than thick and thin filament aggregation? Moreover, why, in differentiating chick skeletal muscle cells, does the amorphous component of the Z line appear before the filamentous component? One possible explanation is that the complexity of the Z line itself is responsible for its slow development. The fact that numerous thin filaments must align with their free ends in the same proximity and with the correct polarity may serve to slow the process. This could be expected because a large filament would not move around in solution as rapidly as an individual molecule. Therefore the movement and alignment of thin filaments in preparation for Z line formation would be a much slower process than the movement of individual actin or myosin molecules in forming thick

and thin filaments. Z lines should therefore be formed later than thick and thin filaments.

If the amorphous component of the Z line is α-actinin, as proposed by Schollmeyer (1976), the early appearance of amorphous Z line precursors may actually represent the alignment and initial cross linking of thin filaments by α-actinin. Because tropomyosin is present, α-actinin cross linking would be restricted to the Z line end of thin filaments. The alignment and cross linking process may be the prerequisite for Z filament formation which completes the Z line assembly process. This would explain the sequential formation of amorphous and filamentous components of the Z line.

This process could be expected to result in well formed straight Z lines within a myofibril, but what forces are responsible for bringing adjacent myofibrils in register? It seems feasible that T-tubule and sarcoplasmic reticulum development play a role in this process, but there is no evidence or even a respectable hypothesis for explaining this role.

A complete molecular understanding of myofibril assembly must await answers to the preceding questions, and many others.

VI. SUMMARY AND CONCLUSIONS

The accumulation patterns of myosin, actin, α-actinin, and tropomyosin were determined in differentiating chick embryo leg muscle cultures. Isotope dilution techniques were used to follow the accumulation of individual myofibrillar proteins. In order to conduct the experiments, several procedures had to be developed including procedures for preparing pure aliquots of myofibrillar proteins from muscle cell cultures and quantitative SDS polyacrylamide disc gel electrophoresis and densitometry.

Light and electron microscopy were used to monitor the state of muscle cell differentiation and to follow the assembly of myofibrillar structures in cultured muscle cells. Muscle cells in culture were capable of fusion into multinucleated myotubes and of myofibril assembly. The assembly of myofibrils appeared to agree with the observations of several other investigators (Firket, 1967; Fischman, 1967, 1970; Shimada et al., 1967). Aggregates of interdigitating thick and thin filaments were the first identifiable myofibrillar structures; Z line precursor material then appeared at the ends of thin filaments. Amorphous, discontinuous Z line precursor material preceded the highly ordered Z line structure characteristic of mature muscle and linked sarcomeres end to end into myofibrils.

All four myofibrillar proteins studied, actin, myosin, tropomyosin, and α-actinin, began to accumulate in large amounts simultaneously following the rapid burst of muscle cell fusion in culture. When myoblast fusion was blocked, the pattern of actin and myosin accumulation

was not altered; this indicates that myoblast fusion is not a prerequisite for myofibrillar protein synthesis.

Rates of accumulation of each protein were determined and ratios of these rates for each combination of two proteins were calculated. These ratios were compared with molar ratios of the respective proteins present in mature pig myofibrils, muscle culture myofibrils, and whole unfractionated muscle cultures. It was observed that the myofibrillar proteins do not accumulate in myotubes in the same proportions as they exist in myofibrils from mature pig skeletal muscle or from muscle culture myofibrils.

The studies of protein accumulation patterns suggest that myofibrils assemble in the presence of all the myofibrillar proteins. With respect to myosin accumulation and thick filament formation, the system is saturated with actin, tropomyosin, and α-actinin. The excess actin and much of the excess tropomyosin and α-actinin probably exist as "free" F-actin filaments with associated tropomyosin and α-actinin. Tropomyosin and α-actinin are even present in excess of actin and may exist as small soluble pools of protein. The excess tropomyosin would be expected to saturate actin filaments and restrict α-actinin binding to the ends of the filament. α-Actinin could then function to cross link the ends of F-actin filaments thereby bringing the ends into close enough proximity for Z filament formation, thus completing the Z line.

The myofibrillar protein accumulation patterns do not correspond to the structural description of myofibril assembly. The ultrastructural studies would have predicted the delayed or sequential appearance of α-actinin and possibly tropomyosin since Z line structures form later

than the initial thick and thin filament aggregates during myofibril assembly. As with metabolic control of other biochemical processes, the accumulation of myofibrillar proteins may be the coarse form of control, and the finer control of assembly may reside in processes such as covalent modification of proteins or selective proteolysis.

The results presented in this thesis warrant the following conclusions:

1. Myosin, actin, tropomyosin, and α-actinin begin to accumulate simultaneously about the time of fusion in differentiating skeletal muscle cells grown in culture. There is no sequence in which these myofibrillar proteins are synthesized prior to myofibril assembly.

2. Inhibition of myoblast fusion by removal of Ca^{++} from complete culture medium does not inhibit myofibrillar protein synthesis. Both actin and myosin accumulation increased during the fusion arrest stage, when less than 1% of the nuclei were in multinucleated cells.

3. The rates of accumulation of actin, myosin, α-actinin, and tropomyosin are not in the same proportion as the stoichiometry of these proteins in mature myofibrils. The relative rates of accumulation indicate that myofibrils assemble in the presence of excess tropomyosin, α-actinin, and actin with respect to myosin. In culture myotubes tropomyosin and α-actinin even accumulate in excess of actin based on the proportions of these proteins in mature myofibrils.

4. The electron microscope evidence suggests that the thick and thin filament aggregate is the initial myofibrillar structure to be assembled. Z lines and M lines assemble later, and distinct I bands

only appear after Z lines are completed. The sequential nature of myofibril assembly does not reflect the simultaneous accumulation of myofibrillar proteins.

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